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Environmental effects on the growth and biochemical composition of four micoalgae, in relation to their use as food for Mytilus edulis larval rearing.

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Environmental effects on the growth and biochemical composition of four microalgae, in relation to their use as food for *Mytilus edulis* larval rearing

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Preface

During the three year PhD research project the prospective candidate not only has to learn specific techniques, conduct experiments and understand methodological approaches relevant to the question in hand but has to fully comply with a scientific way of thinking and finally produce results unknown to the scientific community. All these are limited by the somewhat short amount of time. This is not *in lieu* of excuses for mistakes and omissions that due to our imperfectly human nature will certainly exist; nor is it to play down the reader's expectations in order to provide afterwards an unexpected *tour de force*. This is just to let you know that I am aware of it, slightly troubled by it but overall happy; this is my deposition and I accept full responsibility for it.

It is customary to start with one relatively simple question and seek one equally simple answer and end up after three years of fruitful and hard work with more questions than answers.

But, what is it then all about? One has to ask oneself for this philosophical question; no answer will be provided here and the reader is referred to other sources. This secret I shan't reveal! Time is the fire in which we burn or a companion throughout our journey? I have no illusions of helping anybody with my infinitely small contribution and equally unimportant views. This is only where I stand, now! It goes without saying that through my evolutionary process I deserve the opportunity to change my mind. But certainly, I do not intend to live forever; and I am at easy with the fact that I shall be forgotten.

For this very creative period of my life first and foremost I would like to thank my supervisor Dr. I. Lucas for accepting the challenge of guiding me through this treacherous path. Of course he is not the only one that deserved to be acknowledged during my staying in the School of Ocean Sciences. Andy Beaumont is one of the persons, who besides accepting to become the internal examiner, provided helpful comments and insight into the mussel aspect of the project and helpful encouragement throughout the experimental work. For interesting comments and different perspective given to the project I would also like to thank Dr. David Jones. A very special and big

thanks goes to Mr. John East not only for his invaluable help for the technical part of the fatty acid analysis but also for all our thought provoking conversations.

There are a number of other people that should be mentioned here and an even bigger number of those who shouldn't but I'd rather spare their embarrassment. They truly know where they stand.

This work was made possible by a generous grant from the Greek State Scholarship Foundation (I.K.Y.) and the continuous efforts of Mrs. Metaxa who's help is gratefully acknowledged.

Besides being a student I have been (and still claim to be) an individual person. For totally different reasons, but which help me nevertheless to make it though this period and conclude the work, I want to thank my mother and brother who still bear with me even through the not so very happy times and despite my obnoxious and incomprehensible acts. Ευχαριστώ πολύ.

I do not want the reader to accept the work as the only real truth. It is merely my part of the truth; and as such it is a part of me. You can take away whatever you feel belongs to you. If along the way you try to respect this for what it is, then I would be grateful too.

Abstract

Environmental conditions in form of light intensity, phosphorus and nitrogen limitation were used to manipulate the biochemical composition of continuous cultures of *Skeletonema costatum, Chaetoceros muelleri, Rhinomonas reticulata* and *Pavlova lutheri*. Crude protein, carbohydrate and chlorophyll content as well as the fatty acid profile was determined in the combinations of two light intensities (high and low light, **HL** and **LL**) and three nutrient conditions (no nutrient limitation, **f/2**, phosphorus limitation, **P,** and nitrogen limitation, **N**). They were fed to *Mytilus edulis* larvae over a two week period and the larval size and mortality were assessed; the larval fatty acid profile of various batches of eggs as well as after the end of the feeding trial was also determined. A novel computer aided image analysis technique was used for measuring the length of the larvae. All monospecific diets supported good growth, sometimes equal or better to a control diet which was a mixture of species (*R. reticulata* and *P. lutheri*). In general survival was not affected by the diets and was found to be related more with the specific batch of larvae used. On the contrary growth was correlated with the diet. Ranking of the *S. costatum* diets was: LL N =LL f/2 =LL P =HL N >Control >HL f/2 =HL P. The *C. muelleri* diets were ranked as: LL N =LL f/2 >Control >HL f/2 =HL P =HL N >LL P. The *R. reticulata* diets are ranked, again in decreasing quality order as: HL N =LL f/2 =Control >LL N >HL f/2 =HL P =LL P. The *P. lutheri* ranking order was: HL N =HL f/2 =HL P =LL P >LL N =LL f/2 >Control. The larvae were analyzed for their fatty acid profile and relative content and some fatty acids were significantly correlated with growth thus enabling the usage of certain fatty acids as an index of growth for *M. edulis* larvae. Larval 20:5ω:3 and Polyunsaturated fatty acids (PUFA) were a positive index of growth while 15:0 and Saturated Fatty acids (SaFA) were a negative index. A multidimensional model was used in an effort to correlate algal biochemical components with larval growth. Some fatty acids were found to be the main factors in determining the algal biochemical composition with protein and carbohydrate playing a secondary "modifying" role. In the case of *P. lutheri* the 16:0 and SaFA were positively correlated with larval growth in an almost linear fashion while omega : 3 fatty acids were negatively correlated with larval growth. A positive correlation concerning the 16:0 and a negative one for the PUFA was also established in *S. costatum* and *R. reticulata*.

1. Introduction

The research project that is about to be presented focuses on the use of environmental factors to optimize the dietary qualities of algal cultures which are used as food for larvae of the bivalve *Mytilus edulis*. Before proceeding to the introduction of various related subjects, it was thought necessary to include a short description of the sequence that these subjects will be presented to the reader.

Although the algal part of this project was of primary importance in the completion of this project, the organism that was used to assess the effect that environmental factors have on the nutritional quality of the algal cultures, will be presented first. Naturally, general information regarding the description and geographic distribution, gonadal development and spawning cycles will be also presented. Furthermore, since the work dealt with the larval stages of *M. edulis*, some specific comments dealing with the gametes and the developmental stages of the species will also be included.

The logical sequence of the progression of the subject was to then present a review of the factors that influence larval growth. Focusing on the major factor that influences larval growth that algae are, the introduction will move onto present a set of selection criteria that are usually applied to select an appropriate algal species as food for a larval stage. A brief review of dietary components that are generally accepted to play a pivotal role in determining the nutritional value of algal species, will be included.

Finally, a sharper focus on factors that affect algal biochemical composition, which was the main research area of this work, will conclude the introduction, and present the main objectives of the present research project.

A. The bivalve *Mytilus edulis*

1.1.a Description and geographic distribution

Mussels of the genus *Mytilus* L. are common marine molluscs and constitute an important element in the ecology of coastal waters (Gosling, 1992). Mussels of the genus *Mytilus* belong to the family Mytilidae (Seed and Suchanek, 1992) and have a world wide distribution in temperate waters. The species used in this project, *M. edulis*, extends in the North Atlantic from the northern coasts of France to the White Sea and from the Canadian east coast to North Carolina. In the southern Hemisphere, it has been found only on the coasts of Argentina and Chile (Gosling, 1992). *M. edulis* inhabits typically intertidal habitats.

While the literature on *Mytilus* is extensive, most of the research on this genus has focused on the biology of adults, owing to their importance as food and as fouling organisms (Lutz and Kennish, 1992). It is widely accepted that temperature is the single most important factor that controls the geographic distribution of this species (Seed and Suchanek, 1992).

1.1.b Gonadal cycle and spawning season

Mytilus edulis L. is a dioecious species. The reproductive cycle of *M. edulis* has been widely studied (see also the review of Seed and Suchanek, 1992). Several factors have an influence on this cycle, such as temperature and food availability. In the Menai Strait and the Isle of Anglesey, *M. edulis* has resting gonads during October and November. Throughout the winter active gametogenesis occurs and most mussels have ripe gonads by early spring and spawning occurs only once a year from April to June (Bayne, 1964, Lowe *et al.*, 1982).

In the laboratory however, it is possible to take ripe mussels from the field and keep them in low temperatures in order to suspend spawning. Spawning can then be achieved for up to 5 to 6 months afterwards by use of a thermal shock; an increase in the ambient temperature of the seawater in a batch of mussels will induce the mussels to spawn. This technique which was used in the present work will be described in greater detail in the subsequent Material and Methods Chapter (ch. 2.2.a).

1.1.c Gametes

Mussels produce large numbers of eggs, sometimes up to 8 x 10^6 eggs per individual *Mytilus edulis* (Bayne *et al.*, 1978). The gametes are released into the seawater and the eggs are fertilised externally.The *M. edulis* egg is irregularly oval about 63 μm in diameter. It, typically, becomes spherical after fertilisation (Dan, 1962). Eggs have a vitelline coat and microvilli on the egg surface. Below the microvilli, there is a well defined cortical region, practically free of yolk, lipid and mitochondria, and four layers below it; the lipid layer, a wide layer of hyaloplasm, a layer of mitochondria and the yolk layer (Humphreys, 1967). The spermatozoon is 50-55 μm in length and is divided into three parts: head, middle piece and tail (Hodgson and Bernard, 1986). The head is rounded posteriorly, and tapers forward to a point; it possesses an acrosome (2.03 μm in length) and a nucleus (1.61 μm in diameter), and the mid-piece has a ring of 5 - 6 mitochondria (0.6 - 0.7 μm in diameter) (Hodgson and Bernard, 1986).

1.1.d Development stages

Except for the early work of Wilson (1886) few detailed studies of the development and behaviour of early ontogenetic stages of *Mytilus* were conducted prior to the efforts of Bayne (1964, 1965). The reasons for the paucity of investigations of the early life stages involve the inability of workers to correctly identify individual specimens of various bivalve species isolated from plankton samples, and problems of rearing larvae in the laboratory (Chanley and Andrews, 1971).

As it was pointed out in the previous section, fertilisation occurs in the water. Spermatozoon penetration is initiated by the acrosome reaction (Dan and Wada, 1955). Some time after egg activation the spermatozoon's nucleus, mitochondria and part of its flagellum are found in the egg (Longo and Anderson, 1969). The entrance of a spermatozoon induces resumption of meiotic maturation divisions in the egg (Longo, 1983) which results in the extrusion of the first polar body. Formation of the female pronucleus follows the second polar body extrusion and the fusion of the male and female pronuclei then takes place. The egg then divides by first cleavage into two different segments, the macromere and the micromere (Wilson, 1886) and development continues.

After 4 – 5 h (at 18° C) cilia first appear and the embryo begins to swim. Further development leads to pelagic planktotrophic larvae that spend several weeks in the surface waters. The free-swimming pelagic larval stage can be divided into a development, feeding and growth phase, followed by a "settlement phase" in which development is essentially completed and the larvae select a substratum suitable for settlement and the onset of metamorphosis which marks the start of sessile mode of life (Widdows, 1991). Digitally processed video – photographs of these first stages are shown in Fig. 1.1.1.

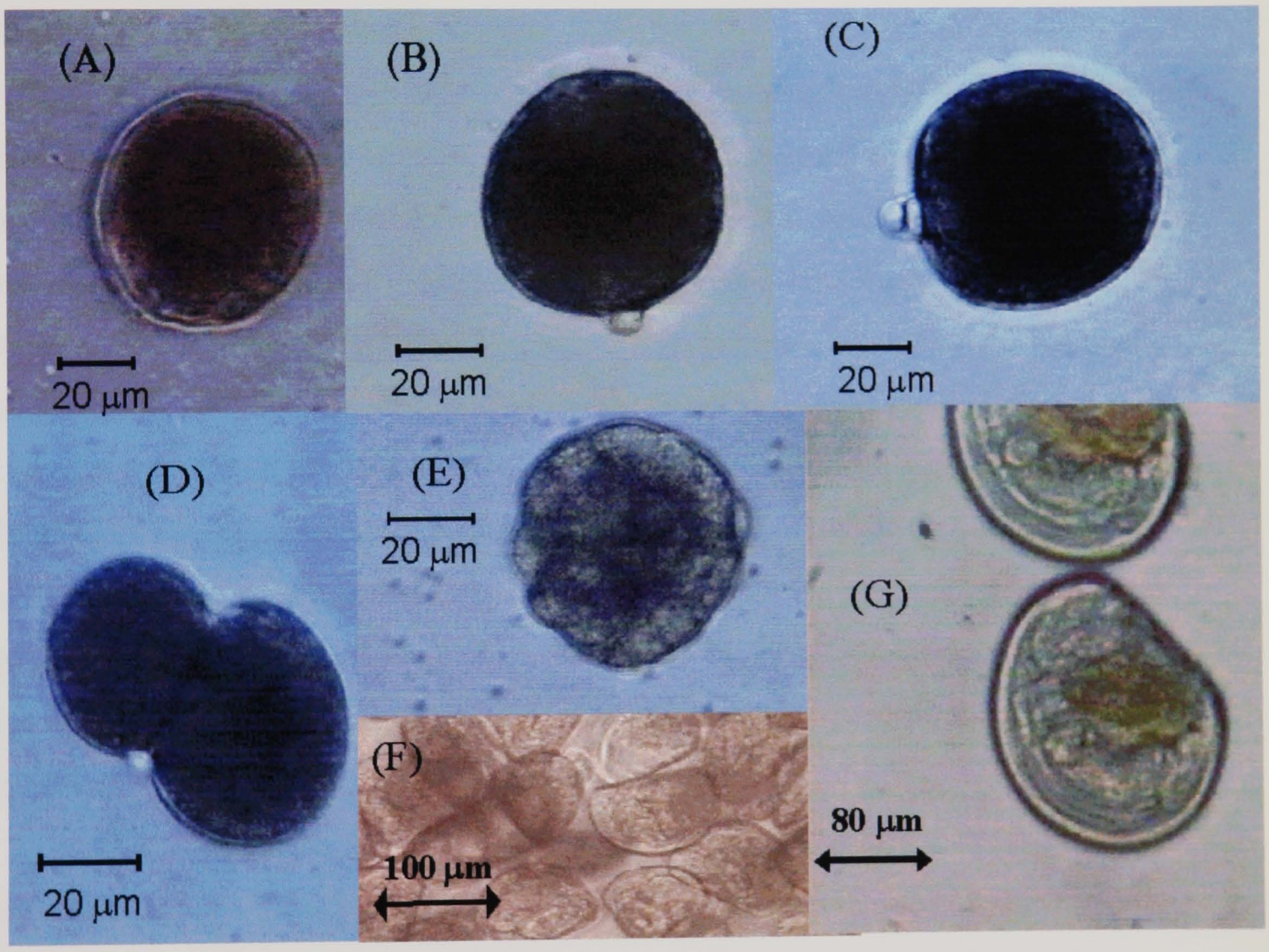

Figure 1.1.1 Development stages of *M. edulis*. (A) Egg with sperm attached. (B) Egg with first polar body. (C) Egg with second polar body. (D) First Division. (E) Trochophore stage. (F) "D – stage" larvae. (G) Larvae two weeks after the D – stage; typically as at the end of the feeding trials.

The sequence of larval development can be divided, in greater detail, into three major phases (Bayne, 1976; Sprung, 1984a):

a) The first stage is the "lecithotrophic" phase. After fertilisation, cleavage occurs and after about 4 – 5 h (at 18 °C), cilia appear and the embryo can swim. This is the "trochophore" stage. After 24 – 48 h, a shell gland starts to secrete the first shell, which is called prodissoconch I. The larvae acquire a "D"-shape and are called "straight-hinge" or D-larvae. It is at the D – stage that the larvae begin to be able to feed on microalgae.

b) The subsequent phase is characterised by active growth of the larva, hence the name "growth" phase although it is also known as the "veliconcha" larval stage. During the growth phase, the larvae grow from 100 to 250 μm in shell length and the mantle secretes the prodissoconch II shell. At about 220 – 260 μm a pair of pigmented 'eye-spots' appear, after which a foot develops.

c) The settling phase or "pediveliger" stage. This stage can be further divided into three stages based on functional, morphological and behavioural characters of the larva: "pediveliger I" still retain a functional velum, has a foot and about 2 – 3 primary gill buds can develop; at a later stage when the foot grows and the velum starts to be reduced, the larva is named "pediveliger II"; finally, when the foot moves forwards in the mantle cavity, more gills are formed, and the velum is reduced to a small mass of ciliated cells, the larva is at "pediveliger III" stage (Bayne, 1965). Pediveligers II and III can be differentiated by their behaviour; pediveliger II larvae swim mostly with the velum and crawl with the foot, whilst pediveliger III larvae move entirely by crawling. At this last stage, the larva is capable of attachment to a suitable substratum and undergoes metamorphosis. After metamorphosis has occurred, postlarvae are called "plantigrades" (Bayne, 1965).

For *M. edulis*, from fertilisation to the plantigrade stage, takes around 2 – 4 weeks (Seed and Suchanek, 1992). However, in non-optimal conditions, this procedure of growth and metamorphosis can be delayed for up to 10 or more weeks (Seed and Suchanek, 1992).

Bayne (1964, 1965) suggested primary and secondary settlement phases in wild *M. edulis*. In these cases, in order to avoid competition with adults, pediveligers settle on filamentous algae for a limited growth period (between 0.9 and 1.5 mm in length) and then, a second pelagic phase occurs which is called bysso-pelagic migration. (Sigurdsson *et al.*, 1976). This phase can take several months and may be repeated several times (Seed

and Suchanek, 1992) through byssus drifting (Lane *et al.*, 1985), until a final settlement occurs in adult beds. In some cases though, direct settlement on adult mussel beds has been reported (McGrath, *et al.* 1988).

The names plantigrade or postlarva are often used to refer to mussels which have recently settled, but there is no single term defined in the literature to indicate the stage between final settlement and the time when the mussel reaches sexual maturity. The term "spat", which is derived from oyster larvae, is very loosely used by most authors and is seldom defined. In this chapter as well as in subsequent chapters, the term spat was transferred directly from the literature and hence, its exact definition will depend on its usage in the original paper.

The present work dealt only with the first stages of *M. edulis* larvae, up to 2 weeks old, which under the experimental conditions enabled the larvae to reach into some way into the growth phase; it should be mentioned that larvae with "eye - spots" were never encountered during these experiments.

1.1.e Relationship between larval growth and spat growth

Most studies which focus on improving the nutritional value of phytoplankton species demonstrate the change by the increase in growth rate achieved by the bivalve larvae.

In commercial hatchery operations it is logical to assume that the primary goal should be improving the growth rate of the animal in all its developmental stages, either by manipulating the quality of the food and/or the culturing conditions. This would have clear economic benefits: reduction in the time animals are kept in tanks, which are more expensive to maintain, or less food needed to complete the operation with the consequent lowering of labour costs associated with these activities.

However, even if significantly faster growing larvae are produced, there is no concrete evidence to suggest that, these faster growing larvae will produce faster growing juveniles or adults. Widely practised nursery management considerations usually dictate that from the tremendous variability of size at all bivalve ages, some or all, of the slower growing juveniles be culled, on the basis that the slow growing portion of the stock

depresses the profitability of the operation by prolonging the time between investment and return (Askew, 1978). The theoretical basis of this model was partially confirmed experimentally by Newkirk *et al.* (1977) who found that larval growth rate and spat growth rate could be significantly correlated for the oyster *Crassostrea virginica*. Similar results were presented for the same species by Haley and Newkirk (1978) and Losee (1979), which indicated that there is a positive correlation between larval growth rate, as indicated by time of setting and spat growth rate.

Later experiments by Newkirk (1981) and Newkirk and Haley (1982) demonstrated that although there might be a positive correlation between the growth rates of these development stages for the first period after settling, this correlation had disappeared completely in subsequent seasons. Which suggests that culling of the slow growing larvae will have no impact on improving growth to market size, although clearly it might improve the economics of intensive nursery culture.

This apparent contradiction on the relationship between the growth rate of larvae and that of juveniles or adult bivalves may in fact be an artefact of the nutritional strategy employed in most commercial and/or laboratory operations. The algal food, if proven to be of good quality for the first, larval, stage of the bivalve, it would then be employed for feeding to subsequent stages. However, as the nutritional requirements of the various developmental stages will almost certainly change, while the quality of the food will not, there is a possibility that a good larval food may be a poor food for juveniles or adults.

It may be observed that there is no clear evidence to indicate that larval growth can be correlated with juvenile or adult growth in bivalves. However, the elucidation of this relationship is beyond the scope of this project; the reader is referred to Del Rio (1996) for a detailed study of this relationship Nevertheless, it was felt necessary to present, albeit briefly, this possible ambiguity before the introduction moves on to investigate the various factors that have been documented, in the relative literature, to influence larval growth.

1.2 Factors influencing larval growth

As has been implied in various points during the description of the developmental stages of *M. edulis*, several parameters influence larval growth. Therefore, at this stage an account of some of the major factors that influence larval growth will be discussed.

Temperature and salinity are two of the most obvious factors, while it has also been shown that in broodstock conditioning, bacteria associated with the larvae as well as algal extracellular products can influence larval growth to a certain extent. But by far the most important factor that determines larval growth, within a salinity and temperature range, is the food that the larvae are receiving. Since this was the main research area on which the project focused, the algal part will be presented in more detail in the subsequent sub – chapter, after an account of the other parameters that influence larval growth have been presented.

1.2.a Temperature and salinity

Of all the environmental factors that may have an influence on the growth of the *M. edulis* larvae, temperature and salinity are the major factors. Temperature has a great influence in mussel larval growth (Pechenik *et al.*, 1990).

The optimum temperature is about 16 °C (Bayne, 1965) and temperatures below this can produce individuals that will metamorphose, but over a longer time period (Lutz and Kennish, 1992). Larvae kept at 5 °C do not grow, but remain capable of reaching metamorphosis once the temperature is increased (Beaumont and Budd, 1982). When the larvae are kept between 13 °C and 16 °C there are no significant differences in growth rates (Bayne, 1965, Widdows, 1991).

Low salinities have a detrimental effect on the larvae (Innes and Haley, 1977). Larval growth stops at 19 ‰, is slow at 24 ‰ and only between 30 - 33 ‰ is growth normal (Bayne, 1964). Salinity has an influence on embryogenesis in mussels and normal development occurs above 15 – 20 ‰ at 15 °C, whilst salinities between 30 – 40 ‰ produce optimum embryonic development to the trochophore stage (Hrs-Brenko and

Calabrese, 1969). Normal larval growth can occur between 18 - 33 ‰ (Innes and Haley, 1977), but the salinity range of 30 - 33 ‰ are considered the best range for larval growth (Bayne, 1965).

However, it remains to be established whether the growth rate remains constant over a wider period of larval development. The rate of change of the growth rate can also be affected to varying degrees by different temperatures. Sprung (1984a,b) reported sigmoidal growth curves for larvae at 6 °C with a food ration of between 1 - 5 cells μl^{-1}. But, higher food concentrations and higher temperatures produced larval cultures which exhibited an almost linear relationship between shell length and time.

1.2.b Broodstock conditioning

Applied aquaculture research has predictably focused for many years now on maximizing growth rate and survival of all developmental stages of the marine organisms of special importance, on the fundamental basis of selecting the best food quality source for that organism. However, most cultured species of commercial importance undergo seasonal cycles of tissue growth and gonad growth concerned with cycle of reproduction. As a direct consequence of that, gametes obtained from the animals being at various stages of that cycle, may produce larvae that differ in their fundamental properties of growth rate and survival.

Broodstock management has been found repeatedly to influence larval survival in a number of ways. Lannan *et al.* (1980) found, by using time course conditioning trials for *Crassostrea gigas*, that there was an optimum conditioning interval during which the proportion of viable gametes was at a maximum. When matings were accomplished outside this optimum period, reduced gamete viability was observed. In this case, spawning and fertilisation may appear to be normal but setting success was substantially reduced. The degree of conditioning required to reach this optimal window was dependent on the stage of gonadal development in the broodstock at the start of the conditioning. The state of the gonads was found to undergo an annual cycle. In later years, the correlation of broodstock conditioning and larval survival and growth, in a closely related species, *Crassostrea virginica*, was further shown by Gallager and Mann

(1986a) to reside specifically in the lipid content of the eggs. Their data suggested that there was a minimum size related threshold lipid level in eggs necessary for optimal survival through the non feeding embryonic stages. They also found that variations in the broodstock conditioning protocol induced a large fluctuation in the lipid content of the eggs, thus providing the necessary link between broodstock conditioning and larval survival and growth. Besides the seasonal cycle of gonadal development and consequent egg quality obtained, Wilson *et al.* (1996) suggested that, broodstock nutrition was also important for the viability of the larvae of *Ostrea chilensis*. More recently, Berntsson *et al.* (1997) displayed the effect of broodstock diet on fatty acid composition, survival and growth rates in larvae of *Ostrea edulis*. They too found that the growth rate was significantly correlated to the content of the total polyunsaturated fatty acids (**PUFA**) and especially the 22:6ω3. The importance of lipids as a major energy source and that larvae with higher levels of neutral lipids at the end of brooding, grow significantly faster than larvae with lower levels was reported for *Ostrea edulis* by Helm *et al.* (1973).

The seasonal variation itself was found to induce seasonal cycles in the content of total tissue lipid, sterol content, fatty acid composition and percent meat weight in *Crassostrea virginica* by Trider and Castell (1980b) and in lipid content of several other bivalve larvae (Gallager & Mann, 1986b). Collectively, these studies suggested the now well established relationship between seasonal cycles and lipid content of the eggs. They found that immediately after spawning there was a significant reduction in the ω:3 PUFA, (particularly the 20:5ω3 and the 22:6ω3) and the 16:1ω7 in the oyster neutral lipid, suggesting that these fatty acids play an important role in embryonic and larval development and sterol metabolism.

Many studies since have provided further insight on the importance of larval lipid and lipid components to the larvae themselves. Lipid was used as an index of growth and viability in three species of bivalve larvae (Gallager *et al.*, 1986) and more particularly triacylglycerol content was more widely used as a condition index for fish, bivalve and crustacean larvae (Fraser, 1989). Triacylglycerol composition of the larvae has long been found to be strongly influenced by the algal diets upon which the larvae were fed (Waldock and Nascimento, 1979).

This completes the cycle, with broodstock diet and seasonal changes influencing broodstock condition, which is expressed by the lipid content that they furnish to their eggs, which is in turn found to be correlated with larval growth and survival.

1.3 Bacteria and dissolved organic products

Before proceeding to describe various aspects of the importance of the algae in the feeding of bivalve larvae, this section of the introduction will deviate slightly by presenting another factor that may influence larval growth, the bacteria associated with the larval cultures and the presence of dissolved organic products.

A major problem in the study of bacterial effects on bivalve larval systems is the difficulty in obtaining axenic larval cultures. Obviously, bacterial contamination will be masking, if not altering, the effects of other food sources (Bull & Slater, 1982). The study of mixed systems has scarcely been attempted in the literature, another reason being that even a single addition on the organismic chain introduces another variable which must be carefully controlled thus increasing exponentially the number of essential experiments.

However, some bivalve larval cultures have been reared axenically, mostly of the oyster *Crassostrea gigas*. These axenic cultures have been achieved (Langdon, 1983; Douillet, 1991) in the past by stripping adult gonads under aseptic conditions and keeping the subsequently fertilised eggs under carefully controlled, but none the less totally artificial, conditions. This is possible for the oyster because its eggs are matured prior to release by the adult, and hence a perfectly functional egg can be removed from the adult gonads. In mussels no fertilisable eggs can be obtained simply by stripping the gonads.

Regularly associated with the usage of axenic cultures are various combinations of antibiotics. The use of antibiotics presents a number of other limitations: the most commonly used antibiotics, such as penicillin and streptomycin, have poor effectiveness against naturally occurring marine bacteria (Helm & Millican, 1977), may have adverse effects on the growth and survival of the larvae (Le Pennec & Prieur, 1977) and also, resistant strains of bacteria are created by systematic use of antibiotics (Jeanthon *et al.*, 1988). Furthermore they might change the physiological properties of the larvae, thus

making the extrapolation of such results taken in these totally artificial conditions of questionable use for applied aquacultural larval rearing practices.

Bacteria have been proposed as playing an important part in influencing larval culture success (Guillard 1957; Walne 1958 & 1966; Prieur, 1982) but historically, hatchery management practices aim at controlling opportunistic pathogens with a number of techniques, like maintaining pathogen-free algal stocks, using sterile seawater (either by sterile filtration or UV radiation) for rearing the larvae, frequently changing the culture water, application of hygienic practices and disinfecting the equipment (Elston, 1984). It has also been observed that bacterial contamination is not only directly harmful to bivalve larvae but also causes particles to form clumps that are too large for ingestion (Masson, 1977).

In bivalve larvae, it is mainly the recent work of Douillet (1991, Ph.D. thesis and subsequent papers, Douillet and Langdon, 1993,1994) that found unequivocally a beneficial bacterial effect on the larval cultures. Bacteria and yeasts have been used to partly substitute algal foods in larval and juvenile bivalve rearing (Coutteau *et al.*, 1994; Nell *et al.*, 1996; Brown *et al.*, 1996a) but in most cases, results are considered successful if the combined algal and bacteria/yeast diet produces as good a growth and/or survival of the larvae as the algae alone diet (Epifanio, 1979a).

Bacteria could serve as a food source for the larvae. Their role in larval cultures, as briefly summarised before, is far from clear, whether as a food source or as a source of organic dissolved organic material (**DOM**).

Many marine organisms have been shown to have a significant capacity to take up **DOM**, sometimes this may comprise up to 40% of their total metabolic requirements (Sorokin & Wyshkwarzev, 1973).

M. edulis larvae, like most marine organisms should be able to feed by "osmotic" nutrition (Pütter, 1909) as has been shown for a number of organisms, with the use of radio labelled organic material (Sorokin & Wyshkwarzev, 1973). This has been documented for *M. edulis* by Manahan & Crisp (1983) again by means of autoradiographic studies coupled with electron microscopy observations. Irrespective of the metabolic role of **DOM**, some algal derived products have been shown to influence other larval behavioural characteristics like grazing rate. Thompson *et al.* (1994b)

showed that some algal filtrates, like ones from *Phaeodactylum tricornutum* and *Gonyaulax grindleyi* significantly reduced the grazing rate of *Crassostrea gigas* larvae. On the other hand filtrates from other algae, such as *Thalassiosira pseudonana*, did not have any effect on the grazing rate of the animals. Concluding this section on **DOM** and more particularly on algal extracellular material, it should be noted that there can be a potential effect on the behaviour of the larvae or even occurrence of dietary substitution, but their role is rather limited and mostly restricted by the specific algal products used and the larval culturing practices.

Fewer reports exist on the effects of nutrients on mixed systems. Goldman *et al.* (1985), Caron *et al.* (1985) and Andersen *et al.* (1986) have studied carbon, nitrogen and phosphorus cycling in batch cultures of algae, bacteria and heterotrophic flagellates, while Güde (1985) has studied phosphate competition and regeneration between the same trophic levels in a 2-stage chemostat system. Thingstad & Pengerud (1985) analysed some mathematical models in bacteria/algae/bacterial predator mixed systems, while Pengerud *et al.* (1987) compared the results obtained from an analogous experimental system with these mathematical models.

Investigations from the aquacultural point of view are mostly concerned with interactions between two trophic levels and are, by and large, concerned with finding bacterial sources for food or food supplement, for the desired animal species (e.g. Intriago & Jones, 1993).

B. Algae as food

The indisputable importance of food supply in the early life stages of *M. edulis* is obvious, thus the interest in assessing the nutritional value and the ability of different algal diets to support larval growth in bivalves (Walne, 1963, 1970; Enright *et al.* 1986a; Wayne *et al.* 1992).

The quality and the quantity of the algal food provided to the animals is expected to influence a number, if not all, of the physiological, morphological and biochemical properties of the bivalvae organism. Not only is growth itself directly determined by feeding regimes (for example: Strömgren and Cary, 1984) but also filtration rate (Riisgård, 1991) and larval differentiation (Pechenik *et al.*, 1990) have been shown to be affected as well.

The initial research which attempted to determine which algal species were suitable for food, was heavily concentrated on finding algae that would grow well in laboratory cultures (Walne, 1956); afterwards studies developed further to determine which of these algae were most suitable for promoting growth in certain bivalvae species, mainly those of commercial interest like *Ostrea edulis* (L.), *Crassostrea virginica* (Gmelin), *Mercenaria mercenaria* (L.), *M. edulis* and others. Species routinely used as food for bivalvae rearing with good results are mainly planktonic algae like *Pavlova lutheri* (Droop) Green [Chrysophyceae], *Isochrysis galbana* Parke [Haptophyceae], *Chaetoceros calcitrans* (Paulsen) Takano [Bacillariophyceae], *Skeletonema costatum* (Greville) Cleve [Bacillariophyceae] and others, while other algae such as *Nannochloris atomus* Butcher [Chlorophyceae] have consistently performed poorly (Walne, 1970; Wayne *et al.*, 1992).

It should be noted however, that of the vast number of algal species found in nature, only around 40 species (De Pauw, 1981), have been used in intensive culture procedures. This is because only a few of them are able to sustain reliable laboratory cultures (Walne, 1970).

A brief account of species used in bivalve larval cultures can be seen in Table 1.B.1.

Table 1.B.1. Some examples of algae used in bivalve larvae rearing.

Algal species	Cell volume (μm^3) *	Larval species	Example of sources
PRASINOPHYCEAE			
Tetraselmis suecica	*240 – 520*	*Crassostrea virginica*	Romberger & Epifanio (1981)
Tetraselmis maculata	-	*Crassostrea virginica*	Wikfors *et al.* (1984)
Tetraselmis chuii	*520*	*Saccostrea commercialis*	O' Connor *et al.* (1992)
Tetraselmis tetrahele	*250*	*Ostrea edulis*	Walne (1970)
CHLOROPHYCEAE			
Chlorella stigmatophora	*30 – 110*	*Mercenaria mercenaria*	Walne (1970)
Nannochloris atomus	*5*	*Ostrea edulis*	Walne (1970)
Dunaliela tertiolecta	*170 – 300*	*Ostrea edulis*	Walne (1970)
Carteria chuii	-	*Crassostrea virginica*	Epifanio & Ewart (1977)
HAPTOPHYCEAE			
Isochrysis galbana	*40 – 100*	*Mercenaria mercenaria*	Riisgård (1988)
Isochrysis galbana var. *affinis*	*40 – 100*	*Crassadoma gigantea*	Whyte *et al.* (1990)
CHRYSOPHYCEAE			
Pavlova lutheri *(= Monochrysis lutheri)*	*32 – 85*	*Mercenaria mercenaria*	Wikfors *et al.* (1992)
CRYPTOPHYCEAE			
Rhinomonas reticulata *(= Rhodomonas baltica)*	*65 – 110*	*Mytilus edulis*	Riisgård (1991)
BACILLARIOPHYCEAE			
Chaetoceros calcitrans	*23 – 38*	*Saccostrea commercialis*	O' Connor *et al.* (1992)
Chaetoceros gracilis	-	*Ostrea edulis*	Enright *et al.* (1986b)
Chaetoceros muelleri	*110 – 600*	*Mercenaria mercenaria*	Nelson *et al.* (1992)
Chaetoceros simplex	-	*Ostrea edulis*	Enright *et al.* (1986a)
Thalassiosira pseudonana	*36*	*Tapes japonica*	Gallager & Mann (1981)
Thalassiosira weissflogii	*500 – 1350*	*Mercenaria mercenaria*	Nelson *et al.* (1992)
Phaeodactylum tricornutum	*60 – 150*	*Crassostrea virginica*	Epifanio *et al.* (1981)
Skeletonema costatum	*60*	*Mytilus edulis*	Strömgren & Cary (1984)

* Cellular volumes when not given by the authors were calculated from other reports.

1.4 Importance of ration size in various larval stages

This small section will briefly discuss some reports that have been published over the years that introduce the concept of ration size.

The concentration of algal food and its availability has a strong influence on larval growth (Sprung, 1984b; Pechenik *et al.*, 1990). On the other hand, it has been reported that food concentration has no influence on larval attachment (Pechenik *et al.*, 1990).

Winter (1973) showed that *M. edulis* controls the rate of phytoplankton intake in to the mantle cavity in relation to the algal cell concentration presented to the animals. The quantity of phytoplankton consumed increases in proportion to the algal concentration, reaching a plateau where it is constant and independent of the particle concentration and finally decreasing with any further increase in algal cell density. Later, Winter (1976) proposed a more general scheme for bivalves. Early studies by Bayne (1965), who cultivated mussel larvae with *Isochrysis galbana* at 25 and 100 cell·μl^{-1}, found pediveligers produced at days 31 and 16, respectively. Sprung (1984b) found a significant increment in growth, when *I. galbana* concentration was increased from 1 to 10 cell·μl^{-1}, and growth reached a plateau at both 6 °C and 18 °C. He also found a slight diminution in growth at 12 °C, and considered therefore that high algal concentrations (above 10 cell·μl^{-1}) at this temperature, might hamper the feeding apparatus, or that algae might produce a high concentration of compounds to which larvae are sensitive.

However in a number of later studies, growth rate of bivalves and algal ration size was found to be almost linear for a wide range of algal concentrations (Langton *et al.*, 1977), or that growth rates increased with increasing food concentration from a range of cell concentrations of 0.5 x 10^4 to 30 x 10^4 cell · ml^{-1} (Pechenik *et al.*, 1990). It could be that the plateau described by Winter (1976) was not reached even at the maximum cell concentration employed in these studies. Although in other studies, this plateau was clearly reached; in the study by Coutteau *et al.* (1994) for juvenile *Mercenaria mercenaria,* where spat fed 1 % of dry weight of algae per wet weight of the animal per day grew significantly slower that animals fed higher feeding ratios. However feeding ratios higher that 2 % did not increase further the growth rate of the spat. A small correction in an equation predicting the maximum daily ration that should be supplied to oysters of various sizes was formulated by Epifanio and Ewart (1977) that should be consulted prior to the design of any study of growth rate experiments and in commercial applications, in order to find the best compromise between maximal bivalve growth rate and smallest feeding ratio to achieve this.

1.5 Selection criteria

From the plethora of algal species that can potentially be used as food for larval stages only few are actually being used. Assuming that the alga can be reliably cultured in the laboratory, which in itself brings down significantly the numbers of algal species that can be used, then there are a number of criteria that should be applied for selecting a species. For the use of various algal species, there are a number or restricting parameters that are dictated primarily by the specific larval species. The most obvious restriction is the size of the algal cell; no food can be useful unless able to be ingested by the larva. For example *Mytilus edulis* larvae can efficiently retain particles ranging from 2-6 μm (Sprung, 1984b) whilst particles larger than 9 μm are not retained by the larvae (Riisgård *et al.*, 1980). A high speed video microscopy technique used by Gallager (1988) showed that larvae select particles and process food at a rate that is a function of particle concentration in the water, degree of gut satiation and particle digestibility.

Digestibility of algae may depend largely on the presence or absence of a thick cell wall. Additionally the algae should not produce any toxins since these induce very high mortalities in the larval cultures.

However, by far the single most important criterion of selection of an algal species relies on its biochemical composition. All the factors described above, together with the biochemical composition of the alga will determine how good a food this species can be for a given larval stage; the combination of all these is known in the literature as the "nutritional value". The variety of factors that were presented before, are involved in determining the food value of a particular alga, including cell size, the growth phase of the algal culture (Wilson, 1979), digestibility and lack of toxicity (Calabrese and Davis, 1970; Webb and Chu, 1983) while of particular importance are the content of dietary carbohydrate (Whyte *et al.*, 1989) and the presence both in terms of quality and quantity of lipids and more particularly of fatty acids (Enright *et al.*, 1986a; Dickey-Collas and Geffen, 1992; Thompson *et al.*, 1993).

1.6 Biochemical composition

Provided that the algae can be ingested and digested, their nutritional value is directly related to their biochemical composition. This means that, even if all of the previous requirements are met by a specific alga, a good food value index is not assured because its biochemical composition may be inadequate for a specific larval species. This aspect of algological research in relation to larval rearing has received comparatively more attention (Webb and Chu, 1983).

The literature has been rather vague in determining the exact nutritional requirements of the larval consumers (De Pauw, 1981). Research has focused on some discrete elements and components, namely amino acids/protein levels, carbohydrate content and lipid composition. Fewer reports exist on the effects of the individual major chemical elements, like carbon, nitrogen and phosphorus.

It has been suggested that the concentration of amino acids plays a role in growth and development of bivalve larvae (Webb & Chu, 1983). However, there are contradictory opinions as to the significance of the role of amino acids. Epifanio (1979b) studying the effects of 15 diets, concluded that growth was not correlated with gross chemical composition or amino acid content, but was correlated with the presence or absence of a particular algal species in the diet. In some cases, the lack of a certain amino acid seems to explain the poor growth obtained using one algal species (Webb & Chu, 1983). For example, Epifanio *et al.* (1981) concluded that growth in juvenile *Crassostrea virginica* is inversely related to the quantity of *Phaeodactylum tricornutum*, a tryptophane deficient species. This deficiency though does not explain the previous suggestion, since other tryptophane deficient diatoms such as *Skeletonema costatum* (Chuecas and Riley, 1969b) give excellent results. Utting (1986) studied the effects of a diet of *Chaetoceros calcitrans* and *Tetraselmis suecica* with different protein levels on *Crassostrea gigas*. Results indicated that larval growth is not controlled by protein quantity, but by the total energy content of the diet.

Gallager & Mann (1981) made use of the C/N ratio as an index of the nutritional value of the diatom *Thalassiosira pseudonana* as food for the clam *Tapes japonica*. They

found a positive correlation between tissue growth and total nitrogen available from the diet rather than total carbon.

Although such algal biochemical components like specific amino acids or C/N ratio may influence larval growth, most of the current research has predictably focused on the major algal dietary components like protein, carbohydrate and lipids, and more particularly fatty acids. Therefore a more specific account on these components is presented next.

1.6.a Protein

In a preliminary study on growth of *Crassostrea gigas* larvae in relation to dietary protein, Utting (1986) found no correlation between dietary protein content and larval growth. In fact growth of spat during the 14 days immediately after settlement was better on a low protein than on a high protein algal diet, when *Tetraselmis* sp. was used, but when *Chaetoceros* diets were used, the differences were not significant.

Kreeger and Langdon (1993) showed that growth rate of juvenile *Mytilus trossulus*, were significantly lower when fed on a low protein algal diet than those achieved when the juveniles were fed on a high protein diet. By carrying out a mixture of supplement experiments they were able to determine a threshold in this relationship between dietary protein and growth of *M. trossulus*, suggesting that dietary protein contents below 40 % w/w and dietary C/N ratios above 10 could qualitatively limit growth rates of the juvenile mussels. Under controlled laboratory conditions, Wikfors *et al.* (1992) carried out a series of feeding trials with young post - set hard clams, *Mercenaria mercenaria*. Of all the algal biochemical characteristics measured, only protein and lipid showed a significant correlation with the growth of the hard clam. It was consequently suggested that both protein and lipid must be present in sufficient quantities to support rapid growth of hard clams.

1.6.b Carbohydrate

It is self-evident that the algae should also be the energy source for the larvae and energy values are largely related to carbohydrate content. Carbohydrate composition is similar amongst quite different algal species, with glucose being the principal component

(Webb & Chu, 1983). Again, no clear relationships have been found between nutritive value and carbohydrate content. For example, Wikfors *et al.* (1984), using the bivalves *Crassostrea virginica* and *Mercenaria mercenaria* (Wikfors *et al.*, 1992), found no correlation with dietary carbohydrate. On the other end of the spectrum, Whyte *et al.* (1989), who used the rock scallop *Crassadoma gigantea*, related growth to the level of carbohydrate content. The diet was a mixture of algae, and there was consistent correlation of dietary carbohydrate rather than dietary lipid or protein with larval growth. Similar importance of dietary carbohydrate was placed by Enright *et al.* (1986a, b) for juvenile *Ostrea edulis*; however it was noted that enhancement of the juvenile growth rate could be achieved with additional carbohydrate provided that adequate protein and essential fatty acids are supplied. Wikfors *et al.* (1984) using juvenile *Crassostrea virginica* suggested a nutritional requirement of the juvenile oysters, for relatively more carbohydrate rather than protein, although a causal relationship between algal lipid and oyster growth was also indicated. This result, for juvenile *Crassostrea virginica* had been verified previously by Flaak and Epifanio (1978), who also found that oysters grew more rapidly when fed diets richer in carbohydrate than proteins.

1.6.c Lipids – Fatty acids

Finally, the quantity and quality of lipid is believed to be of prime importance to the nutritional value of microalgae (Webb & Chu, 1983; De Pauw *et al.*, 1984). This nutritional aspect, along with the others, suffers from a plethora of contradicting reports. However it is undoubtedly the case that lipid quality rather than quantity controls the nutritional value of microalgae. A deficiency in some essential fatty acids, such as some of the ω:3 group, seems to be the major cause of the poor nutritional value of particular algal species (De Pauw, 1981). Langdon & Waldock (1981) demonstrated the importance to *Crassostrea gigas* of polyunsaturated fatty acids, especially the 20:5ω3 and 22:6ω3.

Other scientists point out that the percentages of the various classes of saturated and unsaturated lipids (Thompson & Harrison, 1992) and the degree of saturation (De Pauw *et al.*, 1984) are equally important. Helm *et al.* (1973) assumed that growth and vigour of oyster larvae depends on lipid proportion and, in particular, the proportion of neutral lipids at the moment of their liberation. Also, the potential of spat growth depends

on the quantity of lipid reserves at the moment of settling, as well as on the diet offered to postlarvae (Laing and Millican, 1986). Although the mechanism remains unknown, it seems that survival of larvae is related to the initial content of triacylglycerides furnished by parents to each egg during the conditioning period (Gallager *et al.*, 1986). These aspects have also been discussed before in the section dealing with broodstock conditioning (1.2.b).

Dietary lipid and especially fatty acids have been repeatedly demonstrated to have a significant role in controlling the dietary value of algal species. However there is less agreement on which individual algal FA have a beneficial effect for the consumer's growth. The importance of essential FA especially of the long chained PUFA and the ω:3 series, has long been established for a variety of marine organisms (e.g. turbot larvae, Scott and Middleton, 1979). Trider and Castell (1980a) found a significant role for the ω:3 essential FA requirements of hatchery reared oysters, *Crassostrea virginica*. Similar results were observed for Queen conch larvae, *Strombus gigas*, by Pillsbury (1985) who found that the best algal diets for this species contained more than average lipid content and also contained the long chained PUFA 20:5ω3 and 22:6ω3. However, various publications by Thompson and co-workers (Thompson & Harrison 1992, Thompson *et al.*, 1993, 1994a, 1996), working with larvae of the pacific oyster, *Crassostrea gigas*, and of the Japanese scallop, *Patinopecten yessoensis*, found that increased amounts of the polyunsaturated fatty acids resulted in inferior larval growth. In contrast, a consistent positive correlation of the 16:0 and larval growth was found. The overall negative correlation of the ω:3 series of FA's with growth are also similar to those obtained by Dickey-Collas and Geffen (1992) for plaice larvae, which suggest that while small amounts of these FA may be essential, large amounts may be deleterious.

1.6.d Comments on the algal biochemical composition

The consistency of the performance of the various algal species tested is often ambiguous since there are studies showing the same species having both a "good" and a "bad" food value index when fed on the same or similar bivalves; for example the diatom *Phaeodactylum tricornutum* Bohlin was tested by Walne (1970) on *O. edulis* with fairly

poor results while when tested by Wayne *et al.* (1992) on a closely related bivalve, *Saccostrea commercialis*, it produced quite satisfactory growth. Similar disagreement exists for *Dunaliela tertiolecta* Butcher [Chlorophyceae], with contradictory reports from Walne (1963) and Wayne *et al.* (1992) although this species is more often ranked as inferior food for bivalves (Walne, 1970; Langdon and Waldock, 1981; Enright *et al.*, 1986a).

Bayne (1965) found that *P. lutheri* produced a higher growth rate in larvae than *Isochrysis galbana*, and also that both algae combined as food improved growth rate. In general, higher food value is obtained from mixed diets which are more likely to contain the diversity of biochemical constituents to satisfy most nutritional requirements for growth (Widdows, 1991).

Differences in the nutritive values between mixed and monospecific algal diets may be explained, in the majority of cases, by the type of interaction among algae (Epifanio, 1979b; Romberger & Epifanio, 1981): (a) interactions of a synergistic type, which compensate for the deficiencies, inducing better growth, and (b) non-additive interactions that may be explained by the relative ease of digestion and assimilation of each alga.

Overall, as already stated, until the exact nutritional requirements of the larval species become known in greater detail, there will still be confusion from contradicting literature reports. It seems that some researchers favour the importance of one nutritional element (lipids, carbohydrate or amino acids/proteins) and tend to overlook the others. It should be noted however, that these elements need not be mutually exclusive. This is particularly the case for the long chain polyunsaturated fatty acids, where it seems that their importance has been overgeneralized (Dickey-Collas and Geffen, 1992; Thompson *et al.*, 1994a, 1996). More rationally it appears that there might be a complementary effect between the nutritional elements. Enright *et al.* (1986b) noted that, high levels of essential fatty acids may provide an explanation for high growth rates at high feeding ration, while higher growth rates are possible with additional carbohydrate, provided that adequate protein and essential fatty acids are supplied.

The disagreements concerning the nutritional value of several microalgal species may reflect true differences in the nutritional requirements of various bivalve species. But one must be aware of the fact that different laboratories use animals obtained from

different geographical localities and in addition, almost always, the cultured algae used, were isolated from different sources. To make things more complicated, the culturing conditions of the algae may differ considerably between labs and this may lead to different morphological, physiological and/or biochemical properties of the algae in question (e.g. Gallager and Mann, 1981; Thompson *et al.*, 1992a,b, 1993).

It becomes obvious, that because of the aforementioned reasons direct comparisons between different laboratories may be misleading. Although this fact is commonly known among phycologists (Baars, 1981) its potential importance in mariculture in general has received comparatively little attention until recently. Therefore, since it has been documented that the algal biochemical composition will change depending on the culturing condition this introduction will now examine the factors that can potentially influence the algal biochemical composition and how these can in fact be used to manipulate the nutritional value of the algal species.

1.7 Factors influencing algal biochemical composition

As outlined before, algal biochemical and elemental composition varies between individual species, although similarities are expected between related algae. It is obvious, that algal research has focused mainly on the biochemical components that are thought to play a potentially significant role in larval nutrition and growth, or on human metabolism (Ahlgren *et al.*, 1992).

Early research was directed into providing the essential biochemical profiling information of various algal classes (Ben-Amotz *et al.*, 1985) and trying to establish common trends among them. For example, as early as 1969, Chuecas & Riley (1969a) commented on the differentiated fatty acid content of diatoms. These authors noted a virtual absence of 18:2 and 18:3 fatty acids in diatoms and a high 20:1 content of the Cryptophyta. Ackman & Tocher (1968) also reported analogous fatty acid contents within various algal classes and tried to relate taxonomic groupings with specific fatty acids.

With the advances in analytical techniques, such broad distinctions are nowadays less easy to suggest. On the other hand, research has moved into trying to define the exact effects of environmental parameters on biochemical composition.

The biochemical elements of algae regularly used in larval rearing that have been quite regularly studied are carbohydrate content, elemental cellular composition (C, N, P, Si), proteins and lipids. Because of the importance often attributed to lipids (Webb & Chu, 1983), they have been frequently included in biochemical analyses. However, the influence of physical and chemical factors upon lipid content has not been as extensively studied (Thompson *et al.*, 1992b).

Potentially every single environmental condition can influence algal growth and biochemical composition hence their nutritional value. Out of this plethora of factors, some have been accepted to be more important than some others. A sharper focus on different parameters that have been documented to influence algal biochemical composition is now provided.

1.7.a Temperature

For most organisms, the effect of temperature on growth rate is accepted to be of a bell like shape; the organisms demonstrate little or no growth at all at sub-optimal temperatures, then the growth rate rises smoothly until the optimum temperature is reached, before descending steeply when that optimum is exceeded (e.g. Blinn, 1984).

Temperature exerts a direct control of photoautotrophs in many ways and it has been viewed as a major constraint on their growth in the aquatic environment (Davison, 1991). It is also an important factor influencing the dominance and succession of phytoplankton species in aquatic communities (Goldman and Ryther, 1976). It also has been widely documented to be by far the most important factor in biogeographical dispersion of marine algal species (Breeman, 1988, 1990). However, its role in determining the biochemical composition of phytoplankton is largely undetermined (Thompson *et al.*, 1992a,b) although temperature is known to influence the physiological status, chemical and biochemical composition of phytoplankton cells (Goldman and Mann, 1980; Redalje and Laws, 1983), and consequently it should also influence their nutritional value.

For example, *C. muelleri* has been shown to have a temperature tolerance of >5°C and <40 °C, with maximum division rates occurring in temperatures between 20 °C and 30 °C (Blinn, 1984). In general, it is believed that the increased protein content at lower temperatures (Morris *et al.*, 1974) may reflect the need for more enzymes or for reducing the low-temperature induced loss of fluidity of the cellular membranes (Chapman *et al.*, 1983). Thompson *et al.* (1992a) found that over a range of species tested, *Thalassiosira pseudonana*, *Phaeodactylum tricornutum* and *Pavlova lutheri* had minimum values of carbon and protein at intermediate temperatures. This resulted in a broad U-shaped response over the temperature range 10 to 25 °C, thus indicating similar responses, while carbohydrate showed no consistent trends with temperature. In a previous report Mortensen *et al.* (1988), testing *Chaetoceros gracilis*, a species also tested by Thompson *et al.* (1992a,b), found that levels of C and N followed a pattern of temperature dependence similar to a growth rate which is of a bell – shape type. This apparent inconsistency should be attributed to the variation in the growing techniques of different laboratories and is yet another example of the complex and undetermined effects of temperature on the carbohydrate, nitrogen and protein content of phytoplankton.

On the other hand, the effect of temperature on fatty acids seems to be better documented. It has been repeatedly reported that lower temperatures increase the degree of unsaturation of the fatty acids of algae (Ackman & Tocher, 1968; Mortensen *et al.*, 1988; Pohl & Zurheide, 1982; Thompson *et al.*, 1992b). Recently, the effect of temperature on FA composition of marine phytoplankton has come under increased scrutiny (Cohen *et al.*, 1988; Mortensen *et al.*, 1988), mainly because these biochemical components play a major role in determining the nutritional value of the algal species for aquacultured herbivores (Gallager and Mann, 1981; Enright *et al.*, 1986a). The review of White and Somero (1982) suggested that increased unsaturation of the membrane FA's is a normal response to lower temperature for most organisms. The underlying principle for this response is believed to be the greater fluidity of the membranes composed of more highly unsaturated FA, which are hypothesized to provide a membrane of constant viscosity at low temperatures. However, it is debated that other factors contribute to membrane homeoviscosity as well, like the variation in the cholesterol : lipid ratio (Oldfield and Chapman, 1972), the variation in the protein : lipid ratio (Chapman *et al.*,

1983), the modification of FA chain length (Hadley, 1985) and the variation of the *cis* double bonds within the fatty acid (Hochachka and Somero, 1984). It should be noted however, that the study of the effect of temperature on FA composition is of a complex nature due to the conflicting effects of temperature variation on the cell physiology. Low temperature - induced chlorosis (Geider, 1987) implies a significant degree of light and energy surplus resulting in cells that are high in adenosine triphosphate, reductant, carbon and triacylglycerol containing short chain saturated FA (Sukenik *et al.*, 1989). But lower temperatures may also require more fluid membranes that are usually composed of more of the unsaturated FA (Quinn, 1988), therefore the overall effect is dependent on the balance between these two factors and has been repeatedly shown to be species - specific (Thompson *et al.*, 1992b).

The influence of temperature on the biochemical composition of phytoplankton is complex and still largely undetermined because few studies have been done on the subject, with most of them focusing on one or very few species such as *Phaeodactylum tricornutum* or *Skeletonema costatum* (Yoder, 1979), although some general trends especially in lipid and fatty acid content, as illustrated previously, have been documented. Obviously temperature is an important factor known to influence the physiological and biochemical status of phytoplankton cells (Goldman & Mann, 1980) and therefore also influence their nutritional value.

1.7.b Age of Culture

Another factor, that has been shown to affect the algal biochemical composition and thus, nutritional quality is the age of batch cultures. The effect of the age of the culture on the biochemical composition of the alga, has been studied in a number of reports, all verifying the change in various biochemical components.

For example, Brown *et al.* (1996b), when studying *Thalassiosira pseudonana*, in batch cultures found a considerable effect of the age of the culture on all the major biochemical components of the alga: protein content decreased from the logarithmic phase to the stationary phase, while carbohydrate content increased in the same period. This was also observed for *Tetraselmis suecica* as reported by Utting (1985). Brown *et al.* (1996b) also found that in combination with other factors, in particular light intensity, at

the onset of the stationary phase the cells increased the proportion of saturated and monounsaturated FA while the proportion of PUFA decreased. Su *et al.* (1988) found that the proportion of the 20:5ω3 FA showed a gradual increase during log phase, levelled off during the stationary phase and decreased rapidly during the decline stage of *S. costatum* cultures. Similar results, with respect to the increase of carbohydrate content with the progress of the culture is concerned, was reported by Fernández - Reiriz *et al.* (1989) for a number of marine microalgae. In that paper, they report an increase in lipid content with the age of the culture while this has also been reported for another marine diatom, *Phaeodactylum tricornutum* by Siron *et al.* (1989) who also found an increase in total fatty acid content with the progress of the age of the culture. However the findings of Fernández - Reiriz *et al.* (1989) of the decrease in cellular protein was not uniform in all species with some diatom species and of *Rhodomonas* sp. not complying with the general trend.

In sharp contrast to the results mentioned previously, by Brown *et al.* (1996b) for *Thalassiosira pseudonana*, Fernández - Reiriz *et al.* (1989) found a consistent decrease in the proportion of saturated and monounsaturated FA with the progress of the cultures, while the opposite happened for the proportion of PUFA. For freshwater diatoms, it appears that with the onset of active growth of the logarithmic phase, there is a reduction in total neutral lipids and an increase in all polar lipids and phospholipids, but afterwards at stationary phase the levels of triacylglycerol (neutral lipids) are increased (Sicko – Goad and Andersen, 1991). If the argument that has been reported that, the neutral lipids have a relatively higher content of saturated and mono unsaturated fatty acids than the polar lipids which contain more of the omega : 3 PUFA (Kayama *et al.*, 1989), is followed in these results too, then it would appear that, in freshwater diatoms the proportion of saturated and monounsaturated fatty acids increases with the age of the culture.

However as a whole, there is a major problematic area in these experiments which lies in the underlying properties of the batch culture. The culture will "age" with time but inevitably, the nutrient concentrations will constantly change. Therefore it is very difficult to distinguish whether the response is truly an "ageing" one or is a masked macro- or micro- nutrient stress reaction. The "age" response is invariably due to the change of

other culturing parameters that are seldom defined in experiments that investigate the effect of the age of the culture.

1.7.c Other Factors

In this section, several factors that have also been shown to affect algal biochemical composition will be presented. However due to the few studies regarding the investigations of these factors, in depth comparative analysis cannot be made. Therefore it was felt more appropriate to classify them collectively, although small sub-headings, in italics on top of each factor-related section, should help the reader to make clearer distinction of these different influential parameters.

Effects of light – dark cycles and light quality

Although it has been demonstrated that light intensity itself is a major factor in determining the alga's nutritional quality, both light – dark cycles and light quality, in its spectral wavelengths composition, also influence the algal biochemical composition. Since fewer studies of these factors have been conducted, a general trend of responses has yet to be established.

In isolated studies involving various species, lipid components and/or synthetic pathways have also been shown to vary with respect to light and dark periods (Fisher and Schwarzenbach, 1978). However in a comparative study of various phytoplankton species Shifrin & Chisholm (1981), using synchronised cultures of *Oocystis polymorpha*, found that the total lipid fraction per cell was constant over the cell cycle regardless of the light regime. However, in the light – dark cycle the average cell volume, mass per cell and dry weight, showed a distinctive phased pattern. Dry weight per unit culture volume showed an almost sinusoidal variation over the light – dark cycle, decreasing as soon as the lights went off and increasing during photoperiod. Carbohydrate content of *Thalassiosira pseudonana* has also been shown to be affected by light – dark cycles (Brown *et al.*, 1996b). In that study, protein content was also found to be affected by the light regimes, but total lipid was the organic fraction less affected. However, individual FA and FA groups show considerable variation in their proportion of the Total FA, depending on light regime, with PUFA increasing from the 12 – 12 h regime to the constant light

regime. Total **M**ono **U**nsaturated **F**atty **A**cids (**MUFA**) and **S**aturated **F**atty **A**cids (**SaFA**) also increase under the same circumstances albeit by a smaller percentage.

In a study of the effects of different light quality, Aidar *et al.* (1994) found a number of considerable changes dependent on light wavelength. As anticipated, the concentration of photosynthesising chlorophylls [a, (c_1 +c_2)] and carotenoids differed in the different light qualities used. *Cyclotella caspia* had a higher cellular content of pigments in red and blue-green light, although protein content did not change with light quality. *Tetraselmis gracilis*, on the other hand, had more pigments and protein when grown in mixed wavelength (white) light. Both species used, *Cyclotella caspia* and *Tetraselmis gracilis*, assumed significantly different growth rate. However, since the response of each alga to each light quality regime was different from each other, indicating a strong species specificity of responses.

Effects of silicate

Obviously, the effects of silicate are limited to diatoms since the other algal classes do not have silicate requirements, at least to the extent that diatoms have, due to their silaceous cell wall.

It has been suggested that silicate concentration in the medium does not affect cellular carbon, nitrogen or phosphorus content (Mortensen *et al.*, 1988). However, cellular C:Si ratios increased with increasing degree of silicate limitation. Strangely, some diatoms' growth rates, like *Chaetoceros gracilis* and *Cyclotella* sp., were not affected by silicate deficiency (Taguchi *et al.*, 1987).

Silicate deficiency and lipid metabolism have similar, though not identical, characteristics as nitrogen limitation. Several experiments with marine diatoms have demonstrated that lipid content increases noticeably under silicate-limited conditions (Enright *et al.*, 1986b; Shifrin & Chisholm, 1981). Marine diatoms react faster to a metasilicate limitation than to a reduction of other macronutrients. When metasilicate deprivation prevents them from forming the orthosilic acid frustule, they immediately start to accumulate lipid (Tagushi *et al.*, 1987). The accumulatory production of storage class lipids, such as triglycerides, is triggered by nutrient stress (Lombardi & Wangersky, 1991). But, in contrast with nitrogen-triggered lipid accumulation, the silicate-triggered

reaction appears to be due to two distinct processes: a) an increase in the proportion of newly assimilated carbon partitioned into lipids, and b) a slow conversion of previously accumulated carbon from non-lipid compounds into lipids (Roessller, 1988).

Effects of salinity, aeration rate, pH and culture media

Among the factors documented to have a role in effecting the algal biochemical composition, salinity, pH, culture media and aeration rate are the least studied. As far as pH is concerned, the cells have mechanisms to regulate intracellular pH when the pH of the environment is adverse. The cell membrane are not completely permeable to hydrogen ions, and thus the pH of the cell can differ from that of the medium. Additionally, the external pH determines the relative distribution of types of assimilable inorganic carbon, like CO_2, $^{-1}HCO_3$ and H_2CO_3, and the solubility of salts and other compounds, so its effect can be indirect through the changes that are induced via other factors (Richmond, 1986). The control exercised by various culture media is essentially of different levels of nutrient availability. Aeration rate needs precise equipment in order to be significantly controlled over a long period of time sufficient for any differentiation in cellular biochemistry to appear. Salinity itself, can be studied only by use of totally artificial media. However, these culture media are not only more elaborate and expensive to make, but they have repeatedly been shown not to support good algal growth, and also when they do so, this algal reaction has been limited to a very few species, thus making it difficult to assess the wider usage of such media. Consequently, there is very limited interest in any commercial application of the results of such research.

The main reason for the limited interest in these factors resides though with the fact that in given experimental laboratory conditions, they are held more or less constant over a period of time, dictated by the practicalities of each laboratory's practises.

Blinn (1984), using a defined medium showed that *C. muelleri* will divide at maximum rate at between 2 - 15 ‰ salinity, with growth abruptly decreasing below 2 ‰; however in that report there are no data on the change of the biochemical composition as a response to the change in salinity. In the light of other reports that show a significance variation of the biochemical composition with the variation of the algal growth rate, a

significant change in biochemical composition of the species used by Blinn (1984) is to be expected.

Sánchez *et al.* (1993) using *S. costatum* found a considerable change in growth rate with changing aeration rate of the culture. Growth was maximised using an air flow rate of 1.0 and 1.5 v · v^{-1} min^{-1} (v · v =volume of the air equal to the total volume of the culture) while growth was minimal when the aeration rate was increased to 3.0 v·$v^{-1}$$min^{-1}$. In addition, FA composition was influenced with the change aeration rate. Interestingly, the proportion of SaFA decreased and the proportion of PUFA increased as the aeration rate increased. In the very small list of studies concerned with the effects of various culture media, Sánchez *et al.* (1993), report a preference for Walne's medium (Walne, 1965) of *S. costatum*, on the basis that in these conditions the diatom had the most favourable biomass production and the highest proportion of PUFA in comparison with Ukeles medium (Ukeles, 1980). Sánchez *et al.* (1994), again using *S. costatum* and a batch photobioreactor, tested the responses of this species over a pH range. No appreciable effect on protein content was found but both growth rate and FA composition varied with the pH of the culture. Growth was maximal in pH 8.0 while the highest percentage of PUFA was obtained in the range 6.5 – 8.0. For the reasons mentioned in the beginning of this section and in addition to the ones mentioned on the study of the culture media, the effect of pH on algal growth is both less limited and of smaller significance than the major factors influencing growth and biochemical composition.

Intraspecific and genetic variation on the biochemical composition of algae, and the use of sequential culturing and screening algae to improve their nutritional quality

In the culturing of phytoplanktonic algae for aquaculture, the main objective is to increase the algae's nutritional value for a specific organism. In commercial situations the effectiveness of each method is counted against its simplicity and cost; the more simple and inexpensive method that will yield appreciable results would be readily adopted. However algae, like all other organisms, have an inherent amount of variability in their response which is due to their genetic variation. Genetic variation within marine phytoplankton species in now well established within local populations: individual cells have different reproduction rates and various biochemical and physiological

characteristics that are the result of genetic variability. In some species living in differing habitats or water masses genetic differentiation has developed more strongly (Brand, 1988).

S. costatum is a cosmopolitan species with a well documented intraspecific variation in many physiological and genetical components. Stabile *et al.* (1992) showed a considerable variation in chloroplast DNA and allozyme variation in winter strains of this species. This was even more interesting in the light of the fact that all the isolated strains originated from the same area (Narragansett Bay, USA), and the chloroplast DNA diversity found was expected to represent a minimum level of diversity of the chloroplast DNA diversity present in that area. The fatty acid profile variation of *S. costatum* was investigated in another report by Shaw *et al.* (1989). The results obtained are even more interesting especially in the light of the important dietary role of FA. In this report, the FA composition of triplicate cultures of four clones of *S. costatum*, isolated from various Australian water samples, mainly from the Bass Strait off Melbourne, was found to have significant variation both in the percentage of individual FA and in total acid concentration. For example the relative percentage of 20:5ω5 varied from 10.52 to 18.57, and the 16:0 from 4.65 to 8.94. The authors used selected individual FA components and the relative composition of SaFA, MUFA and PUFA to distinguish coastal and coastal/eutrophic clones from estuarine and oceanic clones. This was consistent with cells of a particular genetical composition being selected because of a favorable FA composition.

The other diatom used in the present work, *C. muelleri* has also been reported to have considerable physiological variability. This species has a wide geographic distribution (Johansen and Rushforth, 1985) and has been reported from waters of varying salinities and temperatures. The alga was also tested in laboratory experiments, displaying strong euryaline and eurythermal characteristics (Blinn, 1984). In a report by Johansen *et al.* (1990) which studied the effects of temperature, ionic composition and conductivity on growth rates and lipid content of 10 strains of this species, it was indicated that these strains fell into two major categories. Although this study was primarily used as a means of testing phylogenetic hypotheses from a taxonomic point of view, the variation of growth rate from 1.4 to 4.0 doublings per day and a variation of lipid content of 14 to

45% of ash free dry weight, between these strains of the same species, should surely be of major importance in many nutritional studies.

Another method that has not been extensively studied but might have interesting implications in the effort to improve the nutritional value of algal species, is by the successive isolation of strains exhibiting favorable characteristics. López - Alonso *et al.* (1993) succeeded in improving the eicosapentaenoic content (20:5ω3) of *Isocrysis galbana* from 2.4% to 7.8% in two generations, which was a significant increase. An increase also occurred for docosahexaenoic acid (22:6ω3) from 0.9 to 3.8 %.

Collectively such results suggest that it is possible to select microalgae with the desirable phenotypic characteristics not only by manipulating the environmental conditions but also by investigating populations of the same species from various environments and by selective isolation of clones which possess desirable characteristics.

However, without underemphasizing the importance of the environmental factors that influence both algal growth and biochemical composition, by far the most important ones are the availability of major nutrients such as phosphorus and nitrogen and light intensity.

1.7.d Nitrogen

Earlier reports have focussed on the effects of nitrogen on growth (e.g. Richardson *et al.*, 1969). The overall picture was blurred due to the fact that the responses were very much species specific. Apparently such "crude" effects had to be studied first, but the limiting analytical techniques available at the time render these reports of limited value for modern research purposes. Advances in analytical and biochemical techniques have led to more detailed and comprehensive understanding. Therefore, these relatively recent reports will be discussed here.

Although nitrogen-limitation reactions may be species-specific, especially as far as growth rates are concerned, it has been reported that nutrient limitation reduces the level of protein (Enright *et al.*, 1986b; Thompson & Harrison, 1992), while it significantly increases the carbohydrate content of algal cells (Wikfors *et al.*, 1984).

Another, obvious, effect of N-deficiency is the decline in nitrogenous photosynthetic pigments such as chlorophylls and phycobilins (Herzig & Falkowski, 1989; Plumey *et al.*, 1989).

Research on the effects of nitrogen on biochemical composition of algae, from an applied point of view, has concentrated mainly on the lipid and fatty acid content, due to their importance in determining the nutritional value of the algal species.

The first to demonstrate a direct effect of nitrogen content (in forms of nitrate or ammonium) on fatty acid biosynthesis in algae were Pohl & Zurheide (1979). These investigators showed that the content of polyunsaturated C_{16} & C_{18} fatty acids in a chlorophyte, *Chlorella vulgaris*, and the euglenophyte *Bracteacoccus minor*, was enhanced by high concentrations of N. In a number of species, representing various algal groups, nitrogen limitation was found to make the algae accumulate lipids. In many cases neutral lipids, primarily triacylglycerol, comprised the bulk of the lipid content in nitrogen-starved cells. Later, other investigators tried to assess interspecific differences and relate the responses to nitrogen concentrations of various algae, applying in a sense, some sort of "nitrogen screening" (Shifrin & Chisholm, 1981). In that comparative study, they reported a 120 - 130 % increase of total lipid content in nitrogen starved cells. It was noticed that green algae increased their lipid content with nitrogen deprivation while diatoms demonstrated variable responses depending on the species; a few strains e.g. *Biddulphia aurita* and *Synedra ulna*, did not accumulate any lipids. The result of lipid increase under nitrogen stress, was later confirmed in the Eustigmatophyte, *Nannochloropsis* sp. QII (Suen *et al.*, 1987); additionally, it was shown that enhanced lipid synthesis resulted principally from *de novo* CO_2 fixation. In a mini review by Roessler (1990) a list of species which were found to accumulate lipids under nitrogen limitation is provided.

However, it has been reported that metabolic pathways of both carbohydrate and lipid production are inhibited by protein synthesis, when nitrogen is in extreme high supply and phosphorus is low (Wikfors, 1986).

It seems that under nitrogen stress microalgae produce substantially less polar lipids and significantly more neutral lipids (Tornabene *et al.*, 1983; Ben-Amotz *et al.*, 1985). More elaborate and detailed culturing and analytical techniques support this, more

complex, image. Intracellular synthesis of triglyceride may be enhanced by nitrogen limitation, but membrane associated polar lipids classes were reduced under these circumstances. It should also be mentioned that different types of dissolved extracellular lipid classes were produced under different nitrogen regimes (Parrish & Wangersky, 1987).

1.7.e Phosphorus

Comparatively, there have been fewer studies concerning the effects of phosphorus limitation, than other major nutrient elements, on the biochemical composition of the algae. Yet, in some natural ecosystems phosphorus can be the main limiting factor of algal production, for example as in some coastal areas (Berland *et al.*, 1980).

It appears that the phosphorus stress reaction is, to a certain extent, similar to that of nitrogen, although fewer data are yet available. In an experiment with combinations of both phosphorus and nitrogen stress, Wikfors (1986) found that increasing phosphorus content in cultures with high nitrogen concentration resulted in an increase in the protein to carbohydrate ratio in one species, *Tetraselmis maculata* [Prasinophyceae] while in *Dunaliela tetriolecta* [Chlorophyceae] this ratio was decreased, although when expressed in absolute cellular concentrations, carbohydrate values increased and protein was decreased under phosphorus limitation, in both these species.

Siron *et al.* (1989) reported that phosphorus limitation on *Phaeodactylum tricornutum* led to the elaboration of triglycerides while it significantly decreased the content of PUFA, and in particular eicosapentaenoic (20:5ω3) acid. The overall degree of unsaturation of microalgal lipids was also altered. Almost identical results were obtained by Lombardi & Wangersky (1991) using another diatom, *Chaetoceros gracilis*. In this species, phosphorus limitation triggered the production of storage lipids such as triglycerides, but reduced the amount of phospholipids.

Reitan *et al.* (1994) investigating the effects of nutrient limitation, which the authors believed to have probably been phosphorus, on the lipid and FA composition of various marine microalgae, reported an increase in relative amounts of total SaFA and a decreased percentage of PUFA under the nutrient stress conditions. The triggering of the

greater production of storage lipids was confirmed as far as Bacillariophyceae and Prymnesiophyceae are concerned, but they (Reitan *et al.*, 1994) reported a different response for two flagellates, *Nannochloris atomus* [Chlorophyceae] and *Tetraselmis* sp. [Pranisophyceae], in which the lipid content was decreased with nutrient limitation; they speculated that phosphorus limitation probably reduced the synthesis of ω:3 PUFA.

Although lipid accumulation under nutrient stress may be partially due to steady lipid synthesis combined with reduced cell division rate and protein synthesis due to reduced availability of nutrients (Sukenik and Livne, 1991), it could also be that in some species this accumulation of lipids is primarily a result of an increase in neutral lipids (Parrish & Wangersky, 1987).

In conclusion, it is generally believed that phosphorus limitation may reduce the formation of phospholipids and trigger the production of triglycerides and other storage neutral lipids (Tornabene *et al.*, 1983; Suen *et al.*, 1987; Lombardi & Wangersky 1991). The neutral lipids have a relatively higher content of saturated (SaFA) and mono unsaturated FA (MUFA) than the polar lipids which contain more ω : 3 PUFA (Kayama *et al.*, 1989). Ratios between SaFA, MUFA and PUFA change with variable nutrient limitation (Ahlgren *et al.*, 1992).

1.7.f Light Intensity

The effects of light intensity on algal physiology and biochemistry have also been extensively studied. Although the relationship seems to have a general trend, some contradicting records have been presented, which will be covered in this section.

In respect to different types of photoadaptation, Jørgensen (1969, 1977), reported two different types of adaptation to light limitation: the "*Chlorella*" type, in which chlorophyll **a** content varies with light intensity and the "*Cyclotella*" type, in which chlorophyll **a** content does not vary. In the "*Chlorella*" type, the actual carbon fixation rate per cell at the light intensity where the cells are grown does not differ much between high and low light intensities, while in the "*Cyclotella*" type this rate is much higher at a high than at a low light intensity.

Thompson & Harrison (1992) found that an increase in light intensity caused a lowering in protein content and an increase in carbohydrate in *Thalassiosira pseudonana*

cultures. In a later report, Thompson *et al.* (1994a), using a different diatom species, *Chaetoceros simplex*, found no significant differences in protein and carbohydrate contents. Similar results for elemental composition were obtained for another *Chaetoceros* species (*C. gracilis*) by Mortensen *et al.* (1988). It may well be that the relationship between light intensity and biochemical components is species specific.

Cohen *et al.*, (1988) testing the effect of various environmental conditions, including light intensity, on the FA composition of the red alga *Porphyridium cruentum*, found that an increase in light intensity resulted in a decrease of SaFA and also a shift from the 20:4 as the predominant PUFA to the 20:5ω3 FA as such. On the contrary, Brown *et al.* (1996b), using batch cultures of the diatom *Thalassiosira pseudonana*, found an increase of SaFA and a decrease of PUFA with increasing light intensity. The later results was also reported for several other microalgal species (Thompson & Harrison, 1992; Thompson *et al.*, 1992b). Therefore one may favour the hypothesis that, for a number of algal species from different classes, an increase in light intensity causes an increase in the degree of saturation of fatty acids (Thompson & Harrison, 1992; Thompson *et al.*, 1994a).

The ratio of triacylglycerol to polar lipids was also found to increase from low to high light intensity for *Cylindotheca fusiformis* and the diatom *Nitzschia alba* (Opute, 1974; Orcutt & Patterson, 1974). Increased levels of triacylglycerols were also observed under high, photoinhibitory levels on the eustigmatophyte *Nannochloropsis* sp. (Sukenik *et al.*, 1989). In the latter case ^{14}C tracer experiments indicated that the percentage of newly assimilated carbon partitioned into triacylglycerols increased at high light intensity, suggesting that *de novo* synthesis is at least partially responsible for the observed triacylglycerol accumulation. It seems that, light intensity affects the total triglyceride content, in an inverse manner; an increase of irradiance results in a decrease of the total triglyceride content (Parrish & Wangersky, 1990).

1.8 Scope of this project

Through this introduction, it has been highlighted that, the quality of algal food is by far the single most important factor that influences larval growth. However it has been difficult to tabulate information from the literature and select some algal species which possess the highest nutritional value. This nutritional value is basically a reflection of the innate biochemical characteristics of the algal species.

However, this biochemical composition appears not to be constant. A major problem area, which may in fact be the one responsible for the various contradictory records of effects, is that there is variation associated with different laboratories and different algal culturing techniques. This in turn, renders direct comparisons between different laboratories unsafe. The exact culture system (e.g. aeration, swirling of the cultures, illumination type etc.) may cause discrepancies between the effects of nutrient starvation on members of the same genus or even clones of the same species.

In general, the problem with all reports is that, from the plethora of factors influencing physiological and biochemical properties of the algae, only some of them are actually controlled each time in "controlled culture" designs, and the others are assumed not to be significant. Although this may well be the case, the potential influence of variable, uncontrolled, parameters may be the source of the reported variable responses.

Therefore this project tries to explore the effect of three of the most important environmental parameters, nitrogen and phosphorus availability and light intensity, on the biochemical composition of four microalgal species: *Skeletonema costatum* (Greville) Cleve, *Chaetoceros muelleri* Lemmermann [BACILLARIOPHYCEAE], *Rhinomonas reticulata* (Lucas) Novarino [CRYPTOPHYCEAE] and *Pavlova lutheri* (Droop) Green [CHRYSOPHYCEAE].

To avoid artifacts in the biochemical composition, maximum care was taken to keep all other parameters constant. More specifically, in order to avoid the well established effect of the age of the algal culture, continuous cultures were used. In these cultures the population density is held constant and hence a desired algal growth phase can be chosen as the specific target for experimentation.

Since all experiments were carried out under the same experimental laboratory facility, direct comparisons between these species can safely be applied.

Furthermore, this approach was used to manipulate the biochemical composition in such a way that these cultures would have improved nutritional value for the early larval stages of *M. edulis*.

Since algal biochemical components were analysed concurrently with the feeding trials, the results can be also used as part of a theoretical model to explain the basis of the algal nutritional value as well as in an effort to explain the way in which dietary components determine subsequent larval growth.

Finally, since larval biochemical properties were also analysed, the results obtained were investigated in order to determine whether these larval biochemical components can be used as an index of growth of the larvae.

2. Materials and Methods

2.1 Algal Cultures

2.1a Isolation and purification

Four algal species were used as food for the larvae of *M. edulis*. These species were: *Chaetoceros muelleri* Lemmermann 1898 (CCAP 1010/3), *Pavlova lutheri* Droop (Droop, 1953 revised by Green 1975; CCAP 931/1), *Rhinomonas reticulata* (Lucas) Novarino (1991; CCAP 995/2), and *Skeletonema costatum* the strain of which was isolated by the author, using a modified micro pipette method by Hoshaw and Rosowski (1973), from a sample taken from Syros island (Greece).

In detail the isolation procedure was as follows: A 1 l sample was taken from the shore; it was stored in a PVC bottle which was placed near sun lit window, in a cool room (ca. 18 °C), until transferred to the laboratory of School of Ocean Sciences, Menai Bridge, UK. The sample was then diluted 1:5 with fresh sterile f/2 (Guillard & Ryther, 1962) medium and left until a change of coloration in the medium, indicating growth of microalgal species, could be detected by the naked eye. This happened after 10 days, then a small volume of the sample ca. 1 ~2 ml was placed in a Petri dish and fresh sterile f/2 medium was added to fill the dish. The Petri dish was observed under a stereomicroscope and individual cells of the desired species were drawn from the sample, using a micropipette. The micropipette was drawn out using a gas burner, so that it had a diameter big enough to take up the cells of the desired species, but not leaving very much space for other, larger species to be included as well (usually the micropipette has an inner diameter of about 4 ~6 times the diameter of the desired species). The contents of the micropipette were discharged into a large drop of sterilised f/2 medium. In order to avoid other micro-organisms being included into the future culture, the washing procedure of transferring the species from one drop of medium to another was repeated at least 10 times. If the transferring procedure was successful, no damage to the diatom was visible and no other organism could be seen. The diatom was finally transferred, with a micropipette to a sterile Petri dish filled with fresh, sterile f/2 medium. The Petri dishes,

each containing a single isolate, were then sealed with Parafilm®. The whole isolation procedure was carried out into a laminar flow chamber.

The sealed Petri dishes were transferred into the culture room (conditions were the same as for the main cultures which are described further on), where they were left until growth could be detected by observing coloration with the naked eye.

To confirm that a culture was uni-algal, just before inoculating larger volume cultures a sample from the Petri dish was taken in aseptic conditions and examined under a microscope. If the examination suggested that the culture was indeed uni-algal, the contents of the Petri dish were transferred aseptically into 250 ml round bottomed glass flasks and these were used as stock cultures.

In order to identify that the species isolated from the Greek sample was indeed *S. costatum*, a sample from the actively growing culture was inspected in a scanning electron microscope (Cambridge Instruments, Stereoscan 120). The sample was prepared as described by Hasle (1978). In detail the sample was rinsed with distilled water and an equal amount of saturated $KMnO_4$ was added. After 24 hours an equal amount of concentrated HCl was added and the sample was heated gently over an alcohol lamp until it became transparent. Finally the sample was rinsed with distilled water until acid free. A small amount of the cleaned sampled was placed on a stub which was dried at 60 °C for 2 hours and then gold-coated for subsequent observation.

The *C. muelleri* culture received by the Culture Collection of Algae and Protozoa arrived contaminated with unidentified micro-flagellates and was purified with consecutive multiple washes, with the micropipette technique, to obtain a unialgal culture.

From these stock cultures adequate volumes were taken when required to start larger scale cultures which were used in the feeding experiments.

2.1b Media preparation and culturing conditions

All glassware used for algal culture was sterilised by autoclaving in 121 °C for 20 min. A Biospherical Instruments® 4π meter, QSL-100 scalar irradiance meter was used for all light intensity measurements. The measurements were taken at the centre of the culture in a culturing vessel containing filtered seawater.

The algae cultured in continuous culture were grown in f/2 medium with no added silicate. The phosphorus-limited medium (symbolised **P**) and nitrogen-limited medium (**N**) were prepared by adding a quarter of the amount of the respective salt. The cultures were kept at 20 ± 1 °C using illumination by "gro-lux" fluorescent tubes, with a light-dark cycle of 14–10 hours. The high light intensity (**HL**) used was 145 ~155 $\mu E \cdot sec^{-1} \cdot m^{-2}$ while the low light intensity (**LL**) was 45 ~55 $\mu E \cdot sec^{-1} \cdot m^{-2}$.

The algae used in the control diet were cultured in 20 l glass round flasks under a light intensity of 117 ~135 $\mu E \cdot sec^{-1} \cdot m^{-2}$ using a semi-batch culturing system and Conway medium (see Appendix Table A.2.1). All cultures were maintained in uni-algal condition.

Densities of all species were measured with a modified Fuchs-Rosenthal haemocytometer.

2.1c Continuous cultures issues and sampling

The inoculate used to start the continuous cultures were taken from batch cultures which were conditioned in their respective conditions for at least 2 months.

The system used in this study was a modified turbidostat. A diagrammatic representation of this system can be seen in Fig. 2.1.1.

As the main culturing vessel a 2 l round bottomed borosilicate flask was used. The stock media were kept in 20 l jars, while for collecting the culture samples, another 2 l glass flask was used. All tubing was of autoclavable quality, high strength, silicone tubing. All materials were autoclaved before use, while routine washings with alcohol were employed when joining tubing or when replacing worn tubes was necessary.

The density of the cultures was determined at least once a day and the necessary adjustments on the flow rate were also done on a daily basis to keep the cell density constant. Typically the cultures were maintained at the selected density ±10% throughout the feeding trials. Only in one occasion during the repeat of the *C. muelleri* in high light conditions feeding trial, the **N**-limited culture experienced a decrease in density more than the 10% level. The collector flask was emptied regularly and algae were left to stand for no more than 3 hours in that vessel. These cells were used either for larval food, for the determination of the cell density or for subsequent biochemical analyses.

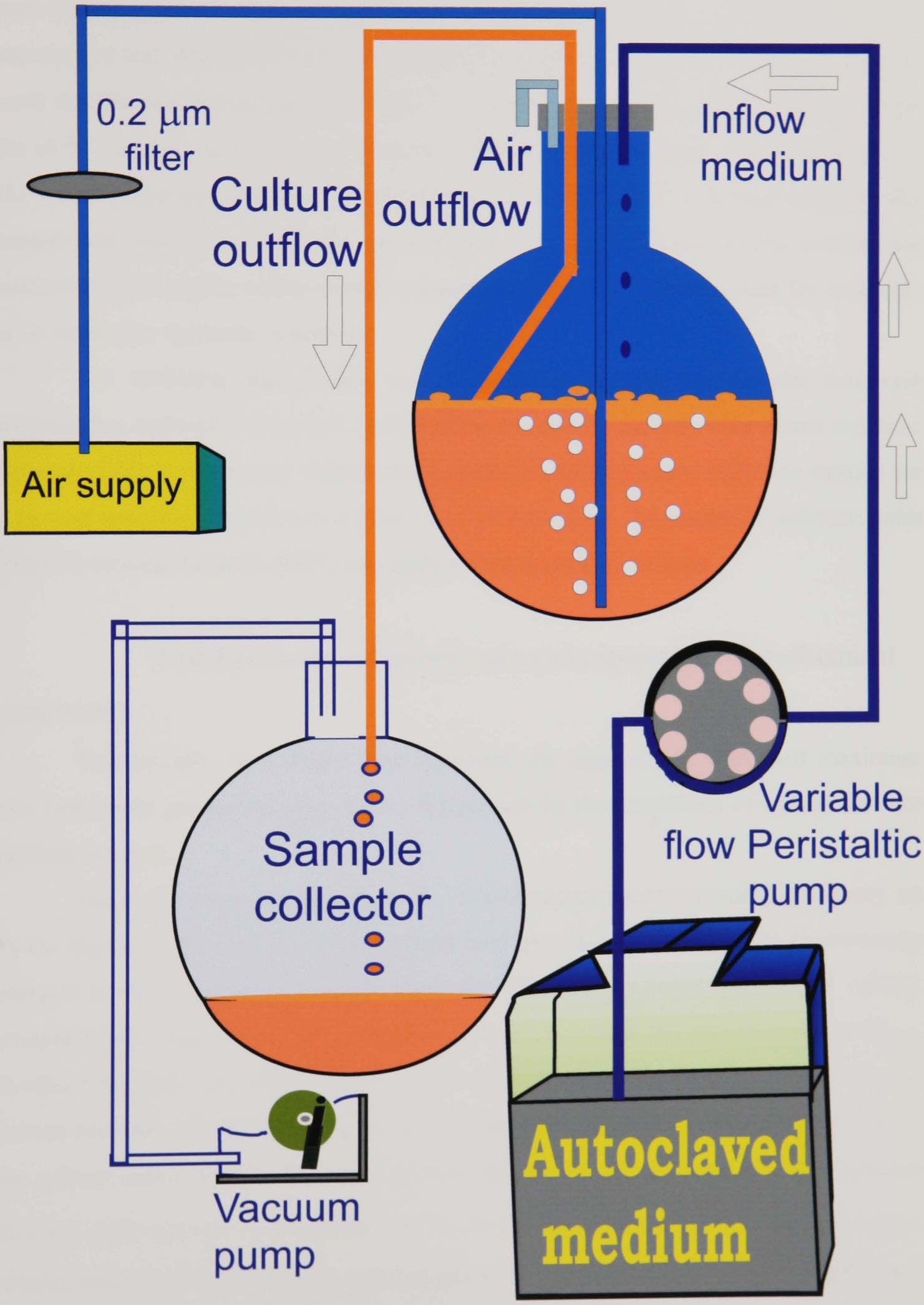

Figure 2.1.1. Diagram of the continuous culture design.

For the chemical and biochemical analyses, 4 samples of 40 ml, from each culture were placed in 50 ml glass centrifuge tubes and centrifuged for 30 min at 2500 rpm. The supernatant was removed by aspiration. The supernatant from the 1st sample was used to wash the filtering device and the storage bottle and then discarded. The supernatant from the next 3 samples of the same culture was pooled together, filtered through a Whatman No 4 filter paper and kept in a plastic bottle in the deep freezer for nutrient analysis; this sample was treated as "**Outflow**" medium and it was assumed that in this medium the nutrient concentrations represented the equilibrium nutrient concentrations for each alga in its respective culturing condition.

The remaining algal pellets were collected and stored. The samples that were scheduled to undergo chlorophyll, carbohydrate and protein analysis were stored in plastic Eppendorf tubes in the deep freezer resuspended in distilled water, while the sample for fatty acid analysis was stored in a glass vial, in Chloroform : Methanol 2:1 solution (with Butyl Hydroxide Toluene, **BHT**) and again stored in the deep freezer.

2.1d Estimation of growth rate and expression of biochemical parameters

Theoretically, in a steady state condition, the algae divide with their maximum rate (minimum generation time, **G**) as determined by the conditions of culture and the limiting nutrient.

The total volume of the culture ($\mathbf{V_t}$, in mls) remains the same and is obviously set by the experimenter. The continuous culture concept is that fresh medium is continuously pumped in the culture ($\mathbf{V_{infl}}$), with a flow rate ($\mathbf{V}f$ ml's per hour), and culture volume containing the algal cells is also continuously harvested from the culturing vessel ($\mathbf{V_{outf}}$, in mls). Obviously, the volume of the inflow medium equals the volume of the harvested culture per unit of time ($\mathbf{V_{infl}}=\mathbf{V_{outf}}$). In a steady-state situation the cell concentration in the culture vessel remains the same. To keep this stable cell concentration the cells are dividing with constant division rate (μ). In the period of time needed to harvest culture volume equal to the total culture volume (therefore the total culture volume would have been 2 x V_t), the cells should have doubled, so that their density inside the culturing vessel remains the same. That period of time equals the species generation time (**G**, in

hours). In this case where $V_{outf}=V_t$, the time required is **G**. Therefore $V_t = V_f * G$ and hence $G = \frac{Vt}{Vf}$ (equation 1).

To calculate the generation time, at first it was established by measuring the cell densities that the cultures were indeed in a steady-state condition. Then the flow rate was measured, under the assumption that Volume of the flowing in medium =Volume of the culture harvested from the culture. At the end of the experiment the total culture volume was measured and **G** was obtained by using eq. 1. The results can also be expressed as specific growth rate μ (divisions per day). It follows that: $\mu = \frac{24}{G}$ (equation 2).

The experiments were designed with the aim of providing a certain concentration of algal cells to the larvae (50 cells·μl^{-1} of larval culture). Therefore all the studied parameters were expressed on a per cell basis.

2.2 Larval cultures

2.2a Spawning of mussels and larval rearing

Ripe mussels, *M. edulis*, collected from the Conwy estuary (N. Wales) each autumn and held suspended over winter on a raft in Menai strait, were used as broodstock for the trials. These were kept at 6 ± 1 °C in flowing sea water until required for spawning.

Throughout the experiment 0.2 μm fine filtered, UV-light treated sea water was used. It will be referred to as "filtered seawater" from here on. All glassware and plasticware used for mussel spawning and larval rearing was washed in dilute chloros, rinsed thoroughly with hot fresh water, followed by a final rinse with filtered sea water just before use.

Mussels of ca. 60 mm in length were selected and epifauna and byssals threads were removed. To induce spawning, 2 ml of 0.5M KCl was injected by a syringe into the animal's mantle cavity and they were then left out of the water for 1 hour at 16 °C. After that period the animals were placed individually in filtered seawater in 250 ml glass jars and left there until spawning was observed. Sperm from all males which spawned was mixed before use. Similarly, eggs from all females which spawned were mixed,

separately from the sperm mixture, just before use. However, this procedure of mixing eggs and sperm from various adults does not necessarily result in larvae of mixed genetic origin since previous work (Del Rio, 1996) found in subsequent genetic analysis that in some cases the bulk of the larval batch were siblings.

The eggs were sieved through a 80 μm sieve, to remove debris, and were placed into a measuring cylinder which was filled to 1 l with filtered seawater. The egg suspension was mixed gently using a perspex stirring rod and six samples were taken in which the number of eggs was counted using a modified Fuchs-Rosenthal haemocytometer. The mean number of eggs·ml^{-1} was estimated and therefore the total number of eggs could be calculated.

A suspension of ca. 100,000 eggs per dish were placed in 7 glass 500 ml dishes, while ca. 1.5 million eggs per tray were placed into 2 plastic 20 l trays, and then sperm was added. The jars and trays initially did not contain any water. The mixture of eggs and sperm was left for 20 min until the formation of the first polar body could be observed; if that happened then the beakers were filled with filtered sea water and left with the same water at 14 °C until the larvae reached the D-stage, normally after three days.

2.2b Feeding trials

After the three-day incubation period the larvae were observed under a microscope to see if they developed according to the normal larval stages as described by Bayne (1965). If that was the case, or the percentage of abnormality was small, the contents of the dishes and/or trays were mixed and sieved through a 45 μm nylon sieve.

A small sample of larvae was taken from the sieve and photographed or videotaped (see later section on image analysis) under a microscope for later calculation of the initial mean length of the larvae. The larvae were then transferred into a volumetric cylinder which was filled with sea water to 1 l. Again, the larval suspension was mixed gently with the aid of a perspex stirring rod and 6 samples of 100 μl were taken. In these samples the percentage of normality and abnormality as well as the total number of larvae was estimated, so that the total number of normal larvae could be calculated.

An appropriate volume was taken from the larval D-stage mixture to be diluted to give an end concentration of 30000 larvae l^{-1} and was transferred into plastic 1 l beakers; in each beaker the respective species and quantities of algae were added and they were then filled to 1 l and left at 14 ±1 °C.

A change of the water of the larvae as well as feeding was done every second day. To do that, the contents of each beaker were poured in a 45 μm nylon sieve, which was prewashed in chloros, hot fresh water and filtered seawater. The beaker was then disinfected by rinsing it with chloros, then with hot fresh water and finally, before adding the larvae back in it, with filtered seawater. The respective algal food was added to the beaker, in such amount to bring the end concentration to 50 cells·μl^{-1}, and then the beaker was filled with 0.2 μm filtered seawater to 1 l.

Every experiment was repeated again with another batch of larvae, from a different spawning, to establish whether the displayed behaviour was dependent on a certain larval batch.

To establish whether the bacteria included in the larval cultures had any effect on the growth of the larvae, an additional experiment was designed, using 5mm filtered seawater. In this case, fresh seawater taken from the Menai Strait (M. Bridge, N. Wales) was filtered using a 5 μm filtering cartridge and that was used to fill up the cultures instead of the ordinarily used 0.2 μm, UV-radiated seawater.

In order to study the effect of algal extracellular products (**A.E.C.P.**), another experimental design was used. For this, the density of the algal culture was first determined and then filtered through a Whatman GR/C filter. The volume of the algal culture which was needed to "feed" the larvae was calculated as if the filtrate contained all the algae. The extracellular products of *S. costatum*, in **high light** and **N** limited conditions were used for this experiment.

The main feeding trials were carried out using four algal species, which were tested against a standard diet (which for reasons of simplicity will be referred to as the "**Control**" diet), a 4:1 mixture of *P. lutheri* and *R. reticulata*, while an additional control measurement was obtained by keeping unfed larvae as well. In all experiments the final concentration of the algae in the beakers with the larvae, that were meant to be fed, was

50 cells μl^{-1}. The water change of the unfed larvae occurred just as the in the other diets. every second day, but no food was added.

For every diet within each experiment three replicate beakers were used. Care was taken to change the position where the beakers were left to stand in the cold room and sequence of the water change of the beakers at every water change at random, so that possible position effects could be avoided.

In each experiment, a set of 6 diets were tested against the described set of reference treatments (Control diet and Unfed larvae). The combination in which the diets were tested and the actual sequence in which the experiments were carried out, can be seen in Table 2.1. The repetition of each experiment was carried out either immediately after the end of each experiment or started half way through the duration of the respective experiment.

Table 2.1. Sequence of feeding trials and combinations of algal species and culturing conditions used in each experiment together with the number of animals from which the gametes were obtained in each case.

	Algal species used	Light Intensity	Nutrient	Spawned animals (first trial)	(repetition trial)
Exp. **1**	*S. costatum* & *C. muelleri*	High Light	F/2 , N , P	**(5 ♂, 3 ♀)**	**(8 ♂, 4 ♀)**
Exp. **2**	*S. costatum* & *C. muelleri*	Low Light	F/2 , N , P	**(8 ♂, 5 ♀)**	**(9 ♂, 6 ♀)**
Exp. **3**	*R. reticulata*	High & Low Light	F/2 , N , P	**(7 ♂, 7 ♀)**	**(7 ♂, 6 ♀)**
Exp. **4**	*P. lutheri*	High & Low Light	F/2 , N , P	**(4 ♂, 2 ♀)**	**(5 ♂, 2 ♀)**

2.2c Estimation of growth and survival

To calculate the number of live larvae at the end of the experiments, the culture was first transferred to a plastic 1 l volumetric cylinder and its volume adjusted to 1 l. The culture was mixed using a perspex stirring rod, and 3, 2ml samples were taken, stored in a glass vial and fixed with few drops of Lugol's iodine solution. Each sample was at a later stage decanted into a sedimentation chamber. The vial was then washed thoroughly at least three times with 0.2 μm filtered seawater and the seawater was decanted into the sedimentation chamber, to ensure that no larvae were left in the vial. The whole chamber was counted using a Nikon® inverted microscope.

Growth was estimated on the basis of length increase of the larvae. Although it was the size of the larvae that was measured, the term "growth" will also be used in a non strict fashion to indicate the increase in larval size that occurred during feeding experiments. To measure length, two image analysis techniques were used. The larvae were measured at the beginning of every experiment (while on D-stage) and at the end, after a two week period. The first technique was based on measuring photographs of the animals, and the second was based on processing videotaped information of the larvae.

The first steps for both techniques were the same: the larvae of every container were concentrated by pouring its contents into a 45μm nylon sieve. A small sample was taken with a pipette and placed onto a slide. The slide with the live larvae was then placed under a Nikon inverted microscope.

In the beginning of the experimental period (experiments 1 & 2) the first technique was used. In this case, the slide was photographed with a Nikon FM2 camera, so that a permanent record of the larvae on film could be obtained. To estimate the mean length of the larvae, the negative pictures were measured at a later stage under the same microscope fitted with an eyepiece scale; 60 normal clearly focused larvae were measured for every culture. The real dimensions of the larvae were calculated using a real magnification factor which was obtained by taking a picture of a graticule of known length and measuring the negative exactly as all the other negatives.

2.2d Digital image analysis technique

The second technique, digital image analysis, was used for experiments 3 and 4. A diagram of this set up can be seen in Fig. 2.2.1.

In detail the technique is as follows: the slide with the larvae were placed on a Nikon inverted microscope which had a microscope videocamera (Hitachi MOS Colour Video Camera, VK-C150ED) attached to it. The resulting picture was recorder with a video recorder (Panasonic, NV-J35HQ) on a videotape. The tape was later projected to a IBM compatible computer, equipped with a Miro PCTV video card. Images of the larvae in clear focus were grabbed with the aid of Microsoft's Media Manager (Video capture 32) and the result was saved as bitmap images on the computer's hard disk. Then an

image analysis software package, Jandel Scientific Sigma Scan, was used to measure the length of 60 larvae for every treatment from the bitmap pictures. The real length of the larvae was obtained by measuring a digitised picture of a graticule under the same microscope magnification.

This technique was tested against the photographic technique by measuring the same slide of larvae. The mean values of the length of the larvae differ by not more than 5% of each other.

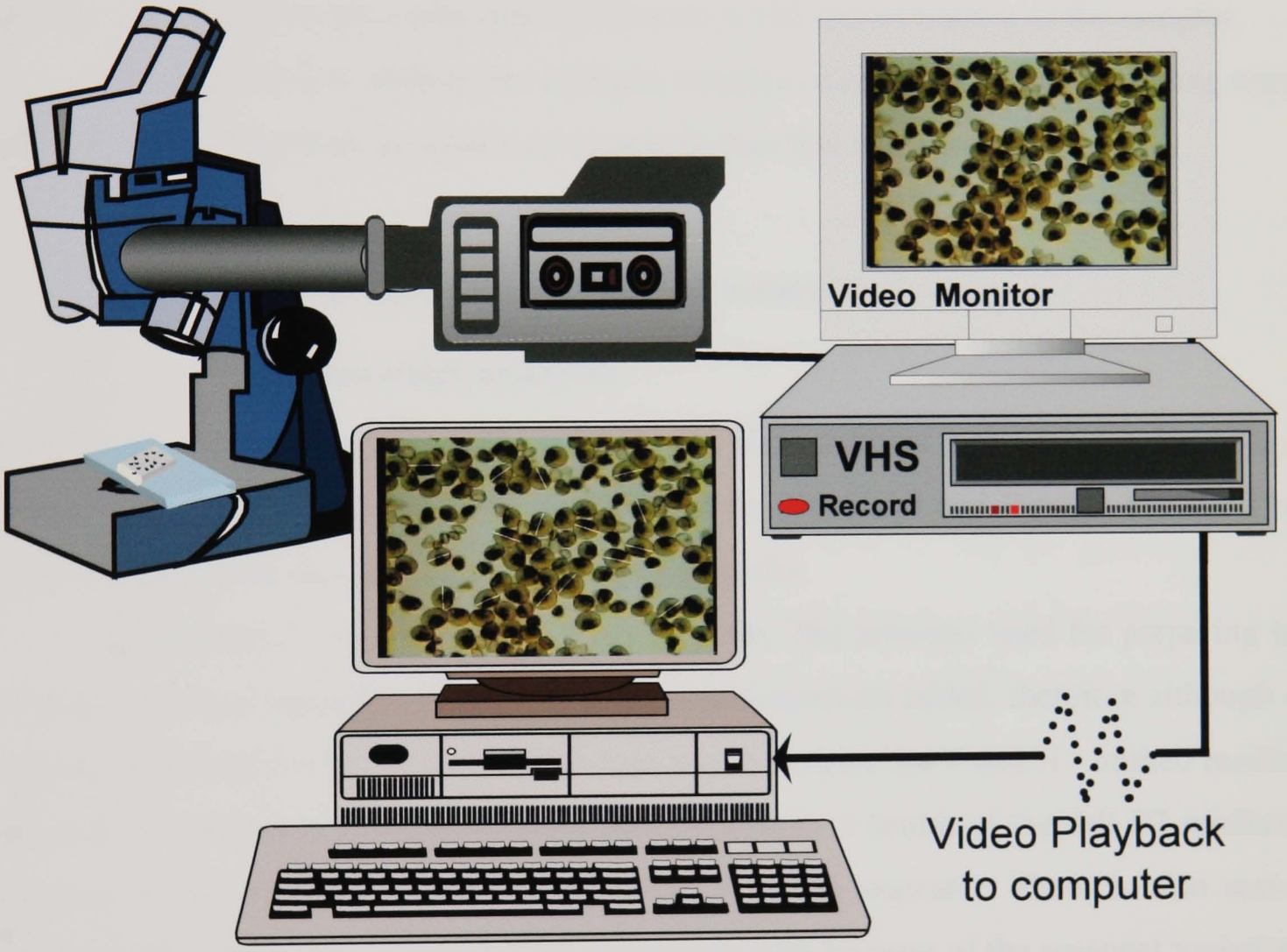

Figure 2.2.1. Schematic representation of the digital image analysis set up.

2.2e Larval Fatty acid sampling

Three stages of larval development were analysed for fatty acids: eggs before fertilisation and 19-days old larvae (after the two-week feeding trial).

The eggs samples were obtained by filtering a known number of through a pre-washed in methanol, Whatman No 4 filter, and then storing the filter in a clean glass vial with chloroform:methanol (+ Butyl Hydroxy Toluene, **BHT**) 2:1 solution (Bligh & Dyer, 1959) kept in the deep freezer at –70 °C, until needed for analysis.

After obtaining the information for the length measurements and having taken the samples for estimation of survival from the 1l volumetric cylinder, its contents were filtered into a Whatman No 4 filter, which has been prewashed in methanol. The larvae on the filter were collected and stored into glass vials, which have been pre-washed with chloroform. The vial was then filled with approx. 10 mls of chloroform:methanol (+BHT) 2:1 solution and stored in the deep freezer (–70 °C).

Care was taken to wash all the necessary equipment used in handling the filters (forceps, filter holders etc.) with chloroform as to avoid contamination of the samples.

It was decided to analyse two replicate samples of each diet for every feeding trial, and leave the 3rd replicate as a back-up sample in case that was needed.

2.3 Analytical methods and related issues

2.3a Seawater analysis

The medium that was collected from the algal cultures was analysed for $^{-1}NO_3$, $^{-1}NO_2$, $^{-3}PO_4$ and Si with the methods described by Strickland and Parsons (1972). The same analysis were also carried out in the inflow media.

f/2 medium is an enriched seawater medium. The seawater used for preparing it, already contained quantities of the macro and micro nutrients added; therefore although a quarter of the amount of $^{-3}PO_4$ and $^{-1}NO_3$ was added to make the **P** and **N** - limited media, the end concentration of these nutrients was not exactly a fourth of the full f/2 medium because of the residual nutrients already present in the seawater. There is also some variation of the end concentration of the macronutrients because of the seasonal variation of the concentration of these nutrients in the Menai Strait.

However, for each alga grown in continuous cultures, the seawater used was taken within a three-week period in time; therefore the residual concentration of the macronutrients was very similar. In addition, the multiple samples analysed from the same culture indicate that the algae utilise the nutrients in such a way that the end concentration of these nutrients in the outflow medium remains stable, even when there is some variation on the nutrient concentration in the inflow medium.

2.3b Protein, Carbohydrate and Chlorophyll analysis

Protein determination was carried out with the technique described by Bradford (1976). A protein standard prepared from bovine serum albumin (Sigma Diagnostics) was used as reference.

Carbohydrate was analysed with the Phenol - Sulphuric acid method (Dubois *et al.*, 1956). A carbohydrate standard curve was constructed with glucose as a reference sugar.

Chlorophyll were determined by the method described by Strickland and Parsons (1972) with the new spectrophotometric equations given by Jeffrey and Humphrey (1975) for chlorophyll analysis in seawater samples. 90% acetone was used for extraction.

For all these biochemical components two samples were independently analysed and the mean value was calculated; in most cases the obtained values did not differ more than 15 % and only the mean will be displayed in the corresponding tables.

2.3c Fatty acid method

Both algal and larval samples underwent lipid analysis. All the glassware used was pre-washed with chloroform to avoid lipid contamination; it will be referred to as "clean" glassware. The lipids were extracted using a modification from the original method by Bligh & Dyer (1959) and fatty acid methyl esters were obtained with methylation using 12% BF_3 in methanol (Metcalfe *et al.*, 1966). In detail the technique was as follows.

To start the analytical procedure, the samples were removed from the deep freezer and a further chloroform:methanol 2:1 solution was added to give a solvent:sample ratio of 15:1 by volume. In this stage, a methanol solution of $C_{23:0}$ in known quantity was added, as an external standard. Usually 125μl of a 100$\mu g \cdot \mu l^{-1}$ of stock solution were added. The samples were left to extract at 4 °C for 20 min and then filtered through a No 4 filter paper, pre-washed with chloroform:methanol 2:1 solution. The volume of the filtrate was measured in a glass, grounded top, measuring cylinder and 0.2 volumes of 0.017 w/v magnesium chloride was added. The solution was flushed with nitrogen and shaken to form an emulsion. It was then quickly decanted into clean glass centrifuge tubes and centrifuged at 2200 rpm for 5 min. The upper phase was carefully removed by

aspiration and discarded; on top of the lower phase approx. 0.5 volumes of Folch upper phase solvent (chloroform:methanol:water 3:48:47 v/v/v), was layered, then gently swirled, and finally removed again by aspiration. This procedure was repeated once more. The remaining lower phase was filtered through a No 4 filter paper filled with anhydrous Sodium Sulphate to remove the water and the filtrate was collected in clean pre-weighted glass vials. The solvent was evaporated in 40 °C under a nitrogen atmosphere and the vial was reweighted to obtain the total lipid weight. The residue was taken up in a small volume of chloroform and placed into glass reactivials for methylation, to transform the Fatty Acids (**FA**) to Fatty Acid Methyl Esters (**FAME**s). To do this, 1 ml of 14 % boron trifluoride in methanol solution, was added to the lipid extract. The mixture was flushed with nitrogen and mixed thoroughly. Then, it was heated for 1 hour at 100 °C, allowed to cool, and 2 volumes of pentane and 1 volume of water were added. The new mixture was shaken and allowed to separate. The top phase (pentane) was pipetted off and saved. The extraction was repeated again keeping the top phase. The sample was evaporated to dryness and appropriate volumes of hexane were added to give an end concentration of the internal standard of 50 $ng \cdot \mu l^{-1}$; in the usual case where 125 µl of the external were added in the beginning, the samples were redissolved with 250 µl hexane. The samples were securely capped and stored in a deep freezer. Within less than a week of the end of analytical procedure the sample was analysed in the HRGC (High Resolution Gas Chromatograph). For this last part of the analysis the samples were removed from the deep freezer, brought to room temperature, and 1 µl of the sample was injected into the HRGC. Traces from the HRGC separation were analysed by comparison with a standard series of peaks produced using Cod Liver Oil as a standard. The percent areas detailed on the readout were matched to the corresponding FAME peaks on the trace and summed; then the final percentages were calculated as a fraction of the total percent area of the identified FAME peaks (% of Total Identifiable Fatty Acids, **TIFA**). In some cases (e.g. *S. costatum* LL f/2, Table 3.2.3) the 18:0, 18:1ω9 and 18:1ω7 fatty acids were not separated properly and were integrated by the instrument as one peak in both samples, therefore the sum of these fatty acids is shown in the table. In other cases (e.g. *R. reticulata* HL N and LL N, Table 3.4.3) these fatty acids were not integrated properly in one of the samples, although they were separated in GC trace; in such cases the mean of

the sum of the non integrated fatty acids was calculated from both samples, while the individual fatty acids were calculated from one sample only. This explains why in these cases the sum of the proportions of all identified fatty acids does not add up to 100 %.

Additional information on the quality of the FA of the samples was obtained by injecting some samples run in HRGC, in a High Resolution Gas Chromatographer - Mass Spectrometer (**GC-MS**) too. The peaks produced in that instrument were analysed for their structure and matched with the peaks present the HRGC traces. A sample from every algal species and the larvae and mussel eggs was run in the GC-MS to obtain a detailed profile of their FA.

The equipment used were, for the HRGC section, a Carlo Erba Gas Chromatograph, Model GC 6000 (Vega Series), which was equipped with an Alltech, Econocap, Carbowax 20m column of 30 meters long, 0.32 I.D. of a film thickness of 0.25μm. The Carrier Gas was hydrogen with a flow rate of 2 ml/min. The GC had a linear flow FID cold in column injector. The integrator used was a Varian 4290. For the GC-MS section, a Carlo Erba 8060 GC which was fitted with a Fisons MD 800 Mass Detector. The column in that instrument was a SGE BPX70 of 50m long, with a 0.2 I.D. and a film 0.25μm. The injection was cold on column and the carrier gas was helium. To determine the quality of fatty acid the Mass Lab software, supplied with the instrument was used.

2.4 Statistical analysis and related issues

Before applying any parametric test each data set was tested to verify whether it had a normal distribution and homogeneity of variances. Normality was tested with a normal probability plot while homogeneity of variance was tested with Barlett's test. Significance was tested at the 95% level.

In general logarithmic transformation of data resulted in more data sets having a normal distribution, therefore the log-transformed data sets were used. However in no instance were all the distributions of all data sets normal although, generally, most were. Even when one of the assumptions necessary for applying parametric tests was violated in a small part of the data set, parametric tests were used. However, additional non-parametric tests were applied.

The experimental design was such that nested ANOVA could be used in all experiments to assess whether significant differences exists between diets.

Only in one case, in the repetition of experiment 4, one of the beakers of the *P. lutheri* f/2 high Light, did not perform as well as the other two. Survival was very poor with only a few larvae surviving and with significantly smaller growth; probably due to some bacterial infection in this beaker. This beaker was excluded from subsequent analysis and the tests applied wherever this diet was tested against the others, was two-way ANOVA.

In order to identify the groups that demonstrated significant differences (95% confidence limit) the multiple comparisons between means T'- method was used (Sokal and Rohlf, 1981a). All tests were carried out with the aid of the statistical software package Minitab®, while preliminary calculations and graphs were made in Quattro® Pro. Probability values were taken from tables in Sokal and Rohlf (1981b).

The combination of nested-ANOVA and the analysis of means test should reveal any significant difference between diets. However it should be noted that this would be the case between the diets specifically included in the analysis. Comparing growth between all these many different experiments is not wise. There is biological variation present within the larval batches, since the experiments were carried out with different batches of larvae and at different moments in time. The controls (Control diet and unfed larvae) serve as a benchmark to compare how different batches of larvae perform. It could be assumed that if the performance of the controls was the same, all the data could be pooled together and one single test performed. The original length of the D-stage larvae was almost identical in all experiments and in any case, there were no significant differences between the two larval batches used in the first experiments and their corresponding repetitions. The same stands for the unfed larvae. However some control diets performed differently with different batches of larvae; it was therefore unwise to pool all the data together in this case. It was preferred that only relative diet performances would be tested. To dissect whether differences exist between different diets one has to chose a combination of diets to compare with each other. If it is necessary to test how the species differ from each other, then the test should be applied between cases of same condition-grown (Light and Nutrient) species. Similarly if the argument is for a certain

condition, say light effects, then the test should be between same species and nutrient diets but different light intensity. If this trial combination was carried out with the same batch of larvae the test poses no difficulty. On the other hand, if that was not the case, the biological variation, as identified by the performance of the controls, should be taken into account. If the difference of the performance of the controls used in the corresponding experiments are not significant the results of the test can be safely assumed to hold true. But if there are significant differences between the controls, care must be taken when drawing conclusions.

Further insight into this methodological (and interpretational) problem is provided by the results obtained by the repetition of every experiment. When the performance of every diet is consistent, which was indeed the case in most repetitions, the statistical tests can be trusted. If that was not the case, then the relative performance of each diet, in relation to each other should be taken into account. The result show that even if the exact length of the larvae achieved after the two-week trial was not the same, the relative performance of each diet was very much consistent, which enable us to be more confident about the conclusions.

Finally for the last part of the analysis, to identify any relationship between algal biochemical component and larval growth, correlation analysis was used.

The algal biochemical components were correlated with larval length. This larval parameter was chosen over larval survival because the latter was shown to be independent from the algal diet and had more to do with the "quality" of the larval batch. More specifically within every experiment survival was more or less the same between diets or varied randomly; even unfed larvae, although they don't grow much, survive just as well as the fed larvae.

It must be emphasised that the experimenter does not have control of the displayed parameters but merely observes and measures their values. In other words, it is not possible to select, say, that the algae have a given concentration of protein. This would be a totally different type of experiment when the biochemical composition of the food can be chosen at will (i.e. in the case when artificial diets are used and the exact amount of each biochemical component can be chosen before hand). In the experimental design used here, it is only possible to analyse the contents and determine, the protein cell

concentration. The same applies for the larval length. It is not possible to force the larvae to assume a certain length although it can then be measured. At the end, the aim is to examine how these parameters covary. In those cases when the degree of association between pairs of variables (interassociation) in a population sample is desired to be established, correlation analysis is the proper approach. Therefore for this type of data, regression analysis is not appropriate and it would be a mistake to use it. The underlying assumption of Model I regression does not hold and fitting such a model to the data is not legitimate, although there is not much problem finding instances of such improper practises in the published research literature. For a more detailed discussion and useful examination of various aspects of regression and correlation analysis the reader is referred to Sokal & Rohlf (1987) ch. 12, pp. 267-270. Another interesting discussion between wrong uses of Model I and Model II regression analysis in marine biological and fisheries modelling research is found in Ricker (1973, 1975).

In summary, data were assessed by nested analysis of variance (ANOVA), while where necessary, multiple comparisons were made by the analysis of Means test (Sokal & Rohlf, 1987) and correlation analysis was used to investigate potential relationships between various biochemical components and larval size.

3. Results and Discussion

3.1 Effects of bacteria and algal extracellular products on larval growth

Prior to assessing the nutritional value of phytoplankton cells to *M. edulis* larvae there are two issues that need to be addressed. The fundamental properties of the algal and larval cultures do possess some characteristics which might potentially influence the demonstrated response of the larvae. These two main elements have to do with the fact that a) *M. edulis* larvae are not reared axenically, meaning that in the rearing vessel there are bacteria present and b) the algal cells are provided as food together with the culture medium in which they were cultured will also contain organic material, as well as some bacteria.

Both aspects are equally important since the bacteria could also serve as a food source for the larvae. Their role in larval cultures is far from clear, whether as a food source or as a source of organic dissolved organic material (DOM). Many marine organisms have been shown to have a significant capacity to take up DOM, sometimes this may comprise up to 40% of their total metabolic requirements (Sorokin & Wyshkwarzev, 1973). Manahan & Crisp (1983) showed the uptake and incorporation of DOM (in form of ^{3}H glycine) by several bivalve larvae, *M. edulis* being among them. The autoradiographic traces indicated that the uptake path was from the velum of the larvae to other organs. The absence of bacteria from the surface of the velum, in Scanning Electron Micrographs, suggested direct epidermal uptake without bacteria acting as an intermediate step in nutrient transport.

The present study is not oriented towards elucidating the role and mechanisms governing the bacterial – larval interactions nor is it to investigate the role of DOM for *M. edulis* larvae. The main issue is the optimization of the biochemical properties of the algal cells to be used as food in the early larval stages of *M. edulis*. Therefore, these issues are dealt with in the context of the given experimental design and conditions and should not be treated as universally applying relationships. In other words, the present study is interested in knowing that the demonstrated behavior of the larvae is due only to

the consumption of algal cells, alone, or that the role of other parameters influencing this behavior can be quantified so that the true effect of the diet can be understood correctly.

The bacterial effect could be demonstrated when the bacteria are included in the larval culture by themselves, as the sole potential source of food for the larvae or when they are included together with the algal cells. The latter options should be included in the design of the feeding trials because differences in the nutritive values between mixed and monospecific algal diets may be explained, in the majority of cases, by the type of interaction among algae (Epifanio, 1979a,b; Romberger & Epifanio, 1981): (a) interactions of a synergistic type, which compensate for the nutritional deficiencies of each food component, inducing better growth, and (b) non-additive interactions that may be explained by the relative ease of digestion and assimilation of each alga.

The problem of investigating the role of bacteria in *M. edulis* larval cultures largely lies in the fact that unless combinations of antibiotic solutions are used, one can not obtain axenic larval cultures. However, bivalve larval cultures have been reared axenically, mostly of the oyster *Crassostrea gigas*. These axenic cultures have been achieved (Langdon, 1983; Douillet, 1991; Douillet and Langdon, 1993, 1994) in the past by stripping adult gonads under aseptic conditions and keeping the subsequently fertilized eggs under carefully controlled, but in non the less totally artificial, conditions. This is possible for the oyster because its eggs are matured prior to release by the adult, and hence a perfectly functional egg can be removed from the adult gonads. In mussels no fertilizable eggs can be obtained simply by stripping the gonads. The use of antibiotics pose a number of other limitations: the most commonly used antibiotics, such as penicillin and streptomycin, have poor effectiveness against naturally occurring bacteria (Helm & Millican, 1977), may have adverse effects on the growth and survival of the larvae (Le Pennec & Prieur, 1977) and also, resistant strains of bacteria are created by systematic use of antibiotics (Jeanthon *et al.*, 1988). Furthermore they might change the physiological properties of the larvae, thus making the extrapolation of such results taken in these totally artificial conditions of questionable use for applied aquacultural larval rearing practices.

Since axenic *M. edulis* larvae cannot be obtained, the study resorted to applying an indirect way of assessing the bacterial effect in the larval cultures: a set of cultures

containing the "average" bacterial fauna normally present would be treated as the control cultures while another set of larval cultures would be provided with an "excess" of natural bacterial populations normally found in Menai Strait sea water. The latter set of cultures would provide an estimate of the effect of added bacteria in the larval rearing system. This system was also used as an addition with algal cultures as food to the larvae in an attempt to investigate any nutritional interaction of algae and bacteria to the larvae. Obviously this design should not be regarded as an in depth investigation of these relationships but as one that merely tries to estimate the effect of other parameters, than algal cells, in the present larval rearing system.

Results concerning the growth (see section 2.2.c about the usage of term "growth" in the present work, p. 52-53) of unfed larvae with and without "excess" bacterial population included in the rearing vessel are presented in Fig. 3.1.1.

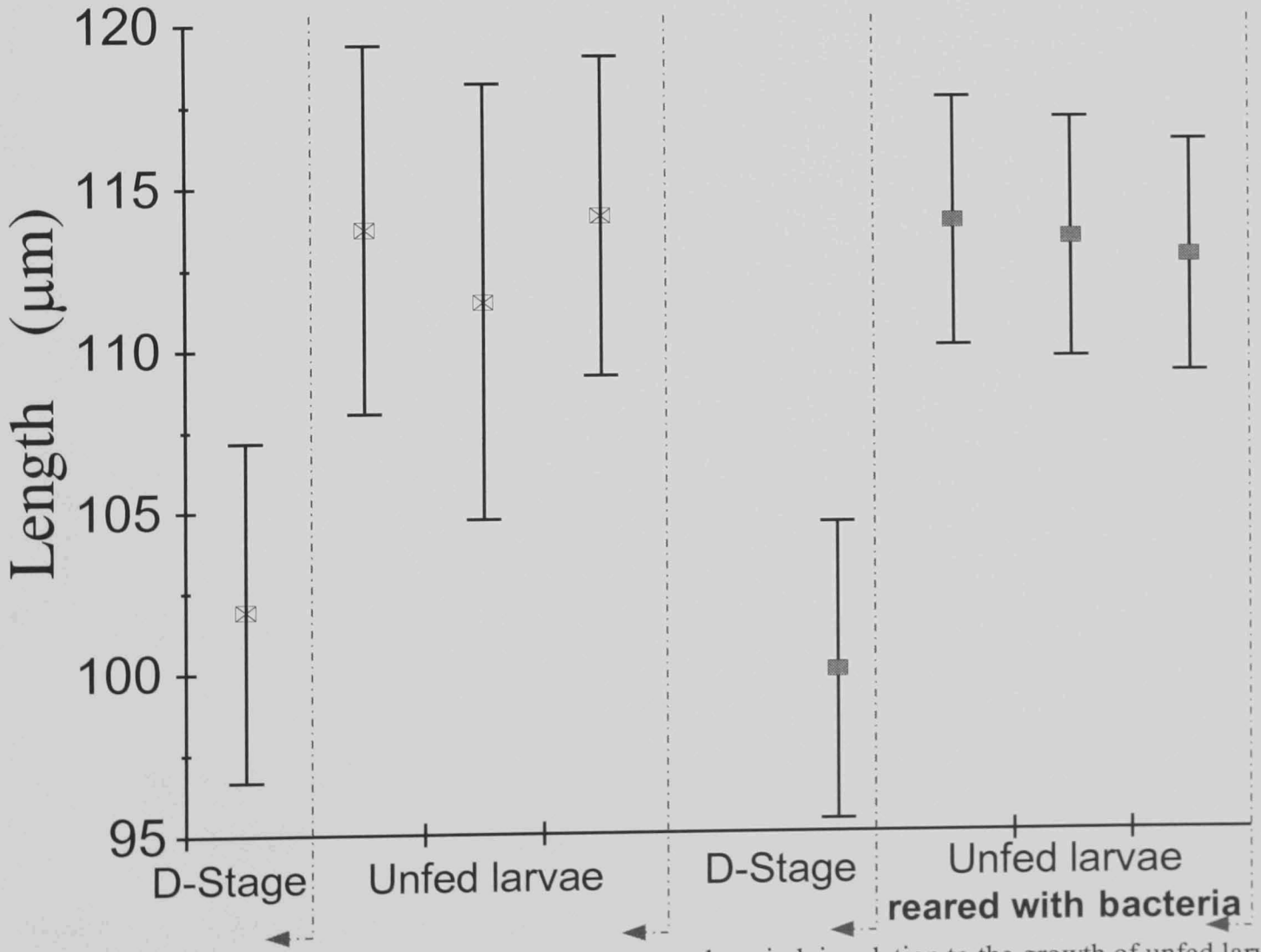

Figure 3.1.1. Growth of unfed larvae over a two – week period, in relation to the growth of unfed larvae reared with added bacterial populations. Growth is shown as Mean ±S.D.

The next time this experiment was repeated with another batch of larvae, all three larval cultures in which excess bacterial populations were included suffered 100%

mortality, while the typically unfed larvae, without the added bacteria grew minimally as in the previous experiment.

From figure 3.1.1 two main things become immediately apparent : a) that unfed larvae grow minimally over the 2-week period, with growth typically less than 15μm; this minimal growth is largely due to depletion of their reserves with which the eggs were furnished on release b) there is no difference whether "excess" bacterial populations are present or not. The results from the detailed statistical test appear in the Appendix, Table A.3.1.1.

Similarly, results concerning the grow of larvae fed on a "control" algal diet and on the same diet but with "excess" bacterial populations also added, are shown in figure 3.1.2.

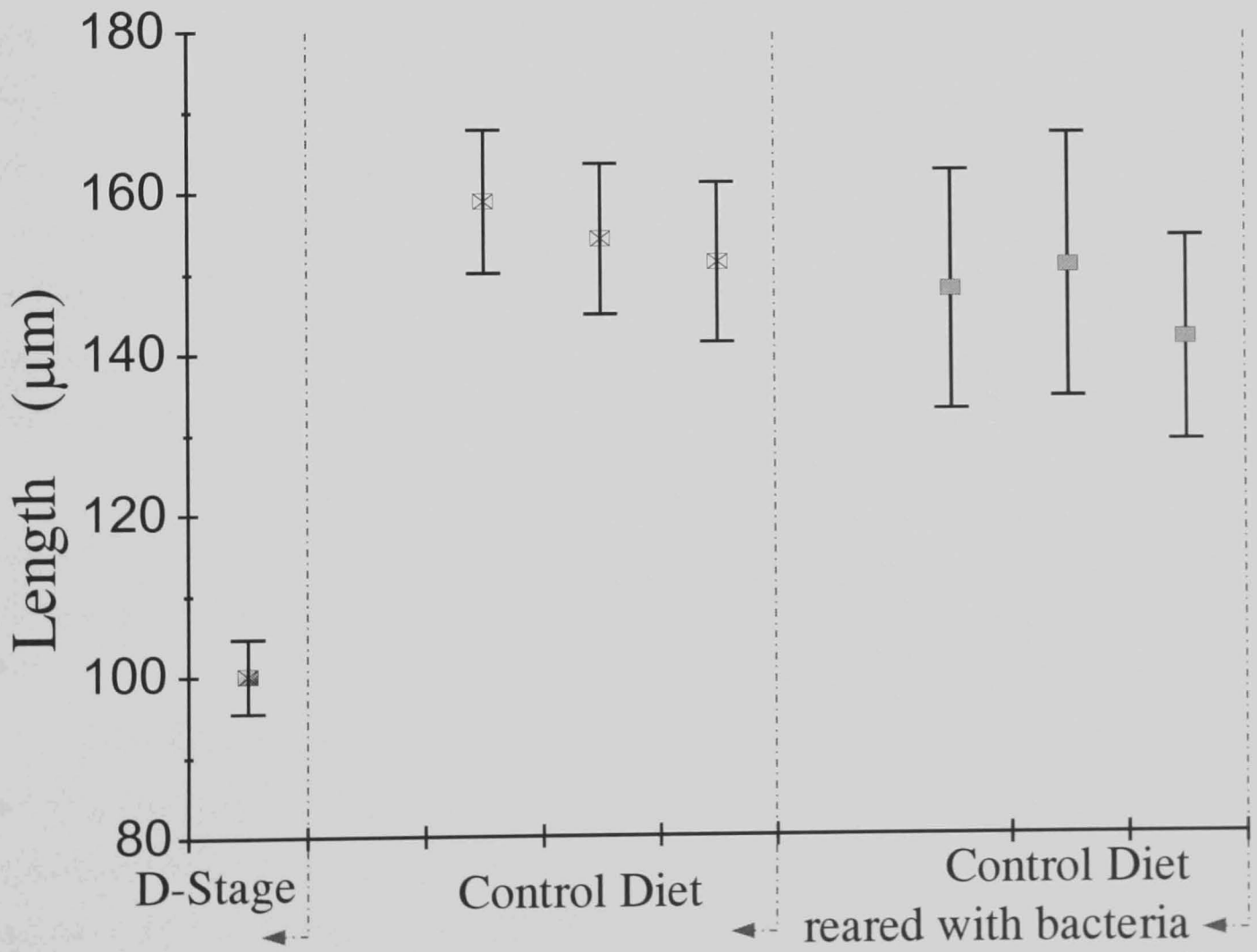

Figure 3.1.2. Growth of larvae fed on a control diet and on the same diet but with added bacterial populations, after the two – week feeding period.

When excess bacterial populations are included with the control diet the larvae appear to grow less. Nested analysis of variance (A.3.1.2) between the diets, suggest that

the difference is significant, however there is also significant variation within beakers which were fed the same diet ($p < 0.05$); therefore statistically we cannot safely conclude whether diets are truly significantly different. Due to the minor difference in mean end length achieved by the larvae at the end of the two – week feeding trial, it is safer to suggest that, there are no differences in the end length of the larvae. If anything, addition of excessive bacterial population does not benefit the growth of the larvae.

In the repetition of this experiment, all the larvae in which the bacterial populations were added with the food, died. The control diet fed larvae grew normally achieving similar end lengths as with the first experiment.

The effects of algal extracellular products on the growth of the larvae was investigated by adding to the larval cultures the filtrate of the algal culture. The volume of the filtrate was estimated as if it contained all the algal cells previously counted in that culture and the end concentration of algal cells would have been 50 cells/µl. Again it must be emphasized that the design was not oriented towards proving or disproving the uptake of DOM by *M. edulis* larvae but towards showing if the extracellular products invariably added to the larval culture did have a nutritional effect, in the subsequent feeding trials. This is why the algal culture filtrate was not added at random or excessive volumes but only with the volumes that would have been used if the algal cells were present in it as well.

Algal extracellular products of *Skeletonema costatum* grown in N- limitation were used for this purpose, because in preliminary experiments larvae fed on this diatom grown under N-limitation had demonstrated very good growth. Results are shown in Fig. 3.1.3.

From Fig 3.1.3, it becomes clear that unfed larvae grow minimally, as in the previous experiments, while the larvae "fed" on algal extracellular products also exhibit minimal growth. In essence, they grow like unfed larvae, which is minimal anyway. Further statistical tests indicate that there is significant differences between the lengths of the larvae (A. 3.1.3) and detailed analysis of means confirms that this significant difference is between the D – stage larvae, at the beginning of the experiments and the unfed larvae at the end of the trial (A. 3.1.4). However, more importantly, between unfed

larvae and larvae in which Algal Extra Cellular Products were added, there was not any significant difference.

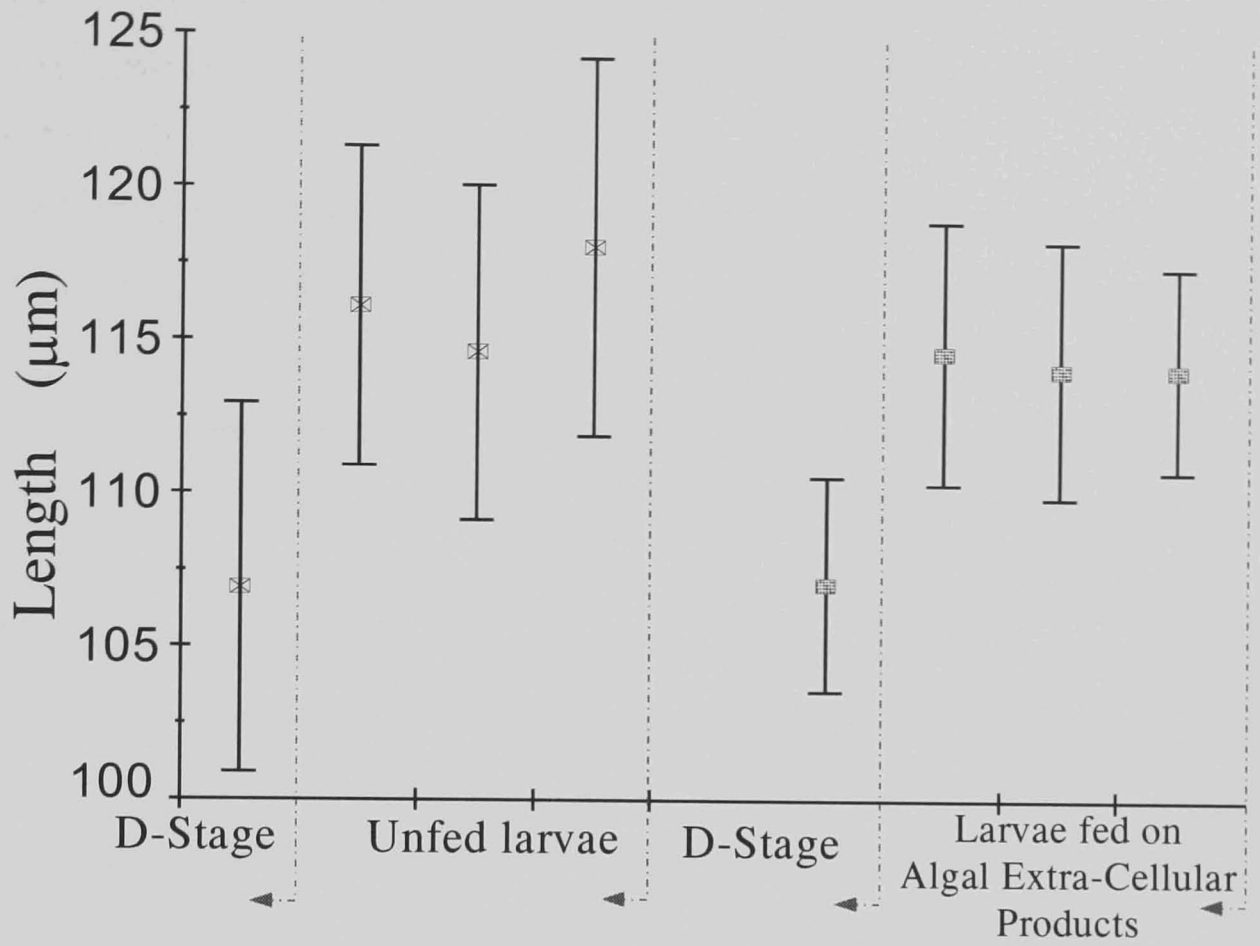

Figure 3.1.3. Growth of unfed larvae in relation to larvae fed on algal extracellular material, after the two – week feeding trial.

Survival rates of all three replicates for all the previously shown experiments are shown in Table 3.1.1.

Table 3.1.1. Survival rates (%) for all experiments. Columns correspond in couples with the growth results shown in fig. 3.1.1, 3.1.2 & 3.1.3, respectively. A.E.C.P.=**A**lgal **E**xtra **C**ellular **P**roducts.

	Unfed	Unfed with excess bacteria.	Control	Control with excess bacteria	Unfed	Unfed with A.E.C.P.
Beaker 1	36.7	68.0	70.7	58.7	30.0	87.2
Beaker 2	29.4	64.0	60.0	59.3	55.0	52.8
Beaker 3	35.6	67.3	74.7	73.3	38.9	90.0

The previous table shows that survival of *M. edulis* larvae varies randomly both within treatments and between different diets, proving to be a non reliable parameter of assessing the effect of various diets. In conjunction with preliminary experiments, not shown here, and with results that will be presented in later chapters, it appears that survival of larvae is influenced more by the condition of the given batch of larvae rather

than with anything else. Since consistent results cannot be obtained by examining the survival of the larvae, the following discussion will be based on the growth performance of the larvae; survival rates will help to assess the general condition of all batches of larvae used, and in some cases will add insight to the differentiated performance of some batches of larvae.

Although strictly axenic larval cultures were not obtained, additions of bacterial populations as "a food supplement" to xenic larval cultures is a valid way of investigating any potential effects of the bacteria on the larvae (Douillet & Langdon, 1994).

In the present experiments no differences emerged between the cultures with added bacteria and the ones without this addition, either when the larvae were left unfed or when algal food was also provided. Furthermore, in the repetition experiments the larvae in which bacteria were added died. In the light of these findings it is inevitable to conclude that *M. edulis* larvae, under the given experimental conditions, do not benefit by the presence of excessive bacteria populations; in fact the risk of introducing potentially harmful bacteria in the larval cultures far outweighs any potential nutritional benefit.

Bacteria have been suggested to play an important part in influencing larval culture success (Guillard 1957; Walne 1958 & 1966; Prieur, 1982) but historically, hatchery management practices aims at controlling opportunistic pathogens with a number of techniques like maintaining pathogen-free algal stocks, using sterile seawater (either by sterile filtration or UV radiation) for rearing the larvae, frequently changing the culture water, application of hygienic practices and disinfecting the equipment (Elston, 1984). It has also been observed that bacterial contamination is not only directly harmful to bivalve larvae but also cause particles to form clumps that are too large for ingestion (Masson, 1977).

The bacterial nutritional value however, is often more complicated to investigate and interpret. In bivalve larvae, it is mainly the recent work of Douillet (1991) that unequivocally found a beneficial bacterial effect on the larval cultures. Bacteria and yeasts have been used to partly substitute algal foods in larval and juvenile bivalve rearing (Coutteau *et al.*, 1994; Nell *et al.*, 1996; Brown *et al.*, 1996a) but in most cases, results are considered successful if the combined algal and bacteria/yeast diet produces as good growth and/or survival of the larvae as the algae alone diet (Epifanio, 1979a). The

rationale behind this is that by substituting the relatively expensive to produce algal diet with cheaper artificial diets, bivalve hatcheries can reduces the running costs of the whole operation (Laing, 1987). However, screening alternative sources for supplementing or substituting algal diets is more difficult largely because the relative food value of the bacteria/yeast diets are not closely correlated with gross chemical composition (Epifanio, 1979a) and thus relationship between most bacteria and yeast species with larvae appear to be very much species specific, thus rendering generalizations meaningless. Another aspect that hinders the wider use of bacteria and yeast diets for bivalve larval rearing is the fact that these organisms lack the Essential Fatty Acids (EFA's) like the 20:5ω3 and the 22:6ω3 (Brown *et al.*, 1996a), often associated with the good nutritional value of a diet.

On the use of Dissolved Organic Material (DOM) by the larvae, results indicate that for the relatively low volumes of algal cultures used to feed the animals, *M. edulis* larvae do not seem to be able to grow at any significant rate, by utilizing any dissolved organic material present. The present study does not dispute the fact that many (if not all) marine organisms can feed by "osmotic" nutrition (Pütter, 1909) as has been shown with radio active labeled organic material (Sorokin & Wyshkwarzev, 1973; Manahan & Crisp, 1983) and that, algal metabolites have an effect on growth, survival or feeding behavior of *M. edulis* larvae (Ward & Targett, 1989; Thompson *et al.*, 1994b). But in acknowledging the fact that most algal species, including *S. costatum*, will excrete organic material, like fatty acids, into the medium into which they are cultured, albeit in small quantities (Kattner & Brockmann, 1990), one has to eliminate any potential effect of this material on the growth and survival of the larvae, under the present experimental conditions.

Thus it has been shown that both the effects of bacteria and DOM in the present experimental design are minimal, the study can proceed into investigating the effects of algae of varying biochemical composition on the growth and survival of *M. edulis* larvae, confident that the displayed response is due to the consumption of the algal cells alone.

3. Results and Discussion

3.2 Effects of light intensity and nutrient limitation on the biochemical properties and nutritional value of *Skeletonema costatum*.

A summary of the effects of changes in nutrient concentration and light intensity on growth, dry weight, chlorophyll, protein and carbohydrate concentration of *S. costatum* are shown in Table 3.2.1.

Table 3.2.1. Responses of *S. costatum* in various conditions. **HL**=High light **LL**=Low light **f/2**=f/2 medium no nutrient limitation **P**=Phosphorus limited medium **N**=Nitrogen limited medium.

Condition	Generation time **G** in **h** (SD)	**Dry weight** pg/cell	**Protein** pg/cell	**Carbohydrate** pg/cell	**Chl A** (pg/cell)	**Chl C** (pg/cell)
HL f/2	21.6 (1.79)	209.48	4.62	1.22	0.237	0.115
HL P	25.8 (2.89)	216.60	6.09	2.71	0.351	0.266
HL N	19.7 (3.30)	262.86	4.89	1.79	0.222	0.159
LL f/2	108.9 (35.6)	66.55	6.43	2.07	0.238	0.133
LL P	122.9 (38.7)	120.70	14.43	5.27	0.211	0.194
LL N	96.5 (40.1)	193.92	3.95	1.06	0.197	0.149

S. costatum is a diatom which is well known for its ability to thrive in a wide range of environmental conditions. This is further confirmed here with the display of the small effects on generation time by nutrient limitation. There was no significant difference ($p > 0.05$, 95% level) between nutrient conditions, but on average the smaller generation time was shown under nitrogen limitation or no limitation, whereas phosphorus limitation seemed to slightly slow down the growth of this species. However, a greater effect was shown under different light conditions, with a substantial increase of **G** under low light. Growth was on average 5 times slower under low light conditions. In general, **G** was within the range reported by numerous other authors (Shifrin & Chisholm, 1981; Sánchez *et al.*, 1994; Blanchemain & Grizeau, 1996).

The per cell dry weight of this species was increased under nitrogen limitation and high light, but it is not clear why this happens. Nitrogen limitation was expected to affect protein concentration and this was again confirmed here. This effect was more obvious in the low light condition. Carbohydrate concentration increased under phosphorus limitation.

In general, the effects on the biochemical components analysed were more obvious in low light conditions.

The chemical analyses for the nutrient concentration of the inflow and the outflow media for *S. costatum* cultures are shown in Table 3.2.2.

Table 3.2.2. Nutrient concentrations of major macronutrients in **Inflow** and **Outflow** media in various conditions used for the *S. costatum* cultures. Figures indicate μgr-at/l. **HL**=High light **LL**=Low light **f/2**= f/2 medium no nutrient limitation **P**=Phosphorus limited medium **N**=Nitrogen limited medium.

Condition	Infl. $^{-3}PO_4$	Outfl. $^{-3}PO_4$	Infl. Si	Outfl. Si	Infl. $^{-1}NO_2$	Outfl. $^{-1}NO_2$	Infl. $^{-1}NO_3$	Outfl. $^{-1}NO_3$
HL f/2	26.26	1.40	252.96	325.16	5.63	0.59	259.3	234.84
HL P	10.35	0.83	297.33	47.43	5.59	9.04	254.55	212.43
HL N	26.21	1.33	81.34	71.02	3.61	0.09	188.52	18.12
LL f/2	26.26	6.30	252.96	263.99	5.63	4.71	250.8	232.27
LL P	10.35	0.59	297.33	331.19	5.59	4.29	254.55	245.49
LL N	26.21	13.73	81.34	565.68	3.61	3.86	188.52	119.80

These results indicate that for the nitrogen and phosphorus limited cultures the $^{-1}NO_3$ and $^{-3}PO_4$ showed limiting concentrations in their Outflow media respectively. Total silicate concentration in the Outflow media was not limiting in all cases, verifying that their assigned limiting nutrient was indeed such. The superficial inconsistency in the silicate concentration can be explained by the fact that the culture vessel was of borosilicate glass which has been known for leaching silicate into the medium over a period of time. Additionally the method for measuring silicate is based on measuring the "reactive" silicate which is different from total silicate contained in the medium. The ratio of reactive/total silicate can change under various conditions and over the duration of the storage of the medium (Strickland & Parsons, 1972).

The fatty acid profile of this species can be seen in Table 3.2.3, for all the different culture conditions.

Table 3.2.3 shows that the 14:0, 16:1ω7, 16:3 and 20:5ω3 being the most abundant fatty acids in this species with the last one being the most predominant. It further confirms the increased content of 16:2 and 16:3 fatty acids in diatoms in relation to other classes of algae (Siron *et al.*, 1989; Volkman *et al.*, 1989; Thompson *et al.*, 1992b). In general, it is also true that nutrient limitation had a more conservative effect on the change of individual fatty acids than light intensity. As far as the effect of culturing conditions on various fatty acids groups, it can be seen that, expect for the Phosphorus limited medium, the *S. costatum* cells decreased their ω:3 and PUFA content with decreasing light intensity. Due to non separation of the 18:0 and 18:1's fatty acids in the

Low Light f/2 condition (see section 2.3.c for more details on this matter) no general trend can be safely established for the total SaFA and MUFA. It is also noticeable that phosphorus limitation caused a considerable increase in the proportion of SaFA and a decrease in PUFA and ω:3 in high light, while this trend was inverted under low light conditions but in this case the change of the relative proportions of these FA groups was not as dramatic as in the high light intensity.

Table 3.2.3. Fatty acid composition of *S. costatum* under various culture conditions. Individual fatty acids (**FA**) are expressed as % of Total Identifiable Fatty Acids (**% TIFA**). **HL**=High light **LL**=Low light **f/2**= f/2 medium no nutrient limitation **P**=Phosphorus limited medium **N**=Nitrogen limited medium. **B.D.** = Below detection levels. **N.S.**=Individual peaks were not separated. Values are means of two samples. In the **LL f/2** case where the 18:0, 18:1^{a}9 and 18:1^{a}7 FA peaks were not separated the values indicate the % of TIFA of the sum of these fatty acids that were coeluded from the column. **SaFA** = Saturated FA. **MUFA**= Mono Unsaturated FA **PUFA** =Poly Unsaturated FA.

Fatty acid	HL f/2	HL P	HL N	LL f/2	LL P	LL N
14:0	11.59	35.62	14.92	6.57	11.05	8.46
15:0	1.09	0.96	3.04	0.77	1.92	1.69
16:0	8.25	5.19	9.10	11.57	12.98	15.91
16:1ω7	11.64	11.81	13.69	15.65	16.25	16.77
16:2	2.32	2.18	2.64	2.92	2.70	2.78
16:2	4.85	7.32	5.45	4.55	4.67	4.74
16:3	12.63	9.33	10.64	3.27	2.68	1.96
16:4	7.03	2.50	4.90	4.12	2.71	3.12
18:0	2.12	0.48	2.07	N.S.	3.95	3.95
18:1ω9	5.50	1.26	5.04	N.S.	4.65	5.89
18:1ω7	1.92	2.10	0.21	N.S.	5.29	4.72
18:0 +18:1ω9 +18:1ω7	10.05	3.84	7.82	19.02	23.38	19.39
18:2ω6	3.51	1.64	1.33	7.47	4.07	6.88
18:3ω3	1.22	0.48	0.54	2.42	1.26	1.12
18:4ω3	2.38	2.04	2.96	1.67	0.78	2.09
20:4ω6	4.21	B.D.	B.D.	B.D.	B.D.	B.D.
20:5ω3	19.66	15.14	19.85	15.66	12.52	12.65
22:6ω3	2.17	1.94	3.11	4.34	3.03	2.45
Total SaFA	23.05	42.25	29.13	⩾18.91	26.55	30.01
Total MUFA	19.06	29.33	18.94	⩾15.65	25.08	40.90
Total PUFA	59.98	32.73	51.42	43.50	48.37	37.79
Total ω:3 series FA	25.43	15.53	26.46	18.86	17.35	13.86

The growth of the larvae fed on *S. costatum* is shown in Fig. 3.2.1. Results show growth of larvae in each individual beaker. The repetition of this experiment with another batch of larvae can be seen in Fig. 3.2.2.

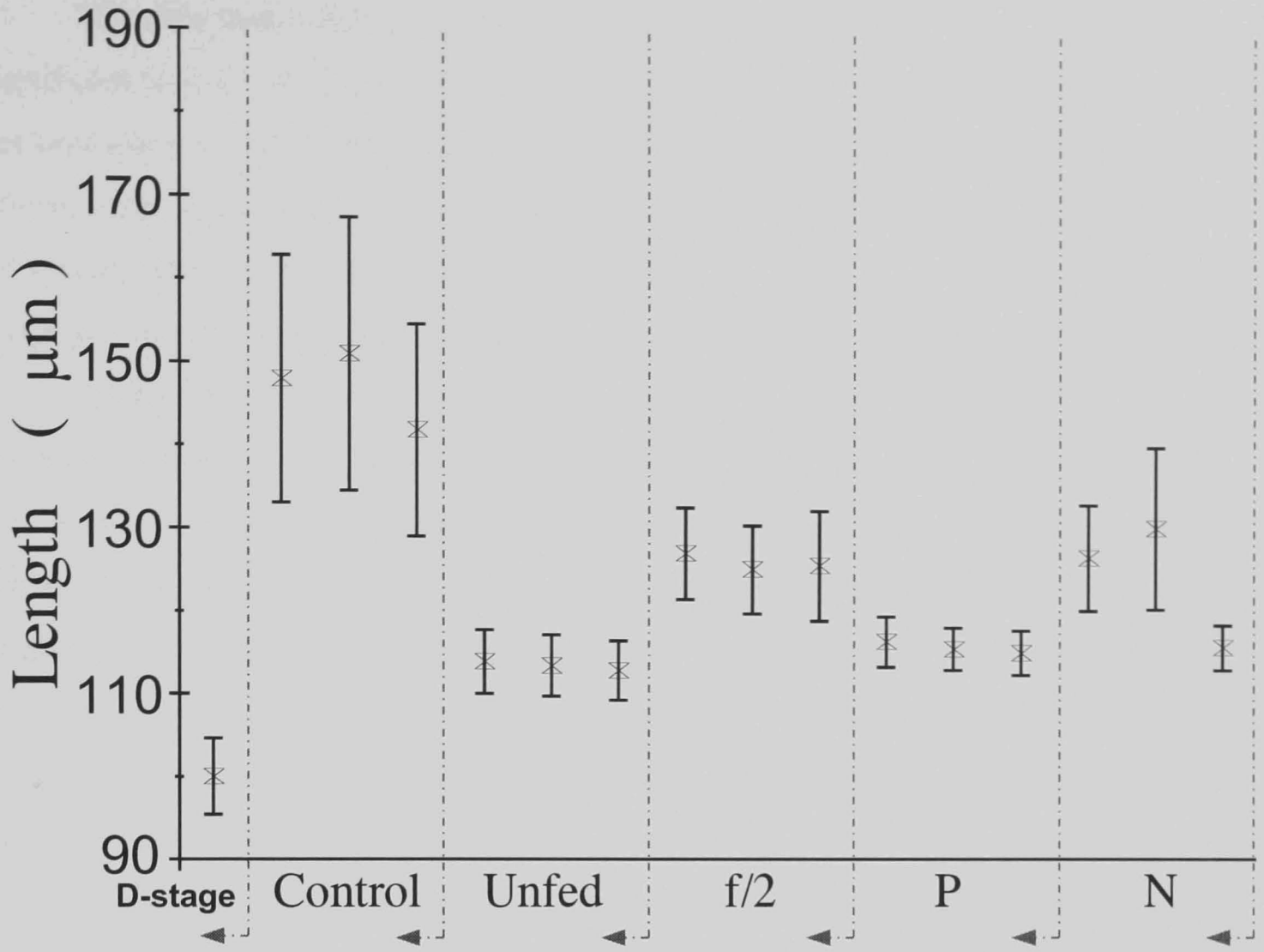

Figure 3.2.1. Size of *M. edulis* larvae fed on *S. costatum* cultured under high light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

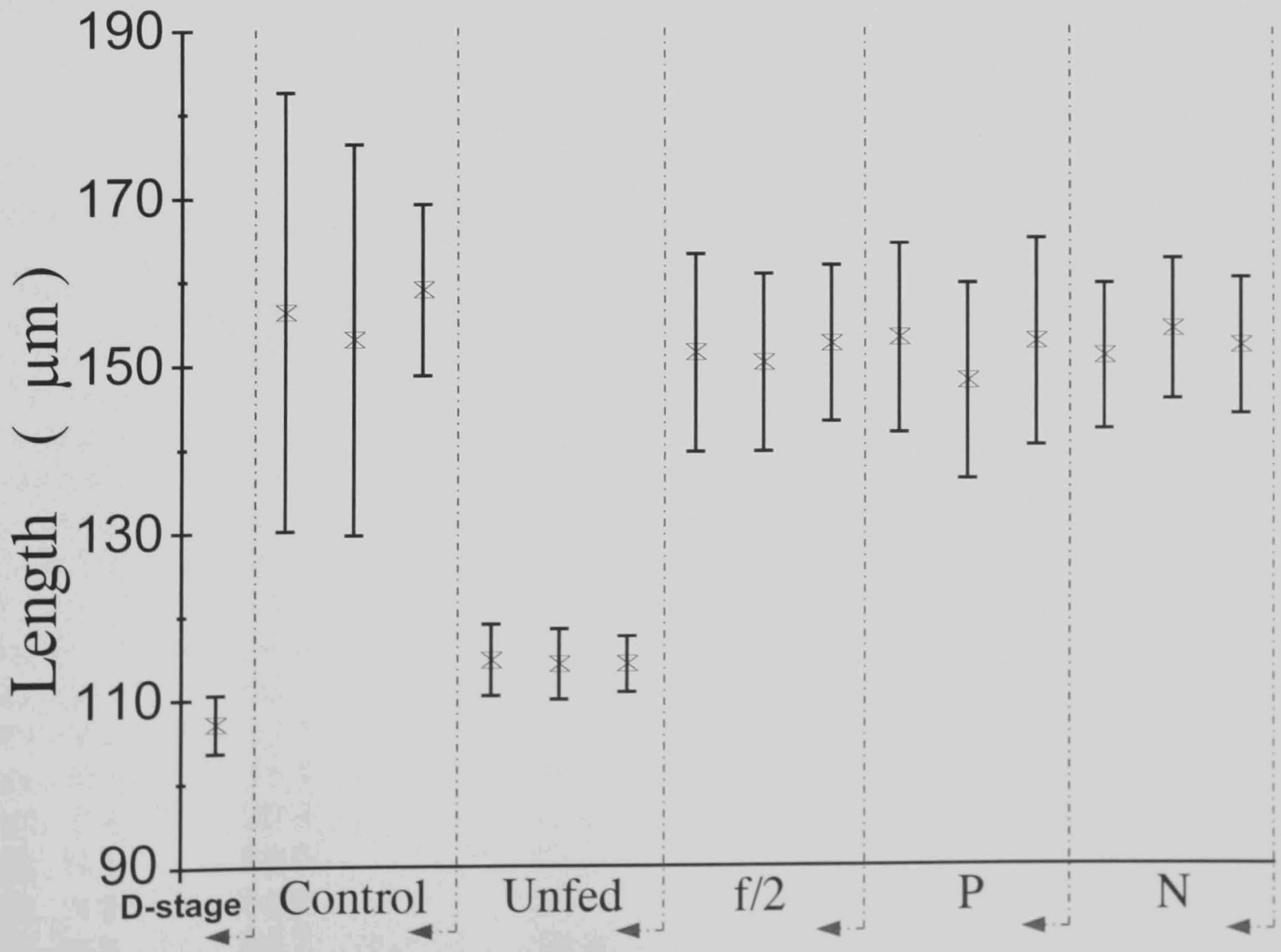

Figure 3.2.2. Size of *M. edulis* larvae fed on *S. costatum* cultured under high light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

It is clear that unfed larvae show minimal growth; this difference is obviously significant ($p < 0.01$, 95% Level). In order not to mask any other potential differences between diets the unfed larvae will not be included in the statistical analysis. The results of every test are given in detail in the Appendix. The number in the parenthesis given after every reference to a test here, will refer to a corresponding table in the Appendix. Variation among the triplicates of the same diet is within acceptable limits ($p > 0.05$).

In the first experiment, nested anova and multiple means test shows that all *S. costatum* diets perform significantly poorer that the control diet ($p < 0.05$, A. 3.2.1 & 3.2.2), and the same result is observed on the repetition of the same experiment with another batch of larvae (A.3.2.3 & A.3.2.4). The statistical analysis from the first experiment also suggests that the variation within triplicates was too high to make any comparisons between diets safe. However, in the repetition experiment exactly the same result was obtained with acceptable variation within triplicates (A.3.2.3) and therefore it can be concluded that under these conditions the *S. costatum* cells were of poorer nutritional value than the control diet. Although mean end length achieved after the two – week long feeding trial by the larvae fed on Phosphorus limited cells, are much smaller than the two other nutrient conditions, it is suggested that these differences are not significant (A.3.2.2 & 3.2.4)

Survival for each experiment after the two – week long feeding trial is shown in Table 3.2.4.

Table 3.2.4. % Survival of larvae fed *S. costatum* cultured in high light conditions

Diet	% Survival of larvae for the experiment of ***S. costatum* HL**	% Survival of larvae for the **repeat** experiment
Control 1	58.7	74.4
Control 2	59.3	92.8
Control 3	73.3	88.9
Unfed 1	68.0	87.2
Unfed 2	64.0	52.8
Unfed 3	67.3	90.0
HL f/2 1	59.3	98.9
HL f/2 2	56.0	91.1
HL f/2 3	60.7	81.1
HL P 1	28.7	91.7
HL P 2	13.3	93.3
HL P 3	20.0	77.2
HL N 1	66.0	87.2
HL N 2	64.0	86.1
HL N 3	32.7	84.4

As is the case with the growth results, survival results also showed that the first batch of larvae was not as robust as the second one. Both survival and growth were less good in the first batch, although the reasons for these differences cannot be qualified with this kind of work, many researchers in the field (Beaumont, pers. com.), believe that this is due to the quality of the

eggs obtained from the mussels that spawned.

Since there is no precise agreement in performance between different larval batches, conclusions concerning these diets would be rather unsafe.

However, it is noticeable that survival shows a wider variation, both among replicates as well as between diets, than growth. It does seem that survival is rather more determined by the genetic composition of a given batch of larvae rather than the diet on which they were fed and follows a random pattern.

It also becomes clear, that the diet concentration (50 cell/μl) was not limiting, since the batch that had greater survival rate also grew more rapidly. This discards the potential argument that if fewer larvae survive they should grow more since there would be more food available per live larva. This hypothesis can be further examined by means of a growth versus survival graph. If the former argument was true, the results should be positioned in relation to the X-axis, in a line with a negative slope (higher survival => smaller length, and, lower survival =>bigger length). If the pattern was random (no relationship between growth and survival) the data points should be distributed in an either vertical or horizontal fashion. This type of relationship for both experiments grouped together, is shown in Fig. 3.2.3.

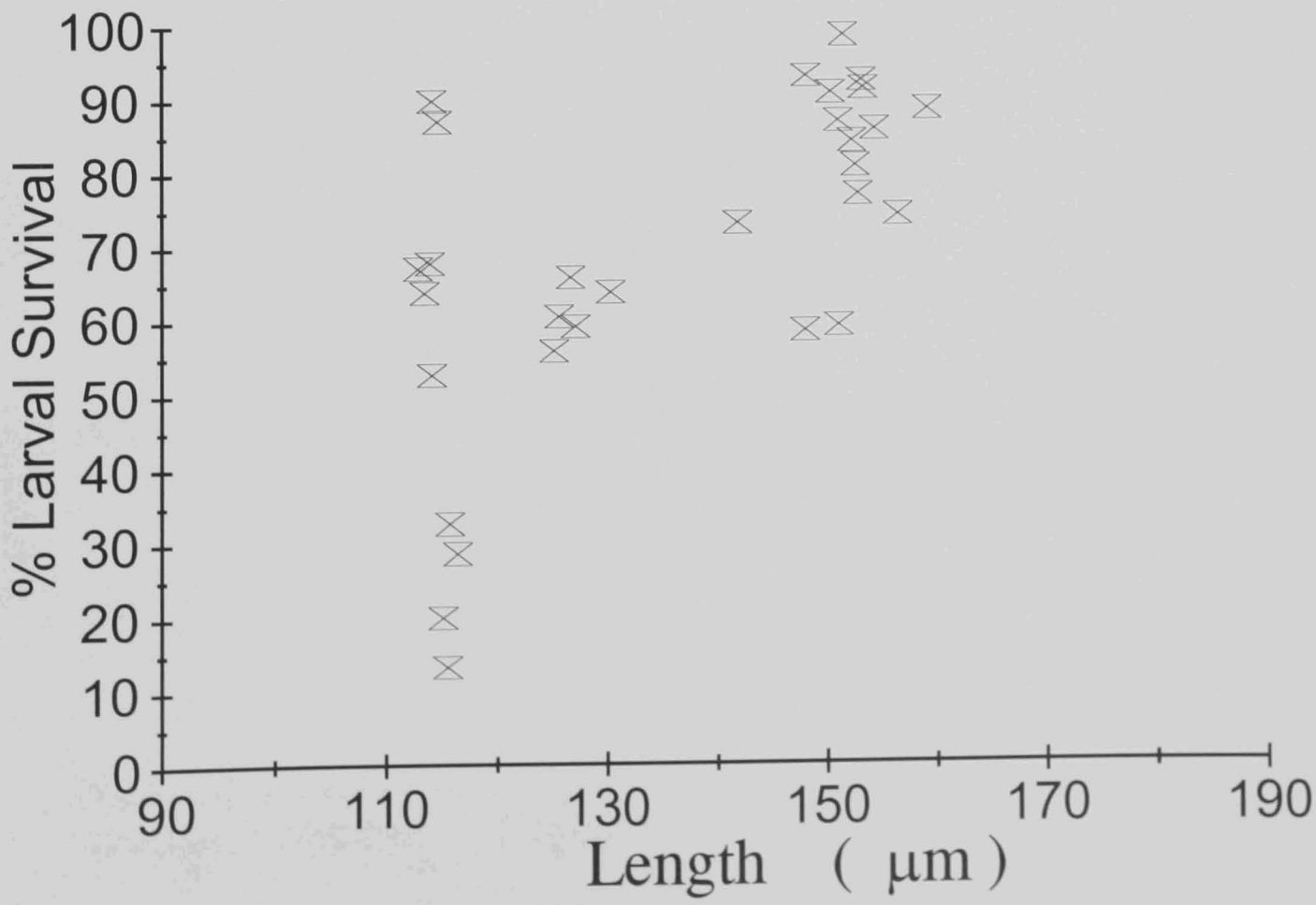

Figure 3.2.3. Length/Survival relationship for the larvae fed on *S. costatum* cultured under High Light conditions. Results shown include the first experiment and its repetition.

From Fig. 3.2.3., it becomes clear that there is no relationship between growth and survival. This graph clearly illustrates the hypothesis put forward when discussing the performance of the two batches of larvae, since it is further clarified that some larval cultures that grew poorly also suffered greater mortality, while on the other hand, larval cultures that performed well in survival rates terms, also grew very rapidly too. However, as stressed before, the overall image remains that there is a random relationship between growth and survival.

Following are the results concerning the feeding trials using *S. costatum* cultured under Low Light conditions, which will be presented with a similar pattern as their high light counterparts. Examining the growth of the larvae of each individual beaker when they were fed *S. costatum* cultured in Low Light conditions, growth results are shown; the first experiment in Fig. 3.2.4 and the repetition of the same experiment, in Fig. 3.2.5.

The combination of nested ANOVA and analysis of means test for these specific culturing conditions that the diet was cultured under (A.3.2.5-3.2.8) suggest that, for the first experiment N-limited or no nutrient limiting conditions significantly improved the

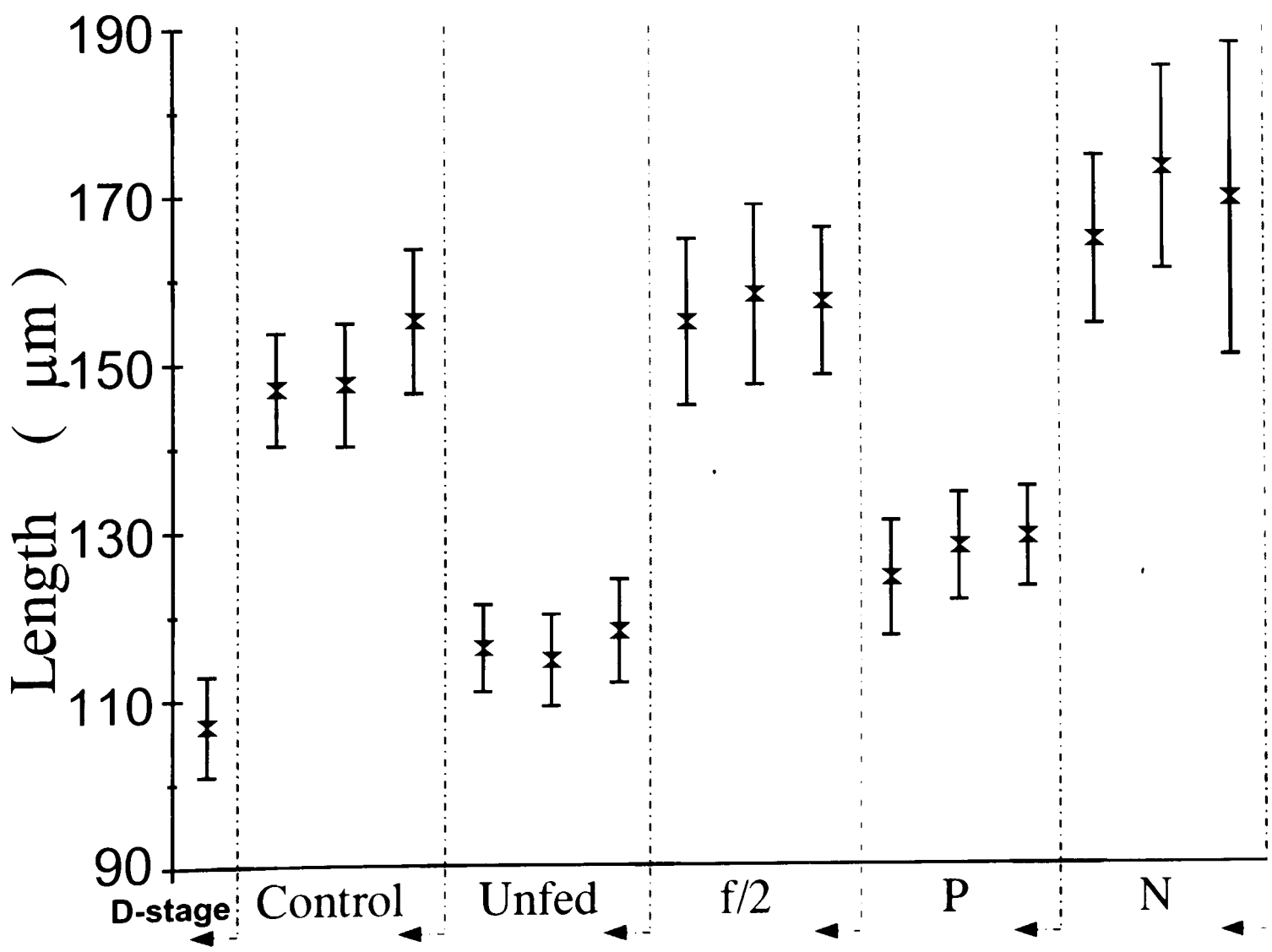

Figure 3.2.4. Size of *M. edulis* larvae fed on *S. costatum* cultured under low light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

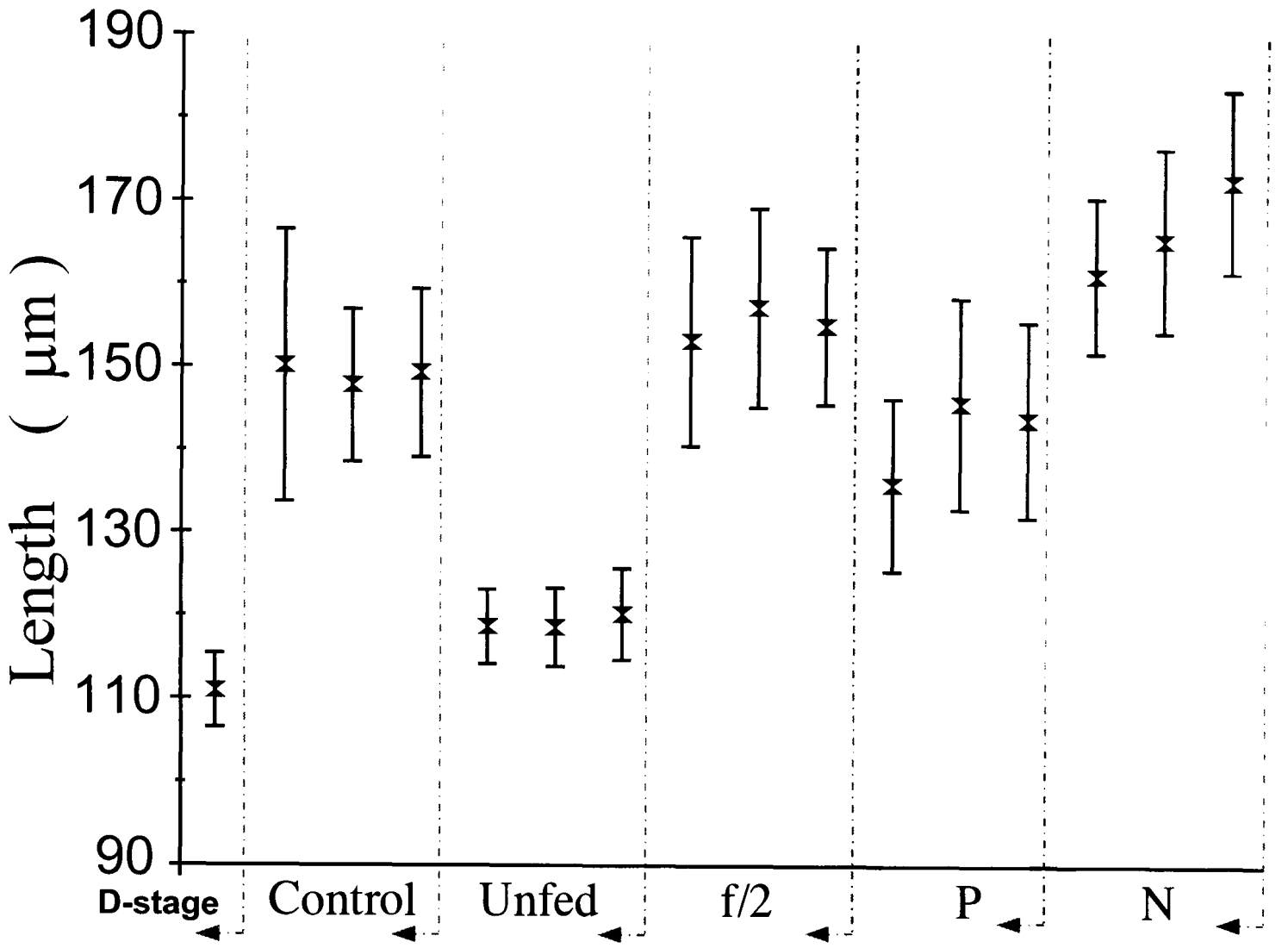

Figure 3.2.5. Size of *M. edulis* larvae fed on *S. costatum* cultured under low light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

nutritional value of the alga in comparison with phosphorus limited conditions or the control diet ($p < 0.05$, A.3.2.5 & 3.2.6). Moreover, the phosphorus limited cells were significantly inferior than the control diet too.

In the repetition experiment, P-limited cells were no different than the control diet (A.3.2.8) but, both of them were inferior than the N-limited and the no nutrient - limited diets (f/2 medium). Additionally, the nitrogen limited cells produced significantly bigger larvae than the non limited cells (A. 3.2.7 & A.3.2.8).

In relation to the control diet, N-limited *S. costatum* cells were consistently of better nutritional value, therefore it can be concluded that nitrogen limited cells cultured under Low light conditions have a better nutritional value than either phosphorus limited or no nutrient limited cells. Although from the statistical tables A.3.2.5 & 3.2.7 the homogeneity of the triplicates is questionable, both the mean end length results together with the consistency of the overall trend between experiments, gives sufficient logical ground to rank the diets, with decreasing nutritional value from N-limited to the non-limited medium grown cells, to the P-limited cells.

Survival rates, for the trials using as a food source *S. costatum* cultured under low light conditions, are shown in Table 3.2.5.

Table 3.2.5 % Survival of larvae fed *S. costatum* cultured in low light conditions

Diet	% Survival of larvae for the experiment of ***S. costatum* LL**	% Survival of larvae for the **repeat** experiment
Control 1	47.2	60.0
Control 2	44.4	53.9
Control 3	35.6	58.9
Unfed 1	30.0	15.0
Unfed 2	55.0	15.6
Unfed 3	38.9	14.4
LL f/2 1	28.9	23.9
LL f/2 2	38.9	44.4
LL f/2 3	30.6	40.0
LL P 1	18.3	31.1
LL P 2	18.9	33.9
LL P 3	17.2	35.0
LL N 1	28.9	46.7
LL N 2	15.0	40.6
LL N 3	12.2	31.7

Again, survival showed a random pattern and does not seem to be correlated with the diet upon which the larvae were fed. For example the greatest survival rate on the first experiment was demonstrated by the 2nd unfed culture, while on the repetition experiment unfed larvae had very poor survival rates.

To investigate whether a growth/survival relationship exists we plotted the length and survival data against each other. Results are shown in Fig. 3.2.6.

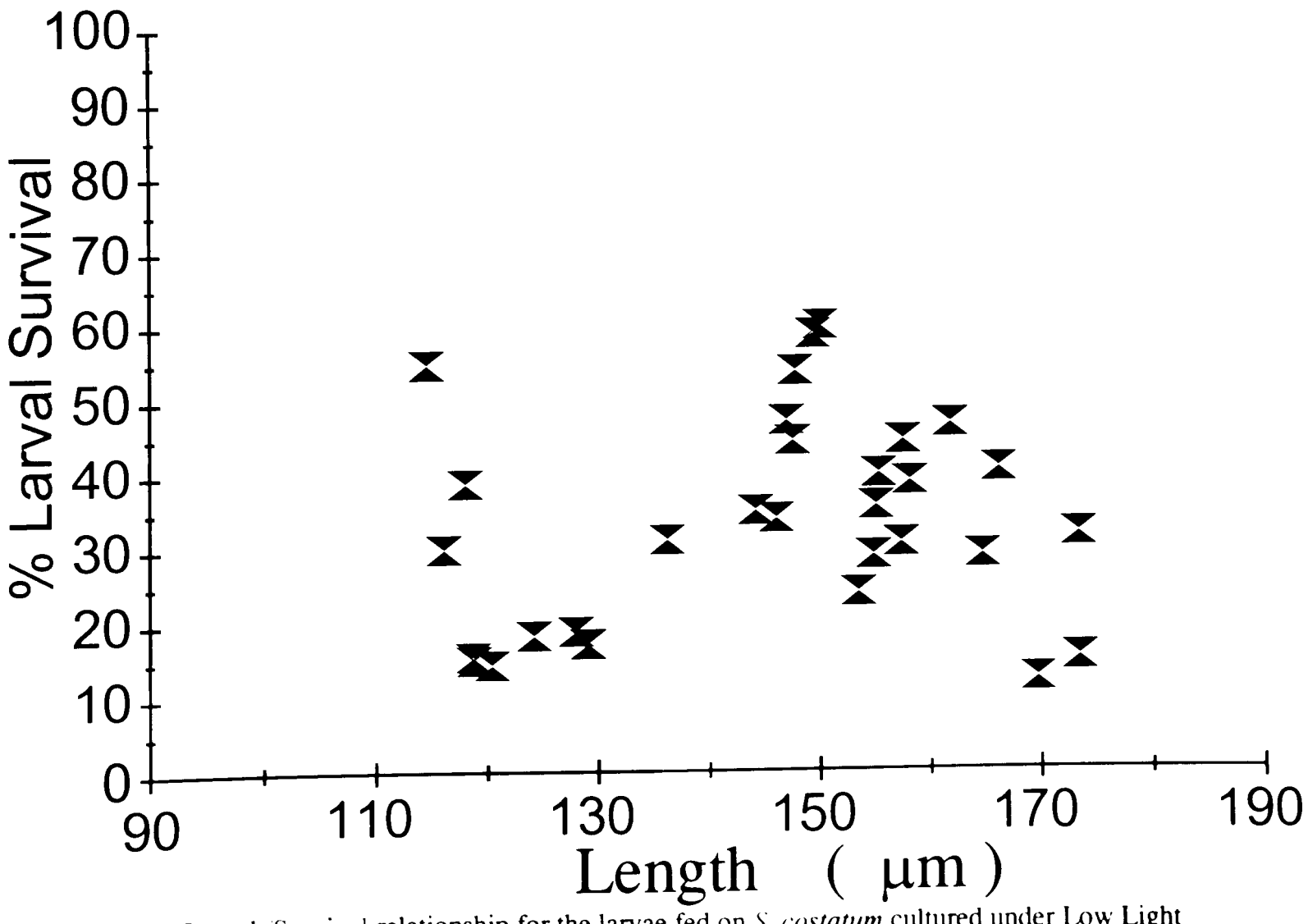

Figure 3.2.6. Length/Survival relationship for the larvae fed on *S. costatum* cultured under Low Light conditions. Results shown include the first experiment and its repetition.

The result illustrates graphically that there is apparently a random relationship between growth and survival. However, there is an added graphical illustration of the fact that generally, under the low light experiments the *M. edulis* larvae suffered greater mortality rates than under the high light experiments. Due to the lack of any consistent trend among or between these experiments and also due to the fact that the corresponding control diet larvae, between the high and low light experiments, perform differently in terms of their survival rate, suggests that this observed trend is probably due the quality of the larvae and hence the overall relationship between survival rate and diet, under the specific conditions used in these experiments, is of random nature.

The larvae from the repetition of the High light experiment as well as the from the first Low Light experiment were analysed for their fatty acid content. Larvae from 2 beakers were processed, individually. Because the larvae were from different batches, the respective control and unfed larvae were analysed individually. Results are summarised in Table 3.2.7.

Table 3.2.7. Fatty acid content of *M. edulis* larvae obtained during the *S. costatum* experiments. Individual FA are expressed as % of Total Identifiable Fatty Acids (**% TIFA**). Values are mean of two samples analysed. Numbers with asterisks (*) on some fatty acids indicate that although the fatty acids (**FA**) was identified in both samples in one of them, it was not integrated; therefore for the respective FA the value is taken from the sample that the FA was integrated. **N.S.**=FAs not separated. **B.D.**=FAs below detection level. **SaFA** = Saturated FA. **MUFA**=Mono Unsaturated FA **PUFA** =Poly Unsaturated FA.

	Control	Unfed	f/2	P	N	Control	Unfed	f/2	P	N
Fatty Acid	*Larvae obtained after the **H.L.** experiment*					*Larvae obtained after the **L.L.** experiment*				
14:0	6.31	5.76	2.96	2.32	3.44	3.78	4.93	6.01	8.27	4.75
15:0	4.36	4.41	2.07	1.46	1.08	4.02	4.92	3.73	5.20	3.98
16:0	38.23	33.12	23.78	12.96	18.69	32.42	36.61	26.50	34.25	30.61
16:1ω7	4.73	8.69	5.39	2.94	6.09	10.70	6.83	10.94	10.03	7.92
18:0	12.21	10.63	11.67	6.41	7.69	9.47	11.67	10.60	11.03	12.96
18:1ω9	4.91	6.94	4.97	N.S.	6.82	10.85	7.97	11.20	10.01	11.25
18:1ω7	3.61	3.01	4.06	N.S.	3.42	2.65	1.08	6.07	2.88	2.75
18:1ω9&ω7	8.51	9.95	9.03	5.76	10.24	13.50	9.05	17.27	12.89	13.99
18:2ω6	0.96	1.52	1.82	0.84	2.19	1.62	0.88	1.88	1.21	1.32
18:3ω3	0.69*	0.53	0.40	0.45	0.83	0.54	1.64	0.13	B.D.	B.D.
18:4ω3	0.55*	0.51	0.90	0.86	2.50	0.24	0.12	0.34	B.D.	0.21
20:1	2.85	0.86	2.38	1.62	4.97	1.06	3.92	2.04	1.38	2.21
20:1	5.00	3.33	5.20	3.51	7.96	1.91	1.23	1.95	1.22	1.05
20:4(ω6?)	2.30	1.80	2.04	1.04	2.77	2.24	3.64	1.21	0.89	1.49
22:0	5.22	4.32	3.76	33.44	2.49	2.92	5.08	3.82	4.50	5.69
20:5ω3	0.73	3.77	13.38	15.89	14.12	4.43	0.93	6.15	1.59	3.78
22:1ω11	3.29*	1.06	1.00	0.57	1.31	1.23	2.14	0.92*	1.56	0.91*
22:5ω3	0.25	0.93	1.33	1.76	1.40	0.81	0.68	0.87	0.96	1.83
22:6ω3	6.08	8.82	12.90	8.16	12.25	9.13	6.62	6.11	5.02	7.76
***Total* SaFA**	66.33	58.24	44.24	56.60	33.40	52.60	63.20	50.66	63.25	57.99
***Total* MUFA**	24.38	23.89	23.00	14.40	30.55	28.40	23.16	33.11	27.08	26.08
***Total* PUFA**	11.56	17.87	32.76	29.00	36.06	19.00	13.64	16.68	9.66	16.39
***Total* ω:3 FA**	8.30	14.56	28.90	27.12	31.09	15.14	9.11	13.59	7.56	13.58

These data suggest that there is a relationship between certain larval fatty acids or fatty acid groups and the corresponding growth of the animals. However, this specific subject will be the main focus of the analysis in a later section of chapter 4 (section 4.1). Therefore, the use of individual F.A. as indices of growth in *M. edulis* larvae, and other related topics, will be discussed there.

As a diet for *M. edulis* larvae, *S. costatum* appears to be an adequate food source. In most cases larvae fed on this diatom grew as well as the control diet which was a mixture of two species. However, in the first experiment in HL condition the larvae did not perform very well with a significantly slower growth rate. The LL results were in very good agreement with each other. It is worth looking at the results after pooling the data from respective experiments together. Preliminary experiments using batch cultures of *S. costatum* (Leonardos, unpubl.) have showed that over a two week period larvae fed *on S. costatum* grown under different nutrient concentration, in the same HL conditions as the ones used for the Continuous cultures, grew up to 150 μm in length. Therefore, it is safer to exclude the results of the first experiment from the analysis on the basis that this batch of larvae was of poor quality. The averaged results of three replicates, for both experiments in LL conditions and the one experiment in HL conditions are shown in Fig. 3.2.7.

S. costatum, as shown in Tables 3.2.1. & 3.2.3 will change its growth rate and biochemical composition, to adapt to environmental conditions. Subsequently, its overall nutritional quality for *M. edulis* larvae has been also demonstrated to change. **P**-limitation in **LL** condition is significantly worse that its **HL** counterpart, while the opposite is true for **N**-limitation. From Figure 3.2.7, it becomes clear that the various nutrient effects do not change the nutritional value of *S. costatum*, when the alga is cultured under high light conditions; this is in clear agreement with results presented in Table 3.2.1 where it has been verified that the biochemical properties studied were found not to change considerably under these high light conditions. On the other hand both the biochemical properties of this alga and its growth rate were found to exhibit a wider change under low light conditions and this was also observed by the performance of the larvae fed on these cells. Consequently, the widely accepted assumption that the nutritional quality of the food is largely depended on its biochemical composition is verified to hold true.

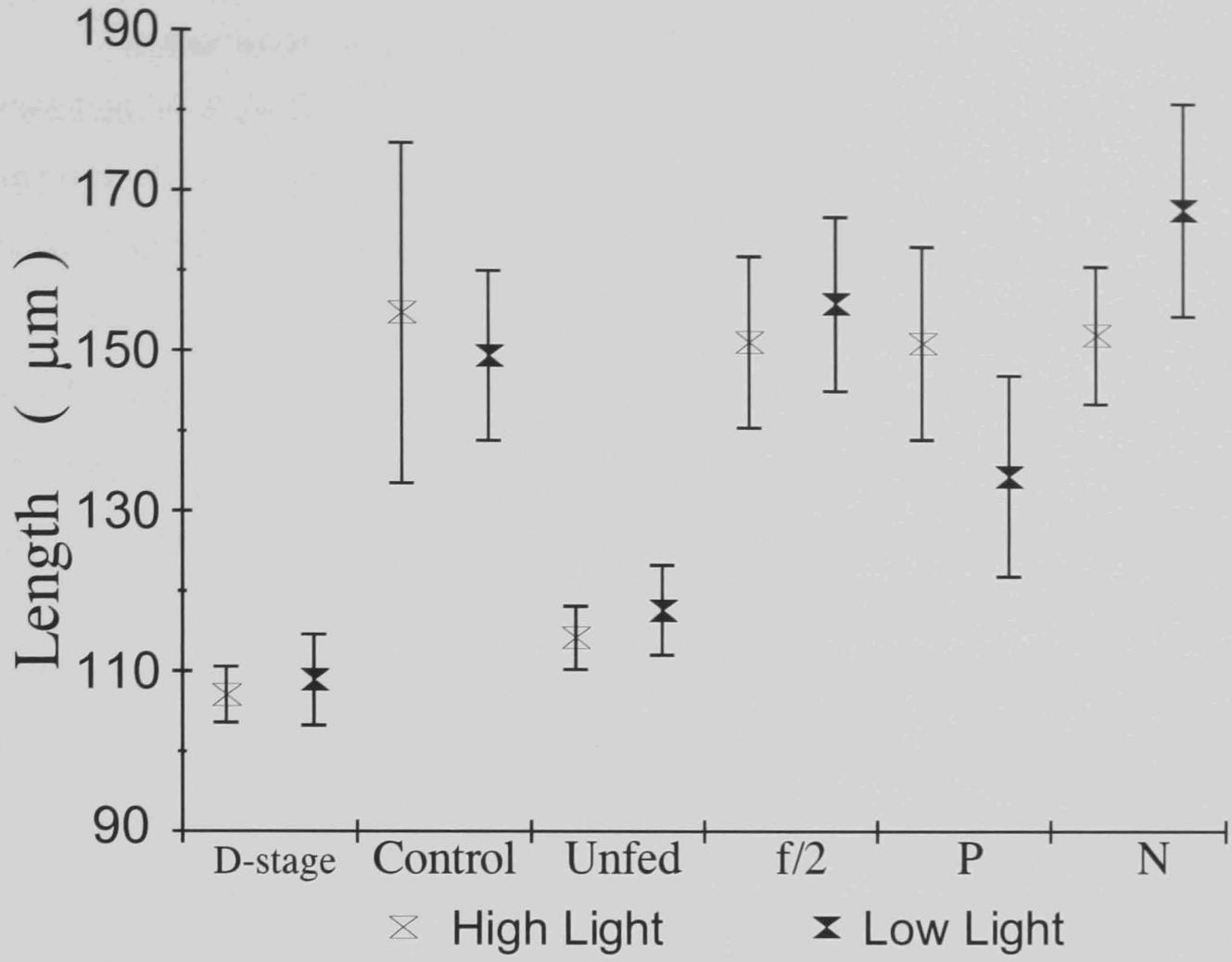

Figure 3.2.7. Averaged results for both LL experiments and the second HL experiment, of *M. edulis* larvae fed on *S. costatum* of various nutrient and light conditions, after two weeks of feeding.

It is difficult to identify which change of which individual component determines the change of the nutritional value of the alga. It would seem reasonable to assume that it will be the combination of the change of various biochemical components that will exercise the overall effect.

However, on more specific components, among the ones analysed here, it is widely accepted that overall variation in Fatty Acid content is responsible for the variation in the nutritional value of any cultured species (Blanchemain & Grizeau, 1996, Sánchez *et al.*, 1993; Volkman *et al.*, 1989). But there is not precise agreement on the role of individual, or groups of, FA's. For example, Webb & Chu (1983), point out that the ω:3 series are essential for spat development whereas the ω:6 family aided the metamorphosis of oyster larvae. Others use the proportion of the eicosapentaenoic acid (20:5ω3) as the basis of evaluation of dietary value (Ackman *et al.*, 1964; Blanchemain & Grizeau, 1996; Sánchez *et al.*, 1994; Su *et al.*, 1988) or more generally the proportion of HUFA (Enright *et al.*, 1986b; O'Connor *et al.*, 1992). *S. costatum*, has a relatively high proportion of PUFA's and eicosapentaenoic acid and from that it is expected that its dietary value is increased. However, increased eicosapentaenoic acid and PUFA's, do not seem to result in faster growing larvae. In fact the opposite is true for **LL**, **N**-limitation.

Another aspect that could be examined from the present type of data is the resemblance of the fatty acid of the *M. edulis* larvae, and its food, since these biochemical components were analysed in both organisms. Concentrating on the fatty acids comprising the biggest proportion of Total Identifiable Fatty Acids, like the 14:0, 16:0, 20:5ω3 & 22:6ω3, it is noticeable that the changes of their relative proportions in the diet does not always result in similar trend of changes in the grazer. There is a mixed set of reactions; for example, increase of the proportion of the 16:0 FA under N-limitation of *S. costatum* under HL does not lead do an increase of this FA of the larvae fed on this alga. Under LL the changes of the 16:0 FA proportion are followed by a similar change in the larval cells. The "similarity in trend" relationship of relative FA proportions exist for the 20:5ω3. The proportion of this FA, among High Light culture conditions for *S. costatum*, shows a decrease under P-limitation, which is followed by another minimum by the larval 20:5ω3 fed on this alga, and under Low Light conditions the maximum peak of algal 20:5ω3 shown when *S. costatum* is grown under no limitation (f/2 medium) is equally followed by a relative peak of the same larval FA. Similar trends can also be noticed for the 22:6ω3.

This complex set of reactions is difficult to interpret since on one hand a relationship between the FA profiles of the organisms is bound to exist, on the other hand this relationship cannot yet be fully explained since the exact biochemical pathways of FA creation and conversion is not clearly understood. Many authors have repeatedly put forward the idea that in food and grazer FA profiles should be similar (Ackman *et al.*, 1964; Chuecas & Riley, 1969a) and although in some cases the amounts of certain fatty acids like the 18:2ω6, 18:3ω3 and 20:5ω3 of the grazer correlated well with their levels in their diet (e.g. Lavens *et al.*, 1989) in a number of others this was not observed (e.g. Navarro & Amat, 1992). These aspects of the discussion will be analysed in detail in the later chapter dealing with the correlation of various parameters (ch. 4.2 & 4.3).

The improvement of the food value of *S. costatum* for *M. edulis* larvae would seem to require **N**-limitation under **LL**, as optimal culture condition for this species. Under these conditions this diatom has an increased proportion of the saturated 16:0 FA and a reduced proportion of PUFA's. It also has the lowest amounts of protein and carbohydrate per cell of all the conditions used.

3. Results and Discussion

3.3 Effects of light intensity and nutrient limitation on the biochemical properties and nutritional value of *Chaetoceros muelleri*

A summary of the effect of nutrient concentration and light intensity on growth, dry weight, chlorophyll, protein and carbohydrate concentration is shown in Table 3.3.1.

Table 3.3.1. Responses of *C. muelleri* to various conditions. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Condition	Generation time **G** in **h** (SD)	**Dry weight** pg/cell	**Protein** pg/cell	**Carbohydrate** pg/cell	**Chl A** (pg/cell)	**Chl C** (pg/cell)
HL f/2	34.0 (3.59)	314.12	9.16	3.19	0.267	0.237
HL P	67.4 (6.07)	217.19	8.57	5.60	0.246	0.199
HL N	47.3 (2.43)	422.84	7.19	4.85	0.246	0.242
LL f/2	98.8 (21.97)	265.58	12.51	3.20	0.616	0.453
LL P	139.9 (14.62)	246.73	10.48	2.61	0.334	0.311
LL N	109.3 (18.66)	426.77	10.32	2.88	0.450	0.425

This diatom, *C. muelleri*, responded to both nutrient and light limitation by increasing its generation time. Low light conditions increase its generation time by a factor of approximately 2.5 (for each respective nutrient condition). When nutrient limitation was taken into account, faster division rate (smaller **G**) was demonstrated, in the non-limiting medium (f/2) as expected, with a slight decrease in N-limiting medium, while the slowest division rate (greatest **G**) was experienced under P-limitation. This pattern was the same for both low and high light. There was no significant difference ($p > 0.05$, 95% level) between f/2 and N-limited nutrient conditions in both light conditions, while P-limited **G** was significantly greater ($p < 0.05$, 95% level) than the previous two, again in both light conditions. In many documented cases in the literature (Blinn, 1984; Nelson *et al.*, 1992; Reitan *et al.*, 1994) it has been shown that in general, *Chaetoceros* species are more affected than diatoms such as *Skeletonema costatum* by varying environmental conditions. In a paper more specific to *C. muelleri* which describes

the physiological variability within ten strains of this diatom, Johansen *et al.* (1990) found that growth rate (at temperatures and in media different from the ones used here) varied from 1.4 to 4.0 doublings per day. Blinn (1984) reported growth rates for one strain of *C. muelleri*, from >1.5 to 0.5 division per day. The optimum temperature was shown to be at 25 °C, which was the temperature used in the present work.

The per cell dry weight of this species increased under nitrogen limitation in both light conditions, while there is a small decrease in P-limited cells. Although it not very clear why this happens, it could be that under nitrogen stress the increase in organic material was produced in an effort to increase the internal energy reserves, especially as nitrogen limitation was expected to affect protein concentration. This was confirmed by the protein analysis which showed a decrease of per cell protein in N-limited cultures.

Carbohydrate concentration demonstrated an apparently random variation between nutrient limitations and light conditions, with no consistent trend established between nutrient limitation, although the per cell carbohydrate content appeared to decrease under the low light conditions.

The cells increased the amount of Chlorophyll a and c when cultured in low light conditions. This is obviously due to the fact that when cultured under low light the cell need to have more chlorophyll to make more efficient use of the ambient light.

The chemical analyses for the nutrient concentration of inflow and outflow media for *C. muelleri* cultures are shown in Table 3.3.2.

Table 3.3.2. Nutrient concentrations of major macronutrients in Inflow (**Infl.**) and Outflow (**Outfl.**) media in various conditions used for the *C. muelleri* cultures. Figures indicate μgr-at/l. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Condition	Infl. $^{-3}PO_4$	Outfl. $^{-3}PO_4$	Infl. $^{-1}NO_2$	Outfl. $^{-1}NO_2$	Infl. $^{-1}NO_3$	Outfl. $^{-1}NO_3$
HL f/2	26.26	5.26	5.63	4.23	259.3	236.27
HL P	10.35	1.19	5.59	5.22	254.55	232.88
HL N	26.21	5.09	3.61	0.05	188.52	6.64
LL f/2	26.26	11.68	5.63	2.25	250.8	236.84
LL P	10.35	0.50	5.59	1.83	254.55	237.25
LL N	26.21	14.44	3.61	1.15	188.52	21.29

Nutrient concentrations were lower in outflow media, since the cells utilise the inflown material to biosynthesise various components. In N-limited cultures the amount of $^{-1}NO_3$ present in the outflow medium was minimal while the other macronutrients were in much greater quantities. Accordingly, $^{-3}PO_4$ concentration in the outflow medium was

minimal in the P-limited cultures. These patterns indicate that, indeed, the assigned macronutrient was the limiting factors for each respective medium.

This diatom responds to changes of environmental conditions more obviously than the other diatom, *S. costatum*, does. The protein content is influenced more by nitrogen limitation among the high light conditions, while under low light there is a maximum concentration peak appearing when the species is cultured with no limiting nutrient (**f/2** medium).

The fatty acid profile of this species can be seen in Table 3.3.3, for all different culturing conditions.

Table 3.3.3. Fatty acid composition of *C. muelleri* under various culturing conditions. Individual fatty acids are expressed as % of Total Identifiable Fatty Acids (**TIFA**). **B.D.**=Below detection levels. **N.I.**= Individual peaks were not integrated properly. Values are means of two samples, except for LL P & LL N, where values are from one sample, due to loss of the other sample during analysis. (*) indicate that the value is taken from one sample because the Fatty Acid peak was not integrated properly in one of the samples. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Fatty acid	HL f/2	HL P	HL N	LL f/2	LL P	LL N
14:0	9.43	14.26	8.77	10.06	5.84	6.83
15:0	0.88 *	0.68	0.69	0.75	0.99	1.77
16:0	11.94	21.14	19.86	9.91	14.86	12.56
16:1ω7	17.29	26.89	26.65	16.49	17.56	16.16
16:2	4.41	2.37	3.27	4.70	4.12	3.66
16:2	4.68	2.65	3.25	3.78	3.58	2.41
16:3	11.49	5.22	7.83	12.64	10.83	6.68
18:0	2.16	1.93	2.18	3.01 *	4.85	4.02
18:1ω9	2.19	2.00 *	N.I.	N.I.	5.78	N.I.
18:1ω7	0.57	0.20 *	N.I.	N.I.	1.74	N.I.
18:1ω9 +18:1ω7	2.76	2.45	2.37	14.26	7.51	24.75
18:2ω6	1.06	2.22	1.21	5.77	10.53	5.13
18:3(ω6?)	0.29	2.31	1.46	0.12	0.32	0.92
18:3ω3	0.35	0.13 *	0.06 *	1.00	0.34	1.83
18:4ω3	0.92	1.46	1.31	0.62	0.21	0.45
20:4ω6	5.28	2.42	2.18	1.23	1.64	1.26
20:5ω3	23.58	11.38	16.76	15.66	15.33	10.17
22:5ω3	0.22 *	0.48	0.18 *	0.08	B.D.	B.D.
22:6ω3	3.82	2.08	2.08	1.42	1.46	1.41
Total Saturated F.A.	24.41	38.01	31.50	23.73	26.55	25.18
Total Monounsaturated F.A.	20.05	29.33	29.02	30.75	25.08	40.90
Total PolyUnsaturated F.A.	56.09	32.73	39.59	47.03	48.37	33.91
Total ω:3 series F.A.	28.88	15.53	20.39	18.78	17.35	13.86

The growth of the larvae fed on *C. muelleri* is shown in Fig. 3.3.1. Results show growth of larvae in each individual beaker. The repetition of this experiment with another batch of larvae can be seen in Fig. 3.3.2.

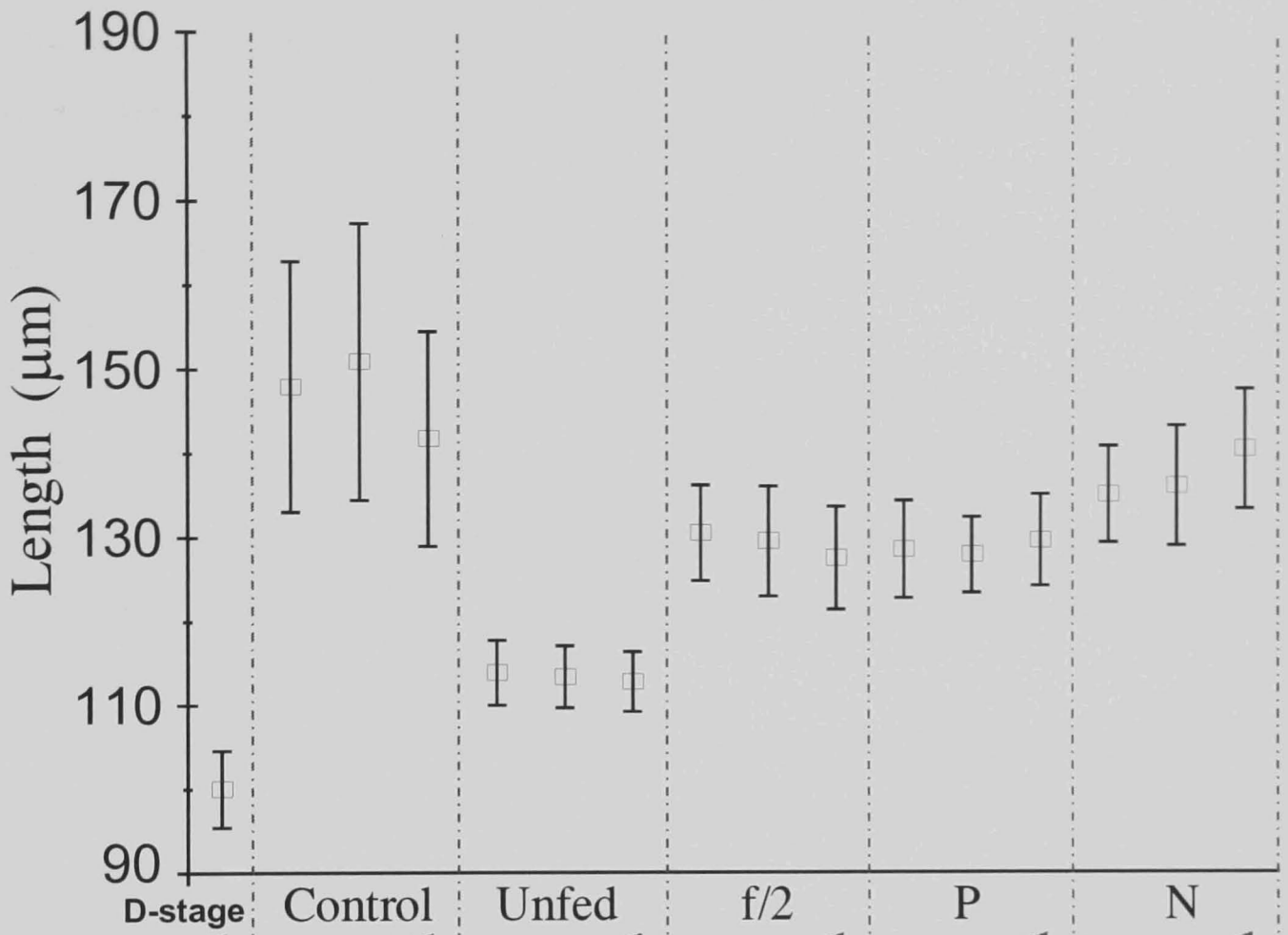

Figure 3.3.1. Size of *M. edulis* larvae fed on *C. muelleri* cultured under high light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

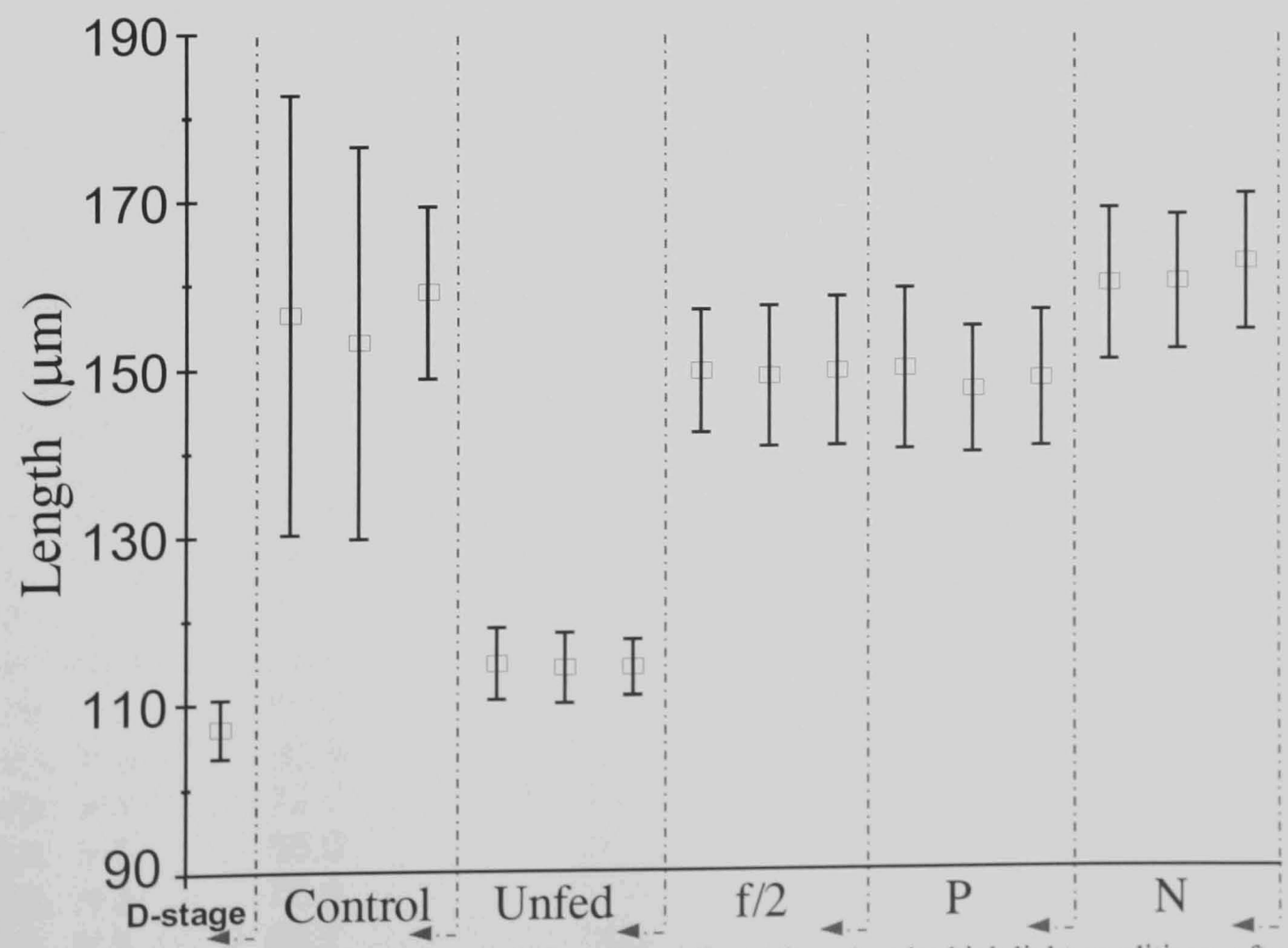

Figure 3.3.2. Size of *M. edulis* larvae fed on *C. muelleri* cultured under high light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

Again, as with the *S. costatum* experiments, it is clear that unfed larvae showed minimal growth; this difference in length between fed and unfed larvae is significant ($p < 0.05$, 95% level). As explained in the chapter describing the performance of the other diatom, in order not to mask any other potential differences between diets the unfed larvae will not be included in the statistical analysis. The results of every test are given in detail in the Appendix. The number in the parenthesis given after every reference to a test here, will refer to a corresponding Table in the Appendix section. Variation among the triplicates of the same diet is within acceptable limits for both experiments (A.3.3.1 & A.3.3.3).

In the first experiment, nested anova and multiple means test, show that all *C. muelleri* diets perform significantly poorer than the control diet ($p < 0.05$, A. 3.3.1 & A.3.3.2). Between the *C. muelleri* diets, N-limited conditions produce a diet that is significantly better than the phosphorus limited or the f/2 conditions. Between the latter two, there are no significant differences.

On the repetition of the same experiment, N-limited cells are similar to the control diet, while both these diets are significantly better than cell grown in f/2 or P-limited media ($p < 0.05$, A. 3.3.3 & A.3.3.4).

Collectively, the statistical analysis reveals that for both experiments, nitrogen limited cells are consistently the best diet among the three different conditions that *C. muelleri* were cultured under. For both experiments f/2 and P-limited diets do not have different effects on the larvae (A. 3.3.2 & A.3.3.4).

The variation between triplicate cultures was found to be statistically acceptable, thus strengthening the above conclusions.

Survival for each experiment is shown in Table 3.3.4.

As it is the case with the growth results, survival results also show that the first batch of larvae

Table 3.3.4. % Survival of larvae fed *C. muelleri* cultured in high light conditions

Diet	% Survival of larvae for the experiment of *C. muelleri* HL	% Survival of larvae for the **repeat** experiment
Control 1	58.7	74.4
Control 2	59.3	92.8
Control 3	73.3	88.9
Unfed 1	68.0	87.2
Unfed 2	64.0	52.8
Unfed 3	67.3	90.0
HL f/2 1	58.7	82.8
HL f/2 2	71.3	84.4
HL f/2 3	80.7	93.3
HL P 1	66.7	73.3
HL P 2	65.3	87.8
HL P 3	72.7	74.4
HL N 1	56.0	85.0
HL N 2	78.0	81.7
HL N 3	62.7	85.6

was not as robust as the second one. Both survival and growth were less good in the first batch.

This was also the case in the first *S. costatum* experiment. This is so because, in fact both the *S. costatum* and *C. muelleri* experiments were conducted with the same batches of larvae every time. Although, as stressed previously, the reasons for these differences cannot be quantified with this kind of work, researchers in the field (Beaumont, pers. com., Del Rio-Portilla, pers. com.), believe that this is due to the quality of the eggs obtained from the mussels that spawned. Quantifying the effect of parental function is very difficult since a number of parameters could potentially effect the quality of the eggs. Some of these factors have been reported in the wider research literature area for a variety of different organisms, from bivalve and fish eggs to *Artemia* sp. cysts, like duration of conditioning period of the broodstock, genetic variation and function of parental fatty acids furnished to the eggs (Trider & Castell, 1980a; Gallager & Mann, 1986a; Navarro & Amat, 1992).

Although in each experiment, the *C. muelleri* diets produced slightly different results in relation to the control diet, the same trend between these diets appeared in both experiments. N-limited cells produce bigger larvae for the same feeding period than the f/2 or the P-limited cells. Between the latter two there is no significant difference.

In addition, it is noticeable that survival showed a wider variation, both among replicates as well as between diets than, growth. It does seem that survival has more to do with the quality of the larvae rather than the diet on which they were fed on and follows a random pattern.

It also becomes clear, that the diet concentration (50 cell/µl) was not limiting, since the batch that had greater survival rate also grew more rapidly. This refutes the potential argument that if fewer larvae survive they should grow more since there would be more food available per live larva. As illustrated before, this relationship can be investigated in a growth / survival graph. This relationship for both experiments is shown in Fig. 3.3.3, which will be shown in the following page.

From Fig. 3.3.3, it becomes clear that there is no relationship between growth and survival. This can easily be observed in the graph, since there are all possible combinations of types of cultures: cultures with larvae that grew well which experienced

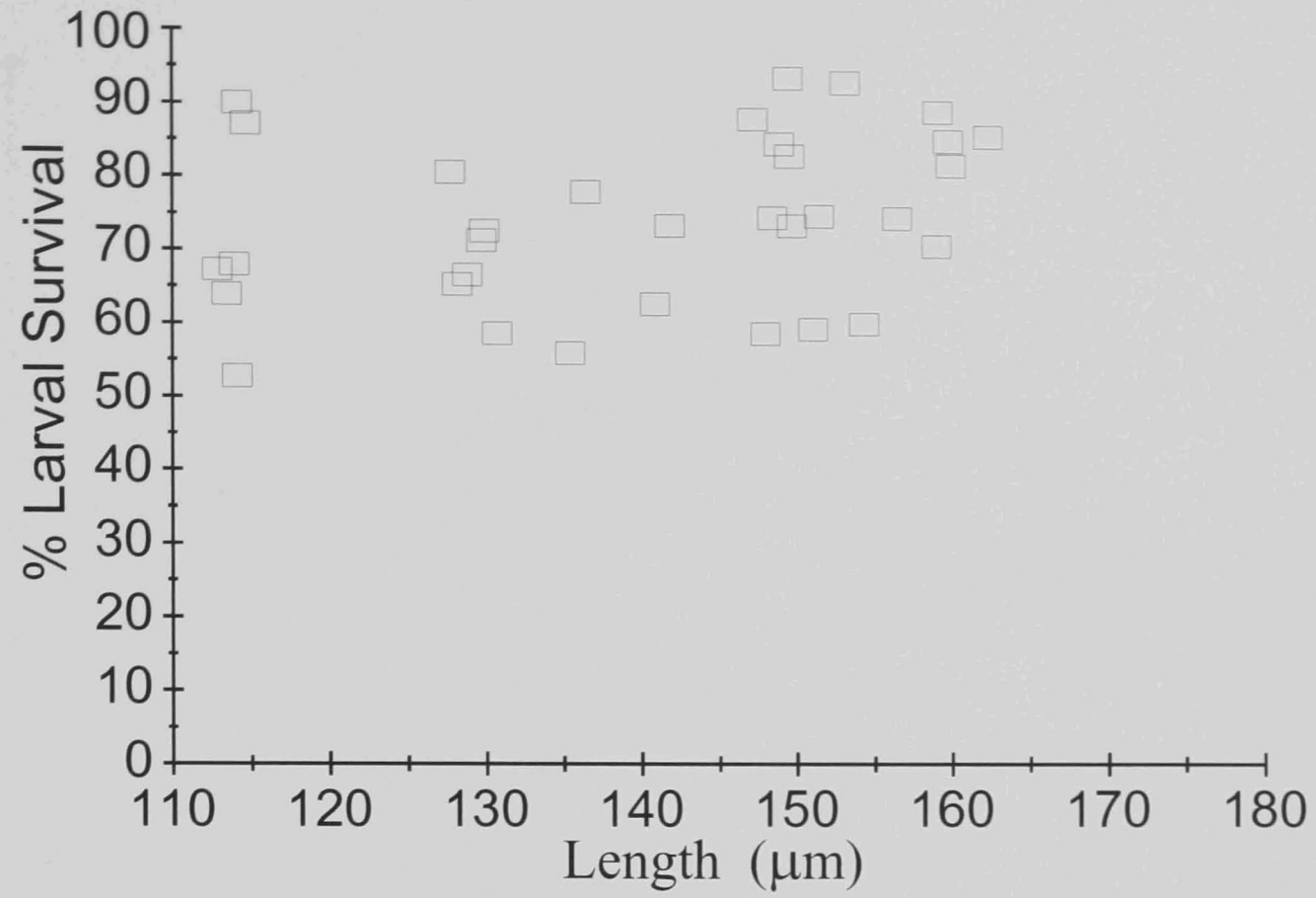

Figure 3.3.3. Length/Survival relationship for the larvae fed on *C. muelleri* cultured under High Light conditions. Results shown include the first experiment and its repetition.

very low mortality rate (more than 90 % survival), cultures that possessed larvae that grew well but suffered high mortality rates, and also cultures that fed on diets that produced small larvae, around 110 µm, with survival rates fluctuating greatly from 50 to 90 %.

For *C. muelleri* cultured under low light, results of the first experiment are shown in Fig. 3.3.4, while for its repetition in Fig. 3.3.5.

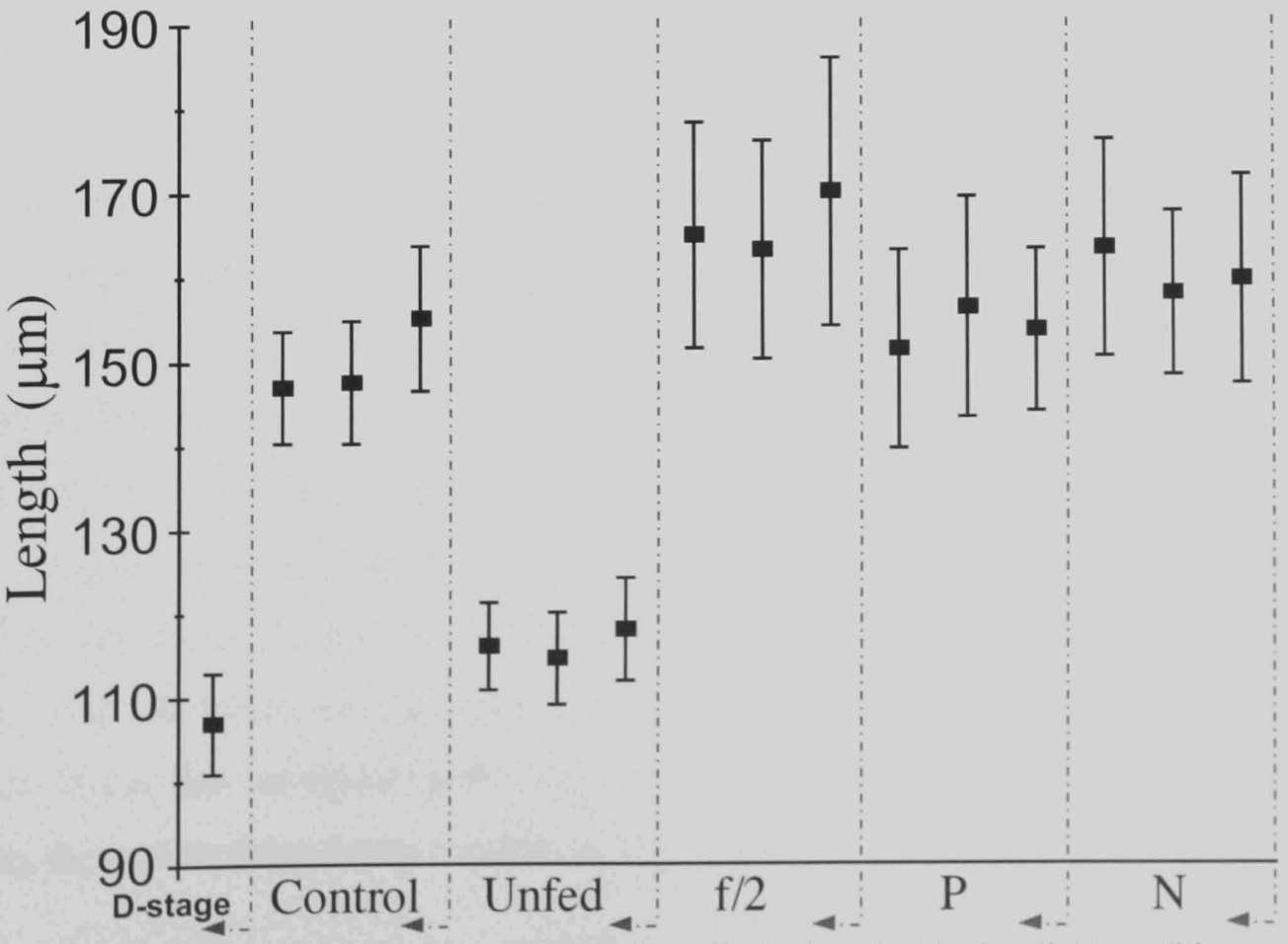

Figure 3.3.4. Size of *M. edulis* larvae fed on *C. muelleri* cultured under low light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

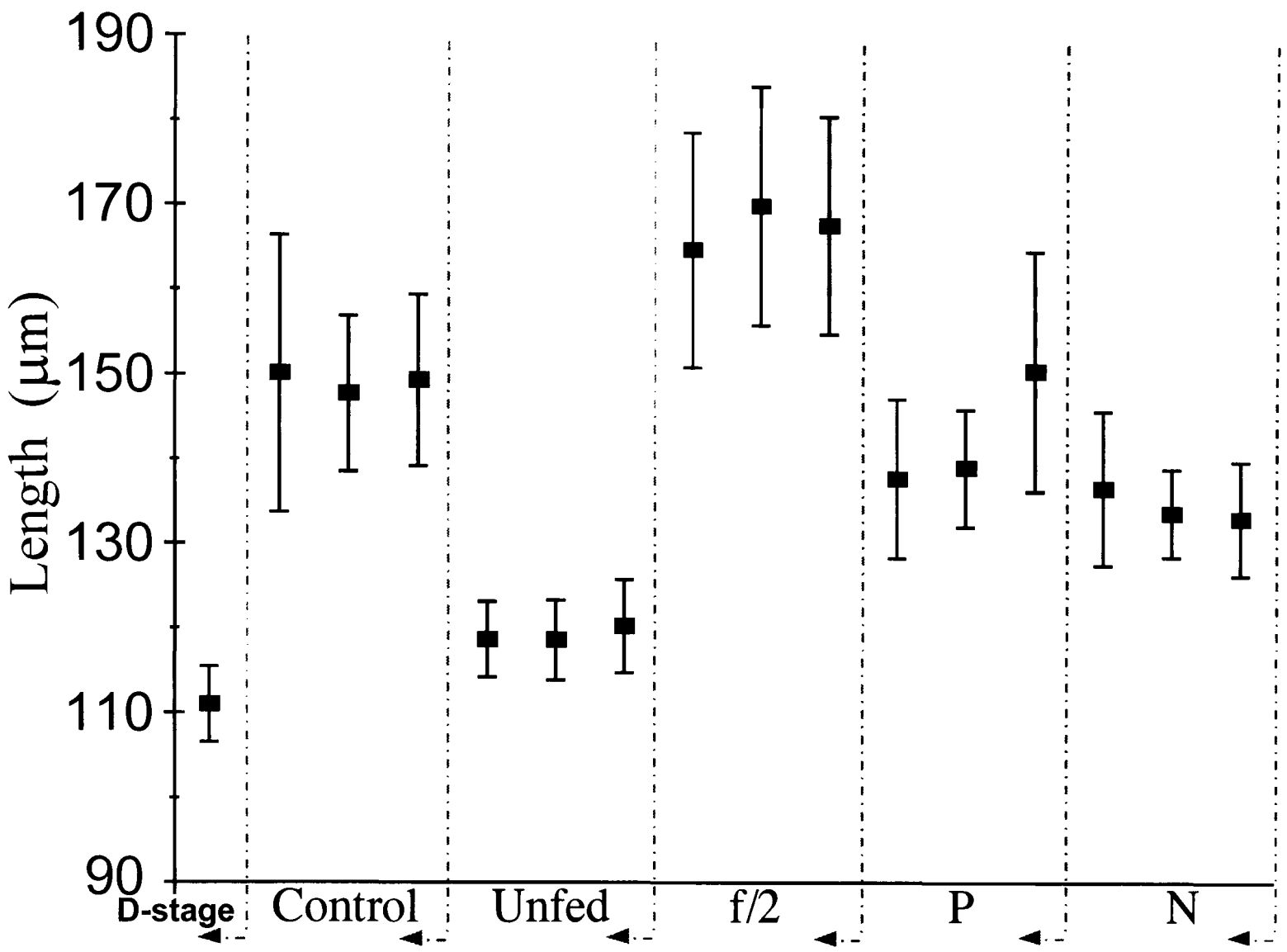

Figure 3.3.5. Size of *M. edulis* larvae fed on *C. muelleri* cultured under low light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

Analysing the results statistically, with a combination of nested ANOVA to reveal at first any significant differences between the diets and then with an analysis of means to identify which diets differ significantly from which, in similar manner as before the following results are obtained: after the end of the first low light feeding trial, *C. muelleri* when grown in a non-limiting medium (f/2) or the nitrogen limited medium, produces larvae that are bigger than the control diet with the difference being significant ($p < 0.05$, A. 3.3.5 nested ANOVA & A.3.3.6, analysis of means). Phosphorus limited cells are of lesser nutritional value in relation to the other two conditions. Non – significant differences between the alga cultured in P-limited medium diet and the control diet appear after the end of this experiment (A.3.3.6).

On the second feeding trial using these diets but with another batch of larvae, the statistical analysis (A.3.3.6 & 3.3.7) suggests that again the f/2 medium – grown cells are superior than the control and the other diets. However in this experiment, the control diet produces larvae that are significantly bigger that either the P- or N- limited *C. muelleri*; between these diets there are no significant differences.

However, there were some additional observations that should be mentioned before interpreting the results from the repetition feeding trial. Because, during that experiment, the N-limited culture appeared to have a very slow, and fluctuating, growth rate with cells not looking "healthy" as in the first experiment where the culture was stable and produced healthy looking cells. This culture's behaviour although unexplained in itself, probably explains the discrepancy between the size of the larvae when fed N-limited cells between the two experiments.

In the light of the previous observation it was considered safer to exclude the results, concerning the N-limited medium from the repetition experiment, due to the specific state of the algal culture at that time rather than treating them as characteristic of the nutritional quality of N-limited cells.

Consequently, more trust is placed in the statistical analysis of the results of the first experiment, which suggest than the alga under low light conditions and N-limitation produce larvae that grow significantly more that the control diet (A.3.3.5 & A.3.3.6) and hence can be characterized as a better food than the control diet, under such conditions.

Therefore, for the low light conditions overall, *C. muelleri*, when cultured without nutrient limitation, will produce bigger larvae than the larvae obtained by the control diet, which is, as described in the Materials and Methods chapter (p. 51), a mixture of algal species. N-limitation will produce larvae of bigger, than the control diet size, although smaller than their f/2 medium counterparts in terms of mean lengths, while P-limitation will produce larvae that are smaller than both its two other nutrient regime diets. However caution should be exercised when interpreting these results because, the statistical analysis (A.3.3.5 & A.3.3.7) also reveals that there is significant unexplained variation within the triplicates of these cultures in both experiments.

Survival rates for this diatom, *C. muelleri*, when cultured under low light conditions are shown in Table 3.3.5.

Again, as with the previous experiment, using *C. muelleri* cultured under High Light conditions, survival shows a random pattern and does not seem to be correlated with the diet upon which the larvae were fed.

To investigate whether a growth/survival relationship exists the length and survival data against were plotted against each other. Results are shown in Fig. 3.3.6.

Table 3.3.5 % Survival of larvae fed *C. muelleri* cultured in low light conditions

Diet	% Survival of larvae for the experiment of ***C. muelleri*** LL	% Survival of larvae for the **repeat** experiment
Control 1	47.2	60.0
Control 2	44.4	53.9
Control 3	35.6	58.9
Unfed 1	30.0	15.0
Unfed 2	55.0	15.6
Unfed 3	38.9	14.4
LL f/2 1	20.6	44.4
LL f/2 2	25.0	41.7
LL f/2 3	32.8	35.0
LL P 1	23.9	18.9
LL P 2	42.2	27.2
LL P 3	19.4	27.8
LL N 1	19.4	29.4
LL N 2	23.3	34.4
LL N 3	22.2	10.0

The result illustrates graphically that there is apparently a random relationship between growth and survival.

As observed when the length/survival relationship was investigated when *C. muelleri* was cultured under high light conditions and used as a diet, all possible combinations of larval cultures exist.

However it is worth mentioning that as a whole, the low light experiments produced cultures with lower survival rates. In the high light experiments survival was

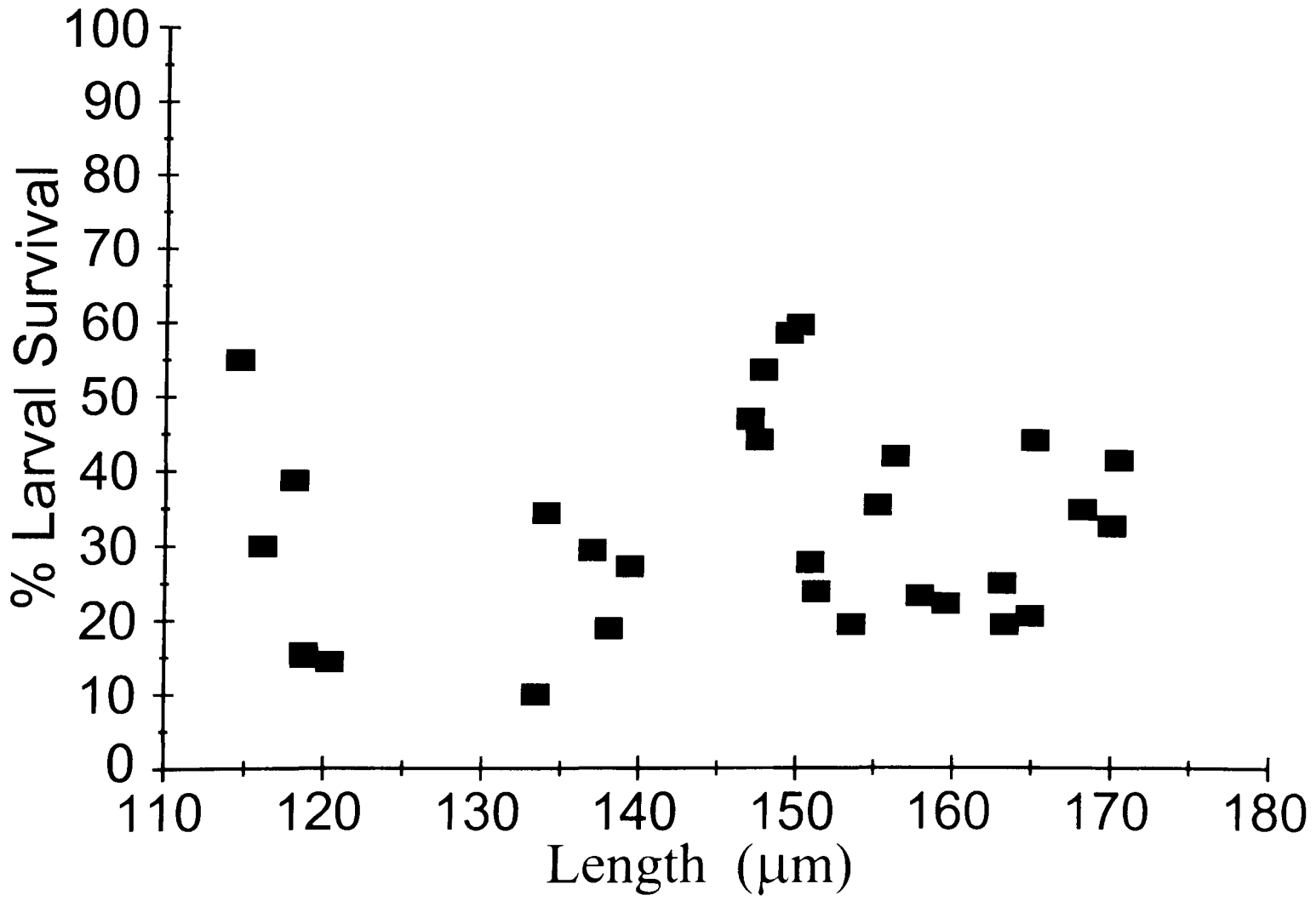

Figure 3.3.6. Length/Survival relationship for the larvae fed on *C. muelleri* cultured under Low Light conditions. Results shown include the first experiment and its repetition.

always above 50 %, whereas in the present light regime, only few of the cultures exhibit survival rates more that 50 %, with some of them experiencing very high mortality rates. sometimes up to 90 %. Nevertheless, as mentioned before within the low light experiments there is no relationship between length and survival.

The larvae fed on algae grown at high light (repetition experiment) and the low light (first experiment) were analysed for their fatty acid content. Two beakers of larvae were individually processed. Because the larvae were from different batches, the respective control and unfed larvae were also analysed separately. Results are summarised in Table 3.3.7.

The use of individual F.A. as indices of growth in *M. edulis* larvae, will be discussed in a later chapter (ch. 4.1).

Table 3.3.7. Fatty acid content of *M. edulis* larvae obtained during the *C. muelleri* experiments. Individual FA are expressed as % of Total Identifiable Fatty Acids (**TIFA**). Values are mean of two samples analysed. Numbers with asterisks (*) on some FA's indicate that although the FA was identified in both samples in one of them, it was not integrated; therefore for the respective F.A. the value is taken from the sample that the FA was integrated. **N.S.**=FA's not separated. **B.D.**=FA's below detection level. **N.I.**=Fatty Acid peak not integrated properly. **Safa**=Saturated Fatty Acids, **MUFA**=Mono unsaturated Fatty Acids, **PUFA**=Poly unsaturated fatty acids.

	Control	Unfed	f/2	P	N	Control	Unfed	f/2	P	N
	*Larvae obtained after the **H.L.** experiment*					*Larvae obtained after the **L.L.** experiment*				
14:0	6.31	5.76	5.09	4.33	3.54	3.78	4.93	6.14	5.62	4.39
15:0	4.36	4.41	3.13	1.86	2.01	4.02	4.92	3.70	3.48	3.74
16:0	38.23	33.12	26.15	23.61	23.21	32.42	36.61	27.75	29.34	30.06
16:1ω7	4.73	8.69	6.45	9.70	6.22	10.70	6.83	13.73	8.47	7.92
18:0	12.21	10.63	8.20	9.30	8.21	9.47	11.67	8.16	9.93	11.41
18:1ω9	4.91	6.94	6.40	4.64 *	6.22	10.85	7.97	9.95	9.72	10.87
18:1ω7	3.61	3.01	3.70	4.12 *	4.07	2.65	1.08	7.53	5.46	8.03
18:1ω9&ω7	8.51	9.95	10.10	9.24	10.29	13.50	9.05	17.49	15.18	18.90
18:2ω6	0.96	1.52	1.14	1.22	1.38	1.62	0.88	1.90	2.47	1.91
18:3ω3	0.69 *	0.53	0.49	0.24	0.38	0.54	1.64	0.16	N.I.	N.I.
18:4ω3	0.55 *	0.51	0.97	0.97	1.04	0.24	0.12	0.15	N.I.	N.I.
20:1	2.85	0.86	1.38	1.17	2.00	1.06	3.92	1.57	1.70	1.75
20:1	5.00	3.33	3.87	2.70	3.90	1.91	1.23	1.65	1.72	1.72
20:4(ω6?)	2.30	1.80	4.98	5.16	5.38	2.24	3.64	1.65	2.07	1.99
22:0	5.22	4.32	3.66	2.01	2.06	2.92	5.08	2.84	4.36	4.23
20:5ω3	0.73 *	3.77	13.12	15.07	17.13	4.43	0.93	7.19	7.75	3.59
22:1ω11	1.65	1.06	1.32	0.92	1.37	1.23	2.14	0.40	0.00	1.34
22:5ω3	0.51*	0.93	1.36	1.99	1.86	0.81	0.68	0.92	1.67	1.14
22:6ω3	6.08	8.82	8.60	10.51	10.01	9.13	6.62	4.61	6.24	5.89
SaFa	66.33	58.24	46.23	41.12	39.03	52.60	63.20	48.58	52.73	53.84
MUFA	22.74	23.89	23.11	23.73	23.79	28.40	23.16	34.84	27.06	31.63
PUFA	11.82	17.87	30.66	35.15	37.19	19.00	14.51	16.58	20.21	14.53
Total ω:3	8.56	14.56	24.54	28.77	30.42	15.14	9.99	13.03	15.66	10.63

As a diet for *M. edulis* larvae, *C. muelleri* appears to be an adequate food source. In many cases larvae fed on this diatom grew as well as, or better than the control diet which was a mixture of two species. However, in the first experiment in HL condition the larvae did not perform very well with a significantly slower growth rate. The LL results were (under the light of the previous discussion, concerning the only disagreeing case) in very good agreement with each other. Accordingly, it is worth looking at the results after pooling the data from respective experiments together. Preliminary experiments using batch cultures of *C. muelleri* (Leonardos, unpubl.) have showed that over a two-week period larvae fed on *C. muelleri* grown under different nutrient concentration, in the same HL conditions as the ones used for the Continuous cultures, grew app. up to 150 ~160 μm in length.

Therefore, it is safer to exclude the first experiment from the analysis on the basis that this batch of larvae was of poor quality. The averaged results of three replicates, for both experiments in LL conditions excluding only the result from the repetition N-limited cultures, and the repetition experiment in HL conditions, are shown in Fig. 3.3.7.

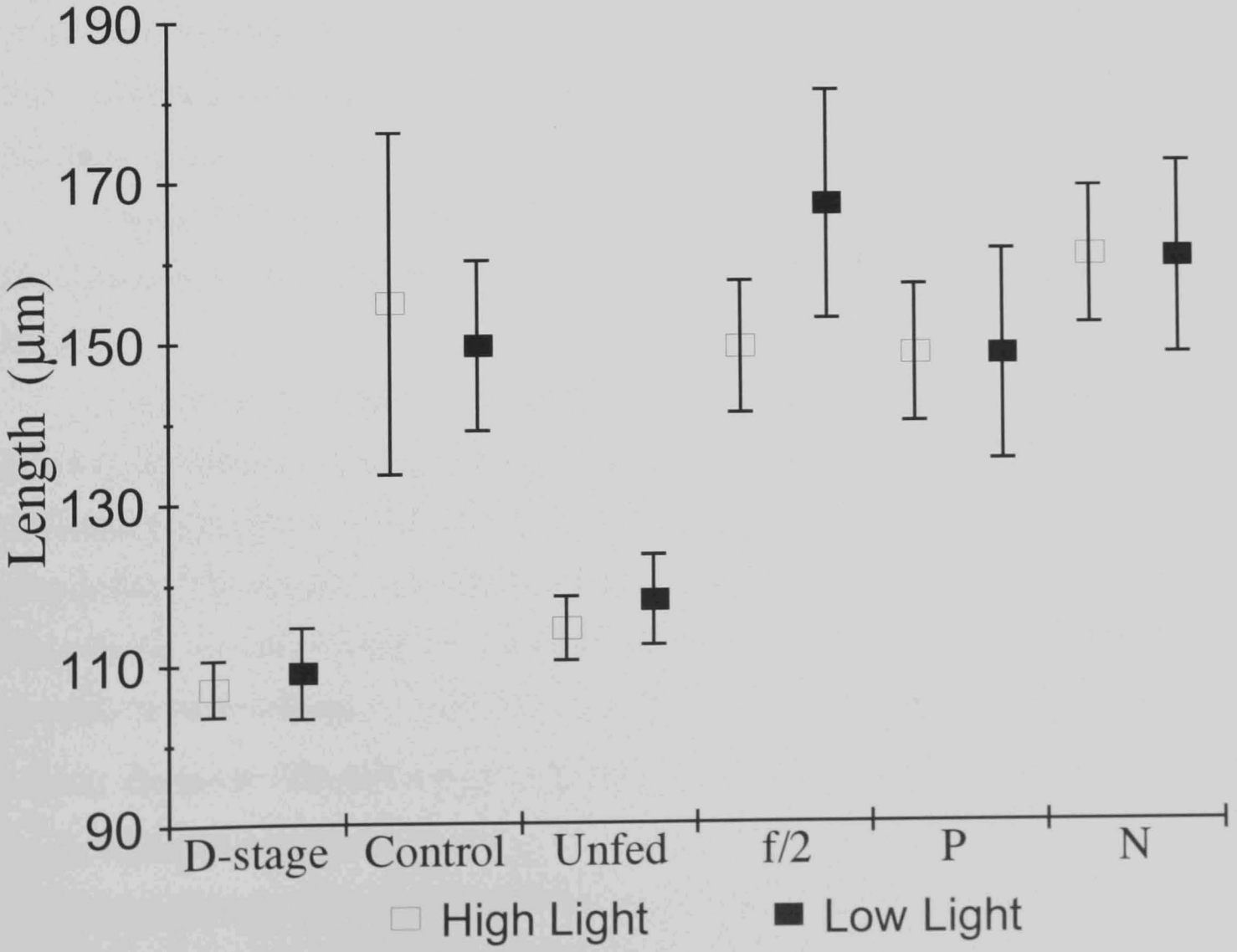

Figure 3.3.7. Averaged results for both LL experiments and the second HL experiment, of *M. edulis* larvae fed on *C. muelleri* of various nutrient and light conditions. Value indicated for LL N is from the first experiment only, for reasons explained in the text.

C. muelleri, as shown in Tables 3.3.1. & 3.3.3 will change its growth rate and biochemical composition, to adapt to environmental conditions. Subsequently, its overall nutritional quality for *M. edulis* larvae is also demonstrated to change. **P**-limitation in both light conditions will produce smaller larvae that the other two nutrient conditions. **N**-limitation will produce significantly better results in high light conditions, among the other two nutrient conditions, while under low light there is no significant difference.

When comparing the two light intensities used, it is clear that low light conditions will produce cells of better nutritional quality, when the non-limiting **f/2** medium is used.

It is difficult to identify which change of which individual component is having this effect. As with all algal species, it is reasonable to assume that it will be the combination of the change of various biochemical components that will exercise the overall effect. For this specific type of analysis, the subsequent chapter 4.3 will try to elucidate the subject.

Notwithstanding the points raised in the discussion of the other diatom, *S. costatum*, on more specific components, i.e. total protein, fatty acid etc., and the literature sited there (Ackman *et al.*, 1964; Webb & Chu, 1983; Enright *et al.*, 1986a; Su *et al.*, 1988; Volkman *et al.*, 1989; O'Connor *et al.*, 1992; Sánchez *et al.*, 1993; Sánchez *et al.*, 1994; Blanchemain & Grizeau, 1996), some observations more specific to the *Chaetoceros* species can be made.

Nitrogen limitation decreases the amount of protein produced per cell; Enright *et al.* (1986b) report similar results in a study of a closely related *Chaetoceros* species, *C. gracilis*.

As was the case with the other diatom studied here *S. costatum*, *C. muelleri* increases the amount of saturated fatty acids and decreases the total amount of polyunsaturated fatty acid with increasing light intensity and/or nitrogen stress. The proportion of the omega:3 series fatty acids also increased with increasing light intensity; this was also the case, reported by Mortensen *et al.* (1988), for *C. gracilis*. However the increase in dietary omega: 3 series does not result in similar changes of this group of fatty acids in the larvae. The diet with the highest relative proportion of 20:5ω3 FA produces larvae with only around average content in this FA. Similar differences, in the relationship between dietary and larval FA content, also exist for other major components of the total FA profile, for example the 16:0 and 22:6ω3.

In many reported cases in the aquacultural literature. the genus *Chaetoceros* is very often considered to be of good nutritional value for bivalve larvae, spat and adults (Enright *et al.*, 1986a,b; Whyte, 1987; Whyte *et al.*, 1990; O'Connor *et al.*, 1992; Thompson *et al.*, 1994a) or larvae and postlarvae of shrimps (Chu, 1989). This observation is further verified with the present study.

The improvement of the food value of *C. muelleri* for *M. edulis* larvae would seem to require a low light intensity with no nutrient limitation, as the optimal conditions for culture. In these conditions the cells contain the lowest amount of protein of all the conditions used. On the other hand, no general trend can be established for its fatty acid profile. Levels of unsaturated fatty acids are between the levels obtained when culturing the species in the other conditions, while for the saturated fatty acids levels are slightly lower that the ones recorded under the other conditions.

For a detailed analysis of these aspects of the discussion the reader will have to wait until sections 4.2 and 4.3 where a correlation analysis of algal dietary components and larval growth will be presented. The analysis of these aspects specific to *C. muelleri*, has been omitted from this section to avoid unnecessary repetition, since subsequent analysis will attempt to analyze the subject collectively.

3. Results and Discussion

3.4 Environmental effects on growth, biochemical composition and nutritional quality of *Rhinomonas reticulata*

R. reticulata (previously known as *Rhodomonas baltica*) was cultured using the previously employed continuous culture system under the same conditions as the other algal species. A summary of the effect of nutrient concentration and light intensity on growth, chlorophyll, protein and carbohydrate concentration of this species, is shown in Table 3.4.1.

Table 3.4.1. Responses of *R. reticulata* to various conditions. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Condition	Generation time **G** in **h** (SD)	**Protein** pg/cell	**Carbohydrate** pg/cell	**Chl A** (pg/cell)	**Chl C** (pg/cell)
HL f/2	76.6 (25.38)	25.86	4.17	0.496	0.367
HL P	175.1 (37.77)	29.03	7.56	0.287	0.333
HL N	150.3 (45.64)	36.23	18.27	0.468	0.387
LL f/2	240.1 (128.95)	25.44	8.04	0.915	0.640
LL P	305.8 (119.91)	28.24	4.67	0.650	0.485
LL N	263.2 (133.68)	32.83	6.31	0.705	0.444

This species, *R. reticulata*, responded to both nutrient and light limitation by altering its generation time. Low light conditions increased its generation time by a factor of approx. 3, when the non-limiting f/2 medium was used, while when P- or N- limited media were used G was more than doubled under low light conditions. When nutrient limitation is taken into consideration, faster division rate (smaller **G**) was demonstrated in the non-limiting medium (f/2) as expected, with a slight decrease in N-limiting medium, while the slowest division rate (greatest **G**) was experienced under P-limitation. This pattern was the same for both low and high light conditions. There is no significant difference ($p > 0.05$, 95% level) between P and N-limited nutrient conditions in high light conditions, while f/2's value for **G** is significantly smaller ($p < 0.05$, 95% level) than the previous two. Under low light conditions, there is no significant difference between nutrient limited and non-limited media. Literature on the physiological properties of this species is rather more limited. The species showed the slowest division rate of all the species tested here. This accords with the general idea that species of larger volumes

usually show slower division rates. Indeed, *R. reticulata* is the biggest of all species used here. The two diatoms, *S. costatum* and *C. muelleri*, have dimensions of approx. 5 μm x 10 μm (width x length) and *P. lutheri* a round shaped species has a radius of around 5 μm, whereas *R. reticulata* is oval and reaches an overall length of around 20 μm.

The variation of generation time is, proportionally, within the range that was demonstrated by the other species cultured under similar conditions.

Protein concentration increased in algal cells grown in f/2 media in comparison to these grown in P-limited media, with a peak under N-limitation. Cellular protein concentration remained approximately the same at the two light intensities, for each respective culture medium.

Carbohydrate concentration increased with nutrient limitation, with a maximum amount found in N-limited cells. The pattern as far as nutrient limitation is concerned was the same under both light intensities, but the carbohydrate concentration increased with increasing light intensity.

The cells increased the amounts of both major chlorophylls when cultured in low light conditions. This is a reflection that when cultured under low light the cells need to have more chlorophyll to make efficient use of the reduced ambient light. Both chlorophyll **a** and **c** per cell concentration almost doubled in each respective culture medium from high to low light intensity. Consequently, its photoadaptation type is a the "Chlorella" type (varying chlorophyll content with light intensity) and not of the "Cyclotella" type, in which chlorophyll content per cell does not vary (Jørgensen 1969, 1977).

The overall view of the effects of the tested environmental parameters on the biochemical properties of *R. reticulata* is that it is mainly generation time that was affected (and that is mostly due to light intensity) but carbohydrate and protein per cell content appeared to be little influenced by them.

The chemical analyses for the nutrient concentration of inflow and outflow media for *R. reticulata* cultures are shown in Table 3.4.2, while the fatty acid profile of this cryptophyte can be seen in Table 3.4.3, for all different culture conditions.

Nutrient concentrations were lower in outflow media, since the cell utilise nutrients to biosynthesise various cell components. In N-limited cultures the amount of $^{-1}NO_3$ present in the outflow medium was minimal while the other macronutrients were in

much greater quantities. Similarly, the $^{-3}PO_4$ concentration in the outflow medium was minimal in the P-limited cultures. These patterns indicate that, the assigned "limiting macronutrient" was the limiting factor for each respective medium.

Table 3.4.1. Nutrient concentrations of major macronutrients in Inflow (**Infl.**) and Outflow (**Outfl.**) media in various conditions used for the *R. reticulata* cultures. Figures indicate μgr-at/l. **HL**=High light, **LL**= Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Condition	Infl. $^{-3}PO_4$	Outfl. $^{-3}PO_4$	Infl. $^{-1}NO_2$	Outfl. $^{-1}NO_2$	Infl. $^{-1}NO_3$	Outfl. $^{-1}NO_3$
HL f/2	24.98	0.26	1.31	15.03	239.60	118.75
HL P	7.28	0.40	0.60	13.88	253.11	226.47
HL N	20.77	19.29	0.66	0.01	257.12	5.13
LL f/2	24.98	18.06	1.31	11.05	239.60	236.03
LL P	7.28	0.26	0.60	13.90	253.11	242.48
LL N	20.77	16.80	0.66	0.09	257.12	4.08

Table 3.4.3. Fatty acid composition of *R. reticulata* under various culturing conditions. Individual fatty acids are expressed as % of Total Identifiable Fatty Acids (TIFA). HL=High light, LL=Low light, f/2=f/2 medium with no nutrient limitation, P=Phosphorus limited medium, N=Nitrogen limited medium. N.I.= Individual peaks were not integrated properly. Values are means of two samples, except for HL P, where values are means from three samples. (*) indicate that the value is calculated from one less sample, because the Fatty Acid peak was not integrated properly in that sample.

Fatty acid	HL f/2	HL P	HL N	LL f/2	LL P	LL N
14:0	8.62	12.31	19.12	3.25	2.17	3.93
15:0	0.31	0.26	0.38	2.28 *	0.45	1.44
16:0	7.08	11.40	25.09	6.10	8.37	13.03
16:1ω7	2.63	2.58	0.98	1.94	1.74	2.88
18:0	0.80 *	0.12 *	5.01 *	1.04	1.18	4.51 *
18:1ω9	0.31 *	N.I.	11.31 *	1.05 *	1.33	3.06 *
18:1ω7	10.87	N.I.	4.15 *	7.66	9.21	8.44
18:1 (ω9 & ω7)	11.18	20.7 *	15.46 *	8.71	10.53	11.50
18:0 & 18:1(ω9 & ω7)	11.98	20.53	21.18	9.75	11.71	16.01
18:2ω6	6.60	14.97	4.45	5.12	9.54	4.14
18:3	1.45	2.15	0.92	1.19	2.32	0.36
18:3ω3	15.92	14.84	13.43	12.31	17.44	12.36
18:4ω3	21.57	10.57	9.79	22.58	24.75	15.36
P.U. $_1$ (22:2?)	1.22	1.50	0.31	16.96	2.25	16.52
P.U. $_2$	0.67	0.73	0.20	2.38	2.27	2.57
20:5ω3	11.67	3.75	2.08	9.95	8.63	8.10
24:0	0.22	0.09	0.04	0.26	0.15	0.24
22:6ω3	10.61	4.32	2.02	7.61	8.20	6.82
Total Saturated F.A.	17.04	24.18	49.64	12.93	12.33	23.16
Total Monounsaturated F.A.	13.81	23.28	16.44	10.65	12.28	14.38
Total PolyUnsaturated F.A.	69.71	52.83	33.21	78.09	75.39	66.24
Total ω:3 series F.A.	59.77	33.49	27.32	52.44	59.01	42.64

The proportions of individual fatty acids and fatty acid groups was greatly influenced by both nutrient and light conditions. Although there is not concrete evidence of the manner in which individual fatty acids are changed due to the influence of light intensity, there is remarkable uniformity in the pattern of change of groups of fatty acids. Specifically, increasing light intensity resulted in a decrease of the relative amounts of SaFA and a notable decrease in the proportion of PUFA and omega: 3 fatty acids. Similar uniformity was exhibited as a result of nutrient limitation. Nitrogen limitation increased the proportion of SaFA and it decreased the proportion of PUFA. Phosphorus limitation had similar effects but the relative change of the proportion of SaFA and PUFA was far more conservative.

The final length achieved by the larvae fed on *R. reticulata* after the end of the two week feeding trials, is shown in Fig. 3.4.1. Results show growth of larvae in each individual beaker. The repetition of this experiment with another batch of larvae can be seen in Fig. 3.4.2.

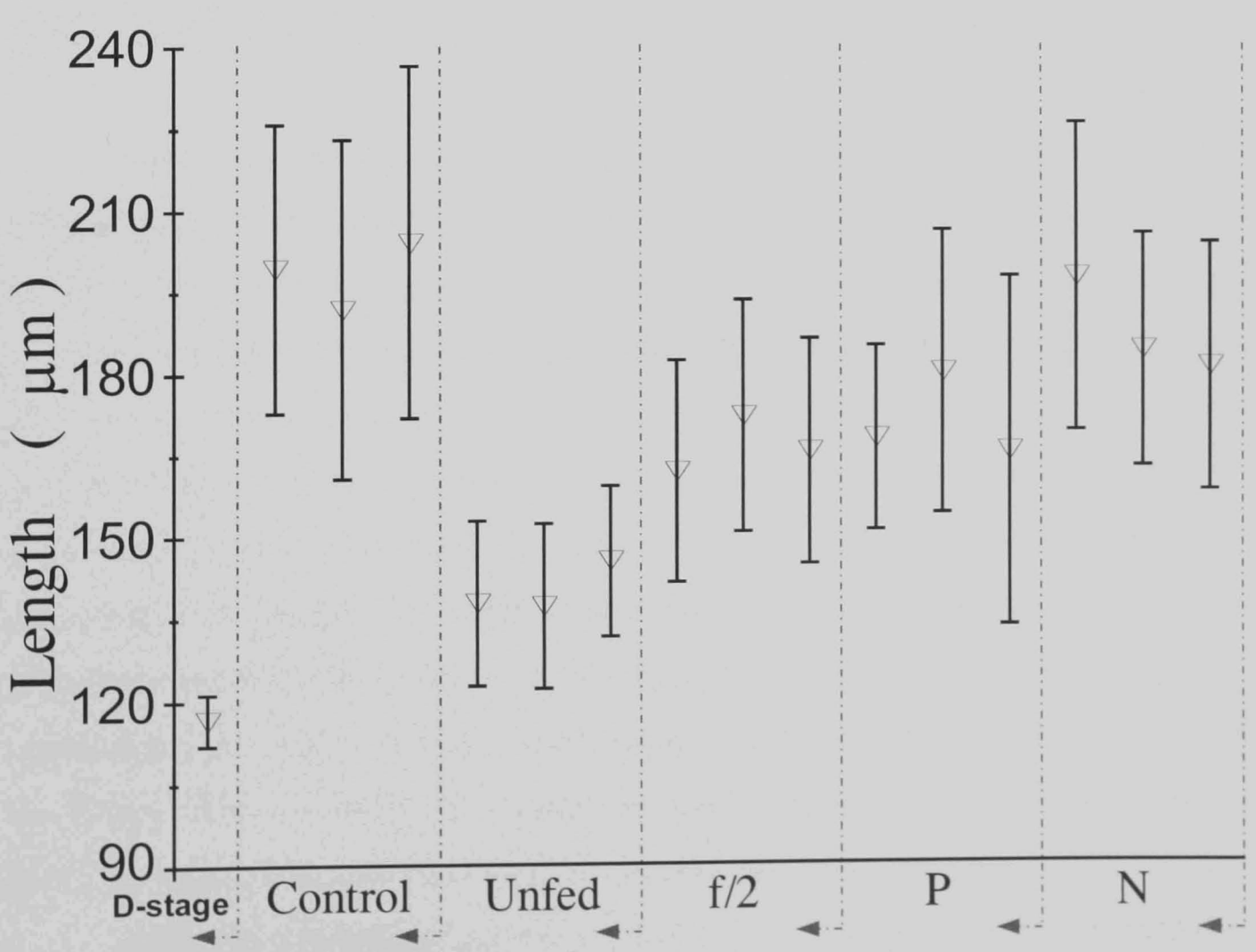

Figure 3.4.1. Size of *M. edulis* larvae fed on *R. reticulata* cultured under high light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

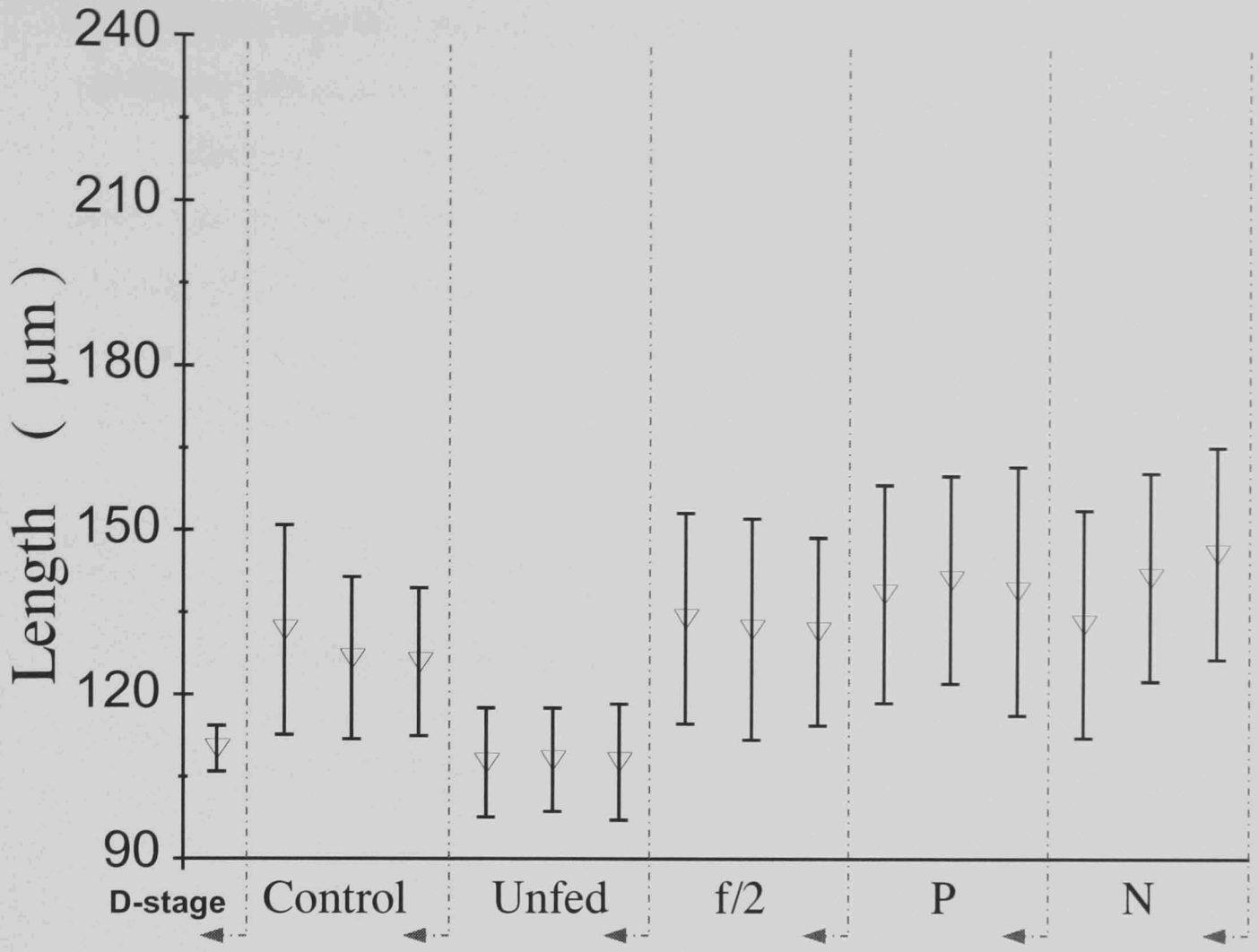

Figure 3.4.2. Size of *M. edulis* larvae fed on *R. reticulata* cultured under high light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

Again, as with all previous experiments, it is clear that unfed larvae showed minimal growth; this difference in length between fed and unfed larvae is significant ($p < 0.05$, 95% level). As explained in the previous species, in order not to mask any other potential differences between diets, the unfed larvae will not be included in the statistical analysis. The results of every test are given, as usual, in detail in the Appendix. The number in the parenthesis given after every reference to a test here, will refer to a corresponding table in the Appendix section. Homogeneity of variance among the triplicates of the same diet is within acceptable limits when tested for significant differences between two variances (Sokal & Rohlf, 1987) with $p > 0.05$. The typical combination of statistical analysis was employed for the interpretation of these results too. Nested ANOVA indicated whether any significant differences between diets existed and if they were, they were identified by the multiple means test, in the 95 % level.

In the first experiment, the f/2 and P-limited *R. reticulata* diets performed less well than the control diet ($p < 0.05$, A. 3.4.1 & 3.4.2), but the N-limited cells were of

better quality than the other two diets; between this diet and the control diet there were no significant differences (A.3.4.2).

However, on the repetition of the same experiment all *R. reticulata* diets were better than the control. Between the test diets, the cells cultured in the no-nutrient limiting medium (f/2) were of inferior quality to the other two nutrient conditions (A. 3.4.3 & A.3.4.4). f/2 and P-limited diets did not have significantly different effects on the larvae (A. 3.4.3 & A.3.4.4).

Table 3.4.4. % Survival of larvae fed *R. reticulata* cultured in high light conditions. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Diet	% Survival of larvae for the experiment of ***R. reticulata* HL**	% Survival of larvae for the **repeat** experiment
Control 1	39.8 (5.79)	56.7 (12.98)
Control 2	38.0 (4.61)	52.8 (8.75)
Control 3	39.2 (7.89)	43.3 (9.53)
Unfed 1	7.0 (1.43)	46.7 (4.08)
Unfed 2	9.4 (0.83)	46.1 (5.15)
Unfed 3	14.6 (5.03)	51.1 (2.83)
HL f/2 1	31.0 (5.79)	42.2 (7.74)
HL f/2 2	35.1 (4.30)	49.4 (12.20)
HL f/2 3	32.2 (5.79)	45.0 (2.36)
HL P 1	40.9 (2.98)	44.4 (11.57)
HL P 2	28.7 (5.79)	64.4 (5.67)
HL P 3	26.9 (0.83)	57.8 (20.61)
HL N 1	19.3 (1.43)	45.0 (12.98)
HL N 2	19.9 (2.98)	51.1 (6.43)
HL N 3	28.7 (5.96)	54.4 (7.74)

Survival for each experiment is shown in Table 3.4.4.

As is the case with the previous survival results, survival rates show a random variation between diets. This pattern is consistent with the patterns observed in the two previously presented species. However it can be noticed that on average survival was less good during the first experiment with the situation improved during the repetition experiment.

As stressed previously, the reasons for these differences cannot be quantified.

Although in each experiment, the *R. reticulata* diets produce different results in relation to the control diet, the same trend, in terms of mean end lengths, between these diets appears in both experiments. N-limited cells produce bigger larvae for the same feeding period than do the f/2 or the P-limited cells.

As has been illustrated in corresponding sections of the other algal species tested, the growth/survival relationship can be investigated by means of a length / survival graph. This relationship illustrated for both experiments concurrently is shown in Fig. 3.4.3, and it clearly implies that there is a random relationship between these parameters.

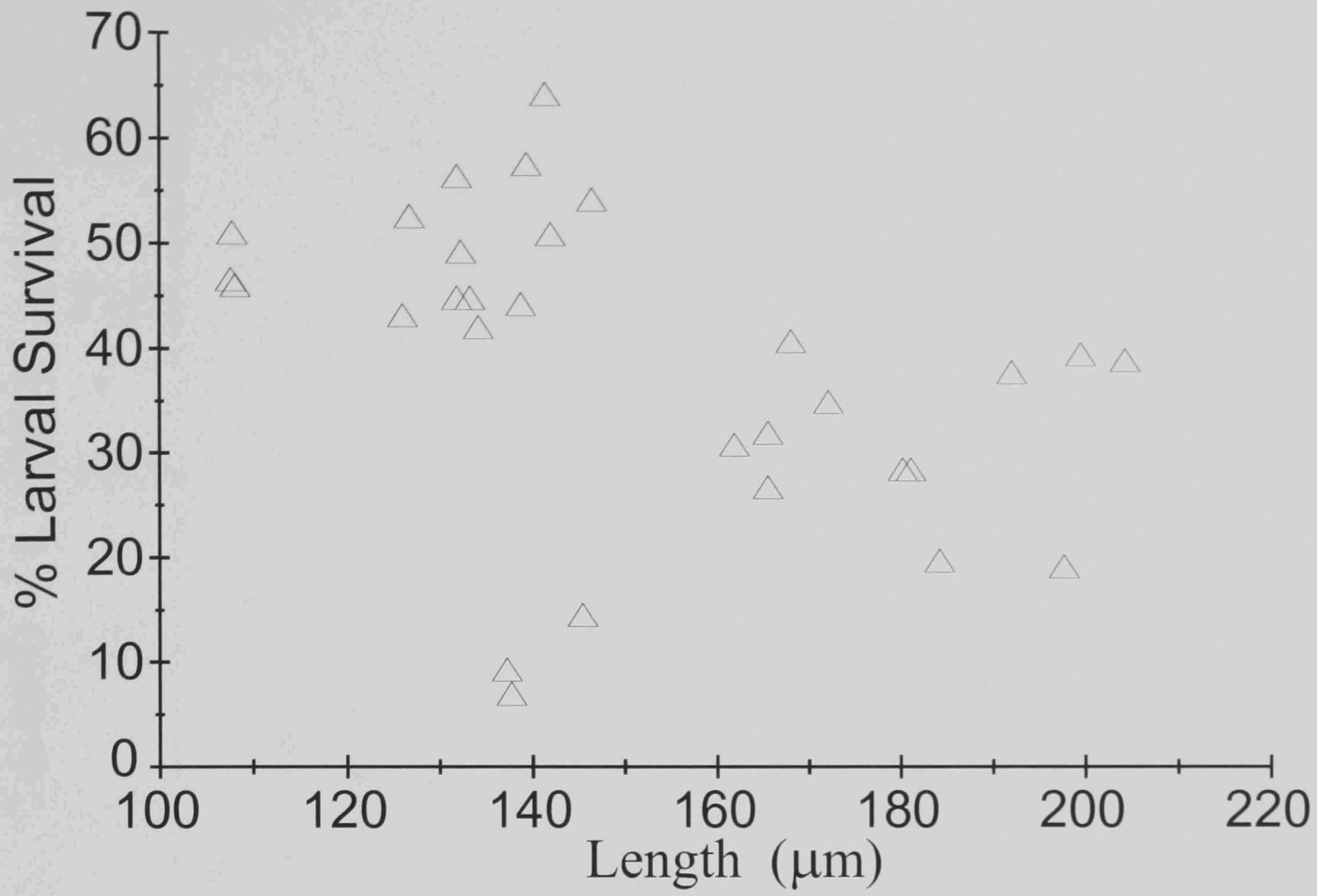

Figure 3.4.3. Length/Survival relationship for the larvae fed on *R. reticulata* cultured at High Light conditions. Results shown include the first experiment and its repetition.

For *R. reticulata* cultured under low light, results of the first feeding trial are shown in Fig. 3.4.4, while for its repetition in Fig. 3.4.5.

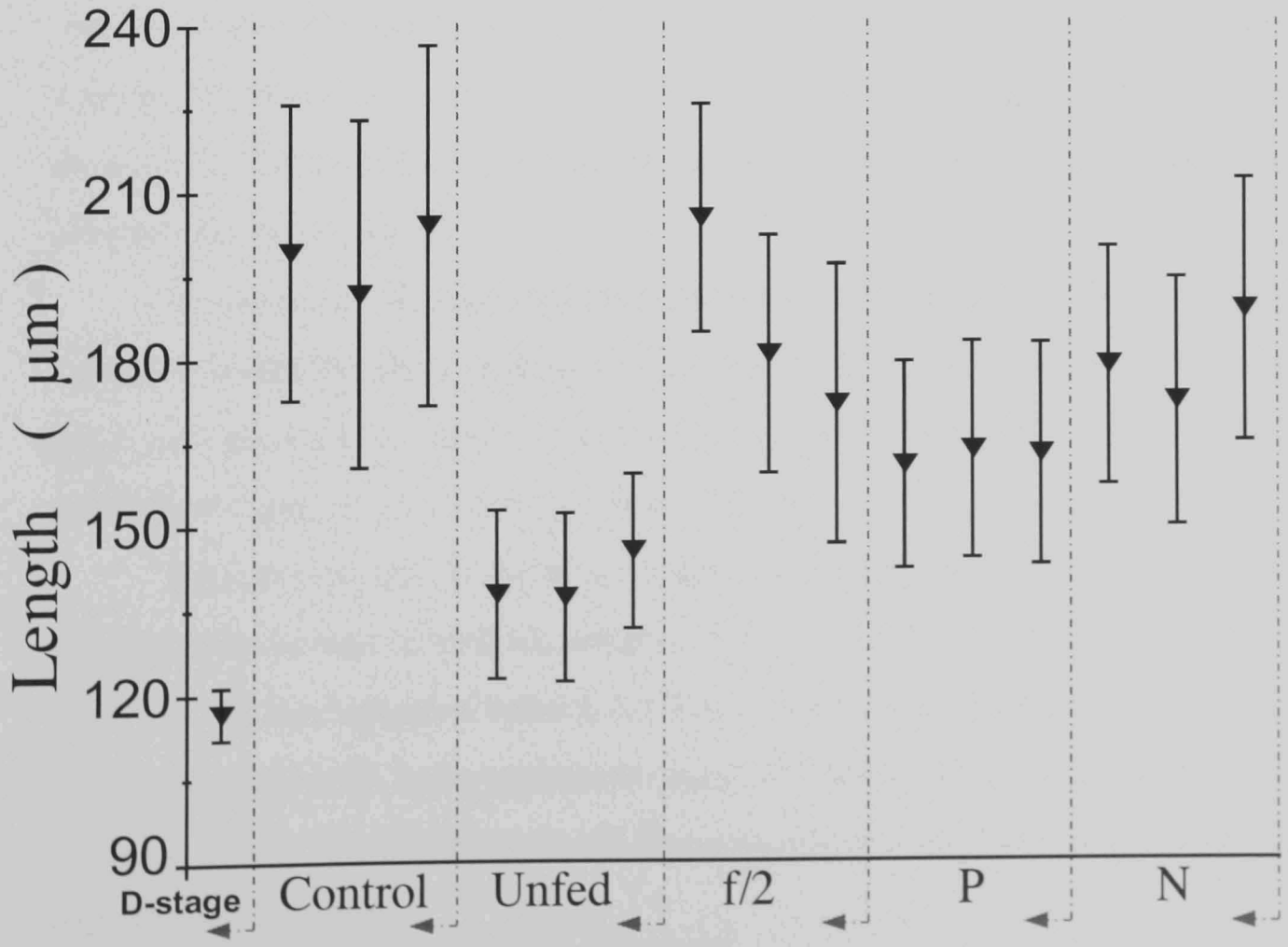

Figure 3.4.4. Size of *M. edulis* larvae fed on *R. reticulata* cultured under low light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

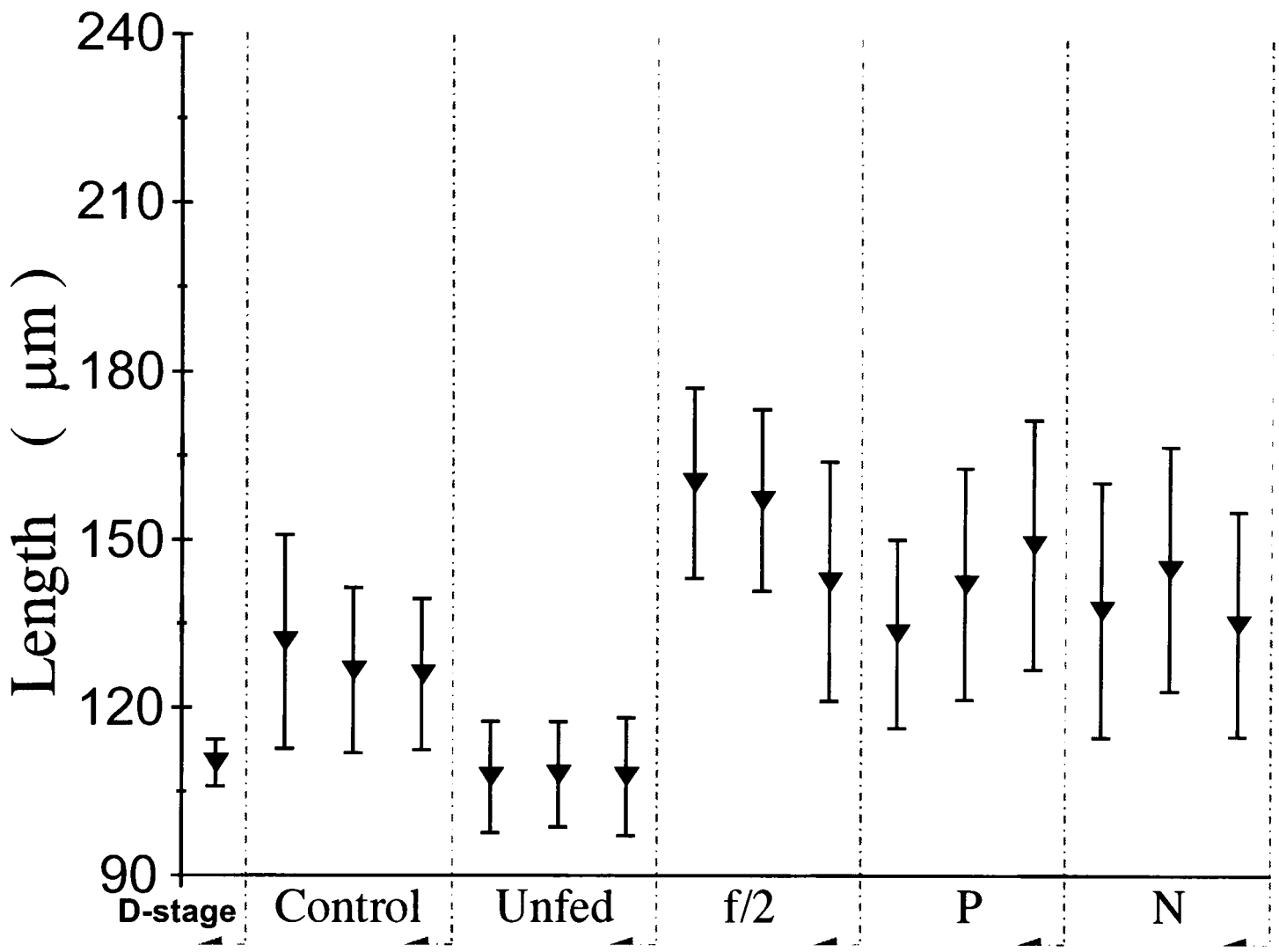

Figure 3.4.5. Size of *M. edulis* larvae fed on *R. reticulata* cultured under low light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

For the first low light feeding experiments all *R. reticulata* diets, are inferior than the control diet. F/2 and N-limited grown cells have significantly better nutritional value than the P-limited cells, while between them there are no significant differences (A.3.4.5 & A.3.4.6). However there is too much variation within the triplicates which renders conclusions rather unsafe.

In the second feeding trial the control diet is inferior to all other test diets. Variation within the three replicates is less than in the first low light experiment. Between the *R. reticulata* diets, P and N- limited diets do not differ significantly from each other, while the f/2 grown cells are better than these two (A.3.4.7 & A.3.4.8).

Comparison among the diets obtained by manipulating the nutrient conditions, it can be observed that, in both experiments the *R. reticulata* cells cultured in the non limiting (f/2) medium are a better food than P limited cells (A.3.4.6 & 3.4.8). Between the nutrient limited cells, in the repetition experiment there are no differences in their nutritional value (A.3.4.8) but in the first experiment N-limited cells are better that P-limited cells (A.3.4.6).

Table 3.4.5 % Survival of larvae fed *R. reticulata* cultured in low light conditions. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**= Phosphorus limited medium, **N**=Nitrogen limited medium.

Diet	% Survival of larvae for the experiment of ***R. reticulata* LL**	% Survival of larvae for the **repeat** experiment
Control 1	39.8 (5.79)	56.7 (12.98)
Control 2	38.0 (4.61)	52.8 (8.75)
Control 3	39.2 (7.89)	43.3 (9.53)
Unfed 1	7.0 (1.43)	46.7 (4.08)
Unfed 2	9.4 (0.83)	46.1 (5.15)
Unfed 3	14.6 (5.03)	51.1 (2.83)
LL f/2 1	34.5 (4.14)	40.6 (4.16)
LL f/2 2	32.8 (5.96)	55.0 (11.14)
LL f/2 3	25.1 (0.83)	47.8 (10.21)
LL P 1	37.4 (3.61)	48.3 (4.91)
LL P 2	32.8 (2.19)	51.1 (6.43)
LL P 3	39.2 (7.21)	40.6 (6.85)
LL N 1	29.8 (4.96)	43.3 (2.72)
LL N 2	30.4 (2.19)	59.4 (7.49)
LL N 3	35.7 (3.31)	44.4 (4.37)

Survival rates for the larvae fed on this species, *R. reticulata*, when cultured under low light conditions, are shown in Table 3.4.5.

As demonstrated by the results obtained in the respective high light experiment, the first feeding trial has on average lower survival rates that the repetition trial. Referring to the Materials and Methods chapter (2.2.b) and the detailed information presented there concerning the feeding trials, this is explained by the fact that the *R. reticulata* High and Low Light experiments were run with the same batches of larvae each time. Again, survival rates of the larvae do not seem to be strictly correlated with the diet upon which they were fed; however in these experiments survival rates seem to be more consistent and show less variation within beakers, especially in the first experiment. The repetition of the feeding trial shows that the variation of survival rates within beakers is bigger than the first experiment. In the light of the lower survival rates and their generally greater end length achieved after the two – week feeding trial, experienced by the first batch, together with the inverted view of both survival rates and end lengths, of the second batch of larvae, the relationship between growth and survival needs to be investigated.

As was employed for the other cases, to investigate whether a growth/survival relationship exists, the length and survival rates data were plotted against each other. Results are shown in Fig. 3.4.6.

The result illustrates graphically that there is apparently a random relationship between growth and survival.

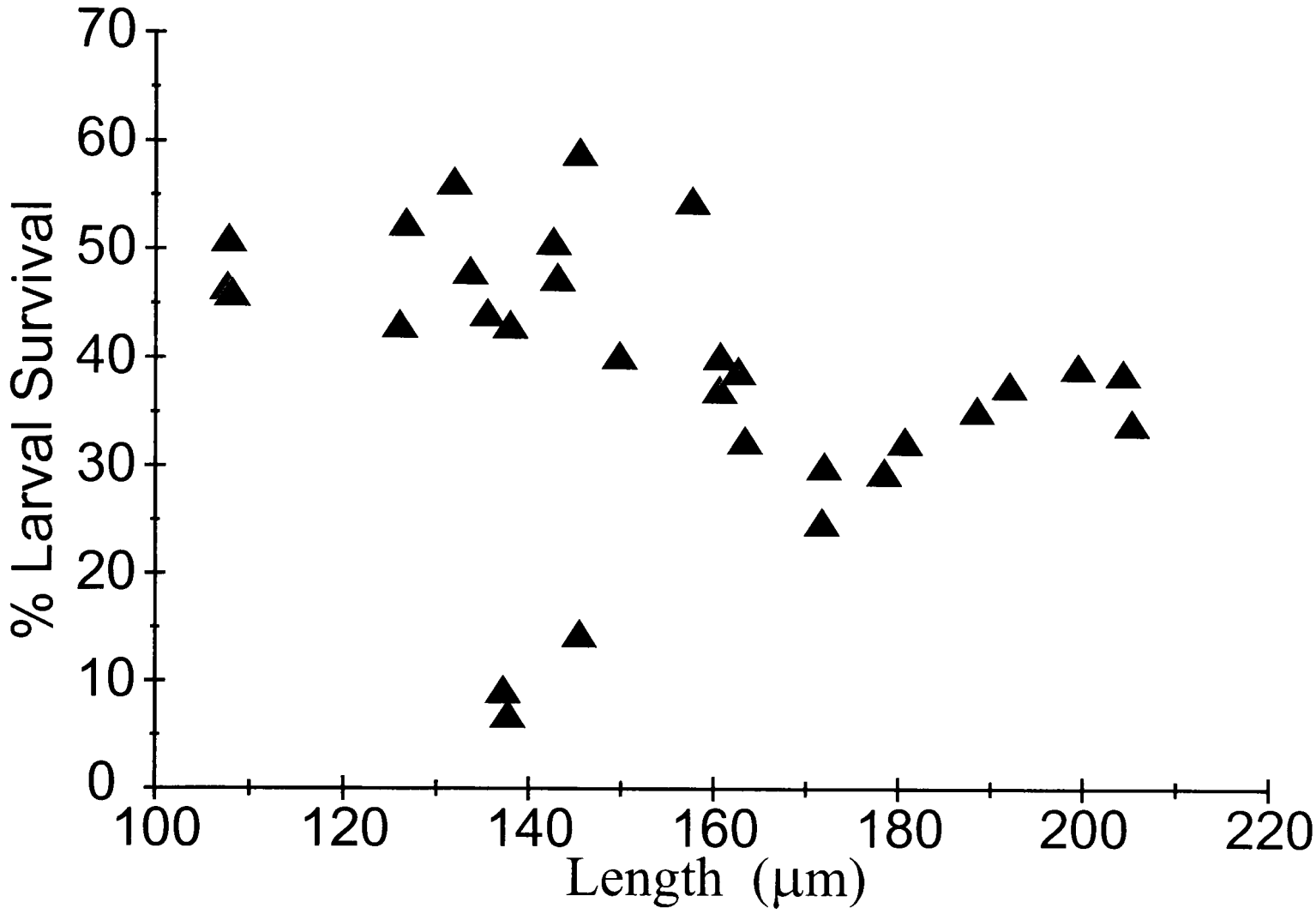

Figure 3.4.6. Length/Survival relationship for the larvae fed on *R. reticulata* cultured under Low Light conditions. Results shown include the first experiment and its repetition.

The consistent lack of relationship between larval growth and survival that has been observed for the various algal diets tested on various larval batches collectively suggest that, these larval parameters cannot be simultaneously correlated with the quality of the food that the larvae were feeding on. As a consequence of this together with the almost random survival rates that the larval cultures exhibited during these experiments also implies that, larval survival cannot be used as a basis of evaluation of the algal diet, whereas the much more consistent larval growth measurements are a valuable index of the nutritional value of the algal diets.

The larvae fed on algae grown at high light and the low light (repetition experiment) were analysed for their fatty acid content. Larvae from 2 beakers were processed separately. Because the larvae were from the same batch, the control and unfed larvae were the same for both high and low light experiments.

Results are summarised in Table 3.4.7. The use of individual F.A. as indices of growth in *M. edulis* larvae, will be discussed at a later chapter. In this stage however, some general observations about the larval FA content can be made.

Table 3.4.7. Fatty acid content of *M. edulis* larvae obtained during the *R. reticulata* experiments. Individual FA are expressed as % of Total Identifiable Fatty Acids (TIFA). Values are mean of two samples analysed. **HL** = High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**= Nitrogen limited medium. Numbers with asterisks (*) on some FA's indicate that although the FA was identified in both samples in one of them, it was not integrated; therefore for the respective F.A. the value is taken from the sample that the FA was integrated.

Fatty Acid	Control	Unfed	HL f/2	HL P	HL N	LL f/2	LL P	LL N
14:0	4.44	4.67	5.54	5.15	4.21	3.59	3.33	4.03
15:0	1.71	3.69	3.67	2.65	3.16	2.49	3.35	1.09
16:0	26.62	31.88	26.48	24.63	26.66	23.53	26.31	22.93
16:1ω7	5.57	7.65	7.93	6.33	5.20	7.41	8.15	4.10
18:0	8.92	10.96	9.21	8.82	9.66	9.46	9.58	7.29
18:1ω9	3.68	11.41	8.72	9.17	6.64	9.71	8.85	14.63
18:1ω7	4.22	4.05	5.95	6.77	10.47	6.79	7.39	6.85
18:2ω6	2.85	2.39	3.01	5.25	1.94	5.94	2.04	4.93
18:3ω3	2.64	0.96	1.32	3.44	1.97	2.10	1.60	9.10
18:4ω3	1.81	0.29	0.99	1.43	1.68	2.17	1.52	4.02
20:1	2.84	1.97	1.88	1.29	1.51	0.73	2.06	3.14
20:1	3.80	1.08 *	2.65	2.91	3.48	2.09	2.99	2.57
20:4(ω6?)	2.73	0.99	1.70	2.74	1.98	1.81	2.12	1.31
22:0	3.57	6.84	5.31	5.13	2.11	4.81	4.42	3.06
20:5ω3	9.46	3.03	4.46	4.12	6.58	6.08	4.83	3.87
22:1ω11	1.33	1.77	1.21	1.47	1.47	1.58	1.80	1.53 *
22:5ω3	0.99	1.60	1.12	0.64	0.64	0.83	0.53	0.25
22:6ω3	12.80	5.32	8.85	8.08	10.66	8.88	9.13	6.06
Total :								
Saturated Fatty Acids	45.27	58.04	50.21	46.37	45.78	43.88	46.99	38.41
Mono Unsaturates	21.45	26.84	28.34	27.94	28.76	28.32	31.24	31.29
Poly Unsaturates	33.28	14.59	21.45	25.70	25.46	27.80	21.77	29.54
ω:3 series Fatty Acids	27.70	11.20	16.74	17.70	21.54	20.05	17.60	23.30

The FA profile of the larvae exhibit rather conservative changes. Overall changes of relative proportions of FAs of larvae fed on different diet are small. The FA with the biggest relative content are the 16:0 and the 22:6ω3. The trends of change depended on nutrient conditions shown for these two FAs are different between the light intensities. When larvae were fed with *R. reticulata* grown under high light, **N** limitation increases the relative proportion of 22:6ω3 but does not change the relative proportion of 16:0, whereas **P**-limitation does not change the amount of the 22:6ω3 but decreases the amount of 16:0. Under Low Light culture conditions for the alga, **N**-limitation decreases the

relative amount of 22:6ω3 but does not have any detectable effect on the amount of larval 16:0, **P**-limitation increases the amount of 16:0 and does not change the relative proportion of 22:6ω3. As noted earlier, aspects of this discussion relative to the larval FA content will be presented in the later chapter dealing with the use of certain FA as indices of growth for *M. edulis* larvae (Ch. 3.6).

As a diet for *M. edulis* larvae, *R. reticulata* appears to be an adequate food source. In many instances the larvae fed on this species alone, grew as well as the control diet which was a mixture of two species. However, in the first experiment for both HL and LL conditions the larvae did not perform as well as the control diet. On the repetition experiment the larvae perform better than the control diet. But the trend within the test diets remained the same for both experiments. This seeming discrepancy is in fact explained by the significantly different performance of the control diet during these two experiments and it is not due to the performance of the *R. reticulata* diets, which were grown employing the continuous culture technique. On a statistical basis it is not reasonable to exclude either experiment from the analysis. However pooling the data from both experiments, poses the problem that since the larvae achieved significantly different end lengths (albeit with the same trend within the test diet) the resulting mean values will have a big standard variation which will clutter the overall view. Again, it has to be stressed that this is not only due to the inherent variation within the population but due to significantly different lengths achieved by the larvae. In statistical terms it could be argued that the precision within experiments is satisfactory but the accuracy between them is not. For reasons of consistency with the layout followed on the previous species the pooled-data graph for both experiments in all conditions is presented in Fig. 3.4.7.

Disregarding the wide standard variation it is noticeable that *R. reticulata* is a good food for *M. edulis* larvae but does not seem to change much its nutritional value under nutrient stress or different light conditions. This can be viewed in conjunction with the results summarized in Table 3.4.1 where the cellular content of various biochemical components was presented; the overall image obtained after that section was that, *R. reticulata* is not very much affected by the tested environmental conditions, at least in a way demonstrated by the change of the analyzed biochemical components. This is most likely the reason that *M. edulis* larvae fed on *R. reticulata* do not exhibit very different behaviour. The only case that the nutritional value of this species was changed for the

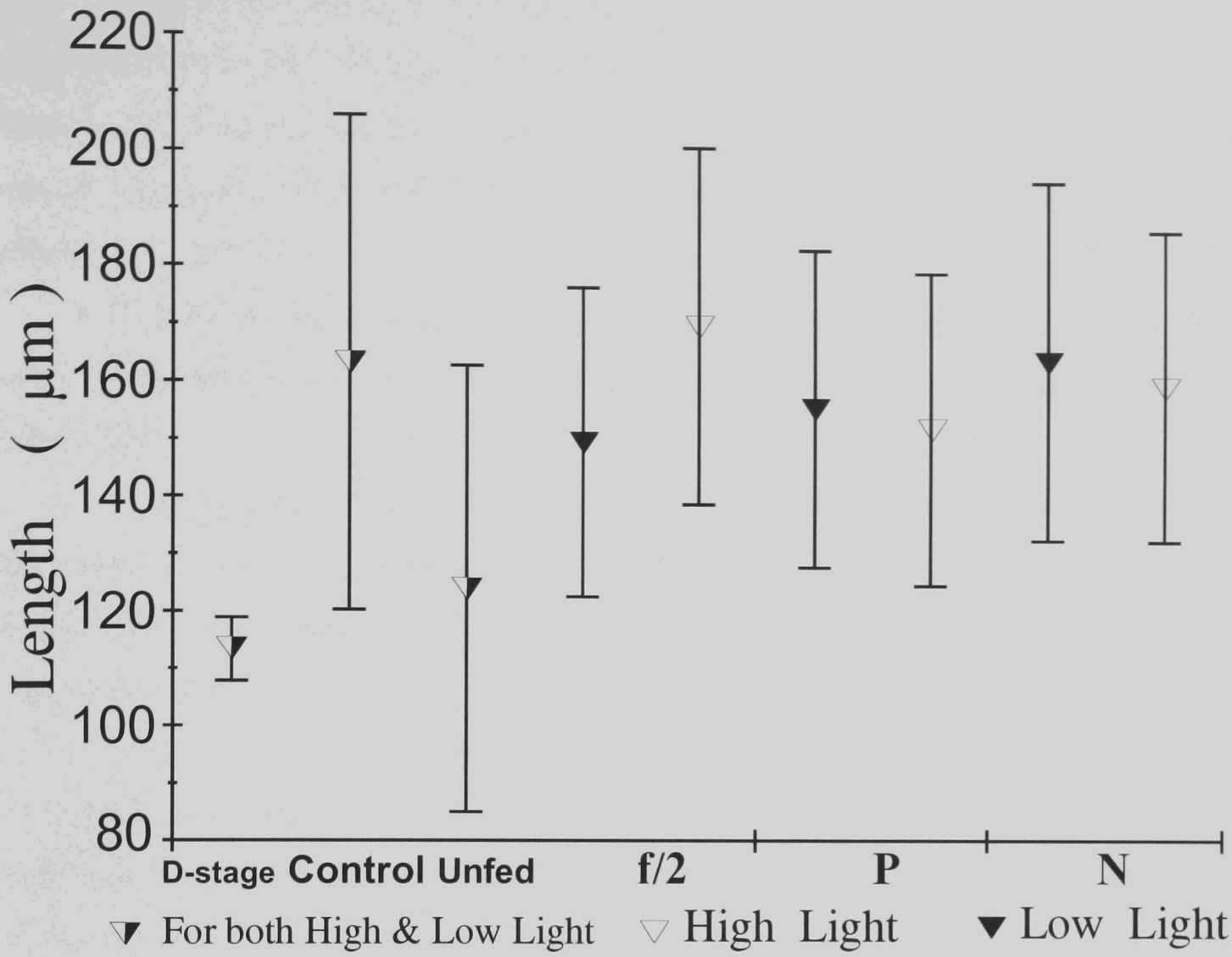

Figure 3.4.7. Averaged results for both HL & LL experiments, of *M. edulis* larvae fed on *R. reticulata* of various nutrient and light conditions.

better, was under low light conditions in the non-limiting f/2 medium, which therefore appears to be the optimum conditions to culture the species if the goal is to improve the nutritional quality for *M. edulis* larvae.

When comparing the two light intensities used, it is clear that low light conditions will produce cells of better nutritional quality, only when the non-limiting **f/2** medium is used. In the aquacultural literature, this species has not been extensively used as a food source and from the results here this cannot be explained since *R. reticulata* is an adequate food source for the larvae used here as bioassay organisms.

Further to the points raised in the discussion of previous species, on more specific components, i.e. total protein, fatty acid etc., and the literature sited there (Ackman *et al.*, 1964; Webb & Chu, 1983; Enright *et al.*, 1986a; Su *et al.*, 1988;Volkman *et al.*, 1989; O'Connor *et al.*, 1992; Sánchez *et al.*, 1993,1994; Blanchemain & Grizeau, 1996), there are few observations more specific to the *R. reticulata* species that can be made.

It has a relatively high proportion of PUFA's which, as advocated by various researchers in the field (Enright *et al.*, 1986b; O'Connor *et al.*, 1992) can be used as a basis for investigating the dietary value of a species. It also has a substantial amount of omega :3 fatty acid which is thought to be of importance for the development of spat (Webb & Chu 1983).

In addition, due to its larger size, it has a higher per cell concentration of carbohydrate and protein which will further enhance its dietary value (Wikfors *et al.*, 1984, 1992)

It is difficult to identify which change of which individual component is having this effect. As with all algal species, it is reasonable to assume that it will be the combination of the change of various biochemical components that will determine the overall nutritional value.

These aspects of the nutritional requirements and the concept of an accepted "balanced" diet (Gallager & Mann, 1981; Langdon & Waldock, 1981; Wikfors *et al.*, 1992) will be analysed in more detail in the chapter dealing with the correlation of various biochemical parameters with larval growth (ch. 4.2 & 4.3).

3. Results and Discussion

3.5 Environmental effects on growth, biochemical composition and nutritional quality of *Pavlova lutheri*

A summary of the effect of nutrient concentration and light intensity on growth, dry weight, chlorophyll, protein and carbohydrate concentration is shown in Table 3.5.1.

Table 3.5.1. Responses of *P. lutheri* to various conditions. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Condition	Generation time **G** in **h** (SD)	**Dry weight** pg/cell	**Protein** pg/cell	**Carbohydrate** pg/cell	**Chl A** (pg/cell)	**Chl C** (pg/cell)
HL f/2	68.3 (18.50)	26.52	3.66	0.51	0.251	0.231
HL P	100.5 (57.53)	44.89	6.56	0.94	0.308	0.236
HL N	69.4 (21.03)	32.08	4.07	0.45	0.206	0.181
LL f/2	136.4 (54.98)	43.29	4.48	0.55	0.381	0.263
LL P	147.3 (41.87)	42.34	4.97	0.54	0.394	0.354
LL N	132.4 (70.56)	19.02	7.08	0.74	0.366	0.263

The generation time of this species, *P. lutheri*, responds to a limited extent to both nutrient and light limitation. For the other test species, generation times were demonstrated to vary by a factor of up to four times, especially between light conditions. *P. lutheri* exhibited a maximum variation of generation time of less than two times, from approximately 70 h (**HL f/2 & HL N**) to <140 h (**LL f/2 & LL N**). The Low light conditions, used in these experiments, did not affect its generation time significantly, since under these conditions **G** was only slightly increased. However, it is noticeable that, probably due to the smaller size of this species, with the much greater cell numbers achieved in these culture conditions, the overall calculated generation time showed a greater standard deviation. **F/2** and **N**- limited media produced almost identical growth rates, while **P**- limited medium increased slightly (not significant, $p > 0.05$) the species' generation time. The per cell dry weight of *P. lutheri* was much smaller than that of the two diatom species tested here (*S. costatum* & *C. muelleri*), although they were roughly of comparable size, due to the lack of a silica cell wall, which increased significantly the dry weight of the diatoms, but has no nutritional value to the larvae. Various division rates of *P. lutheri* tabulated from the literature (e.g. Blinn, 1984; Nelson *et al.*, 1992) range from

20 h in moderately limited medium, to 353 h in a more strongly limited medium (Reitan *et al.*, 1994) and in relation to temperature, from 12 h at 25 °C to 48 h at 10 °C, as reported by Thompson *et al.* (1992a). Growth rates calculated in the present work are within the range reported by other investigators. The temperature at which the species shows a maximum division rate is 25 °C (Thompson *et al.*, 1992a), which was the temperature used in the present work.

Protein analyses of the cells cultured in all the various conditions used showed a moderate variation, with no consistent pattern emerging either between nutrient or light conditions. The biggest increase in protein content happened in the **P**-limited culture under High Light conditions, while there was only minimal increase of per cell protein content in the respective medium under Low Light conditions. **N**-limitation increased the protein content of the *P. lutheri* cells only under Low Light conditions. The overall variation of carbohydrate content was smaller than the one of protein for the applied culture conditions.

The cells increased the quantities of all chlorophylls when cultured in low light conditions. Obviously, when cultured under low light the cells need to have more chlorophyll to make more efficient use of the less light. It is also noticeable that among nutrient conditions, P-limited media, produced cells containing more of all chlorophylls, at both high and low light conditions.

The chemical analyses for the nutrient concentrations of inflow and outflow media for *P. lutheri* cultures are shown in Table 3.5.2.

Table 3.5.2. Nutrient concentrations of major macronutrients in Inflow (**Infl.**) and Outflow (**Outfl.**) media in various conditions used for the *P. lutheri* cultures. Figures indicate μgr-at/l. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Condition	Infl. $^{-3}PO_4$	Outfl. $^{-3}PO_4$	Infl. $^{-1}NO_2$	Outfl. $^{-1}NO_2$	Infl. $^{-1}NO_3$	Outfl. $^{-1}NO_3$
HL f/2	25.17	11.13	0.07	9.77	259.30	240.70
HL P	4.99	0.17	0.14	8.78	254.55	249.94
HL N	16.70	15.49	0.38	0.73	188.52	0.96
LL f/2	25.17	21.78	0.07	3.74	259.30	266.23
LL P	4.99	0.84	0.14	2.67	254.55	259.05
LL N	16.70	21.62	0.38	0.76	188.52	28.06

As is to be expected, the $^{-3}PO_4$ and $^{-1}NO_3$ nutrient concentrations were lower in outflow media. In N-limited cultures the amount of $^{-1}NO_3$ present in the outflow medium was minimal while the other macronutrients were in much greater quantities.

Accordingly, $^{-3}PO_4$ concentration in the outflow medium was minimal in the P-limited cultures. These patterns indicate that, the assigned macronutrient was the limiting factors for each respective medium.

The fatty acid profile of this species can be seen in Table 3.5.3, for all different culturing conditions.

Table 3.5.3. Fatty acid composition of *P. lutheri* under various culturing conditions. Individual fatty acids are expressed as % of Total Identifiable Fatty Acids (**TIFA**). Values are means of three samples, except for HL N, where values are mean of two samples. **B.D.**=Below detection level. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium. **P.U.?**=unidentified Poly Unsaturated FA. FA into parenthesis with a question mark mean that although there was not exact identification of the FA from the Gas Chromatographer traces, additional data from the Mass Spectrometer-Gas Chromatographer indicate that this FA was, almost certainly, the one that was assumed to be.

Fatty acid	HL f/2	HL P	HL N	LL f/2	LL P	LL N
14:0	5.68	6.49	5.60	6.03	4.10	5.13
15:0	0.87	0.27	0.88	0.52	0.49	0.80
16:0	18.50	24.64	17.62	14.95	16.44	16.29
16:1ω7	12.99	18.66	11.34	8.89	9.33	10.17
18:0	2.29	0.83	1.92	1.88	1.87	1.36
18:1ω9	3.13	4.01	4.15	2.12	3.23	2.40
18:1ω7	0.47	0.59	B.D.	1.16	0.53	0.82
18:2ω6	4.81	5.39	4.76	2.12	5.65	2.19
18:3ω3	0.99	1.66	1.88	1.25	2.17	1.53
18:4ω3	7.78	3.77	6.90	10.23	5.22	10.76
(P.U.) ?	6.79	5.07	12.09	12.71	9.06	12.55
(20:4ω6)?	0.50	0.78	0.86	0.58	0.93	0.55
(22:0)?	0.37	0.44	1.09	0.47	0.49	0.50
20:5ω3	27.88	18.71	17.66	22.38	31.14	27.45
22:5ω3	0.45	0.42	2.06	1.13	0.59	0.43
22:6ω3	6.50	8.26	11.19	13.56	8.76	7.07
Total Saturated F.A.	27.71	32.67	27.11	23.86	23.37	24.07
Total Monounsaturated F.A.	16.59	23.26	15.49	12.18	13.10	13.39
Total PolyUnsaturated F.A.	55.71	44.07	57.40	63.96	63.53	62.53
Total ω:3 series F.A.	43.61	32.83	39.69	48.55	47.89	47.24

P. lutheri seemed to increase the relative content of saturated FA (mainly 16:0) and decrease the relative quantities of Poly Unsaturated FAs, under High Light and P-limited conditions, while in the other conditions the FA profile remained remarkably similar. Another FA that was noted for increasing its relative proportion in the **HL P** condition was the 16:1ω7. The rest of the identified FA appeared either not to change their relative amounts or when they did (by not much though, in any case), not to have any consistent pattern among nutrient conditions or light intensities.

The growth of the larvae fed on *P. lutheri* grown in all the previously mentioned conditions is shown in Fig. 3.5.1. Results show growth of larvae in each individual beaker. The repetition of this experiment with another batch of larvae can be seen in Fig. 3.5.2.

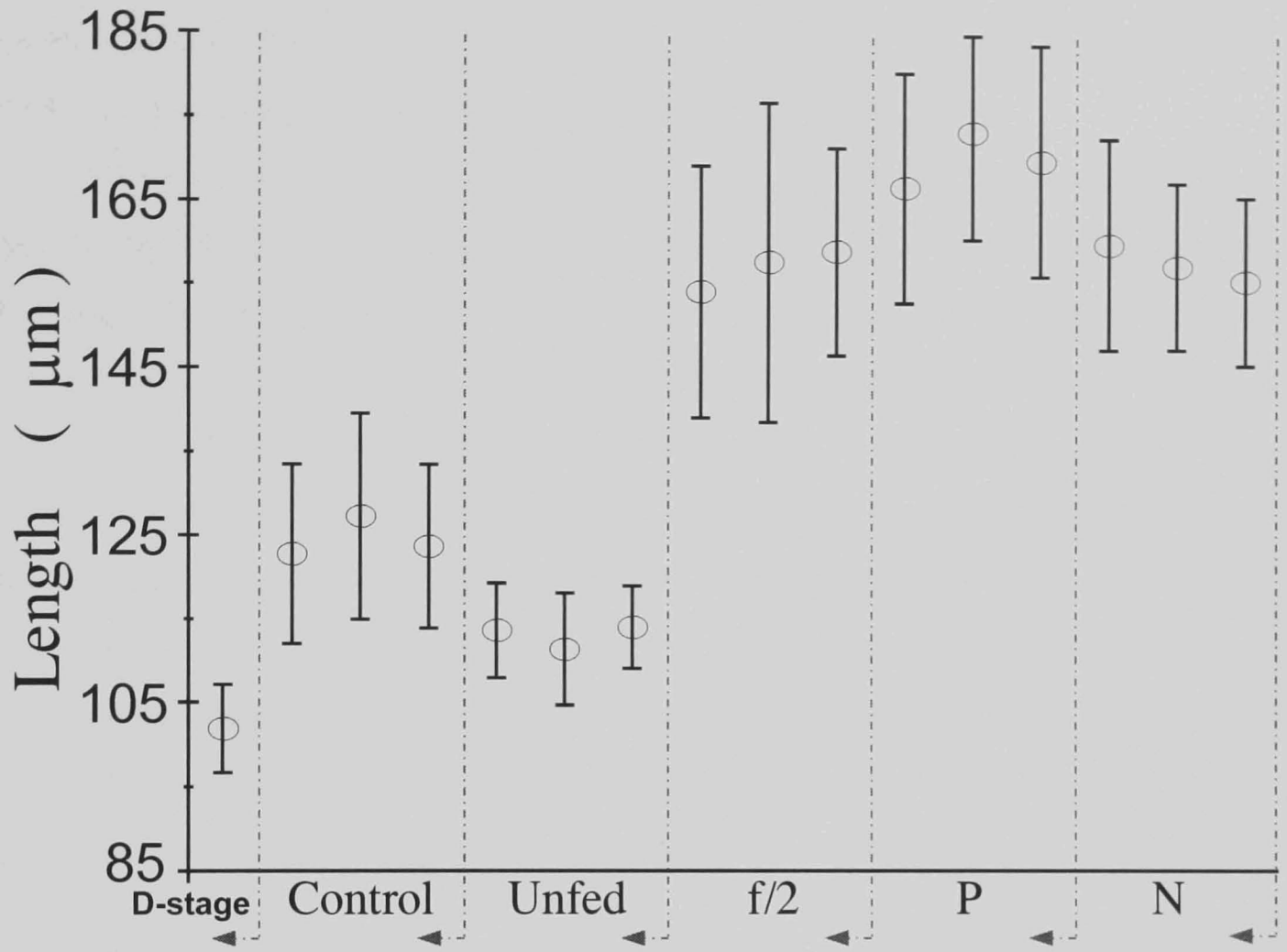

Figure 3.5.1. Size of *M. edulis* larvae fed on *P. lutheri* cultured under high light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

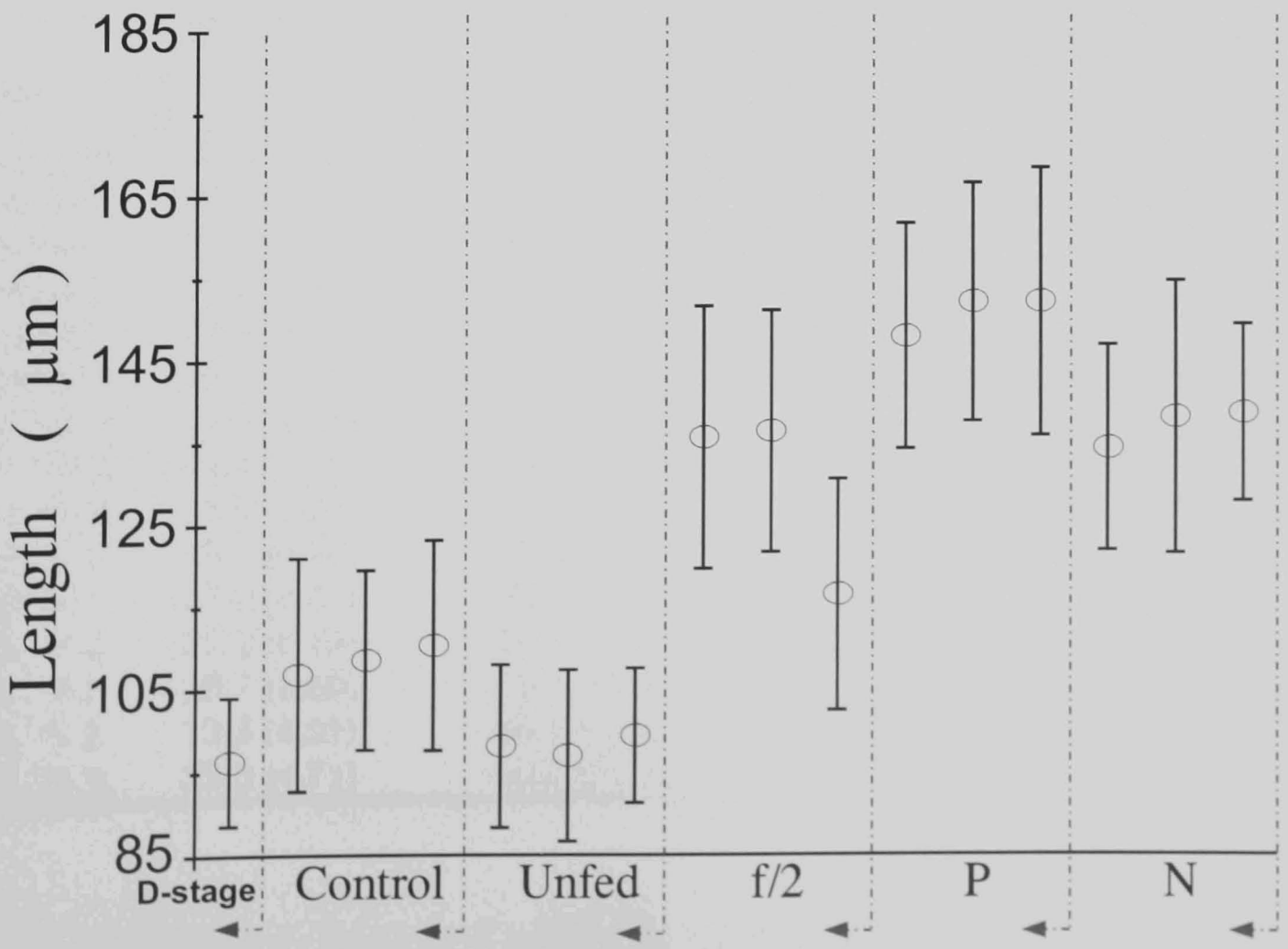

Figure 3.5.2. Size of *M. edulis* larvae fed on *P. lutheri* cultured under high light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

Again, as with previous experiments, it is clear that unfed larvae showed minimal growth; this difference in length between fed and unfed larvae is significant ($p<0.05$, 95% level). As was explained in the case of the previous species, in order not to mask any other potential differences between diets the unfed larvae will not be included in the statistical analysis. The results of every test are given in detail in the Appendix with a Table number inside parenthesis after every reference to a test here.

For both experiments, nested anova and multiple means test show that all *P. lutheri* diets perform significantly better that the control diet (A.3.5.1 – A.3.5.4); as in other cases, the reader is referred to section 2.2.b for explanation that although the term "control" diet is used for the standard diet of *P. lutheri* and *R. reticulata* in a 4: 1 ratio, this diet is not *sensu stricto* a control diet but it was used as a reference diet. Multiple Means comparison test reveals that, after the two-week feeding period, the larvae achieved greater lengths when fed on the P- or N- limited diet, for both experiments (A.3.5.2 & A.3.5.4). For both feeding trials, the cells cultured in the f/2 medium although better than the control diet, were inferior to the P- and N- limited diets (A.3.5.2 & 3.5.4). It should be noted that the variation within triplicates was much higher in the second experiment and just marginally unacceptable in the first experiment ($p=0.04$, A.3.5.1).

Table 3.5.4. % Survival of larvae fed *P. lutheri* cultured in high light conditions. **HL**=High light, **LL**=Low light, **f/2**=f/2 medium with no nutrient limitation, **P**=Phosphorus limited medium, **N**=Nitrogen limited medium.

Diet	% Survival (SD) of larvae for the exper. of ***P. lutheri* HL**	% Survival (SD) of larvae for the **repeat** experiment
Control 1	35.0 (2.36)	71.9 (2.48)
Control 2	40.0 (7.20)	80.7 (12.24)
Control 3	43.3 (3.60)	69.0 (8.64)
Unfed 1	36.7 (1.36)	38.0 (2.98)
Unfed 2	29.4 (1.57)	49.1 (2.87)
Unfed 3	35.6 (4.16)	42.7 (7.07)
HL f/2 1	43.3 (8.28)	73.1 (10.56)
HL f/2 2	38.9 (8.75)	79.0 (6.24)
HL f/2 3	35.6 (3.42)	5.8 (0.83)
HL P 1	36.1 (5.50)	77.8 (10.06)
HL P 2	27.2 (4.37)	70.8 (2.19)
HL P 3	27.2 (0.79)	74.3 (1.65)
HL N 1	26.7 (3.60)	59.1 (6.46)
HL N 2	33.3 (4.91)	68.4 (9.93)
HL N 3	33.3 (4.71)	60.2 (9.54)

Survival for each experiment is shown in Table 3.5.4. From this table, it is further confirmed that survival shows a random pattern and cannot be correlated with the quality of food that the larvae were receiving.

Since there is extremely good agreement between the different batches of larvae used it can safely be concluded that *P. lutheri* alone is a much superior diet than the control diet, with larvae having a maximum growth rate from all the other diets used.

The length / survival relationship, which is shown, for both high light experiments, in Fig. 3.5.3 confirms that there is a random relationship between these two parameters. This discards the potential argument that if fewer larvae survive they should grow more since there would be more food available per live larva.

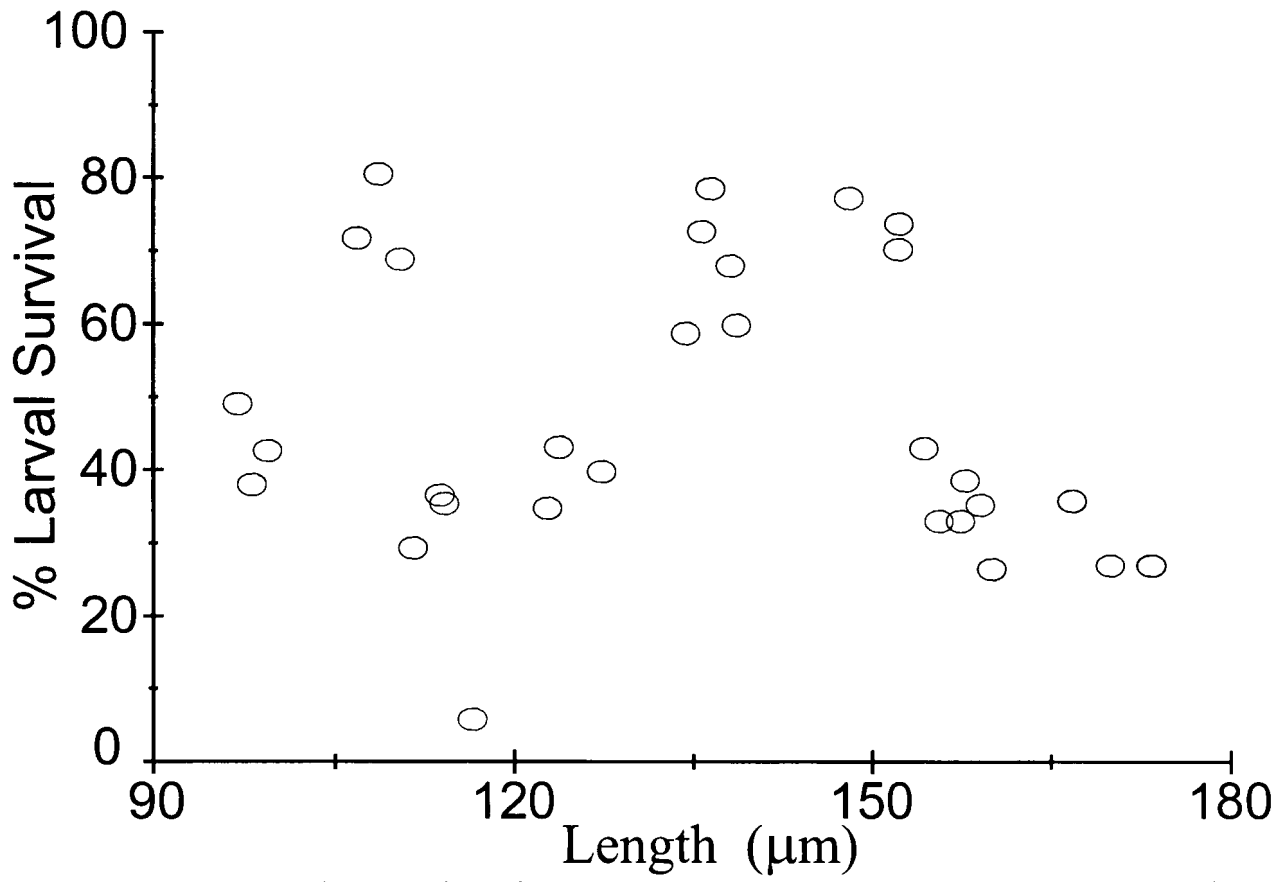

Figure 3.5.3. Length/Survival relationship for the larvae fed on *P. lutheri* cultured under High Light conditions. Results shown include the first experiment and its repetition.

For *P. lutheri* cultured under low light conditions, results of the first experiment are shown in Fig. 3.5.4, while for its repetition in Fig. 3.5.5.

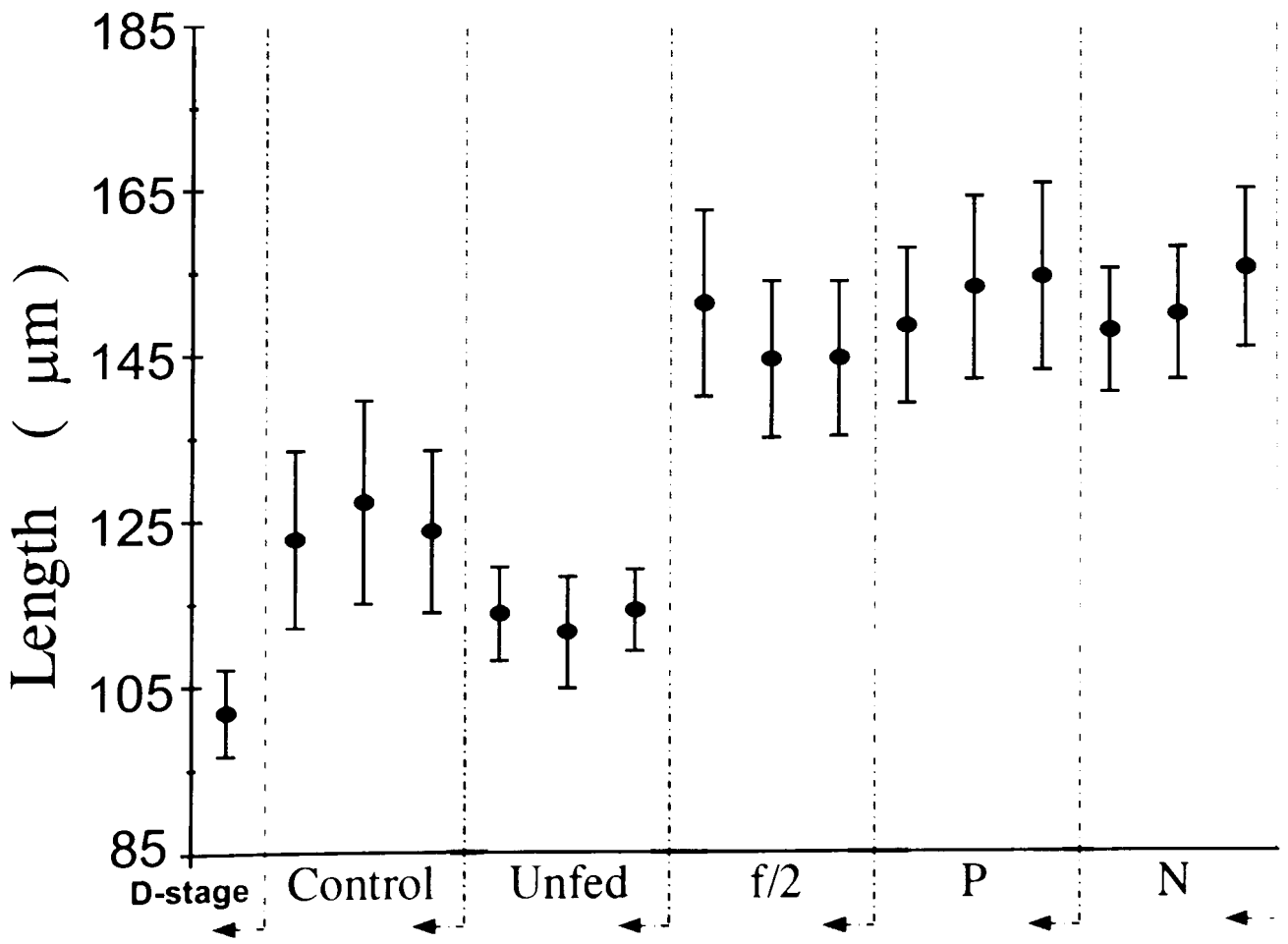

Figure 3.5.4. Size of *M. edulis* larvae fed on *P. lutheri* cultured under low light conditions, after two weeks. Results are from the first trial and vertical lines indicate ± SD.

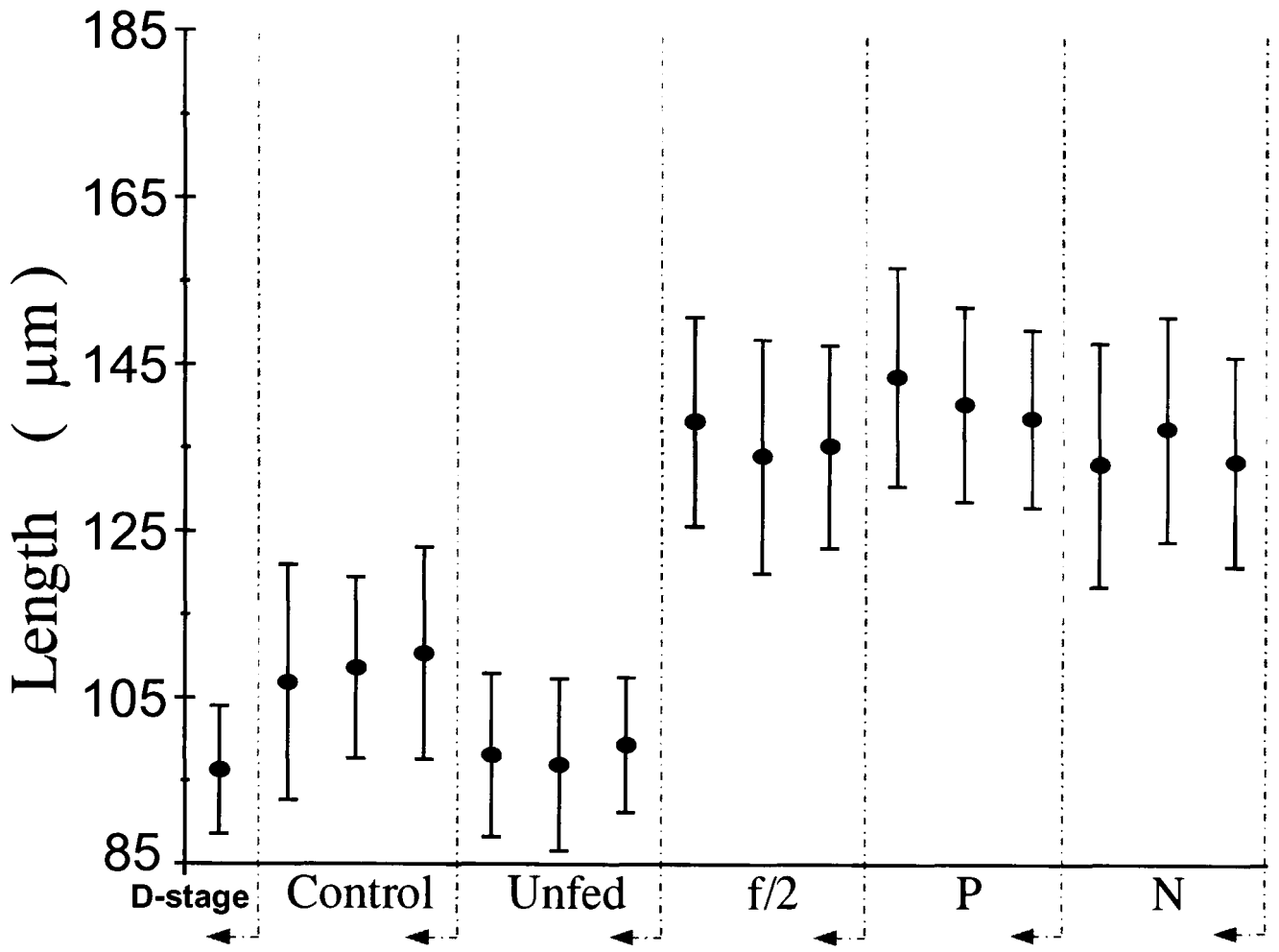

Figure 3.5.5. Size of *M. edulis* larvae fed on *P. lutheri* cultured under low light conditions, after two weeks. Results are from the repetition trial and vertical lines indicate ± SD.

For both the Low light experiments, variation among the triplicates of the same diet was within acceptable limits, as supported by the test for homogeneity of variance (Sokal & Rohlf, 1981a). In both experiments all *P. lutheri* diets produced larvae that were bigger than the control diet; the difference is significant ($p<0.05$, A.3.5.5 & A.3.5.7). However, among various nutrient conditions, the differences were not significant, (A.3.5.6 & A.3.5.8). These results, confirm that, overall, *P. lutheri* is a very good food for *M. edulis* larvae. In the low light conditions the alga did not seem to change its nutritional quality.

Table 3.5.5 % Survival of larvae fed *P. lutheri* cultured in low light conditions

Diet	% Survival of larvae for the experiment of ***P. lutheri* LL**	% Survival of larvae for the **repeat** experiment
Control 1	35.0 (2.36)	71.9 (2.48)
Control 2	40.0 (7.20)	80.7 (12.24)
Control 3	43.3 (3.60)	69.0 (8.64)
Unfed 1	36.7 (1.36)	38.0 (2.98)
Unfed 2	29.4 (1.57)	49.1 (2.87)
Unfed 3	35.6 (4.16)	42.7 (7.07)
LL f/2 1	37.2 (6.71)	64.9 (5.17)
LL f/2 2	44.4 (3.14)	65.5 (7.89)
LL f/2 3	32.8 (4.37)	61.4 (3.79)
LL P 1	39.4 (3.42)	66.1 (5.79)
LL P 2	37.8 (13.22)	71.9 (10.82)
LL P 3	37.8 (2.83)	81.9 (4.38)
LL N 1	42.2 (12.93)	58.5 (6.62)
LL N 2	31.1 (9.06)	64.9 (6.57)
LL N 3	40.6 (6.43)	88.3 (11.58)

Survival rates for *P. lutheri*, when cultured under low light conditions, are shown in Table 3.5.5.

Again, survival shows a random pattern and does not seem

to be correlated with the diet upon which the larvae were fed.

To investigate whether a growth/survival relationship exists the length and survival data were plotted against each other. Results are shown in Fig. 3.5.6.

The result illustrates graphically that there is apparently a random relationship between growth and survival.

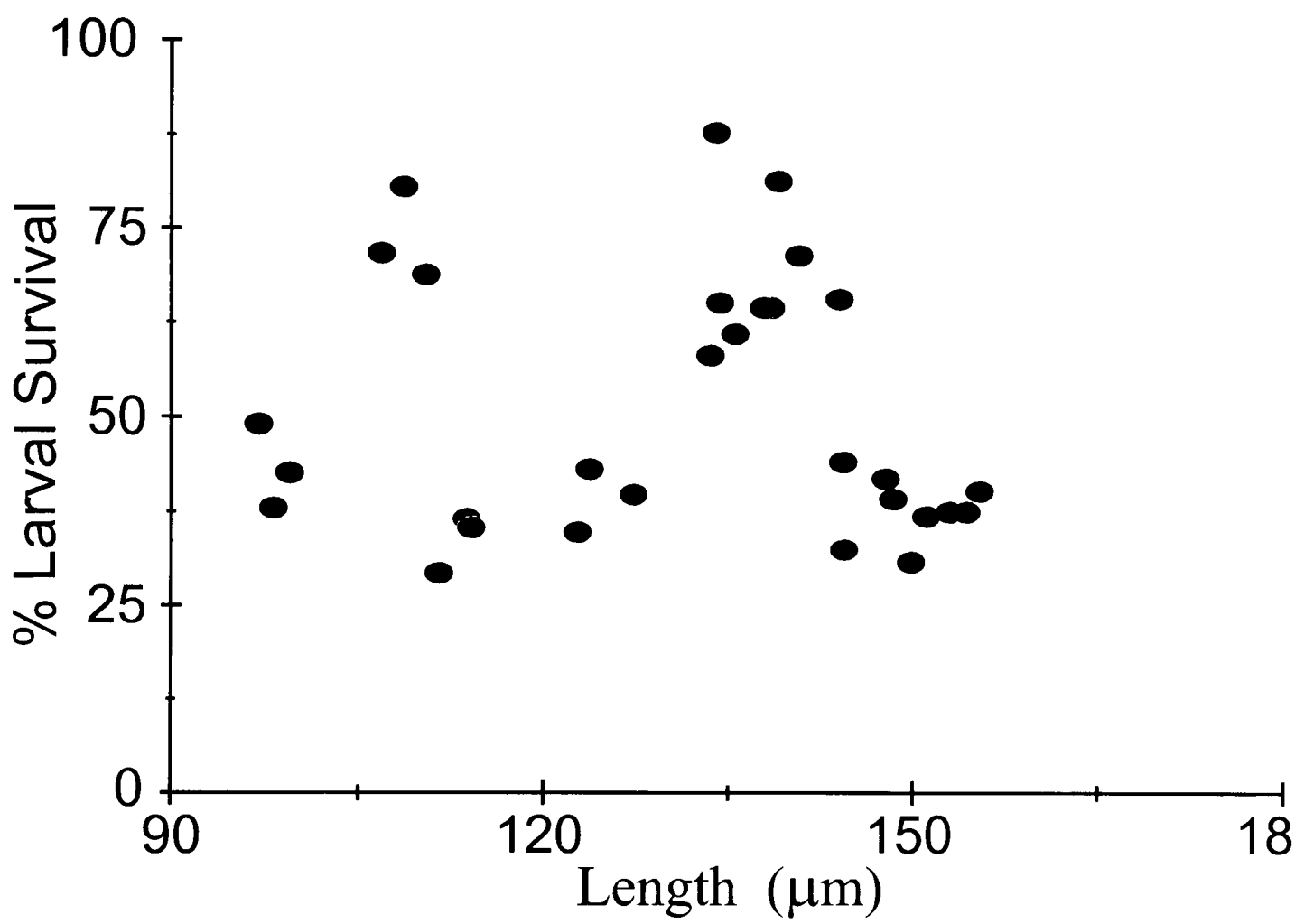

Figure 3.5.6. Length/Survival relationship for the larvae fed on *P. lutheri* cultured under Low Light conditions. Results shown include the first experiment and its repetition.

The larvae fed on algae grown at high light (first experiment) and the low light (first experiment) were analysed for their fatty acid content. Larvae from 2 beakers were individually processed. Because the larvae were from the same batch, the control and unfed larvae are the same for both high and low light larvae. Results are summarised in Table 3.5.7.

The use of individual F.A. as indices of growth in *M. edulis* larvae, will be discussed in a later chapter. In general, the major FA components are the 16:0, the 18:1ω9, the 18:1ω7, the 20:5ω3 and the 22:6ω3, as was the case with the FA profiles obtained by the other batches of larvae used in the feeding trial for the rest of the tested algal species. These FA are observed to change their relative proportions but the pattern of change of the FAs does not appear to have any consistent theme.

Table 3.5.7. Fatty acid content of *M. edulis* larvae obtained during the *P. lutheri* experiments. Individual FA are expressed as % of Total Identifiable Fatty Acids (**TIFA**). Values are mean of two samples analysed. Numbers with asterisks (*) on some FA's indicate that although the FA was identified in both samples in one of them, it was not integrated; therefore for the respective F.A. the value is taken from the sample that the FA was integrated (see also section 2.3.c). **B.D.**=FA below detection level. **N.I.**=Fatty Acid peak not integrated properly.

Fatty acid	Control	Unfed	High f/2	High P	High N	Low f/2	Low P	Low N
14:0	2.64	3.28 *	2.10	3.43	N.I.	0.68 *	2.63	3.46
15:0	1.41	1.69	1.09	0.87	N.I.	0.32 *	1.38	1.20
16:0	21.72	23.86	21.10	21.43	12.10	11.18	21.85	19.78
16:1^{a}7	7.58	5.15	10.24	13.64	1.76	2.90	7.48	9.58
18:0	9.16	4.79	6.77	5.53	11.32	9.23	8.25	6.62 *
18:1^{a}9	6.75	11.37	5.77	5.59	7.44	8.82	6.34	6.89
18:1^{a}7	4.45	8.89	10.06	4.33	5.14	5.30	4.14	8.31
18:2^{a}6	1.52	2.52	1.26	3.86	1.83	1.34	1.85	1.71
18:3^{a}3	1.17	B.D.	0.39	1.07	1.16	1.58	1.61	1.95
18:4^{a}3	2.12	1.13	0.82	1.09	0.67	1.46	0.93	3.59
20:1	3.57	B.D.	2.13	3.01	N.I.	7.68	3.96	N.I.
20:1	4.41	7.02	4.21	2.78	6.16	5.83	2.94	3.94*
20:4(^{a}6?)	1.88	2.35 *	1.63	1.61	1.64	3.03	2.91	1.59
22:0	0.86	3.97	4.16	0.86	2.58	1.06	1.15	1.62 *
20:5^{a}3	11.41	4.72	10.51	13.50	15.15	16.08	13.35	15.26
22:1^{a}11	2.72	B.D.	1.51	0.95	N.I.	N.I.	N.I.	N.I.
22:5^{a}3	0.90	5.24	0.85	1.16	3.20	2.36	3.16	3.44
22:6^{a}3	15.72	16.84	15.41	15.28	29.85	21.65	16.05	17.14
Total :								
Saturated Fatty Acids	35.80	34.31	35.22	32.12	26.00	21.46	35.27	24.44
Mono Unsaturated	29.48	32.43	33.91	30.30	20.50	30.53	24.87	24.78
Poly Unsaturated	34.72	30.45	30.87	37.58	53.50	47.50	39.86	44.69
ω:3 series Fatty Acids	31.32	27.93	27.98	32.10	50.03	43.13	35.10	41.39

As a diet for *M. edulis* larvae, *P. lutheri* is a very good food source. In all cases larvae fed on this species grew better than the control diet which was a mixture of two species (*P. lutheri* and *R. reticulata*). Results of both experiments are in excellent agreement with each other, which strengthens the previous argument. The averaged results of three replicates, for both experiments in all conditions, are shown in Fig. 3.5.7.

It also further confirmed, not only that the diet concentration (50 cell/μl) was not limiting, but also that larvae grow more when fed a diet of nutritional quality more suited to their demands. This can be verified by the fact that this species has the smallest per cell dry weight of all the species used and therefore growth of the larvae is not due to consumption of more material, but only due to consumption of material of better quality.

P. lutheri, as shown in Tables 3.2.1. & 3.2.3 will change its growth rate and biochemical composition, to adapt to culture conditions. Subsequently, its overall

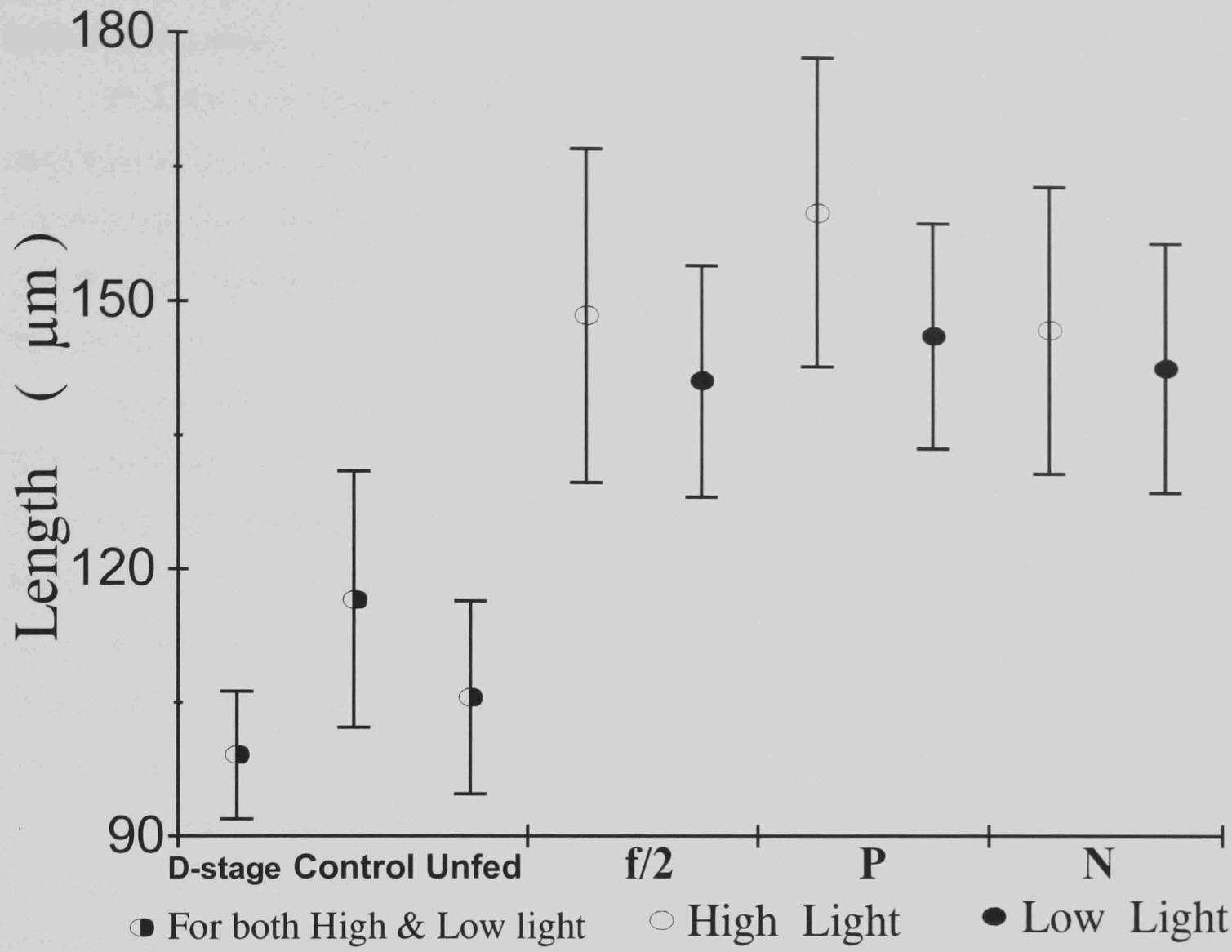

Figure 3.5.7. Averaged results for all replicates of both experiments in all conditions.

nutritional quality for *M. edulis* larvae is also shown to change. **P**-limitation in both light conditions will produce bigger larvae that the other two nutrient conditions.

N-limited and non-limited cells, when presented as food to the larvae will produce larvae of equal length in both light conditions.

When comparing the two light intensities used, in contrast to the previous two diatoms used, high light conditions will produce cells of better nutritional quality, in all nutrient conditions, but the differences becomes more conspicuous when P-limited medium is used. It is difficult to identify which change of which individual component is having this effect. As with all algal species, it is reasonable to assume that it will be the combination of the change of various biochemical components that will exercise the overall effect.

Notwithstanding the points raised in the discussion of the previous species, on more specific components, i.e. total protein, fatty acid etc., and the literature cited there (Ackman *et al.*, 1964; Webb & Chu, 1983; Enright *et al.*, 1986a; Su *et al.*, 1988;Volkman

et al., 1989; Blanchemain & Grizeau, 1996), some observations more specific to the *P. lutheri* can be made.

P. lutheri is a species widely used as food for larval rearing usually with good results for various bivalve larvae i.e. hard clam, *Mercenaria mercenaria*, Pacific oyster. *Crassostrea gigas*, Japanese scallops, *Patinopecten yessoensis*. In the present experimental work, this species was, as ranked in relation to the control diet, the best of the species used.

In contrast to the two diatom species studied here, *P. lutheri*, increases the amount of saturated fatty acids and decreases the total amount of polyunsaturated fatty acid with decreasing light intensity and/or phosphorus stress. Levels of the omega:3 series fatty acids also increased with decreasing light intensity; this is in very good agreement with the result reported by Thompson *et al.* (1992b, 1994a, 1996). In general the overall fatty acid composition reported here for *P. lutheri* is in very good agreement with independent results obtained by Volkman *et al.* (1989, 1991).

The improvement of the food value of *P. lutheri* for *M. edulis* larvae would seem to require a high light intensity with P-limited medium, as the optimal conditions for culture. In these conditions the cells contain the very high amount of protein among all the conditions used, while they also have the maximum amount of carbohydrate from all the conditions used.

In this optimum condition, the cells contain a maximum amount of the saturated 16:0 fatty acid, and generally of saturated fatty acids. On the other hand, the percentage of unsaturated fatty acids are the lowest, from all the conditions used.

These aspects of the discussion will be analysed in more detail in the following chapter dealing with the correlation of various parameters (ch. 4.1 & 4.2).

3. Results and Discussion

3.6 Summarized effects of N - & P- limitation and Light intensity

The scope of this chapter is to try to summarize the effects that phosphorus and nitrogen limitation, together with light intensity, have on the biochemical parameters of the four algal species tested here. Since results have been presented previously for each single species, this chapter will also try to avoid being unnecessarily repetitive. The results of the growth of the *M. edulis* larvae have been also presented graphically before, consequently this chapter will avoid any graphical presentation, and only mean values will be quoted here. This is because issues of variation between replicates of the same diet and between trials has been discussed in their corresponding chapter, and have been shown to be acceptable. Similarly, the statistical tests carried out then will be omitted here. This chapter will mainly focus on the trends displayed by the biochemical parameters of the algae and to a minor extent the response of the larvae. In essence this is a more abstract way of assessing the results, and therefore the reader may need to refer to the previous sections where the statistical tests were displayed, for a more meticulous analysis of the larval responses.

3.6.a Effects of Phosphorus limitation

Before any attempt is made to summarize any effect of Phosphorus limitation, the averaged results of the effects of Phosphorus limitation on all the algal species biochemical properties and the corresponding growth of the larvae fed on those diets, are presented. The original data were presented in Tables 3.2.1, 3.3.1, 3.4.1 and 3.5.1. To avoid unnecessary repetition of lengthy tables, only the major biochemical components and FA are included in these tables. The complete FA profile of each species, from which the following values were quotes were shown in Tables 3.2.3, 3.3.3, 3.4.3 and 3.5.3 for each of the four algal species respectively. However, for easing the comparative purposes that are needed in this perspective, the profile of each species when cultured under the non limiting conditions, in the f/2 medium, is also included in each light condition. Protein, carbohydrate, chlorophyll a and c content, along with generation time of each

species under P-limitation are presented in Table 3.6.1, while the major FA and FA groups are presented in Table 3.6.2.

Table 3.6.1. Biochemical profile and generation time of all algal species under Phosphorus limitation and no limitation. Larval length achieved after the two week feeding trial for each of both experiments are also provided. **HL**=High Light conditions, **LL**=Low Light conditions, **f/2**=f/2 medium (no limiting nutrient), **P**=Phosphorus limited medium.

		Generation Time (h)	Protein (pg/cell)	Carbohydrate (pg/cell)	chl. A (pg/cell)	chl. C (pg/cell)	1st Larval Length (μm)	2nd Larval Length (μm)
S. costatum	HL f/2	21.6	4.62	1.22	0.237	0.115	**126.0**	**151.4**
	HL P	25.8	6.09	2.71	0.351	0.266	**115.7**	**151.3**
S. costatum	LL f/2	108.9	6.43	2.07	0.238	0.133	**156.8**	**155.5**
	LL P	122.9	14.43	5.27	0.211	0.194	**127.1**	**142.2**
C. muelleri	HL f/2	34.0	9.96	3.19	0.267	0.237	**129.4**	**149.2**
	HL P	67.7	9.31	5.60	0.246	0.199	**129.0**	**148.4**
C. muelleri	LL f/2	98.8	12.51	3.20	0.616	0.453	**166.1**	**168.0**
	LL P	139.9	10.48	2.61	0.334	0.311	**153.7**	**142.8**
R. reticulata	HL f/2	76.6	25.86	4.17	0.496	0.367	**166.5**	**132.8**
	HL P	175.1	29.03	7.56	0.287	0.333	**171.2**	**139.8**
R. reticulata	LL f/2	240.1	25.44	3.42	0.915	0.640	**185.8**	**153.7**
	LL P	305.8	28.24	4.67	0.650	0.485	**162.2**	**142.0**
P. lutheri	HL f/2	68.3	3.66	0.51	0.251	0.231	**157.1**	**136.0**
	HL P	100.5	6.56	0.94	0.308	0.236	**170.0**	**150.7**
P. lutheri	LL f/2	136.4	4.48	0.55	0.381	0.263	**146.6**	**136.1**
	LL P	147.3	4.97	0.54	0.394	0.354	**151.9**	**141.2**

Table 3.6.2. Fatty acid profile of all species under P-limitation and f/2 medium. **HL** =High Light conditions, **LL** = Low Light conditions, **f/2**=no limiting nutrient, **P**=Phosphorus limited medium. **SaFA**=Saturated Fatty Acids, **MUFA**=Mono Unsaturated Fatty Acids, **PUFA**=Poly Unsaturated Fatty Acids, **ω:3**=Total ω:3 series Fatty Acids. Numbers indicate percentage of the Total Identifiable Fatty Acids.

Fatty acid :		14:0	16:0	16:1ω7	20:5ω3	22:6ω3	SaFA	MUFA	PUFA	ω:3
S. costatum	HL f/2	11.6	8.2	11.6	19.7	2.2	23.0	19.1	60.0	25.4
	HL P	35.6	5.2	11.8	15.1	1.9	42.3	15.2	42.6	19.6
S. costatum	LL f/2	6.6	11.6	15.7	15.7	4.3	18.9	15.7	46.4	24.1
	LL P	11.1	13.0	16.2	12.5	3.0	29.9	26.2	34.4	17.6
C. muelleri	HL f/2	9.2	11.7	17.0	23.2	3.7	23.9	19.7	57.1	30.1
	HL P	14.3	21.1	26.9	11.4	2.1	38.0	29.3	32.7	15.5
C. muelleri	LL f/2	10.1	9.9	16.5	15.7	1.4	23.7	31.6	47.2	18.9
	LL P	5.8	14.9	17.6	15.3	1.5	26.6	25.1	48.4	17.3
R. reticulata	HL f/2	8.6	7.1	2.6	11.7	10.6	17.0	13.8	69.7	59.8
	HL P	12.3	11.4	2.6	3.7	4.3	24.7	23.3	52.8	33.5
R. reticulata	LL f/2	3.3	6.2	2.0	10.0	7.7	12.6	9.8	78.6	52.9
	LL P	2.2	8.4	1.7	8.6	8.2	12.3	12.3	75.4	59.0
P. lutheri	HL f/2	5.7	18.5	13.0	27.9	6.5	27.7	16.6	55.7	43.6
	HL P	6.5	24.6	18.7	18.7	8.3	32.7	23.3	44.1	32.8
P. lutheri	LL f/2	6.0	15.0	8.9	22.4	13.6	23.9	12.2	64.0	48.6
	LL P	4.1	16.4	9.3	31.1	8.8	23.4	13.1	63.5	47.9

In all the species tested in the present work, phosphorus limitation forces the algae to increase their generation time (G), thus slowing their growth rate. This happens in both light intensities and the increase of G is significant in most cases. The effect however is more pronounced when the cells were growing with faster growth rates, that is in the high light intensity. The increase of G leads to the assumption that either the lower phosphorus concentration used for the phosphorus – limited media was more effective in altering the algal growth rates or that indeed phosphorus exercise a more profound effect than the other limiting nutrient tested here, nitrogen, on the physiological properties of the algae.

In all species, expect *C. muelleri*, phosphorus limitation causes an increase of the cellular protein content. The differentiating diatom shows minimal changes albeit with a minor decrease in its per cell protein concentration. This difference between the two diatoms indicates that in these species there may be different mechanisms governing the effect of phosphorus limitation as this is expressed in the protein biochemistry of the cells.

A more uniform response to phosphorus limitation is observed in the carbohydrate content of the cells; there is only one minor exception, again in *C. muelleri* but this time only in the low light intensity. In general, this limitation triggers the cells to contain more carbohydrate. This could be due either to decreased consumption of readily usable carbohydrate or increased storage of carbohydrate classes probably as a preferred energy storage medium.

It is noticeable that as far as the photosynthetic pigments are concerned there is no uniform response to phosphorus limitation by the algal species. *C. muelleri* and *R. reticulata* show a decrease in chlorophyll a content, *P. lutheri* shows minimal response, while *S. costatum* appears to have slightly more chlorophyll a content per cell. So, if one had to generalize, one would tend to favour the conclusion that phosphorus limitation decreases the amount of chlorophyll a per cell. What is noticeable however is that, the response of each species, of the alteration of the chlorophyll a and c content is the same irrespectively of the light intensity used. This indicates a species specific influence on photosynthesis – related physiology and biochemistry of phosphorus limitation. Although the elucidation of the mechanisms governing this relationship were far from the objectives of this work, this distinct difference between phosphorus and nitrogen

limitation should nevertheless be mentioned. The reason must reside on the fact that, nitrogen exercises a direct effect on the nitrogenous derived photosynthetic pigments, whereas the effect of phosphorus should be exercised indirectly probably in conjunction with other physiological and biochemical elements and in combination with other environmental parameters, which were found to be species specific.

From the summarized data concerning the FA profile of the species under phosphorus limitation, presented in Table 3.6.2, it appears that generally, there is a less uniform response of the algae as far as certain individual FA are concerned, but on the contrary, there is a noticeable uniformity on the response to phosphorus limitation of FA groups. Phosphorus limitation appears to increase the degree of saturation of FA in the algal cells whereas it decreases the relative amount of PUFA. Among the four test species, the two diatoms have a more closely matched FA profile and also the response to phosphorus limitation change in relative FA content, as it is to be expected are again more similar that to those in the other two species. *P. lutheri* and *R. reticulata*, exhibit a variable response in change as a result of phosphorus limitation, suggesting that the biochemical pathways that are affected by this limitation are probably different among these species or there is a different effect on the same pathway.

In the literature, there have been fewer studies concerning the effects of phosphorus on the biochemical composition of the algae. Even though in some natural ecosystems, e.g. some coastal areas, phosphorus can be the main limiting factor to algal production (Berland *et al.*, 1980). It appears that the algal response to phosphorus stress is, to a certain extent, similar to that of nitrogen, although fewer data are yet available. The decrease in chlorophyll a content per cell under phosphorus limitation has also been observed in *Phaeodactylum tricornutum* by Lombardi and Wangersky (1991) and by other researchers for a number of different species (e.g. Kuhl 1974; Healey & Hendzel, 1975) and has been discussed in detail by Cembella *et al.* (1984). There are however some reports that indicate an increase in this pigment under other kinds of stress, for example of silicate (Holmes, 1966) and this is what happened to *C. muelleri*, under phosphorus stress in these experiments. Other reports have findings which suggest the opposite response under similar silicate stress (Werner, 1977; Volcani, 1978; Lombardi & Wangersky, 1991).

In a diagonal design experiment with combinations of both phosphorus and nitrogen stress, Wikfors (1986) found that increasing phosphorus content in cultures with high nitrogen concentration resulted in an increase of the protein to carbohydrate ratio in one species, *Tetraselmis maculata* [Prasinophyceae] while in *Dunaliela tetriolecta* [Chlorophyceae] this ratio was decreased, although when expressed in absolute cellular concentrations, carbohydrate values increased and protein was decreased under phosphorus limitation, in both these species. It was also reported that a probable luxury consumption occurs for the remarkably high protein nitrogen conversions achieved by both species in the high nitrogen media. As with nitrogen, uptake of phosphorus cannot be equated with metabolic need; freshwater and estuarine microalgae have been shown to accumulate excess phosphorus in the form of inorganic polyphosphates which are thought to function in phosphorus storage (Harold, 1966; Sakshaug *et al.*, 1983). The differences in protein content changes between the diatoms and the two other species, *P. lutheri* and *R. reticulata,* can be investigated in the light of the fact that uptake does not necessarily denote metabolic need. Under conditions of nitrogen abundance, luxury consumption and storage as protein have been observed in a number of algal species (Werner, 1970; Dortch, 1982; Sakshaug *et al.*, 1983). The varying effect of phosphorus limitation on carbohydrate metabolism in the four species tested here might be explained on the different reaction of these species to the high nitrogen inhibitory effect suggested previously.

Reitan *et al.* (1994) investigating the effects of nutrient limitation, which the authors believed to have probably been phosphorus, on the lipid and FA composition of various marine microalgae, including *P. lutheri* and *Chaetoceros* sp. reported a similar effect to the present work with a noted increase on relative amounts of total saturated FA and a decreased percentage of PUFA under the nutrient stress conditions. They support the idea that lipid accumulation under nutrient stress is partially the result of steady lipid synthesis combined with reduced cell division rate and protein synthesis due to reduced availability of nutrients (Sukenik and Livne, 1991), while Parrish and Wangersky (1987) showed that this accumulation of lipids in *C. gracilis* was primarily a result of an increase in neutral lipids. However in some other species like *Tetraselmis* sp. lipid content decreased with phosphorus limitation (Reitan *et al.*, 1994) and thus it could be argued

that the accumulated photosynthetic products were not stored as lipid compounds but probably as carbohydrates (Ben – Amotz *et al.*, 1985; Harrison *et al.*, 1990). These patterns, and indeed the ones found in the present work indicate a different accumulation pattern of carbon in nutrient limited microalgae in that not all species accumulate lipids during nutrient limited growth.

Siron *et al.* (1989) reported that phosphorus limitation in *Phaeodactylum tricornutum* led to the elaboration of triglycerides while it also significantly decreased the content of PUFA's, and in particular eicosapentanenoic acid. The global unsaturation of microalgal lipids was also altered. Almost identical results were obtained by Lombardi & Wangersky (1991) using another diatom, *Chaetoceros gracilis*. In this species phosphorus limitation triggered the production of storage lipids such as triglycerides, but reduced the amount of phospholipids. Reitan *et al.* (1994) confirmed these results as far as Bacillariophyceae and Prymnesiophyceae are concerned, but reported a different response for two green flagellates, *Nannochloris atomus* and *Tetraselmis* sp., in which the lipid content was decreased with nutrient limitation; they speculated that it probably reduced the synthesis of ω:3 PUFA's.

However, it is generally believed that phosphorus limitation may reduce the formation of phospholipids and trigger the production of triglycerides and other storage neutral lipids (Tornabene *et al.*, 1983; Suen *et al.*, 1987; Lombardi & Wangersky 1991). The neutral lipids have a relatively higher content of saturated (SaFA) and mono unsaturated FA (MUFA) than the polar lipids which contain more omega : 3 PUFA (Kayama *et al.*, 1989). The findings of this work support the idea that ratios between SaFA, MUFA and PUFA change with variable nutrient limitation (Ahlgren *et al.*, 1992).

Summarizing the effects of phosphorus limitation on the combination of the biochemical properties, and thus their nutritional quality, as is expressed by the growth of *M. edulis* larvae fed on phosphorus limited algal cells, it can be noted that for the two diatoms, this limitation decreases their nutritional value, while for *P. lutheri*, its food value is significantly increased. Finally for *R. reticulata* the nutritional value is dependent on the combination of phosphorus stress and light intensity with the higher of the two light intensities providing better quality food while an opposite effect with a lowering of its food value is observed at the low light intensity.

3.6.b Effects of Nitrogen limitation

In a format similar to the presentation of the phosphorus limitation, the averaged results of the effects of nitrogen limitation on the biochemical properties of all the algal species and the corresponding growth of the larvae fed on those diets, are presented. For comparative purposes the profile of each species when cultured under the non limiting conditions, in the f/2 medium, is also included grown under each light condition. Protein, carbohydrate, chlorophyll a and c content, along with generation time of each species under N-limitation are presented in Table 3.6.3. The original data used to construct this table were presented in the previous Tables 3.2.1, 3.3.1, 3.4.1 and 4.5.1. The major FA and FA groups are presented in Table 3.6.4. The complete FA profile of each algal species was presented in Tables 3.2.3, 3.3.3, 3.4.3 and 3.5.3, for each of the four algal species respectively.

Table 3.6.3. Biochemical profile and generation time of all algal species under Nitrogen limitation and no limitation. Larval length achieved after the two week feeding trial for each of both experiments are also provided. **HL**=High Light conditions, **LL**=Low Light conditions, **f/2**=f/2 medium (no limiting nutrient), **N**=Nitrogen limited medium.

		Generation Time (h)	Protein (pg/cell)	Carbohydrate (pg/cell)	chl. A (pg/cell)	chl. C (pg/cell)	1st Larval Length (μm)	2nd Larval Length (μm)
S. costatum	HL f/2	21.6	4.62	1.22	0.237	0.115	126.0	151.4
	HL N	19.7	4.89	1.79	0.222	0.159	124.2	152.5
S. costatum	LL f/2	108.9	6.43	2.07	0.238	0.133	156.8	155.5
	LL N	96.5	3.95	1.06	0.197	0.149	169.3	167.1
C. muelleri	HL f/2	34.0	9.96	3.19	0.267	0.237	129.4	149.2
	HL N	47.3	7.52	4.85	0.246	0.242	137.6	160.6
C. muelleri	LL f/2	98.8	12.51	3.20	0.616	0.453	166.1	168.0
	LL N	109.3	10.32	2.88	0.450	0.425	160.3	134.8
R. reticulata	HL f/2	76.6	25.86	4.17	0.496	0.367	166.5	132.8
	HL N	150.3	36.23	18.27	0.468	0.387	187.6	140.5
R. reticulata	LL f/2	240.1	25.44	3.42	0.915	0.640	185.8	153.7
	LL N	263.2	32.83	6.31	0.705	0.444	179.6	139.6
P. lutheri	HL f/2	68.3	3.66	0.51	0.251	0.231	157.1	136.0
	HL N	69.4	4.07	0.45	0.206	0.181	157.7	137.0
P. lutheri	LL f/2	136.4	4.48	0.55	0.381	0.263	146.6	136.1
	LL N	132.4	7.08	0.74	0.366	0.263	151.0	135.1

Table 3.6.4. Fatty acid profile of all species under N-limitation and f/2 medium **HL**=High Light conditions, **LL**=Low Light conditions, **f/2**=no limiting nutrient, **N**=Nitrogen limited medium. **SaFA**=Saturated Fatty Acids, **MUFA**=Mono Unsaturated Fatty Acids, **PUFA**=Poly Unsaturated Fatty Acids, **ω:3**=Total ω:3 series Fatty Acids. Numbers indicate percentage of the Total Identifiable Fatty Acids.

Fatty acid :		14:0	16:0	16:1ω7	20:5ω3	22:6ω3	SaFA	MUFA	PUFA	ω:3
S. costatum	HL f/2	11.59	8.25	11.64	19.66	2.17	23.04	19.07	59.99	25.44
	HL N	14.92	9.10	13.69	19.85	3.11	29.14	18.93	51.43	26.46
S. costatum	LL f/2	6.57	11.57	15.65	15.66	4.34	18.91	15.65	46.42	24.09
	LL N	8.46	15.91	16.77	12.65	2.45	30.00	27.38	37.79	18.31
C. muelleri	HL f/2	9.18	11.74	17.02	23.23	3.73	23.94	19.71	57.08	30.06
	HL N	8.77	19.86	26.65	16.76	2.08	31.50	29.02	39.60	20.40
C. muelleri	LL f/2	10.06	9.91	16.49	15.66	1.42	23.73	31.59	47.23	18.86
	LL N	6.83	12.56	16.16	10.17	1.41	25.18	40.90	33.91	13.86
R. reticulata	HL f/2	8.62	7.08	2.63	11.67	10.61	17.04	13.81	69.71	59.77
	HL N	18.61	24.39	0.95	2.01	1.95	48.42	16.41	35.08	26.42
R. reticulata	LL f/2	3.285	6.16	1.96	10.02	7.67	12.61	9.84	78.64	52.86
	LL N	0.46	6.06	1.70	8.81	7.66	6.93	12.82	80.25	46.50
P. lutheri	HL f/2	5.68	18.50	12.99	27.88	6.50	27.71	16.59	55.71	43.61
	HL N	5.60	17.62	11.34	17.66	11.19	27.11	15.49	57.40	39.69
P. lutheri	LL f/2	6.03	14.95	8.89	22.38	13.56	23.86	12.18	63.96	48.55
	LL N	5.13	16.29	10.17	27.45	7.07	24.07	13.39	62.53	47.24

In general, it can be seen than nitrogen limitation has little, if any, effect on the algae, in terms of the displayed generation time; this is true for both light conditions. However, nitrogen limitation seem to affect the biochemical properties of the algal species in different ways. It can be seen that the two diatoms, *C. muelleri* and *S. costatum*, show similar responses in that the per cell amount of protein is decreased. This could be directly related to the lowered rate of amino acid synthesis, for which nitrogen is one of the necessary components. In the other two species, there is an increase in the per cell amount of protein, especially in *R. reticulata*; again the same effects is displayed irrespective of light intensity. This distinct difference between these algal species only adds to the expected result that, diatom biochemistry and physiology should be more similar among them, than in relation with the other non-diatom species. Similar grouping can be established for the carbohydrate content with the diatoms showing a light intensity dependant response; in high light carbohydrate is increased whereas the opposite happens under low light conditions. The other two species, in most cases appear to increase their carbohydrate content under nitrogen limitation. As far as the photosynthetic

pigments are concerned there is a remarkable uniformity on the responses of all species: chlorophyll a is reduced while chlorophyll c is increased, thus prompting a drastic increase in the chlorophyll c:a ratio. This could reflect a more fundamental influence on algal physiology of all algal groups, by nitrogen limitation.

Of the major fatty acid groups, namely the Saturated (SaFA) and Poly Unsaturated Fatty acids (PUFA), again the two diatoms, *S. costatum* and *C. muelleri*, appear to react in much the same way to nutrient stress irrespective of light intensity, while the two other species show variable responses. In detail, the diatoms increase the percentage of PUFA and decrease the percentage of SaFA. *R. reticulata* does the same in high light intensity; but under low light levels this species decreases the percentage of SaFA and shows minimal change of the PUFA percentage. Finally, *P. lutheri* shows only minimal changes under nitrogen limitation for both of the Fatty Acid groups.

Although nitrogen limitation reactions may be species-specific, especially as far as growth rates are concerned, it has been reported that nutrient limitation reduces the level of protein (Enright *et al.*, 1986b; Thompson & Harrison, 1992). Enright *et al.*, (1986b) using another *Chaetoceros* species, *C. gracilis*, found a substantial decrease in cellular protein. In the light of the combined effect of nutrient stress and either temperature or light intensities, Rhee and Gotham (1981a,b), found that the simultaneous effects on growth of three phytoplankton species (*Scenedesmus* sp., *Fragilaria crotonensis* and *Asterionella formosa*) were greater than the sum of individual effects and were not multiplicative. The differences in protein content changes between the diatoms and the two other species found here, can be investigated in light of the possibility of luxury consumption under conditions of nitrogen abundance. Luxury consumption and storage as protein have been observed in a number of algal species (Werner, 1970; Dortch, 1982; Sakshaug *et al.*, 1983).

Enright *et al.* (1986b), Wikfors *et al.* (1984) and Wikfors (1986) report an increase the carbohydrate content of various algal cells under nitrogen limitation. Out of the four test species of the present work, only *R. reticulata* shows a consistent and substantial increase of carbohydrate content under nitrogen limitation, while the other species show either little change or various changes depending both on the nutrient regime and the light intensity used.

Another obvious effect of N-deficiency is the decline in nitrogenous photosynthetic pigments such as chlorophylls and phycobilins (Herzig & Falkowski, 1989; Plumey *et al.*, 1989); the present work supports previous results as far as the reduction of chlorophyll a is concerned. A detailed review of the effect of nitrogen in photosynthesis and the coupling with carbon metabolism was presented by Turpin (1991), were it was clearly shown although the chlorophyll content may vary as a result of varying nitrogen, the ratio of chlorophyll a to auxiliary photosynthetic pigment decreases under nitrogen deficiency. A comprehensive and detailed explanation of the involved biochemistry and photobioreactions were presented there, and it has to be noted that the results obtained in the present work are in very good agreement with the hypothesis presented therein (Turpin, 1991).

The first to demonstrate a direct effect of nitrogen content (in forms of nitrate or ammonium ions) on fatty acid biosynthesis in algae were Pohl & Zurheide (1979). These investigators showed that the content of polyunsaturated C_{16} & C_{18} fatty acids in a chlorophyte, *Chlorella vulgaris*, and the euglenophyte *Bracteacoccus minor*, was enhanced by high concentrations of nitrogen. Later, other investigators tried to assess interspecific differences and relate the responses to nitrogen concentrations of various algae, applying in a sense, some sort of "nitrogen screening" (Shifrin & Chisholm, 1981). It was noticed that green algae increase their lipid content with nitrogen deprivation while diatoms demonstrated variable responses depending on the species. The same result was later confirmed for the Eustigmatophyte *Nannochloropsis* sp. QII, where nitrogen deficiency promoted lipid synthesis (Suen *et al.*, 1987); additionally, it was shown that enhanced lipid synthesis resulted principally from *de novo* CO_2 fixation. It seems that, under such stress, microalgae produce substantially less polar lipids and significantly more neutral lipids (Tornabene *et al.*, 1983; Ben-Amotz *et al.*, 1985). The neutral lipids have a relatively higher content of saturated and mono unsaturated FA and the polar lipids contain more omega : 3 PUFA (Kayama *et al.*, 1989). More elaborate and detailed culturing and analyzing techniques support this, more complex, image. Intracellular synthesis of triglyceride may be enhanced by nitrogen limitation, but membrane-associated polar lipid classes were reduced under these circumstances. It should also be

mentioned that different types of dissolved extracellular lipid classes were produced under different nitrogen regimes (Parrish & Wangersky, 1987).

In a number of species representing various algal groups, nitrogen limitation is found to make the algae accumulate lipids. In many cases neutral lipids, primarily triacylglycerol, comprise the bulk of the lipid content in nitrogen-starved cells. Shifrin and Chisholm (1981) in a comparative study, report a 120 - 130 % increase of total lipid content in nitrogen starved cells; in that study, a few strains including some diatoms, like *Biddulphia aurita* and *Synedra ulna*, did not accumulate any lipids. In a mini review by Roessler (1990) a list of species which were found to accumulate lipids under nitrogen limitation is provided. However, it has been reported that metabolic pathways of both carbohydrate and lipid production are inhibited by protein synthesis, when nitrogen is in extreme supply and phosphorus is low (Wikfors, 1986).

The report of Enright *et al.* (1986b) does not provide a detailed analysis of all individual Fatty Acids of the algal test species *C. gracilis*. Information is given only for some of the major Fatty Acids and the only substantial difference between FA in cells cultured under Nitrogen limitation and without limitation (again f/2 medium was used as control), is in the increase of Total saturated FA (especially the 16:0) and a small decrease in the total ω:3 series of FA, mainly the 20:5ω3 and the 22:6ω3 fatty acids.

Finally, in relation to the effect of nitrogen limitation in the various biochemical components as this is expressed by variation of end length achieved by the larvae consuming this food, it can be noted that both the diatoms seem to increase their nutritional quality under this limitation, especially under low light conditions, whereas larvae fed on *P. lutheri* showing little or no effect and finally, the larvae consuming *R. reticulata* seem to demonstrate changes indicating an interaction of the nutritional value of the nitrogen – limited cells of this species with the light intensity used: in high light, nitrogen limitation is beneficial while on low light intensity the non limited cells are superior to their nitrogen limited counterparts.

3.6.c Effects of Light Intensity

Since the summarized results concerning all the biochemical parameters studied have been presented in the previous nutrient limitation sections, including those for both light intensities, repetition was not thought to be necessary.

However there is an added element that does need to be discussed prior to interpretation of the results from the perspective of the light intensity effect. There were both nutrient limited and non limited cultures tested under both light intensities, with irradiance levels potentially limiting the growth of these cultures. Therefore, in theory, there might be at least two distinctly different activities exercising control over the displayed behavior of the algae, nutrient limitation and light limitation. Only one parameter however, at any one time, should exercise overall control. Although it is obvious that comparisons should only be made between the corresponding pairs of nutrient conditions, it should also be emphasized that when interpreting results concerning light limitation in combination with nutrient limitation, the result could be mainly due to the effect of one of the other parameters, not necessarily light intensity.

Another aspect that needs clarification is that although the light intensities used in the present work were not arbitrary, they do not necessarily represent high or low light levels for each individual algal species, but rather intensity levels achieved under the experimental conditions. Since different species might have distinctly different threshold levels of irradiance that a light limitation or a photoinhibitory effect takes place the experimentally used light intensities will most likely differ from the "physiological" irradiance under which an algal response is observed. Obviously the experimental approach to study the effect of light intensity in a given algal species would be to try to determine the response of the alga to a wide range of irradiance, and then select the extreme intensities under which the effects could be more readily observed and effectively studied. However, from an aquacultural perspective, where most often a number of algal species is routinely cultured, the focus must be on experimenting with light intensities that are easily available and that should exercise an effect of varying extent, to most of the algal species used.

By far the most striking effect of light intensity is on the growth of all four species tested here, as expressed by means of the observed generation time. Lower light intensity slows the growth rate of the species typically by a factor of two to four times. *P. lutheri* is less affected by lower irradiance, increasing its generation time by slightly less twice. Results remain comparable even when nutrient limitation is taken into consideration. The trend of growth rate values remains the same between light intensities under the three nutrient regimes. In this case, it follows that the assumed combination of both nutrient and light limitation was additive but not multiplicative for the tested algal species.

Whenever the "non limiting" f/2 medium was used, cellular protein concentration was not substantially altered in the two light intensities used. Typically there were only minor differences, with only *C. muelleri* showing a slightly bigger increase at the lower light intensity. When phosphorus limitation is examined, only the two diatoms, *S. costatum* and *C. muelleri*, showed some increase in their cellular protein content when the lower light intensity is used. It is only under nitrogen limitation that there is some noticeable change in per cell protein content; however the results are not consistent for the four species between the light intensities. It appears that light intensity itself does not have a notable effect on the protein concentration of the tested algal species.

The effect of light intensity on carbohydrate content of the algal cells is also not conclusive. In the non limiting medium, irradiance does not alter in any meaningful way the per cell carbohydrate content of the cells. Greater effects, in terms of percentage of change, are observed in conjunction with phosphorus limitation. Except for *S. costatum*, all the other species decrease their carbohydrate levels under lower light intensity and phosphorus limitation. The same is true for nitrogen limitation, in this case with only *P. lutheri* becoming a minor exception.

An obvious effect of light intensity is, as expected, exercised on the photosynthetic pigments of the cells. It needs no further explanation that at the lower irradiance, the cells of all the tested algal species contained more chlorophyll a and c.

Interestingly, the effect of irradiance on the major FA groups is consistent among the various algal groups. When examining the effect of light intensity under no nutrient limitation it becomes evident that the percentage of PUFA is decreased with decreasing light intensity for the two diatoms, while it is increased for *R. reticulata* and *P. lutheri*. At

the same time, the SaFA ratio of all algal species is, on the contrary, increased with decreasing irradiance. In general the changes observed in the FA profile of the diatoms are much more similar than the changes observed for the two other species. However, the trend of change in the combination of nutrient limitation and light intensity is not the same between the two light intensities used, indicating that the combined effect of nutrient and light limitation is not additive, as far as the FA profile of the algal species is concerned. This indicates that probably it is one of the two (nutrient or light) factors that exercises the overall control on the FA content of the species. Extending this further, it could be that the cause – effect relationship is of a threshold type, meaning that if one of the factors exceed a species – specific threshold, than the observed biochemical response of the algae is changed, irrespective of the of the status of the other factor, as long as the latter is within given tolerance limits. By changing the quantitative properties of the control factors it could result in the latter factor exceeding its threshold value, thus exercising the overall control, and the effect of the former one reduced, or even not observed at all. Since more similarities are observed among the diatoms, it follows that the threshold for these factors is more similar among members of the same group than between species of different groups.

The effects of light intensity on algal physiology and biochemistry have been extensively studied. Although the relationship seems to have a general trend, some contradicting records have been presented.

Rhee and Gotham (1981b) studying the effects of light and the interactions of light and nitrate limitation on *Scenedesmus* sp. and *Fragilaria crotonensis*, report light limited growth with decreasing irradiance. They also found that cellular chlorophyll content increased as irradiance decreased below saturation. Under nutrient sufficient conditions, cell quotas of carbon, nitrogen and phosphorus increased with decreasing irradiance. This indicates that nutrient requirements increase as light intensity decreases. Moreover, they report that the combined effects of light and nutrient limitation were greater than the sum of individual effects, something which was not found in the present work. When testing the diatom *Chaetoceros gracilis*, Mortensen *et al.* (1988) found no change in elemental composition in the range from 83 to 1428 $\mu E \cdot s^{-1} \cdot m^{-2}$. Similar

elemental composition at various light intensities was also reported for *Thalassiosira pseudonana* by Thompson *et al.* (1996).

Thompson & Harrison (1992) found that an increase in light intensity caused a lowering in protein content and an increase in carbohydrate in the same species. However, in a later report Thompson *et al.* (1994a), using a different diatom species *Chaetoceros simplex*, found no significant differences in protein and carbohydrate contents but recently, when testing *Thalassiosira pseudonana* again (Thompson *et al.*, 1996) under similar irradiance levels as in their previous report (Thompson & Harrison, 1992), found no significant difference on both carbohydrate and protein content between the tested light intensities. Rhee & Gotham (1981b) found that an decrease in irradiance, below saturation levels, resulted in an increase in protein content for *Scenedesmus* sp. and *Fragilaria crotonensis*. On the contrary, Santosa (1990), who used *Nannochloris atomus*, *Nannochloris* sp. and *Nannochloropsis oculata*, found that an increase in light intensity increased the protein content. It may well be that these biochemical components are species-specific. The apparent lack of a consistent trend in protein and carbohydrate changes in the present report, would therefore seems to favor the latter conclusion. But, it may well be that unless irradiance saturation levels are investigated, the apparent lack of change of both carbohydrate and protein content might be attributed to the fact that the light intensities chosen for experimentation may be within the algal species saturation levels, as was demonstrated by Rhee & Gotham (1981b).

As mentioned in the introduction (ch. 1.7.f), Jørgensen (1969, 1977), reported two different types of adaptation to light limitation: the *Chlorella* type, in which chlorophyll a content varies with light intensity and the *Cyclotella* type, in which chlorophyll a content does not vary. In the *Chlorella* type, the actual carbon fixation rate per cell at the light intensity where the cells are grown does not differ much between high and low light intensities, while in the *Cyclotella* type this rate is much higher at a high than at a low light intensity. All species tested here have been found to change, more precisely to increase, their per cell chlorophyll a content under lower light intensity, and therefore their adaptation must be of the *Chlorella* type. It remains unclear whether the tested species change the size of the photosynthetic unit or the number of photosynthetic units. Detailed work on *S. costatum* by Falkowski and Owens (1980) showed that this species

adapts to changes in irradiance by changing the size of the photosynthetic unit. Rarely, researchers have investigated the effects on growth or biochemical composition of light quality in laboratory conditions. Recently, Aidar *et al.* (1994) assessed such effects on the diatom *Cyclotella caspia* and the green alga *Tetraselmis gracilis*. As anticipated, the concentration of photosynthesizing chlorophylls [a, (c_1 +c_2)] and carotenoids differed in the different light qualities used. *Cyclotella caspia* had a higher cellular content of pigments in red and blue-green light, while protein content did not change with light quality. *Tetraselmis gracilis*, on the other hand, had more pigments and protein when grown in mixed wavelength (white) light.

On the combined effects of both nutrient and light limitation, Davis (1976) reported evidence that growth was limited in an "either – or" fashion or in a threshold fashion, when both light and silicate were simultaneously limiting for *Skeletonema costatum*. Falkowski (1977) proposed a theoretical model to treat limitations on the basis of bisubstrate enzyme reaction kinetics. Nyholm (1978) used a multiplicative formula of light and nutrient – limited kinetics. Rhee & Gotham (1981a, b) suggest that the combined effect observed in two algal species tested therein are neither additive nor multiplicative, hence the nutrient and light interaction was not of a threshold or of an "either – or" type. Although the scope of the present report is far from a detailed analysis in enzyme kinetics or proposing a theoretical model of nutrient and light interactions, the combined effects of these parameters on the algal growth studied here were of an additive type and thus the threshold or the "either – or" type seem to describe better the observed patterns on the four tested species.

On the other hand, lipid content and light intensity appear to have a more consistent pattern, although again there are some contradictory records.

Cohen *et al.*, (1988) testing the effect of various environmental conditions, including light intensity, on the FA composition of the red alga *Porphyridium cruentum*, found that an increase in light intensity resulted in a decrease of SaFA and also to a shift from the 20:4 as the predominant PUFA to the 20:5ω3 FA as such. It has been repeatedly shown, for a number of algal species from different classes, that in general, increase in light intensity causes an increase in the degree of saturation of fatty acids (Thompson & Harrison, 1992; Thompson *et al.*, 1994a) and seems to decrease the total triglyceride

content (Parrish & Wangersky, 1990). Mortensen *et al.* (1988) using *Chaetoceros gracilis* in batch cultures under quantum flux densities ranging from 83 to 1395 $\mu E \cdot s^{-1} \cdot m^{-2}$, found an increase of the levels of the omega : 3 groups of FA with increasing light intensity, although FA composition was quite uniform. Thompson and coworkers in a series of papers investigating the effect of light intensity on the food value of various algal species for bivalve larvae found that *Thalassiosira pseudonana* consistently had a higher proportion of 16:0 and Total SaFA and a lower proportion of 20:5ω3 and PUFA in general, with increasing light intensity (Thompson & Harrison, 1992; Thompson *et al.*, 1996) and the same was observed for *P. lutheri* (Thompson *et al.*, 1994a, 1996) and *Chaetoceros simplex* (Thompson *et al.*, 1994a). The results for *Thalassiosira pseudonana* have been further confirmed by Brown *et al.* (1996b), who by using batch cultures of this species, found an increase of SaFA and a decrease of PUFA with increasing light intensity. It has to be emphasized though, that these results were only confirmed when cells were harvested from the logarithmic phase of growth. On the contrary, these results were inverted for cells harvested in the stationary phase. In a report specific to *S. costatum* Blanchemain & Grizeau (1996) tested four different light intensities ranging from 20 to 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and found a minimum proportion of PUFA in the lowest of the tested light intensities but, the expected increased proportion of SaFA was not observed. It appeared that the proportion of both individual FA and of groups of FA fluctuated among the light intensities used, with the only exception being the proportion of 16:0 which decreased with increasing irradiance. The latter results concerning *S. costatum* were also observed in the present work, while results concerning *P. lutheri* are in very good agreement with the ones obtained by Thompson *et al.* (1994a, 1996). Although results concerning *C. muelleri* cannot be independently confirmed, the present work is in agreement with the results obtained for *C. gracilis* by Mortensen *et al.* (1988).

The ratio of triacylglycerol to polar lipids was also found to increase from low to high light intensity for *Cylindotheca fusiformis* and the diatom *Nitzschia alba* (Opute, 1974; Orcutt & Patterson, 1974). Increased levels of triacylglycerols were also observed under high, photoinhibitory levels in the eustigmatophyte *Nannochloropsis sp.* (Sukenik *et al.*, 1989). In the latter case ^{14}C tracer experiments indicated that the percentage of newly assimilated carbon partitioned into triacylglycerols increased at high light

intensity, suggesting that *de novo* synthesis is at least partially responsible for the observed triacylglycerol accumulation.

Finally, to just briefly bring in focus the effect of light intensity on the nutritional value for *M. edulis* larvae, of the algal species tested here it can be noted that, the diatoms are on a separate group with their nutritional value increasing with lower light intensity; *R. reticulata* does not noticeably change its nutritional value under any of the light regimes, while *P. lutheri* increases its nutritional value with increasing irradiance.

4. Relationships between biochemical and biometrical parameters

4.1 Fatty acids of *M. edulis* as indices of growth

It has been shown in the past that lipid content of many marine larvae can be correlated with the viability of these larvae (Helm *et. al.*, 1973; Gallager *et al.*, 1986; Fraser, 1989). The last author, in reviewing the relative research field tried to establish a condition index of environmentally stressed larvae, of fish, bivalves and crustaceans, with lipid content. The early larval development of many marine organisms is characterized by a post hatching period during which larvae are largely dependent upon endogenous energy reserves. Invariably much of these energy reserves are in lipids, which have been shown to be depleted during early developmental stages; this has been documented for a variety of marine larvae like Atlantic salmon, *Salmo salar* (Cowey *et al.*, 1985), European sea bass, *Dicentrarchus labrax* (Quessada & Pionetti, 1987), American lobster, *Homarus americanus* (Sasaki, 1984) and several bivalve larvae (Gallager *et al.*, 1986). It has also been demonstrated (Lannan *et al.*, 1980) that broodstock management strongly influences subsequent larval viability. The influential mechanism was not known by the authors at that time but it may be related to the initial triacylglycerol content furnished to each egg by the parent during the conditioning period (Gallager & Mann, 1986a). Additionally, it has been shown that broodstock nutrition is important for the viability of the larvae and spat of many marine bivalves (Wilson *et al.*, 1996; Berntsson *et al.*, 1997). But it is not only larval growth that is influenced by egg quality; in some cases, settlement, spat growth and juvenile survival are also influenced by egg quality, particularly of the egg lipid content (Bayne, 1972; Helm *et al.*, 1973; Bayne *et al.*, 1975,1978; Kraeuter *et al.*, 1982).

In the present experimental procedure, the conditioning period for different batches of larvae was of different durations. Once the broodstock in every experimental period was taken from the field, around January-March, it was kept in the laboratory tanks in running sea water. Mussels from this stock were taken whenever needed. The *S. costatum* and *C. muelleri* high light experiments were carried out in March - June of the first experimental season. The low light *S. costatum* and *C. muelleri* feeding trials were carried out in July, but immediately after that, the *R. reticulata* high & low light experiments were

carried out. Finally, on March – April of the following season, the last *P. lutheri* high and low experiments were carried out.

Therefore the natural variation occurring due to different broostocks, but mainly due to the different length of the conditioning period each time does render analyzing the sum of all the pooled fatty acid data of all experiments rather unsafe. The only case that this can be done safely is for the *R. reticulata* high and low light experiment with the low light *S. costatum* and *C. muelleri*, because they were run in very close proximity with each other, and were offspring of broodstock taken from the field at the same time and were kept under the same conditions.

The previous hypothetical suggestions are in fact substantiated by the fatty acid analysis of various batches of eggs. Before actually fertilizing the eggs to obtain the larvae to be used for the various feeding experiments, a sample containing a known number of eggs was obtained. Although the mass mating technique used in the present work (see also section 2.2a) should theoretically ensure a uniform genetic variability of the larval batch, it has been shown (Del Rio, 1996) that the yield of offspring from each parent can be very different. Some progenitors may not give any detectable offspring, whilst others may produce a high proportion. Hence the variations of the fatty acid profile of the pooled batch of eggs might not necessarily have been translated into similar variation of fatty acids in the larvae. Nevertheless, it is possible to have an estimate of the variation of the fatty acid profile of the various batches of eggs during different times of the experimental season. The results concerning the eggs used for the feeding experiments of *S. costatum* and *C. muelleri* in high light conditions (repetition experiment) and low light conditions (first experiment) and *R. reticulata* under high and low light conditions (repetition experiment) are shown in Table 4.1.1.

Besides the usual information presented in the fatty acid analysis tables, in this case, the date in which the *M. edulis* egg sample was obtained is also stated, together with the feeding experiment for which these eggs were subsequently used. This is to give further information for the seasonal variation of the fatty acid profile of the eggs. As explained in the Materials and Methods chapter (ch. 2.2.a), the laboratory spawning season for *M. edulis* usually starts around March-April and finishes towards the end of the summer months (July-August). Therefore, the *S. costatum* & *C. muelleri* high light experiments were run around the middle of the spawning season, while the same species'

low light experiments together with the *R. reticulata* (high and low light) experiments were run towards the end of the season and in closer temporal proximity with each other.

Table 4.1.1. Fatty acid profile of various batches of *M. edulis* eggs, sampled prior to fertilization. Values indicate % of Total Identifiable Fatty Acids. Mixt. P.U.F.A.=Mixture of Poly Unsaturated Fatty Acids. B.D.=Below Detection. FA into parenthesis with a question mark mean that although there was not exact identification of the FA from the Gas Chromatograph traces, additional data from the Mass Spectrometer-Gas Chromatograph indicate that this FA was, almost certainly, the one that was assumed to be.

Fatty Acid	***S. costatum & C. muelleri***, repetition of high light experiment, 12/6/97	***S. costatum & C. muelleri***, first low light experiment, 26/7/97	***R. reticulata***, repetition of high & low light experiment, 10/8/97
14:0	2.63	2.32	2.74
15:0	0.58	0.54	0.56
16:0	20.52	18.84	17.47
16:1ω7	13.91	11.18	10.95
17:0	0.73	0.93	1.03
16:4	0.20	0.29	0.37
18:0	2.11	0.98	0.61
18:1ω9	3.11	6.44	6.29
18:1ω7	5.42	2.13	1.49
18:2ω6	1.58	1.79	1.91
18:2	0.98	0.37	0.79
18:3ω3	1.19	1.46	1.35
18:4ω3	3.28	3.90	3.95
20:1	2.04	4.02	4.05
20:1	5.03	3.86	3.85
20:2	3.57	1.79	3.21
20:4(ω6)?	1.34	1.55	1.74
20:3ω3	0.03	2.04	0.27
20:4ω3	0.29	0.37	0.42
20:5ω3	17.53	19.40	20.55
22:1ω11	B.D.	0.80	0.31
22:2	1.81	1.94	2.21
22:2	1.16	1.25	1.41
mixt. P.U.F.A.	0.26	0.24	0.35
22:5ω3	0.97	1.06	1.17
22:6ω3	9.15	10.52	10.54
Total Saturated	26.57	23.60	22.41
Total PUFA	43.35	47.97	50.24
Total ω:3 series	32.44	38.75	38.25

These results confirm the fact that there is a seasonal variation of the fatty acid content with which the eggs are furnished by their parents, which in turn depends on the duration of the conditioning period of the broodstock (Gallager & Mann, 1986a). Notice that the eggs used for the first low light experiment of *C. muelleri* and *S. costatum* have a much more similar fatty acid profile to the ones used for the repetition experiment of *R. reticulata* (both conditions) than to the repetition experiment of high light conditions of the two diatoms (*C. muelleri* and *S. costatum*), which was run much earlier on, in the spawning season. For example the total Saturated and total Poly Unsaturated fatty acids

are more closely matched between these two experiments than with the *S. costatum* and *C. muelleri* high light experiment. The same is true for almost all fatty acids. Therefore, the initial similarity of the fatty acid content of these two different batches of eggs, as can be verified by the results shown in Table 4.1.1, will not add very much to the overall variation of the end content of the larvae obtained after the feeding experiments.

Thus, the pooling of these experimental data might reveal a more coherent relationship between the fatty acid content of the larvae and their growth, not least because it will add strength to the statistical analysis, since the number of data points will be greater, (enabling for greater confidence limits) than if only each experiment was taken into consideration separately, which could be a one-off relationship appearing by chance due to the greater number of statistical tests.

As explained in the statistical consideration section of Materials and Methods (2.4) with this type of data, that the experimenter has no control over the values of the measured parameters but merely can measure their values, regression analysis, although statistically more powerful, is not appropriate. However regression analysis has been erroneously used in the past by many researchers in the field. For example see Ricker (1973) for an elucidating discussion upon the confusion and the wrong application of Model 1 vs. Model 2 regression analysis.

Another problem arises from the fact that, regression analysis assumes a linear relationship between the parameters. Many growth curves are not linear. Unless there is strong evidence, by multiple growth rate measurements over many short time units, to suggest otherwise, there is no reason to assume that the underlying relationship is linear. On top of that, organisms that undergo fundamentally different developmental stages e.g. through metamorphosis or moulting, most likely exhibit different non-linear growth rates. Therefore a regression-obtained growth rate should only be calculated when all the above assumptions are met (Sokal & Rohlf, 1987) and only for a given developmental phase.

Although the feeding trials here were carried out during the same, relatively short, developmental phase of the mussel larvae the first assumption was not met and hence correlation analysis was used. In this case, the correlation value (ranging from -1 to 1) indicates that the higher the absolute value of the number the stronger the relationship between the examined parameters; negative values indicate an inverse-type relationship; note that there is no description (linear or otherwise) of the nature of this relationship.

As demonstrated in all feeding experiments, survival of larvae shows a random variation among individual beakers and diets. Therefore, a trial to correlate other parameters with survival would reveal nothing but this random relationship, thus masking any potential relationship between these parameters.

On the other hand, it has been shown previously, that some algal diets would consistently produce larvae of significantly different end lengths. Although biological and experimental variation are not eliminated, the larval population can be adequately described by the mean length achieved at the end of the 2-week feeding trial. Therefore, it is obvious, that as a correlation parameter, larval size, as a measurement of growth (as already explained in section 2.2.c) and vigour of the animals, would provide a much better parameter, in contrast to larval survival, to dissect any potential underlying relationship between larval fatty acids and growth.

It has been shown before (Gallager & Mann, 1986b; Gallager *et al.*, 1986) that total neutral lipids, as quantified by histochemical staining, can be correlated with the viability of *Crassostrea virginica*, *Ostrea edulis* and *Mercenaria mercenaria* larvae. The underlying assumption is that the faster/better growing larvae will have a certain quantitative and/or qualitative profile of a fatty acid; therefore, if the latter was measured, it could be correlated with the larva's growth. This relationship could be of any type, but it has been more commonly treated, over short period of time and for a given larval stage (as explained before) as linear. A positive correlation would basically mean that the faster the larvae grow in size the more they have of this fatty acid, or fatty acid family; a negative correlation would indicate that the faster the larvae grow the less of this fatty acid they would contain. If there is no apparent trend between the two parameters, then the relationship could be described as random. Because faster growing larvae would achieve greater end lengths after a given period of time they should inevitably weigh more and consequently contain more material, in absolute terms, of any given biochemical component. The previous hypothesis about correlation of biochemical parameters, without any mathematical doubt, could also apply to the relative FA content of each larva, and this is the reason that the results for content of each identified FA were calculated as a percentage (in essence, a ratio) of that FA to the Total Identified Fatty Acids (TIFA).

By rearranging the previous fatty acid data from the *R. reticulata* high and low light experiment and the *C. muelleri* and *S. costatum* low light experiment (for reasons

described before) the mean length of each individual larval culture (each beaker) against any measured fatty acid (given as percentage of Total Identifiable Fatty Acids) can be plotted. Most of the fatty acids seem to be correlated with length in a random fashion. Such a typical example is shown in Fig. 4.1.1, describing the relationship between the 16:1^{a} 7 fatty acid and length. In this case the correlation value is an insignificant 0.303.

The code of the labels of all graphs, unless otherwise specified, is as follows: First capital letter indicate the algal species, with **C**=*C. muelleri*, **S**=*S. costatum* and **R**=*R. reticulata*. Middle section indicate nutrient and light conditions with **f/2**, **P** and **N** being the f/2, phosphorus and nitrogen limited media respectively. **ll** or **hl** indicate low and high light conditions correspondingly. The last number (1 or 2) is the beaker from which the larvae were sampled. **Con** =larvae from the control diets, **Unfed** =unfed larvae.

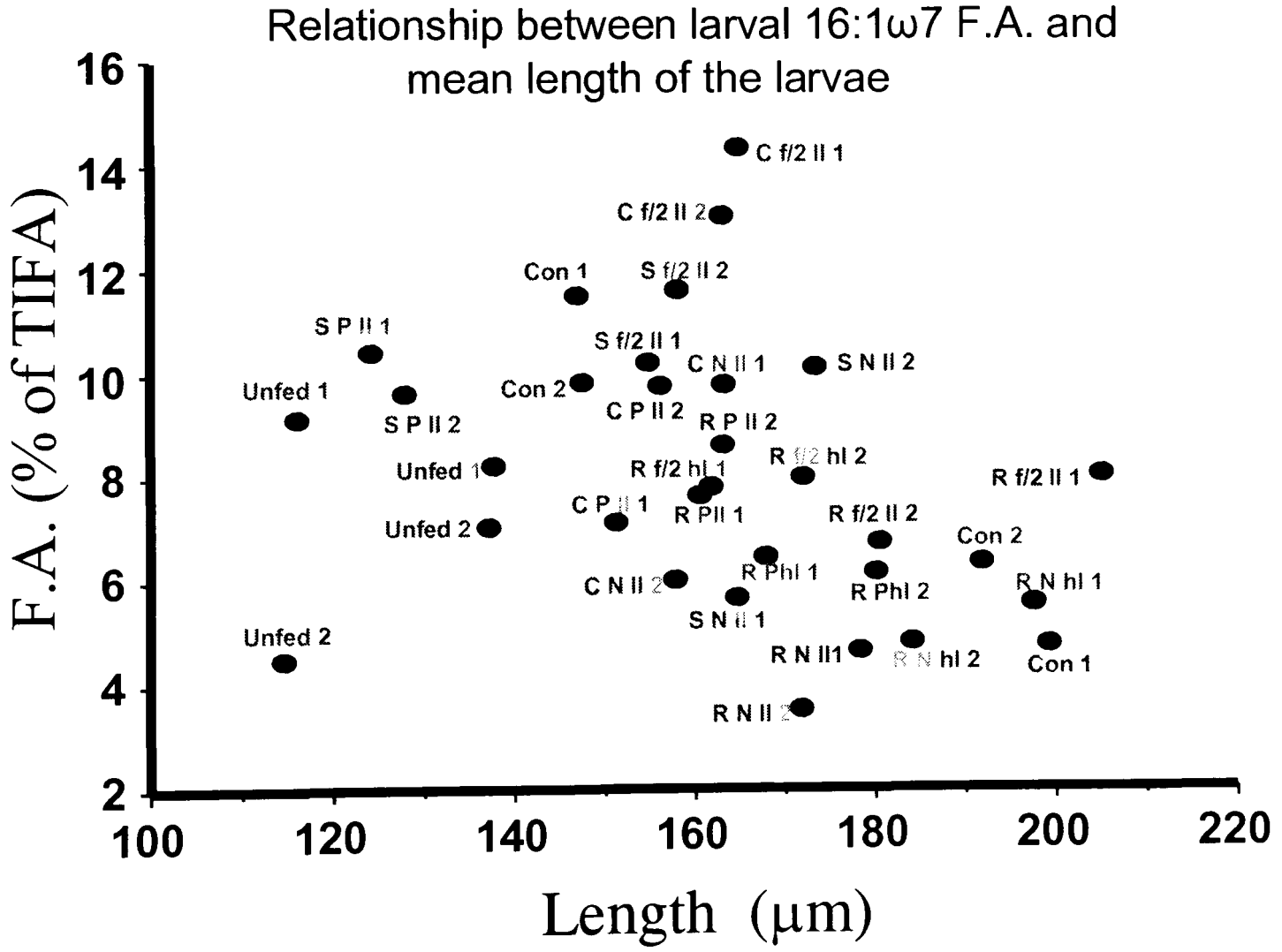

Figure 4.1.1. Relationship between larval 16:1ω7 fatty acid and mean length of the larval culture

However there are few occasions that a trend can be established between the two parameters. A positive correlation existing between the 20:5ω3 F.A. and length with a strong correlation factor 0.65 is graphically illustrated in Fig. 4.1.2.

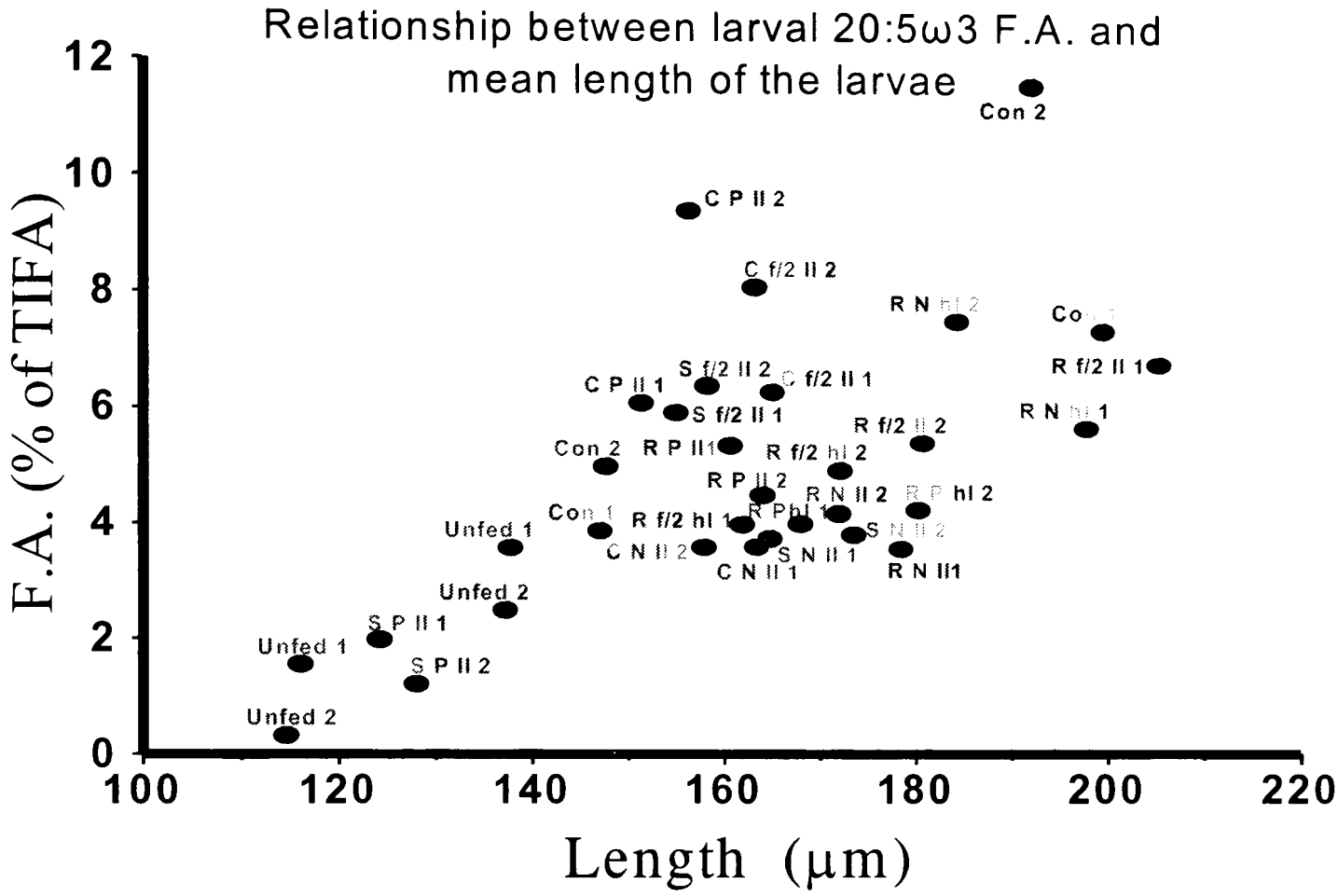

Figure 4.1.2. Relationship between larval 20:5ω3 fatty acid and mean length of the larval culture

The only case for individual fatty acids, that a negative correlation was found, was between length and the 15:0 F.A. In this case the correlation factor was -0.7. The corresponding result is displayed in Fig. 4.1.3

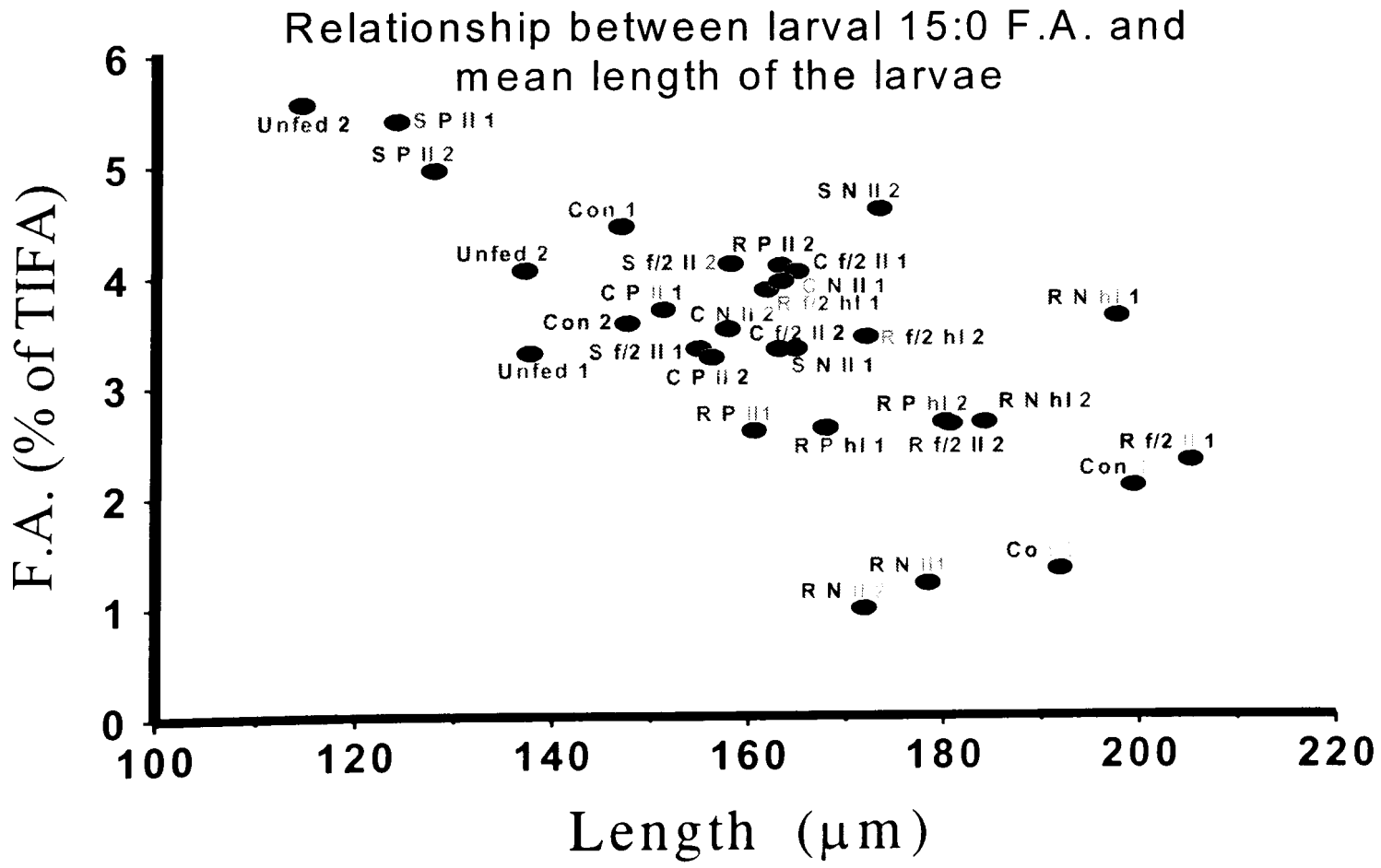

Figure 4.1.3. Relationship between larval 15:0 fatty acid and mean length of the larval culture

This correlation analysis does not use the assumptions of a predictive model and therefore it is not correct to predict that if, for example, a larva has such an amount of that fatty acid, which was found to be related with growth, it should be expected to grow up to a certain length described by the basic equation:

$$\mathbf{L = a * c + b}$$

where L=length, a=the correlation factor, c=the measured percentage of f.a. and b=a constant.

Although there is not much doubt of the biological importance of individual fatty acid, there is additional scope in trying to determine any potential relationship between groups of fatty acids and growth of larvae. The importance of various groups of fatty acids has been described by many researchers in the past and is generally an acceptable way to summarize results. The basis of this grouping of fatty acids under a biological (that is, biochemically and/or physiologically logical) hierarchy, is that by doing so, it is expected to overcome the problem of the inherent variability of individual fatty acid content within the larval cells. If there is a relationship between the studied parameters, it would further enhance our understanding of the significance of these groups of fatty acids. However there is a tempting trap that should be avoided. Grouping of fatty acids can be done a posteriori in order to establish a strong relationship between the artificial group of FA's and growth. Unless there is some evidence that, for example, there is a biological (biochemical, physiological or functional) relationship between the 14:0, the 18:3ω3, the 18:1ω7 and 20:4ω6 fatty acids, one should not group them together just because it happens that, if they are grouped together, there is a very strong correlation between this group and larval growth. Biological significance obviously comes before statistical significance.

After all this points have been raised, the results of the correlation of various groups of fatty acids with growth are presented, bearing in mind the limitations of the presentation method.

Total saturated fatty acids show a very strong negative correlation with growth: correlation factor is -0.67 and results are shown in Fig. 4.1.4. On the other hand, there is a very strong positive correlation of total polyunsaturated fatty acids and the ones of the omega:3 family with growth. Correlation values are 0.74 & 0.75 respectively; these results are illustrated in Fig. 4.1.5 & 4.1.6.

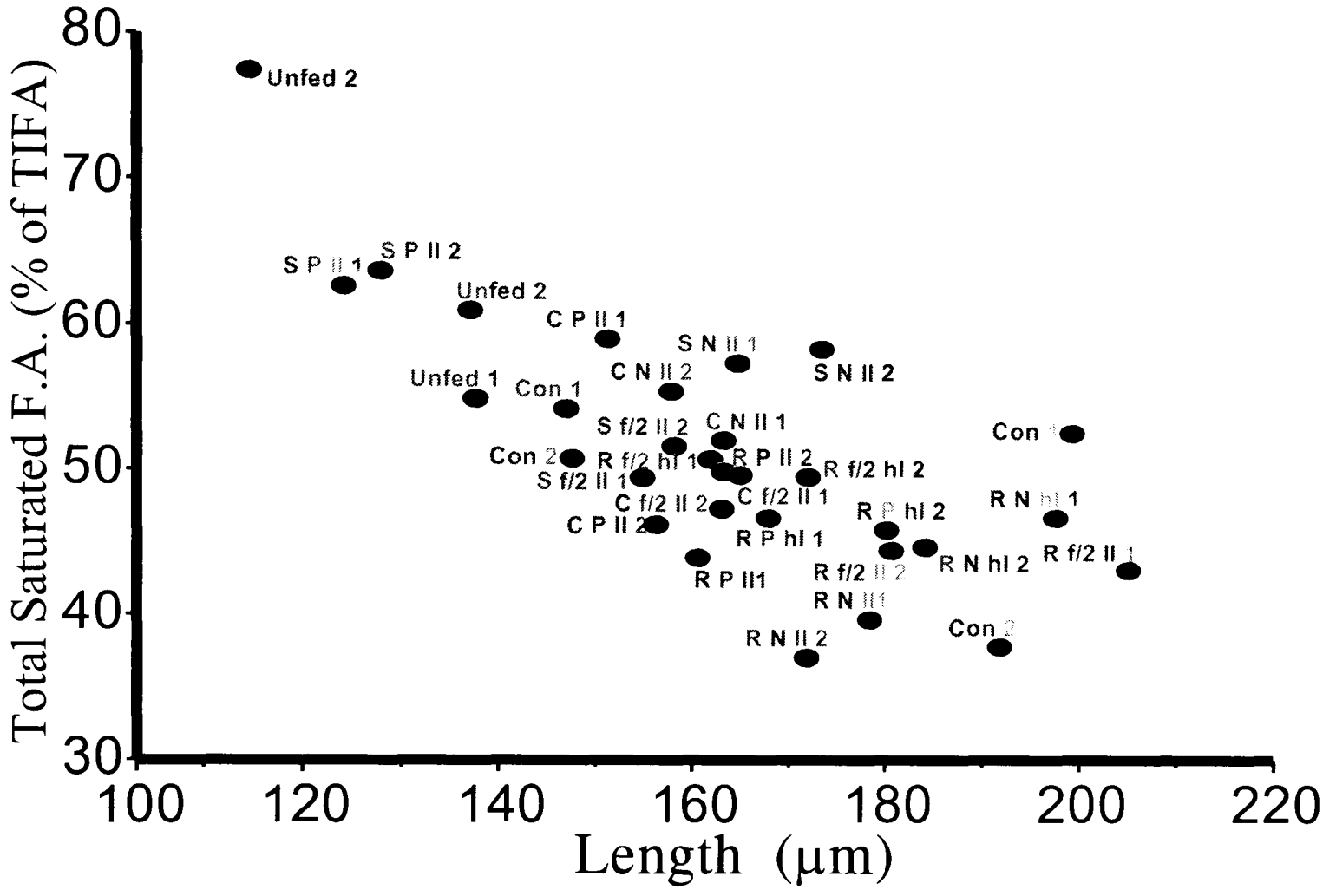

Figure 4.1.4. Relationship between total saturated larval fatty acids (expressed as % of Total Identifiable Fatty Acids, **TIFA**) and mean length of the larval culture.

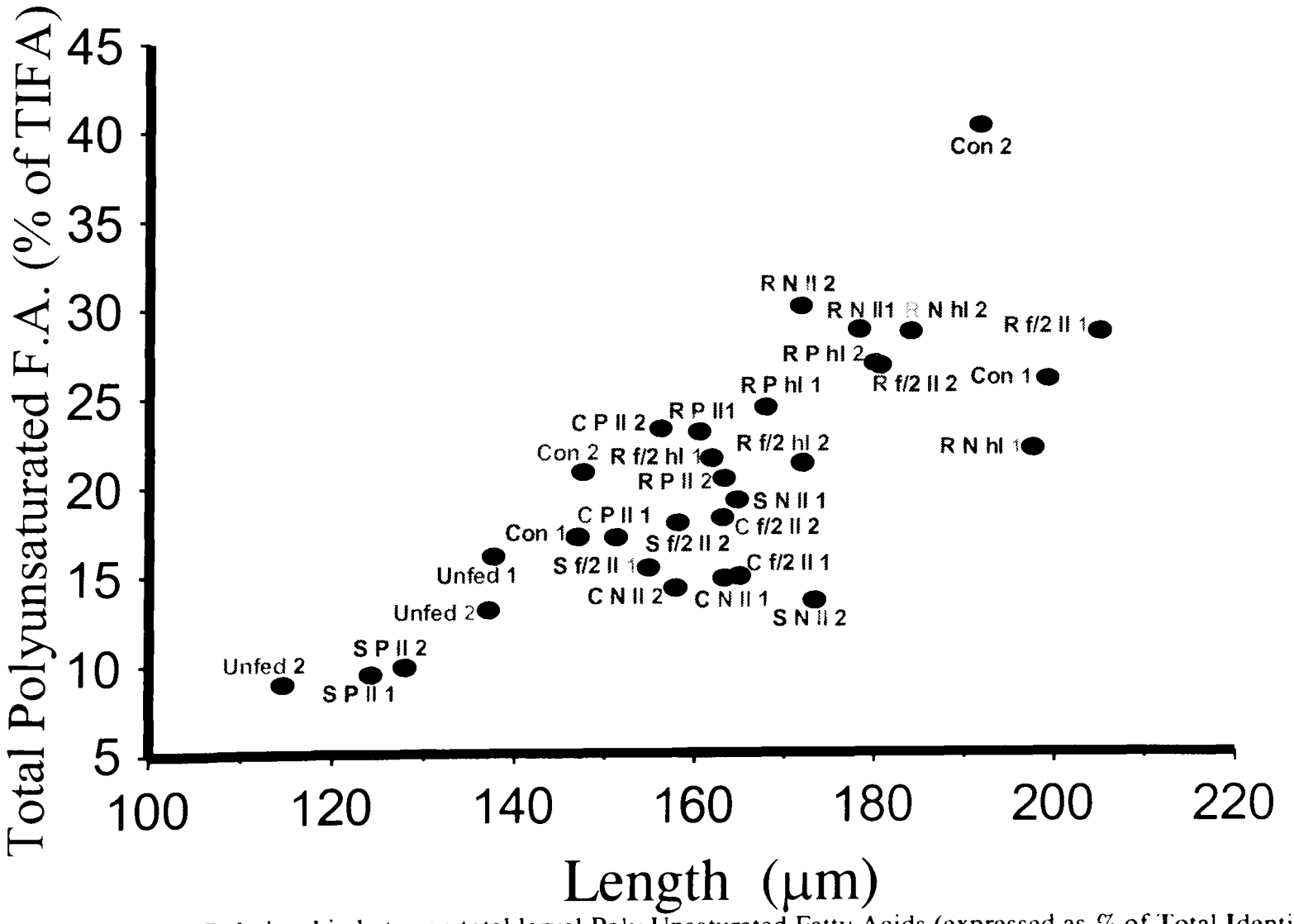

Figure 4.1.5. Relationship between total larval Poly Unsaturated Fatty Acids (expressed as % of Total Identifiable Fatty Acids, **TIFA**) and mean length of the larval culture.

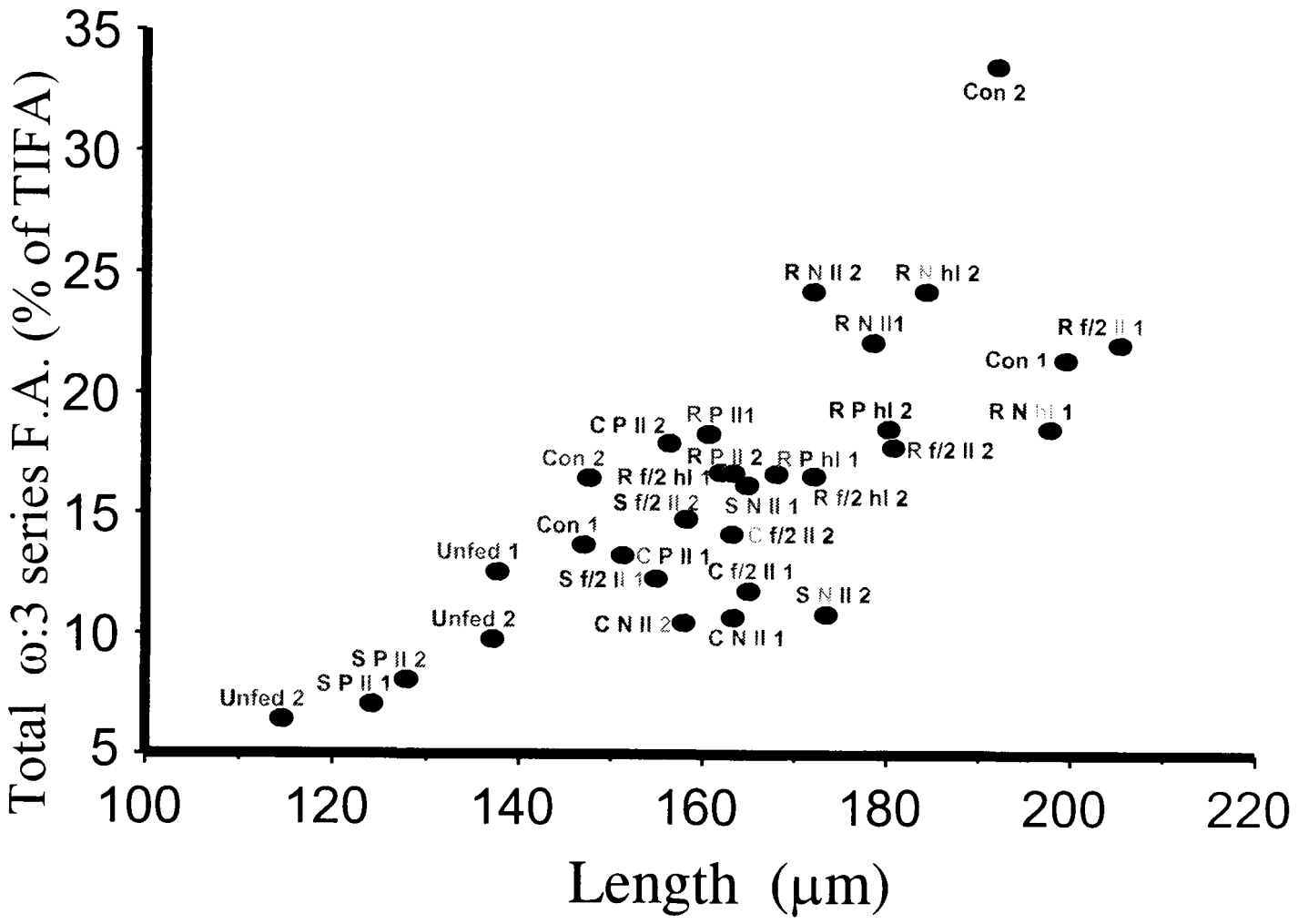

Figure 4.1.6. Relationship between total larval ω:3 family of Fatty Acids (expressed as % of Total Identifiable Fatty Acids, TIFA) and mean length of the larval culture.

As mentioned previously, it has been shown before (Gallager *et al.*, 1986) that indeed, neutral lipids can be used as an index of viability of three bivalve species. A combination of histochemical staining of lipids, in conjunction with survival trials, prompted these researchers to conclude of the biochemical significance and metabolic role of the lipid content. Lipid has been shown by numerous investigators to be an important energy source during stress for larvae of many planktotrophic invertebrates (e.g. review of the early literature by Holland, 1978; Waldock & Nascimento, 1979; Mann & Gallager, 1985).

The 20:5ω3 fatty acid is a major constituent of the larval total lipid fraction, sometimes reaching up to 20% of the total lipid fraction. Therefore it is reasonable to assume that this fatty acid does play a significant role within the cell, probably as a main energy storage medium. The exact way in which this fatty acid influences the larval growth rate and survival remains unknown. However other types of research have promoted the idea that this fatty acid, not only is a major constituent of the total lipid fraction and therefore can be a main source of energy for the animal, but it can also be

important just prior to metamorphosis. Langdon & Waldock (1981) demonstrated the importance of polyunsaturated fatty acids, especially the 20:5ω3, to *Crassostrea gigas*. Other workers, further substantiate this claim by trying to explain the nutritional deficiency of particular algal species in some essential fatty acids, such as some of the ^{a}:3 group (De Pauw *et al.*, 1984). Although this relationship will be discussed later on, a clear distinction should be made between the nutritional elements and the way they influence larval growth / development / biochemical composition from one hand and the larval biochemical components themselves. A deficiency on one nutritional element, like a certain fatty acid, does not necessarily mean that it is exactly the same element in the larval cells that causes the deficiency, since the animals will transform almost all of the components that they digest, through their metabolic pathways, into something else; a deficiency in the input element will translate into a deficiency of the end product inside the animal cells. Therefore it is not entirely correct to substantiate the correlation of a larval fatty acid with larval growth by observing the effect that it has if it is excluded or inadequately present in the diet. The sole significance is related to the nutritional quality of the diet for that given organism and not as to the importance as an growth index for the animals themselves. The opposite does not hold true either, meaning that, if the biochemical profile of an organism was known, it could not be inferred that the organism's food must have exactly the same profile. The transformations happening through the cell's metabolic pathways should be brought into consideration.

Many researchers in the aquacultural field, most likely due to this strong correlation of the larval 20:5ω3 fatty acid with their growth, use this as "a rule of thumb" to rank algal diets (e.g. Su *et al.*, 1988 and many others). For reasons explained above, but as it will be further discussed on the next sub-chapter (4.2, Correlation of diet's components with larval growth), this overgeneralized practice ranges from the misleading to the incorrect.

It is somehow surprising to find out that there is a negative correlation of the larval 15:0 fatty acid with their growth. This fatty acid is generally suggested (e.g. Volkman *et al.*, 1989) to be of bacterial origin; bacterial fatty acids include iso- and anteiso-branched C_{15} and C_{17} saturated and monounsaturated fatty acids. If this is taken into consideration there are two fundamental options that need to be examined: a) is it truly a fatty acid-

specific negative correlation (since as it was also presented, there is a negative correlation in the whole saturated fatty acid family as well)? b) does that imply that there is a negative microbial effect in the larval cultures?

Before any of these questions are dealt with, it should be noted that although there were no direct counts of the bacteria present in the larval cultures, it has been suggested (Volkman *et al.*, 1989) that, due to the specific bacterial fatty acid signatures, these fatty acids distributions can provide a quick semiquantitative indication of the extent of the bacterial load of mass cultures.

The first question falls into the general previous discussion about statistical and true biological significance. These two should not be confused with each other; however if we accept that statistics and the biological analysis for the previous sections, there are no grounds to reject this one.

The role of the bacterial fauna present in the rearing vessels is a matter of ongoing debate. On the one hand, the historical approach in aquacultural practices was to try to eliminate as many of the bacteria as possible in order to avoid potentially detrimental microbial effects to the larvae (Loosanoff & Davis, 1963). On the other hand, it has been documented that some bacteria can have a beneficial effect when included in the larval rearing vessel (Intriago & Jones, 1993; Douillet & Langdon, 1994). However the exact way that this takes place is rather more obscure. Possibilities for this to happen are a) the bacteria have a "conditioning" effect on the water quality in which the animals are reared thus making the environmental conditions more favorable (indirect effect) b) some bacteria which are neutral to the larvae, might compete with some which can be toxic therefore minimizing the number of harmful bacteria in the culturing vessel and c) they might have some nutritional value (Douillet, 1991; Douillet & Langdon, 1993,1994). These possibilities can coexist with one another and hence the difficulty in checking any of these hypotheses.

As shown in the preliminary experiments (chapter 3.1, pp. 62–69), and in accordance with various reports on particle size selection for *M. edulis* (Defossez & Hawkins, 1997 and the very up to date review by Jørgensen, 1996), the larvae are not utilizing the bacteria, at least in a way that there is a difference in their growth rate, and cannot be reared on a bacterial food source alone. For example, early attempts to culture *Artemia* on a diet consisting solely of bacteria failed (D'Agostino, 1964; Seki, 1964).

However bacteria have been used as a food supplement, albeit with varying success. Some bacterial strains when added with microalgae in the larval rearing vessel promote larval growth more than when the alga is fed to the larvae by itself (Yasuda & Taga, 1980; Douillet, 1987; Intriago & Jones, 1993). But, it should not be underemphasized that the risk of introducing potentially harmful bacteria in the culture can have devastating effects, and thus outweigh the potential benefit. (Volkman *et al.*, 1989, present work).

By grouping the fatty acids together in a biochemically rational way, a stronger correlation is unmasked. This could reflect a more realistic way in which the role of the fatty acids is exercised within the cells. But it could also be associated with the methodological random errors of the analytical procedure, however small, balancing each other out and hence, they do not blur the underlying relationship and make its detection possible. Methodological errors of the analytical technique are a) the correct identification of the individual fatty acid peaks in the chromatogram b) their precise integration by the instrument, and c) the random errors associated with the precision and accuracy of the Gas Chromatograph itself. For example, as noted when the fatty acid profile of the algal species was presented, some fatty acids peaks were either not separated or not integrated properly, resulting in incomplete fatty acid profile description.

The Poly Unsaturated Fatty Acids (PUFA) of each single species have been discussed in various previous sections and they have been shown to be both nutritionally important (e.g. Helm *et al.* 1973; Webb & Chu, 1983; De Pauw *et al.*, 1984; Kanazawa, 1985; Enright *et al.*, 1986a,b) and to represent the physiological state of the larvae (Langdon & Waldock, 1981).

The role of PUFA's is expected to be important in many different developmental stages of the larvae, not least because many marine animals appear to have a limited ability to synthesize the polyunsaturated fatty acids 20:5ω3 and 20:6ω3 (Langdon and Waldock, 1981; Enright *et al.*, 1986a,b). The strong positive correlation found between PUFA's and growth clearly supports this theory.

Similar notions can be raised for the ω:3 group of fatty acids. This type of fatty acids is considered to be nutritionally important to adult *Crassostrea virginica* and juvenile *Crassostrea gigas* (Langdon & Waldock, 1981) and has been regarded as nutritionally essential for vigorous growth (Joseph 1982; Watanabe *et al.*, 1983; Levine &

Sulkin, 1984; Waldock & Holland, 1984). The dietary role of this group has also been demonstrated by the poor nutritional value of algal species with a small percentage of this fatty acid family (De Pauw *et al.*, 1984; Helm & Laing, 1987; Whyte *et al.*, 1990). With the present type of analysis this can be further extrapolated to the use of this group as a direct index of growth for *M. edulis* larvae.

The exact physiological role of the saturated fatty acids is little known in marine larvae. It is unclear whether some saturated fatty acids like the 16:0 and the 18:0 can be considered as truly essential fatty acids, with an indication as to their possibly essential nature being their accumulation by the larvae of *Patinopecten yessoensis* (Whyte *et al.*, 1989). Although, due to their relatively greater difficulty to be converted to other forms of more readily usable energy, they are expected to indicate a non-optimal physiological condition. This is confirmed here, by their negative correlation with larval growth.

Although the exact significance of most single fatty acids or fatty acid families is unclear or unknown (Volkman *et al.*, 1989), they can nevertheless be used to describe the physiological state of *M. edulis* larvae. Notwithstanding the fact that protein as a source of stored energy should not be underemphasized, the value of lipids as an indicator of physiological condition is apparent. Holland & Spencer (1973) and Mann & Gallager (1985) have considered in detail the catabolism of energy substrates other than lipid and they concluded that, while catabolism of carbohydrate is negligible in most cases, protein may provide up 40 to 70 % of the calories expended during short starvation periods. Holland (1978) reviewing the literature suggested that during metamorphosis of planktonic bivalve larvae to settled benthic adults, the energy requirements are met by lipid reserves stored prior to metamorphosis. Analogous situations exist in the many other marine larvae; for example the non feeding cyprid stage of the barnacle *Semibalanus balanoides* accumulates large oil reserves, in the form of oil droplets which are catabolized and disappear as the animal attempts to locate a suitable substrate on which to metamorphose (Lucas *et al.*, 1979)

It is obvious that more studies are required to identify the role of most (mainly polyunsaturated) fatty acids in larval biology, if further insight into the mechanisms governing these relationships are to be understood.

4.2 Correlation of larval growth with discrete algal biochemical components

The nutritional value of algal diets depends on a number of parameters, like digestibility, palatability, toxicity and more importantly, biochemical composition. Contrary to earlier experimentation in bivalve aquaculture where nutritional value did not appear to be related to the general biochemical composition of the algae (Walne, 1963, 1970), it is now generally accepted that the algal biochemical properties are of primary importance in determining their nutritional value. However, within the large group of phytoplankton species used as food in bivalve aquaculture, there remains considerable variability in their nutritional value (Enright *et al.*, 1986a) not only because of the different biochemical composition but also because of interspecific differences in digestibility, toxicity and cell size (Webb & Chu, 1983). It is anticipated that dealing on a per species basis, which have a varying biochemical composition, as previously shown (ch. 3.2 – 3.5), should minimize differences caused by interspecific variation, other than biochemical composition and therefore the observed larval properties may be related to biochemical composition alone (Thompson & Harrison, 1992).

Before any attempt is made to correlate the algal biochemical components with larval growth, the relationship between food biomass and the larvae must be investigated.

The experimental approach in the present work was to keep the number of organic particles (algal cells) available to the larvae constant. However, one could argue that larval growth does not depend only on the number of particles ingested and digested. It may have been that the various diets were formulated in such way that their energy content was the same. Another possible constant may have been the wet or dry weight of the algal diet. In all these approaches, the experimenter maintains one parameter constant while the others, since the food is in fact live algal cells each of them with different properties, will change. It is practically impossible to find a live algal diet with all the assumed important nutritional properties constant (Thompson & Harrison, 1992).

As has been stressed previously, it is generally accepted that constant particle number is an appropriate way of testing various algal diets for bivalve larval cultures (Walne, 1963). The present experimental model indeed assumes the constant particle number as a platform for evaluation of the diets. However, since the algal cells have different dimensions and thus different volume and weight, the following argument may

hold true: in any case where the larvae grew more effectively, fed on one algal species than on another diet, this may have been not due to the alga being of better nutritional value, but because, if it was, hypothetically, of larger volume, that in fact the larvae grew more because they were fed with more material.

To investigate whether a relationship between algal dry weight and larval growth exists, the algal dry weight data from Tables 3.2.1, 3.3.1 & 3.5.1 have been plotted against the corresponding mean length achieved by the larvae in each feeding experiment. The results are illustrated in Fig. 4.2.1 & 4.2.2 for the first feeding trial and a repetition. The underlying assumption for comparison is that the larvae were fed on the same number of algal cells, in the three different algal species trials every time.

From these graphs, it becomes clear that the dry weight of the algal species cannot be correlated with larval growth, thus strengthening the argument that indeed, the algal biochemical composition is by far the single most important property that larval growth depends upon. Additionally, it can also be noted that dry weight does not have much significance either between the cells of the same algal species cultured under different conditions, or between different algal species.

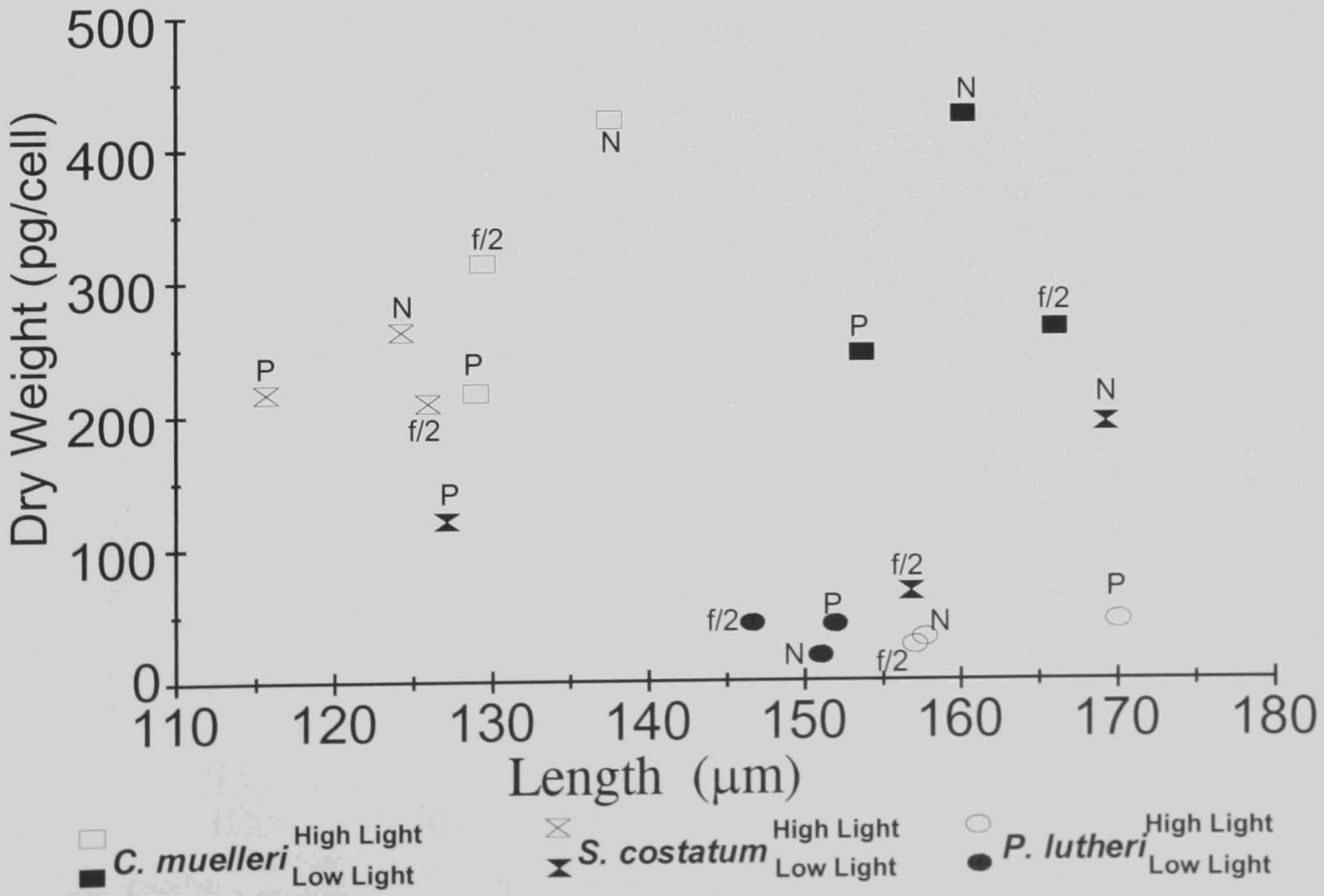

Figure 4.2.1 Relationship between algal dry weight and mean length of larvae of the first trial, fed on the corresponding alga.

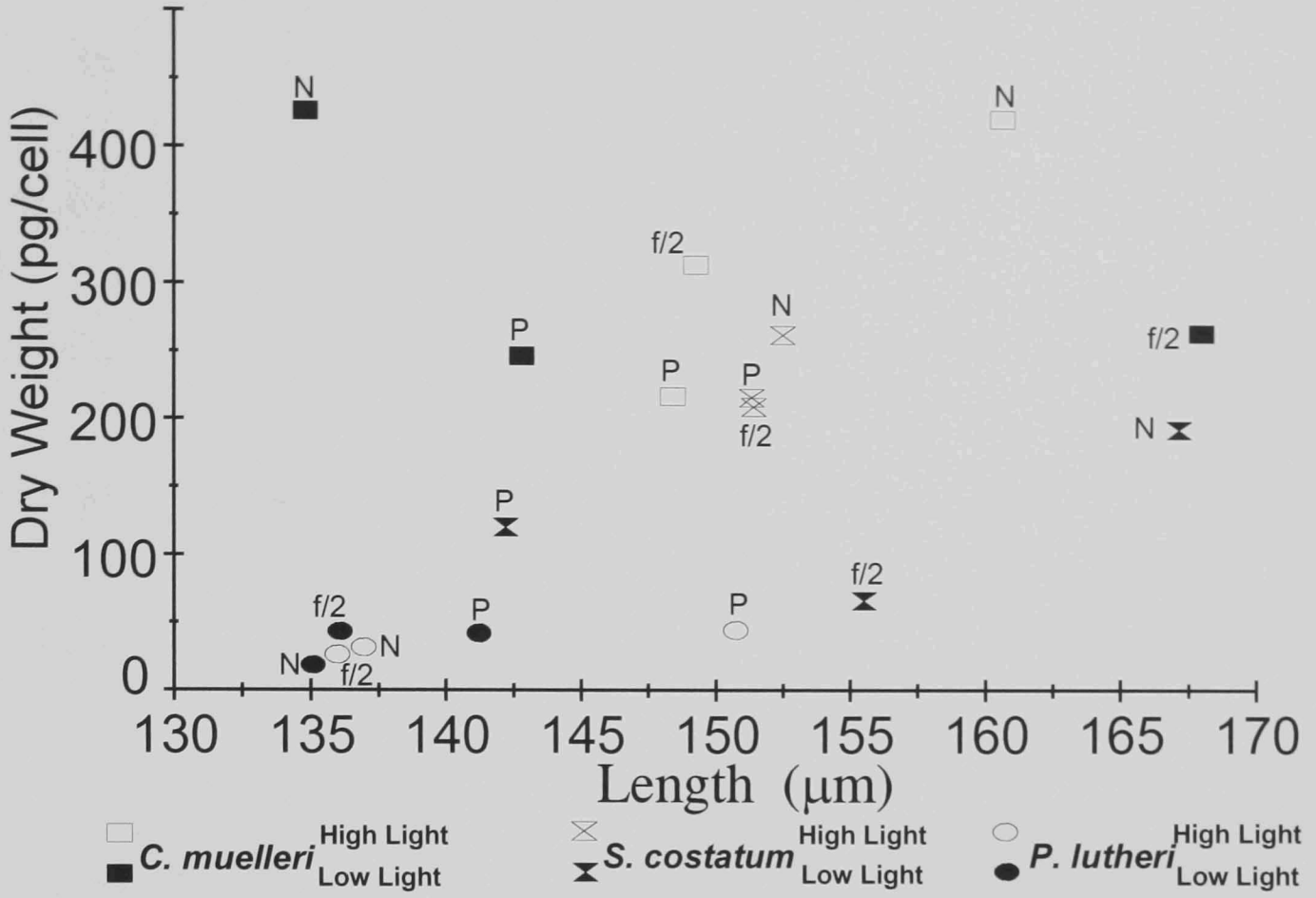

Figure 4.2.2. Relationship between algal dry weight and mean length of larvae of the repetition trial, fed on the corresponding alga.

In very similar way, the photosynthetic pigments chlorophyll a & c do not seem to be correlated with larval growth either, as in general they are assumed to have little or no nutritional role for larvae. Results concerning Chlorophyll a for each feeding trial are shown in Fig. 4.2.3 & 4.2.4, while the ones concerning Chlorophyll c in Fig. 4.2.5 & 4.2.6 respectively.

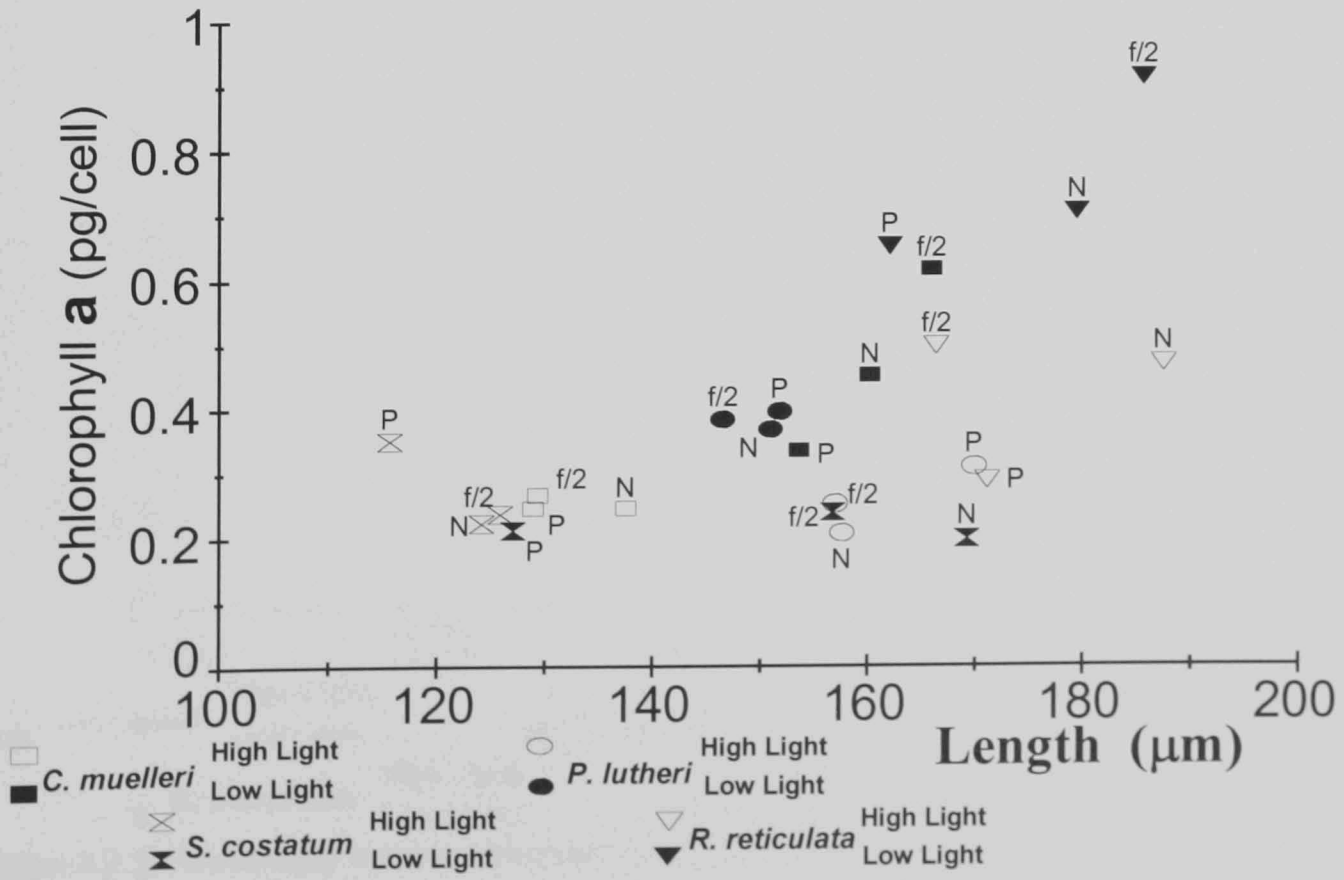

Figure 4.2.3 Relationship between chlorophyll **a** cellular content and mean length of the larvae of the first trial, fed on the corresponding alga.

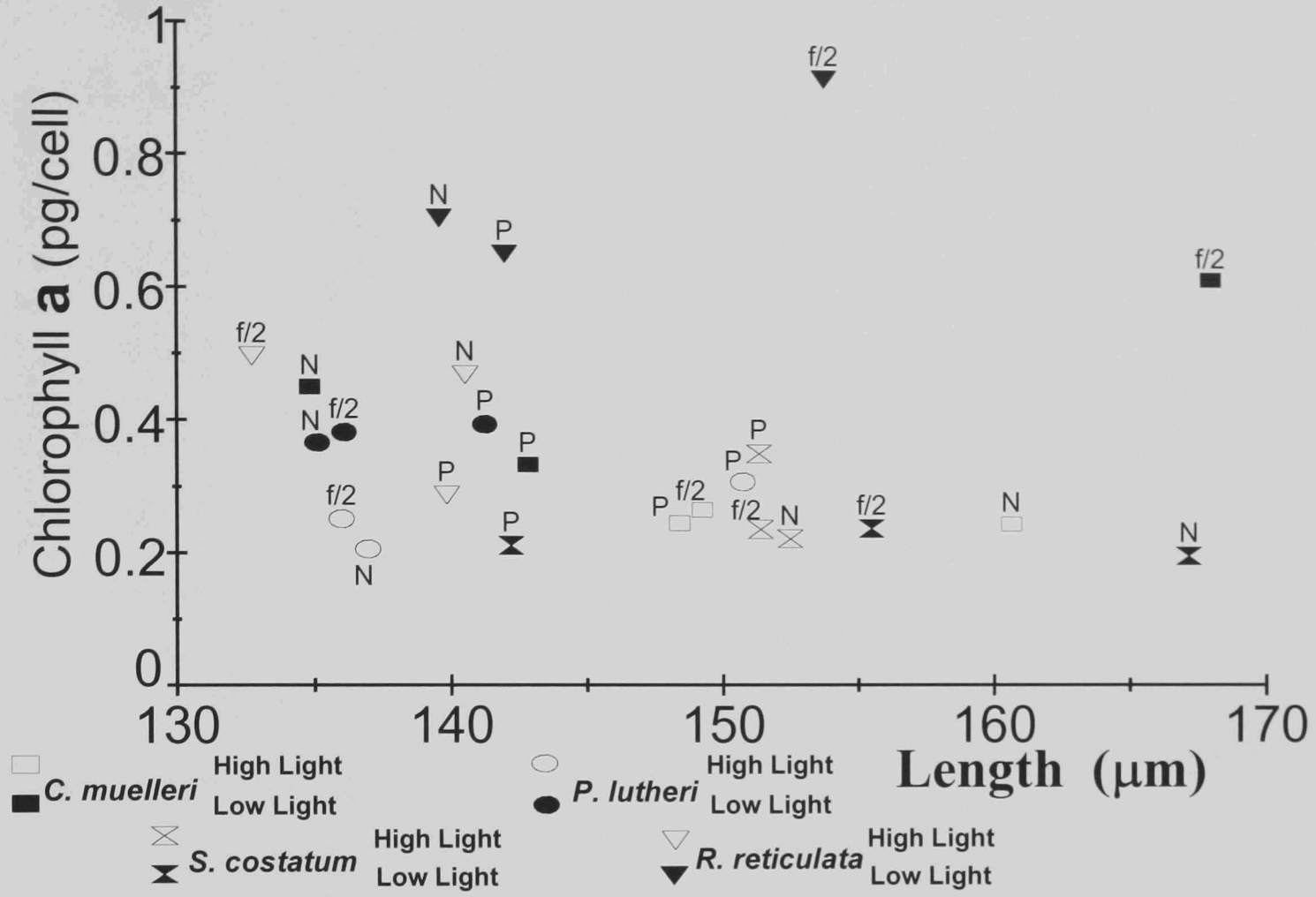

Figure 4.2.4. Relationship between chlorophyll **a** cellular content and mean length of the larvae of the repetition trial, fed on the corresponding alga.

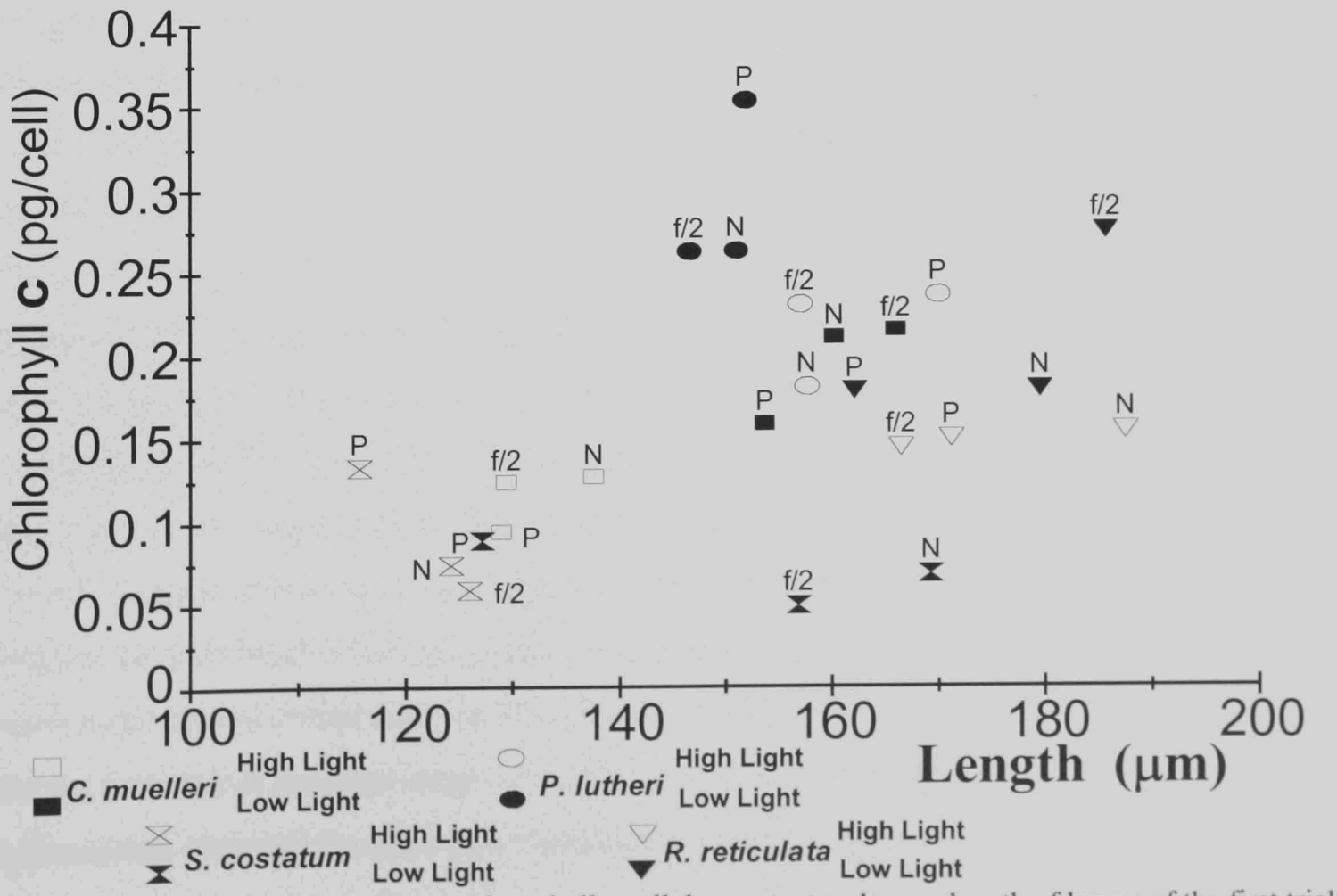

Figure 4.2.5. Relationship between chlorophyll **c** cellular content and mean length of larvae of the first trial, fed on the corresponding alga.

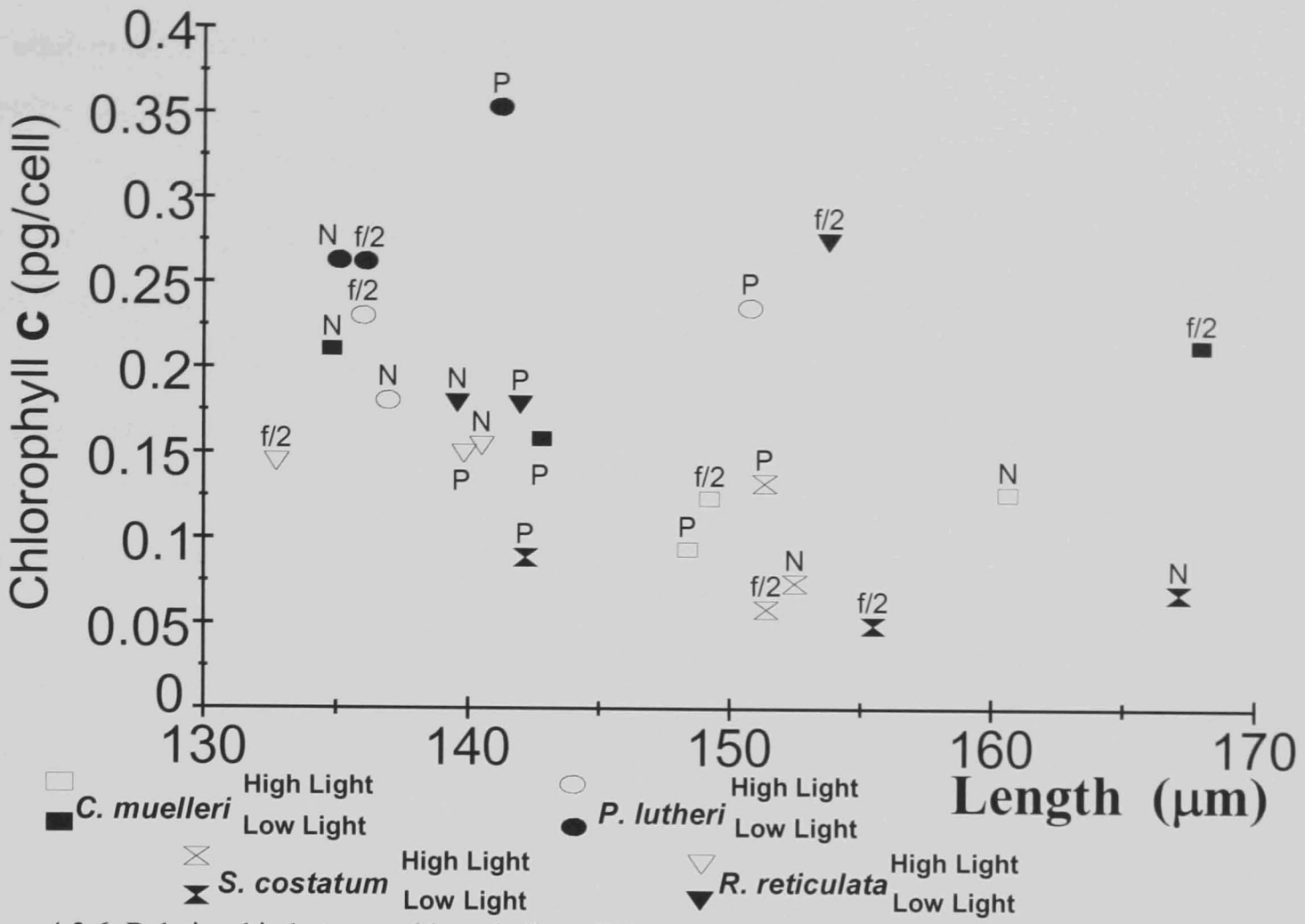

Figure 4.2.6. Relationship between chlorophyll **c** cellular content and mean length of the larvae of the repetition trial, fed on the corresponding alga.

Summarizing the previous correlation analyses, it can be seen that neither dry weight of the cells (combination of biomass and biovolume of the algal cells) nor the photosynthetic pigments, play an important nutritional role as food components for *M. edulis* larvae; correlation values were below any significant value (<-0.65, >0.65). However, as far as the photosynthetic pigments are concerned, it can be assumed that these could, irrespectively of an absence of a nutritional role, be treated as indicative of general growth condition of the algal cells, in a similar way that lipid and fatty acid content has been used as an index of growth for the larvae. The description of the use of a condition index for the algae is not the purpose of the present work; however, as has been discussed in the chapters dealing with the description of each algal species (ch. 3.2 – 3.5), the information about the physiological condition of each algal culture can be used in relation to their biochemical properties in the light of their effect on larval growth. The main biochemical components analyzed here, carbohydrate, protein and fatty acids, will not explain in full the physiological condition of the algal culture. The additional information obtained by chlorophyll measurements and generation time calculations can

be used to give more perspective in the correlation analysis and also explain some superficial discrepancies that could not be otherwise be explained.

After all these preliminary points have been raised, the more nutritionally important, biochemical elements, namely carbohydrate, protein and fatty acids of the algal cells, are examined to find out whether any of them can be correlated with larval growth.

The same pattern of presenting each biochemical component with the corresponding response of every batch of larvae every time will be presented as before. Do note that the statistical notions about correlation vs. regression analysis obviously apply here as well.

Results concerning the potential relationship of dietary, algal, carbohydrate with larval growth are shown in Fig. 4.2.7 & 4.2.8 for the first and the repetition of the feeding trials. As usual, results for the carbohydrate measurement units are expressed in a per cell basis.

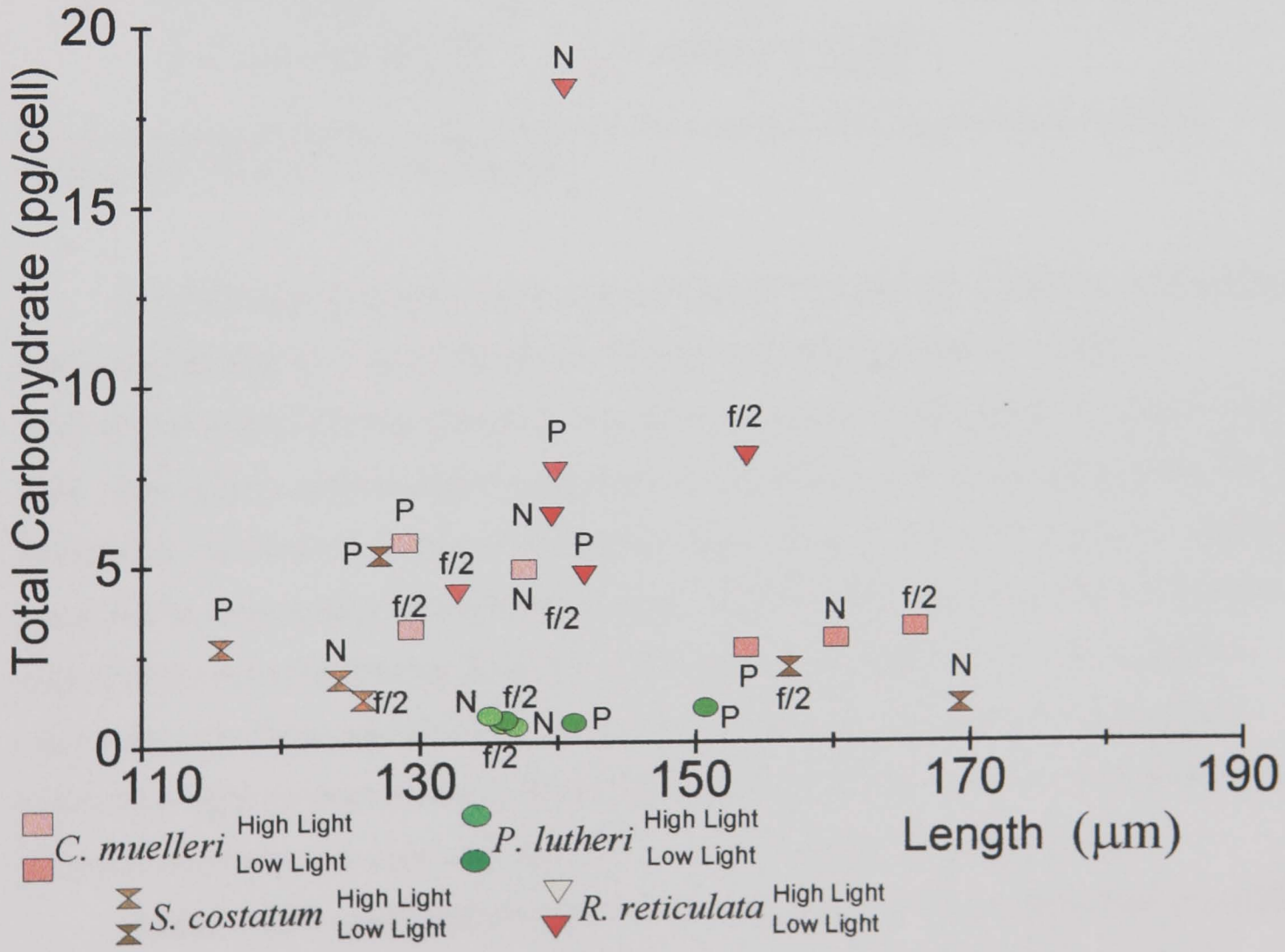

Figure 4.2.7. Relationship between algal carbohydrate content and mean length of the larvae of the first trial, fed on the corresponding alga.

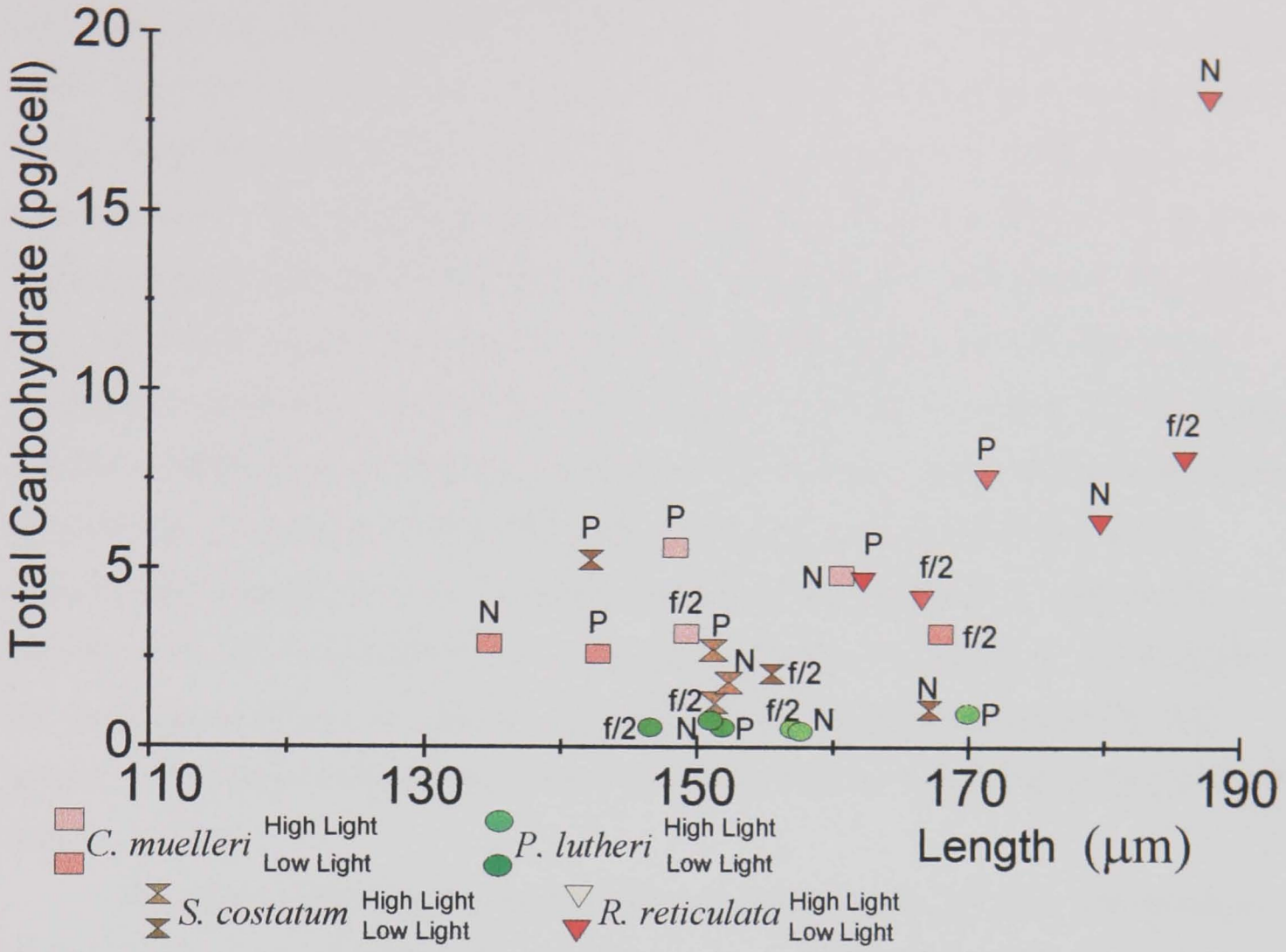

Figure 4.2.8. Relationship between algal carbohydrate content and mean length of the larvae of the repetition trial, fed on the corresponding alga.

From the two previous figures concerning the relationship of dietary carbohydrate and larval growth, it is very difficult to establish any clear trend as to whether a relationship exists between these two parameters. Looking at the graphs, concentrating on a per algal species carbohydrate content and larval growth, a random relationship seems to be in place. As far as *P. lutheri*, *R. reticulata* and *C. muelleri* are concerned, no consistent trend can be seen neither between cells grown under different light conditions nor (more importantly) between feeding trials. However there does seem to be a minor negative correlation of cellular carbohydrate in the *S. costatum* case; although the cells grown under high light produce slower growing larvae (as discussed in ch. 3.2) among the three nutrient conditions, the ones with less carbohydrate produce the biggest larvae.

These results could indicate that indeed the dietary role of carbohydrate is not of primary importance as far as *M. edulis* larvae, in this stage of their development are concerned. If anything, increased carbohydrate content indicates a poorer nutritional

quality food. This could have been expected since it has been shown that carbohydrate is not a main energy source for bivalve larval metabolism (Holland 1978; Mann & Gallager, 1985). Nevertheless it has been shown by Whyte *et al.* (1990), that dietary requirements for essential fatty acids were easier to satisfy than the critical dietary complement of macronutrients, with carbohydrates playing a pivotal role in larval conditioning prior to metamorphosis. However the authors state that there should be a twin edged threshold level, governed by rates of metabolism for specific species and under specific culture conditions, where below its lower end, growth and/or viability is severely compromised and above which these parameters are not improved. This does in fact apply to all dietary biochemical components and it could be the reason that in the present case, dietary carbohydrate does not seem to be directly correlated with growth. If the carbohydrate concentration was enough (above the upper threshold limit) in most algal species under the culturing conditions, it would not reveal any underlying relationship with larval growth, which in turn would appear to be at random with dietary carbohydrate, as indeed is the case here.

The relationship of total algal cellular protein and larval growth is illustrated in Figures 4.2.9 & 4.2.10 for the first and the repetition of the feeding trial respectively.

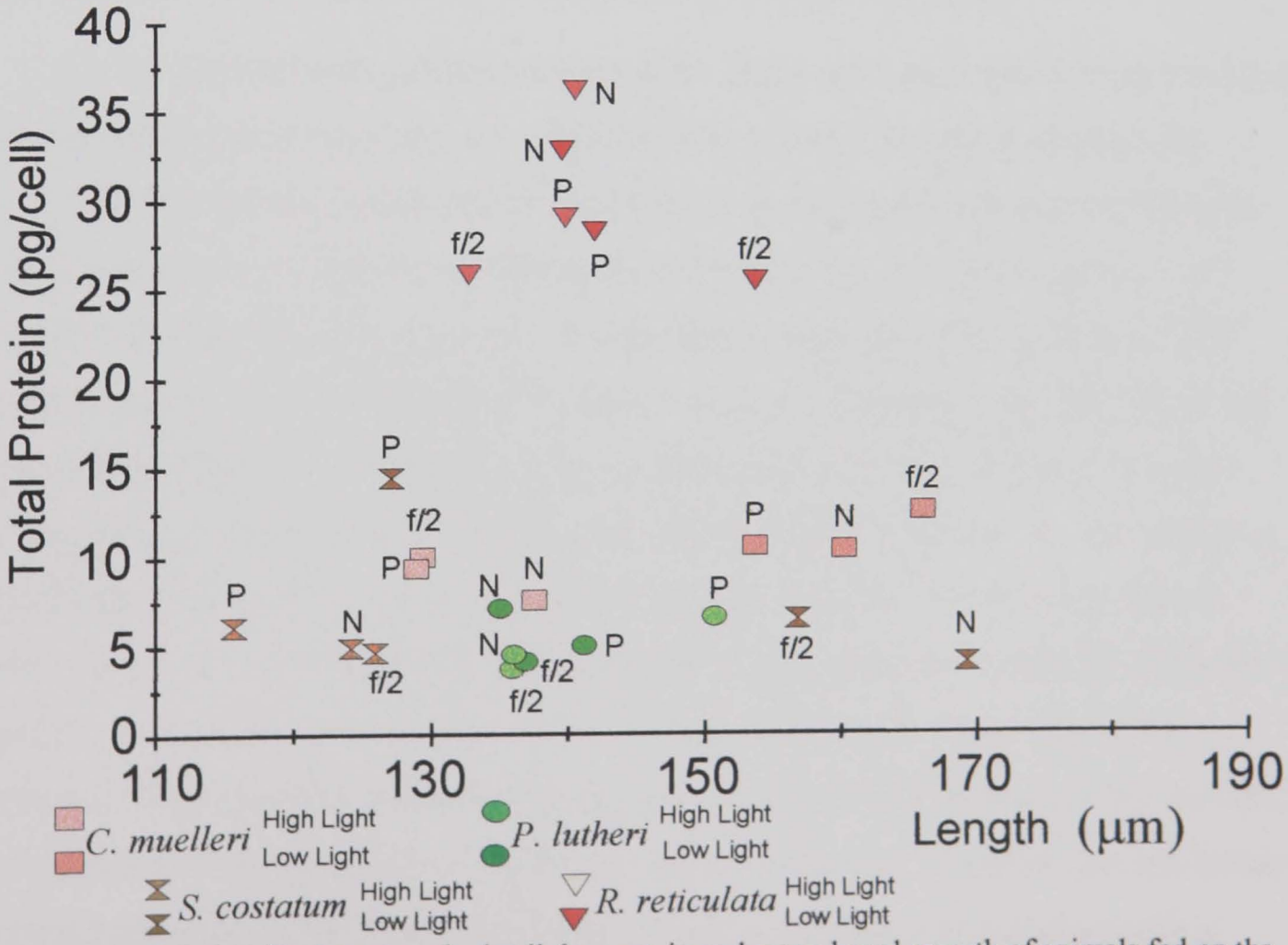

Figure 4.2.9. Relationship between algal cellular protein and mean larval growth of animals fed on the corresponding algal species. Results shown concern the first feeding trial.

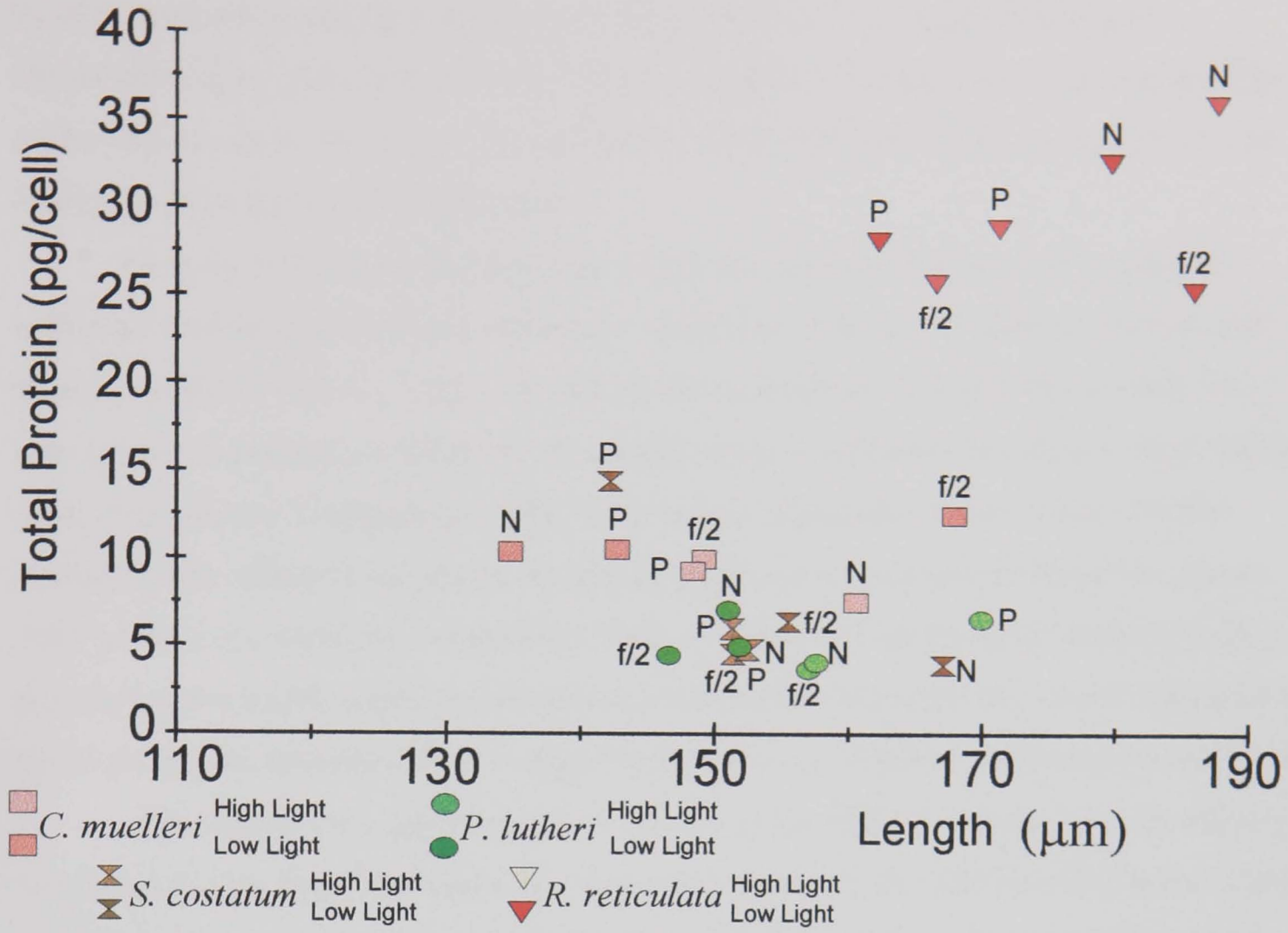

Figure 4.2.10. Relationship between algal cellular protein and mean larval growth of animals fed on the corresponding algal species. Results shown concern the repetition of the feeding trial.

On a per species basis, protein content of the algae does not seem to be related in a meaningful fashion with larval growth. The only case where a pattern emerges is for *S. costatum*. For this species, it appears that the increase in its protein content decreases its nutritional value for *M. edulis* larvae although statistically, the correlation value is non significant in the first experiment (-0.38) but becomes significant in the second (-0.78). This pattern is more clear for algae grown under low light conditions. As discussed in the relevant chapter about *S. costatum* (ch. 3.2), nutrient stress decreases its protein content but also produces cells that are of superior nutritional quality for the larvae. The evidence produced here should not be treated as conclusive, since not only are there few data to apply any meaningful statistical test, but the biological relationship between the three part relationship: nutrient stress $\Rightarrow$ protein content $\Leftrightarrow$ nutritional value for larvae still is inconclusive. There are many contradicting reports as to the importance of dietary protein for bivalve larvae. Some researchers confirm a lack of importance of dietary protein levels with bivalve growth (e.g. Wikfors *et al.*, 1984), while others stress its importance (e.g. Enright *et al.*, 1986a,b). It is more likely that the dietary requirements of the larvae are met

by the algal food source as a whole, with all biochemical components having an interacting relationship with larval growth; this relationship may also be species-specific. In the present study, the lack of any consistent relationship of dietary protein levels and larval growth supports this assumption.

General work on algal physiology suggests that nutrient stress most probably influences amino acid synthesis deficiency within the cells, which leads to a lower total protein content (Wikfors, 1986). The physiological response of algae vary greatly from one species to another, so these results must be treated with extreme caution when dealing with other species. Comparing results from various researchers is not always fruitful because of the effect of variations in methodology used in different laboratories (Baars, 1981). But, even when the comparative work is undertaken by the same research team so that the experimental variation that cannot be controlled is minimized, results concerning different species indicate different algal physiology (e.g. Wikfors, 1986 and present work).

If protein is not a preferred source of energy for this larval stage, then its increase would not have a beneficial impact to the larvae. However, the decrease in protein content itself does not explain the increase in larval growth unless it is explained in the context of its relationship with other biochemical components, such as lipids. It would then mean that the nutritional value of an algal species is more relevant with the ratio of various biochemical component rather than with the content of each individual component. However, as Enright *et al.* (1986a,b) and Whyte *et al.* (1990) pointed out, a threshold relationship is likely to exist.

In the same way as the analysis applied to determine the potential use of larval fatty acids as indices of larval growth, the algal fatty acids can also be examined in order to investigate if any relationship between the two parameters exists. Similar statistical limitations also apply here.

The percentage of single algal fatty acids (as expressed in percentage of Total Identifiable Fatty Acids) in the algae was shown to change with both nutrient stress and light intensity (Tables, 3.2.2, 3.3.2, 3.4.2 &3.5.2) for all species. However, it is not expected that every change recorded is either statistically or biologically significant. The analysis could be restricted to the fatty acids, or groups of fatty acids, whose change is statistically significant. But in doing so the potential nutritionally important changes, which may not be detected as "statistically significant", would be ruled out. To explore

more possibilities, a "graphical" approach was adopted, but without compromising the statistical analysis. If there was a consistent trend appearing between a fatty acid and larval growth, both in feeding trial and the averaged mean lengths of both trials (the latter as a measure of consistency among different larval batches), then a statistical test was also applied and the subsequent results are discussed on the basis of the statistical tests.

As expected, most of the fatty acids seem to be related with the nutritional value of the algal cells, as expressed by the growth of the larvae fed on these cells, in a random fashion. For reasons of parsimony, only the graphical illustrations that imply a relationship of algal fatty acids and larval growth are presented. For reasons of clarity, standard deviations are omitted in the summarizing figures.

A typical example of random relationship between fatty acids and larval growth is shown in Fig. 4.2.11 and concerns the 16:1ω7 fatty acid.

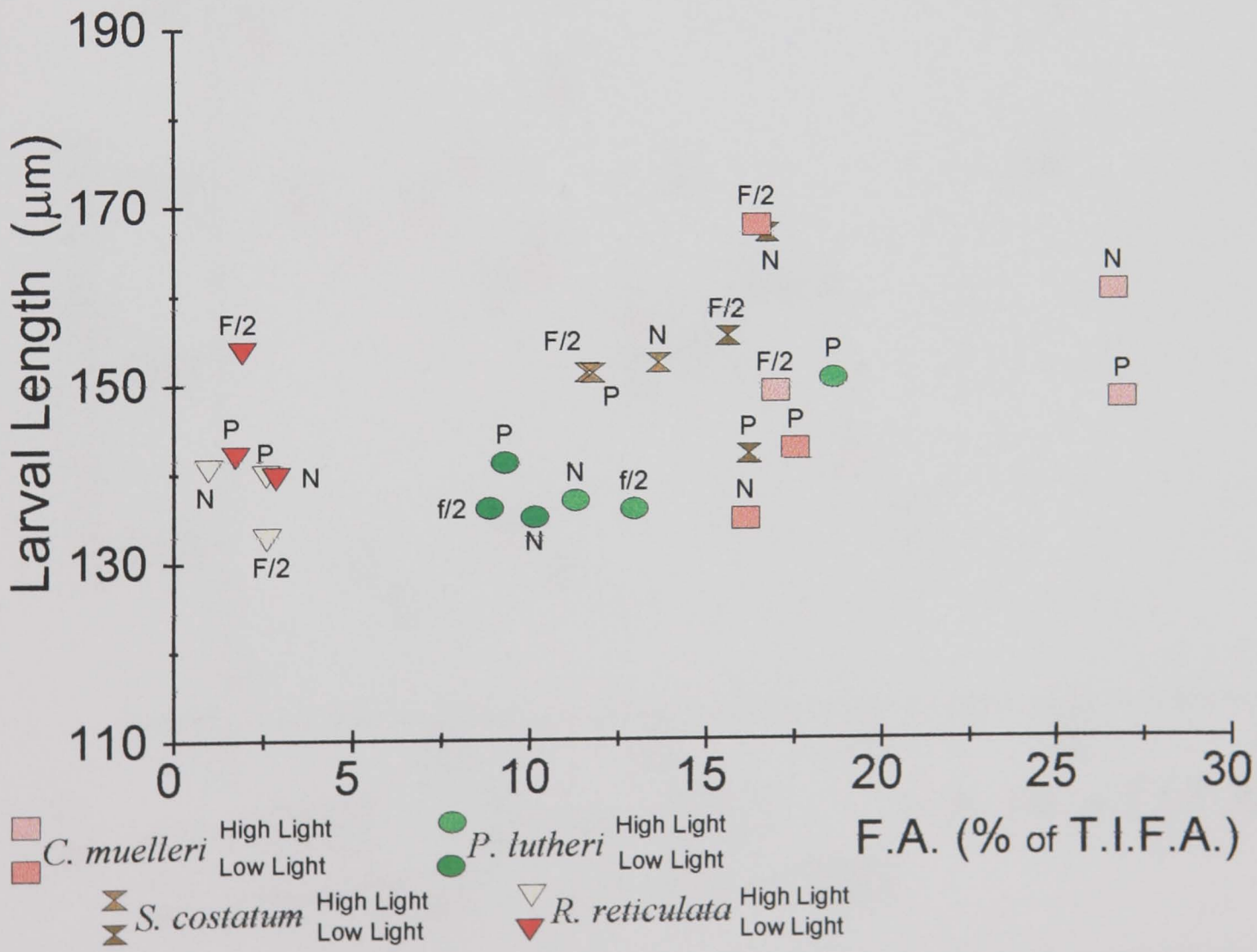

Figure 4.2.11. Relationship between the algal 16:1ω7 Fatty Acid and larval growth of animals fed on the corresponding algal species. Results shown concern the first the feeding trial.

Similar random relationship would emerge if the mean lengths of the larvae from both feeding trials were pooled together. It would seem therefore that, the algal 16:1ω7 fatty acid cannot be correlated in any biologically (nor statistically) significant way with larval growth; this is true for all of the algal species tested.

However, this was not the case with all fatty acids. After running all the possible combinations of plots of all the identified algal fatty acids against larval growth, some were found to be correlated either positively or negatively with larval growth. Results shown concern only the fatty acids that the same correlation could be found in both feeding trials singly and after averaging larval lengths of both feeding trials. The first significant correlation was found for the 16:0 fatty acid both for *S. costatum* and *P. lutheri*. The relevant graphs are shown in Fig. 4.2.12 & 4.2.13 concerning the first feeding trial (singly) and the averaged feeding trials, correspondingly.

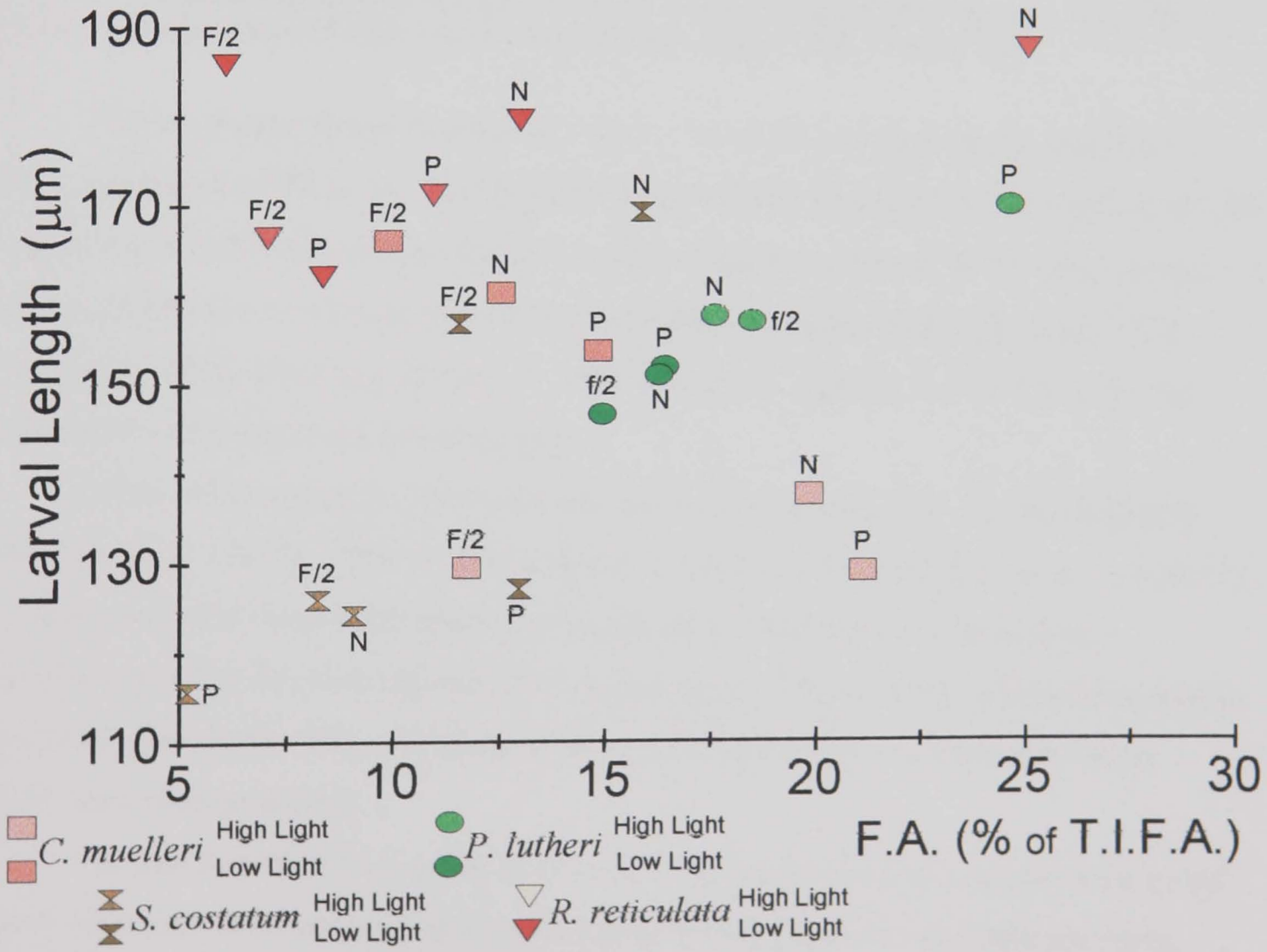

Figure 4.2.12. Relationship between the algal 16:0 Fatty Acid and larval growth of animals fed on the corresponding algal species. Results shown concern the first feeding trial.

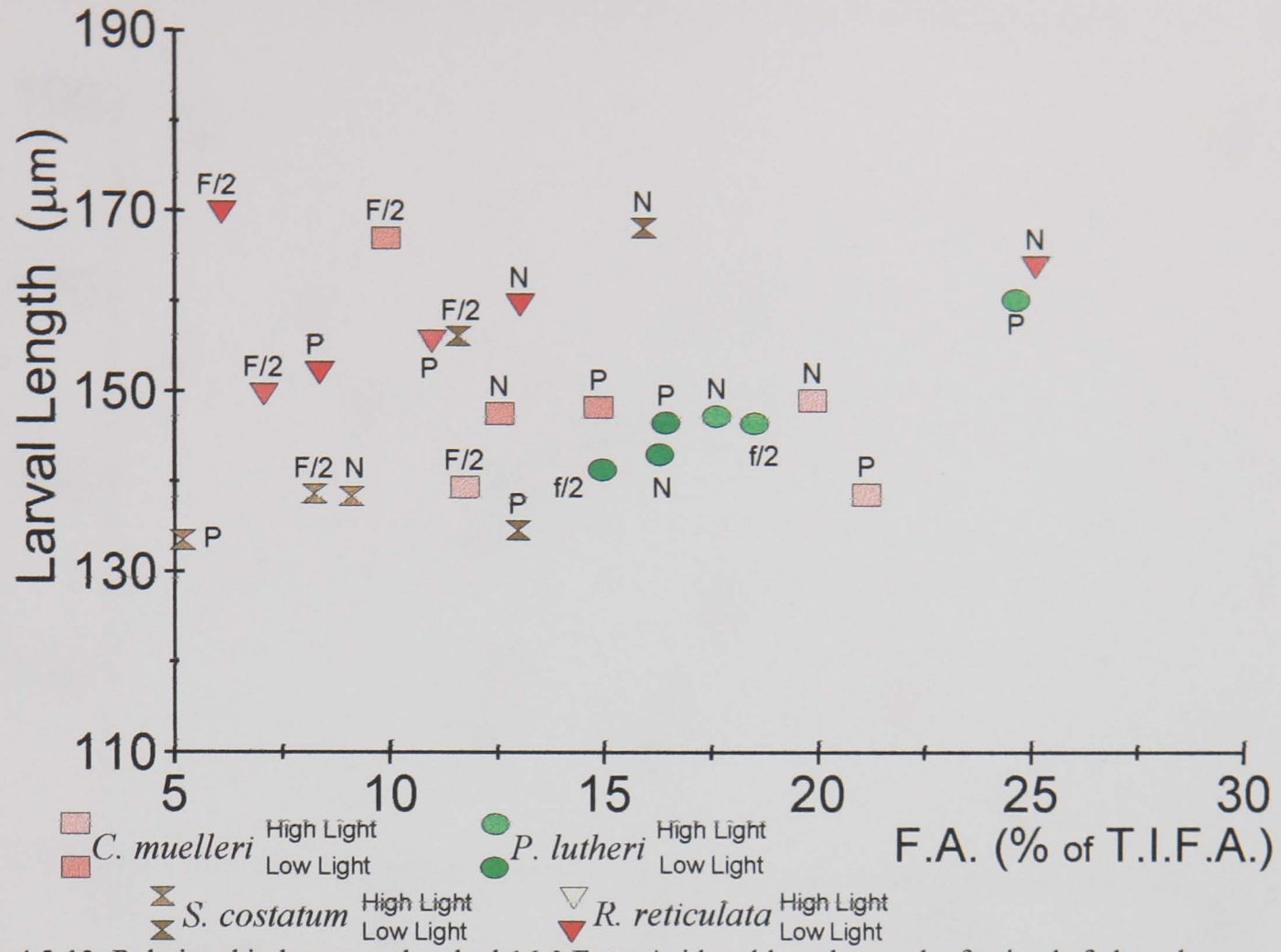

Figure 4.2.13. Relationship between the algal 16:0 Fatty Acid and larval growth of animals fed on the corresponding algal species. Results shown are of the mean values of both feeding trials.

The correlation factor between *P. lutheri* 16:0 fatty acid and larval growth is a highly significant 0.978 on the first experiment and on the averaged experiments 0.979. In *S. costatum*, its 16:0 fatty acid proportion is also strongly correlated with larval growth; correlation factors are somewhat lower for this species ranging from 0.810 and 0.738 respectively. In the other two species, *R. reticulata* and *C. muelleri*, 16:0 FA's are not significantly correlated with larval growth.

Results concerning *P. lutheri* are in excellent agreement with the ones found by Thompson *et al.* (1994a, 1996) who also found a strong positive correlation of *P. lutheri* 's 16:0 fatty acid with the growth rate of both Japanese scallop larvae, *Patinopecten yessoensis*, and Pacific oyster larvae, *Crassostrea gigas*. These results will be discussed in more detail after all the fatty acid data which were found to be correlated with larval growth have been presented.

The total saturated fatty acids of *P. lutheri* were also positively related with larval growth. However in no other species could a relationship between total saturated fatty acids and larval growth be established. Results are shown in Fig. 4.2.14 & 4.2.15 for the first feeding trial and the averaged results of both experiments respectively.

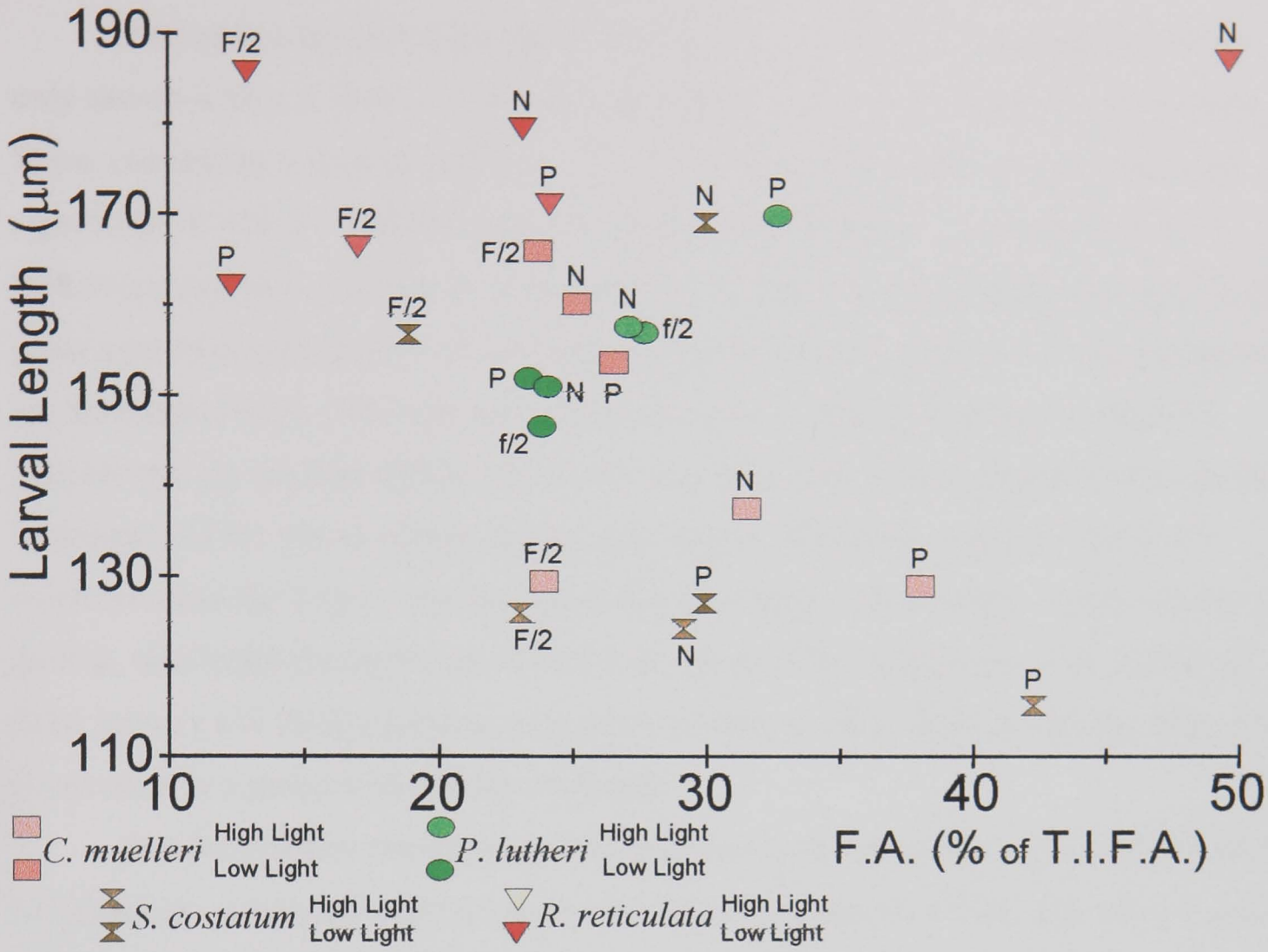

Figure 4.2.14. Relationship between algal Total Saturated Fatty Acids and larval growth of animals fed on the corresponding algal species. Results shown concern the first feeding trial.

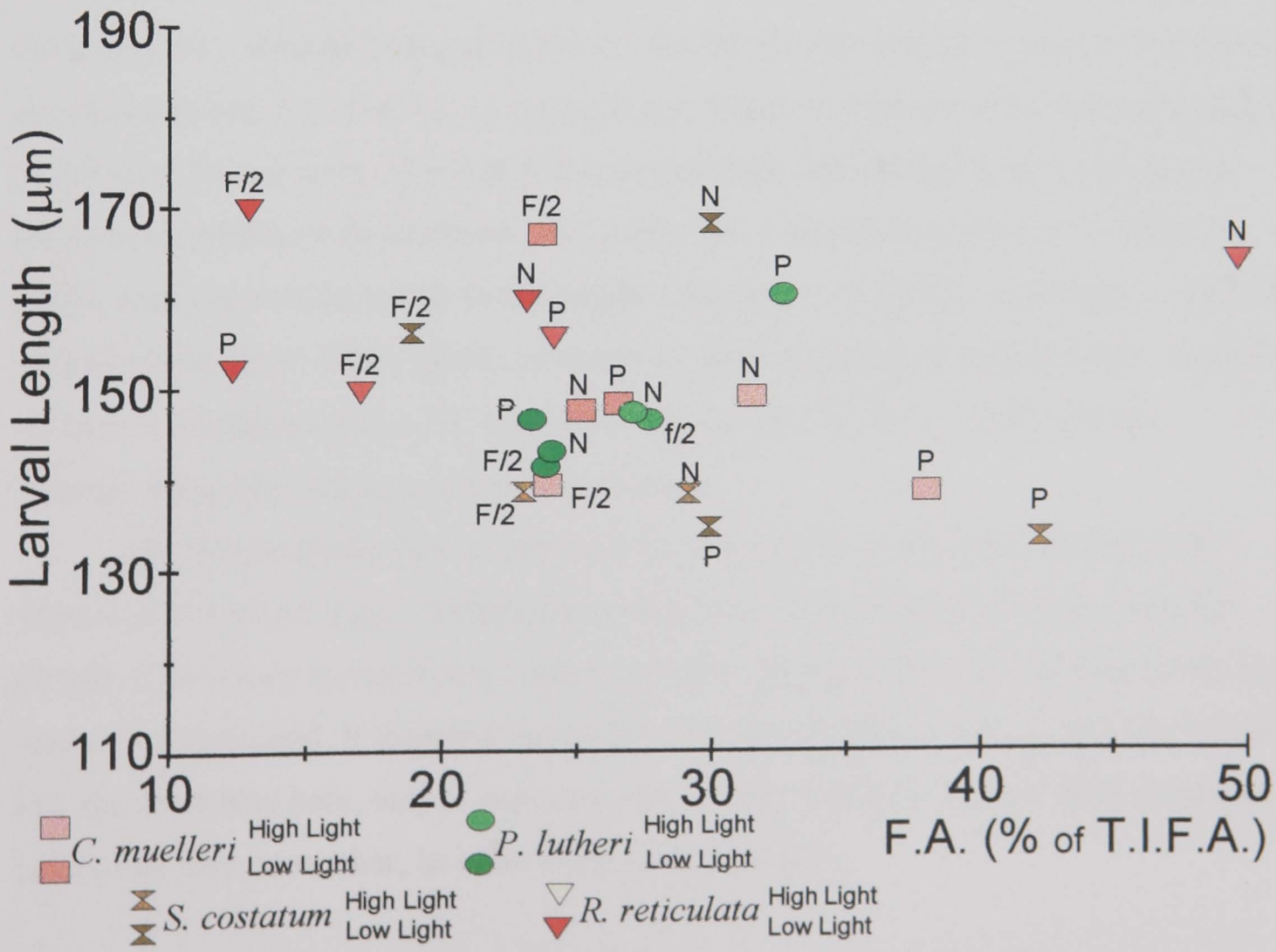

Figure 4.2.15. Relationship between algal Total Saturated Fatty Acids and larval growth of animals fed on the corresponding algal species. Results shown are of the mean values of both feeding trials.

The interesting notion arising from these two plots is that indeed the 16:0 is the only saturated fatty acid of *S. costatum* that is positively correlated with larval growth. Since, correlation values for SaFA of *S. costatum* and length of the larvae were a non significant -0.428 & -0.322 for the two graphs correspondingly. However as far as *P. lutheri* is concerned, the sum of all saturated fatty acids within the cell of this species still gives a positive correlation with larval growth (0.964 and 0.912 in the 1st and the pooled results respectively). Although the correlation factor is slightly smaller, it could still indicate that for this haptophyte the 16:0 is correlated with growth as part of the nutritional importance of the whole saturated fatty acids family. The same cannot be said for *S. costatum* since the 16:0 is only fatty acid that was found to be directly related with larval growth, thus highlighting the nutritional importance of this single fatty acid. Again, all these aspects will be discussed in more detail further on when dealing with the fatty acid component as a group with discreet elements.

Besides positive relationships there was one case where fatty acids were found to be negatively correlated with larval growth. This concerned the PUFA and the ω:3 series of fatty acids (of whose the 20:5ω3 is a major constituent) of *P. lutheri*. The ω:3 series fatty acids of the other species were not found to be related with larval growth in any detectable way. Results concerning the ω:3 FA for the first and the averaged feeding trials are shown in Fig. 4.2.16 & 4.2.17 respectively, which will appear in the following page. Correlation factors were -0.968 & 0.920 respectively, indicating a highly significant negative correlation between these two parameters. Correlation values of *P. lutheri*'s PUFA with the corresponding larval lengths were also very highly significant: -0.977 & -0.942 respectively. Other species correlation values were non significant. For example *S. costatum* correlation of ω:3 FA with larval lengths are -0.437 & -0.419 for the corresponding first and pooled data experiments.

The first result that can confidently be extracted from the presentation of the relationship between algal biochemical components, fatty acids in particular, and the growth of *M. edulis* larvae is that a universal trend among all the algal species tested here, cannot be established. It therefore seems that this relationship is species specific. Of the four species tested here, only *S. costatum* and *P. lutheri* seem to possess fatty acids that can, in one way or another, be related with larval growth.

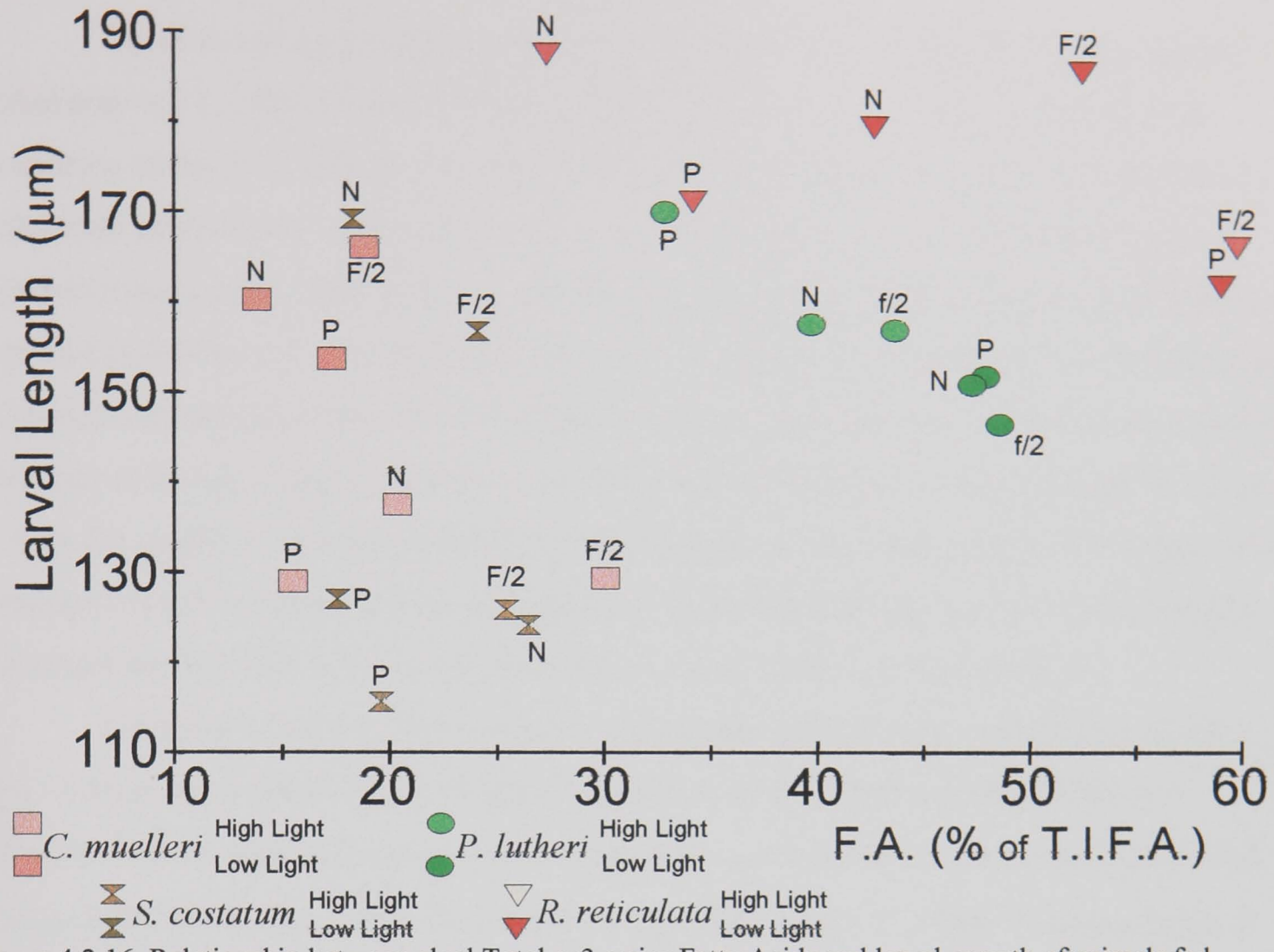

Figure 4.2.16. Relationship between algal Total ω:3 series Fatty Acids and larval growth of animals fed on the corresponding algal species. Results shown concern the first feeding trial.

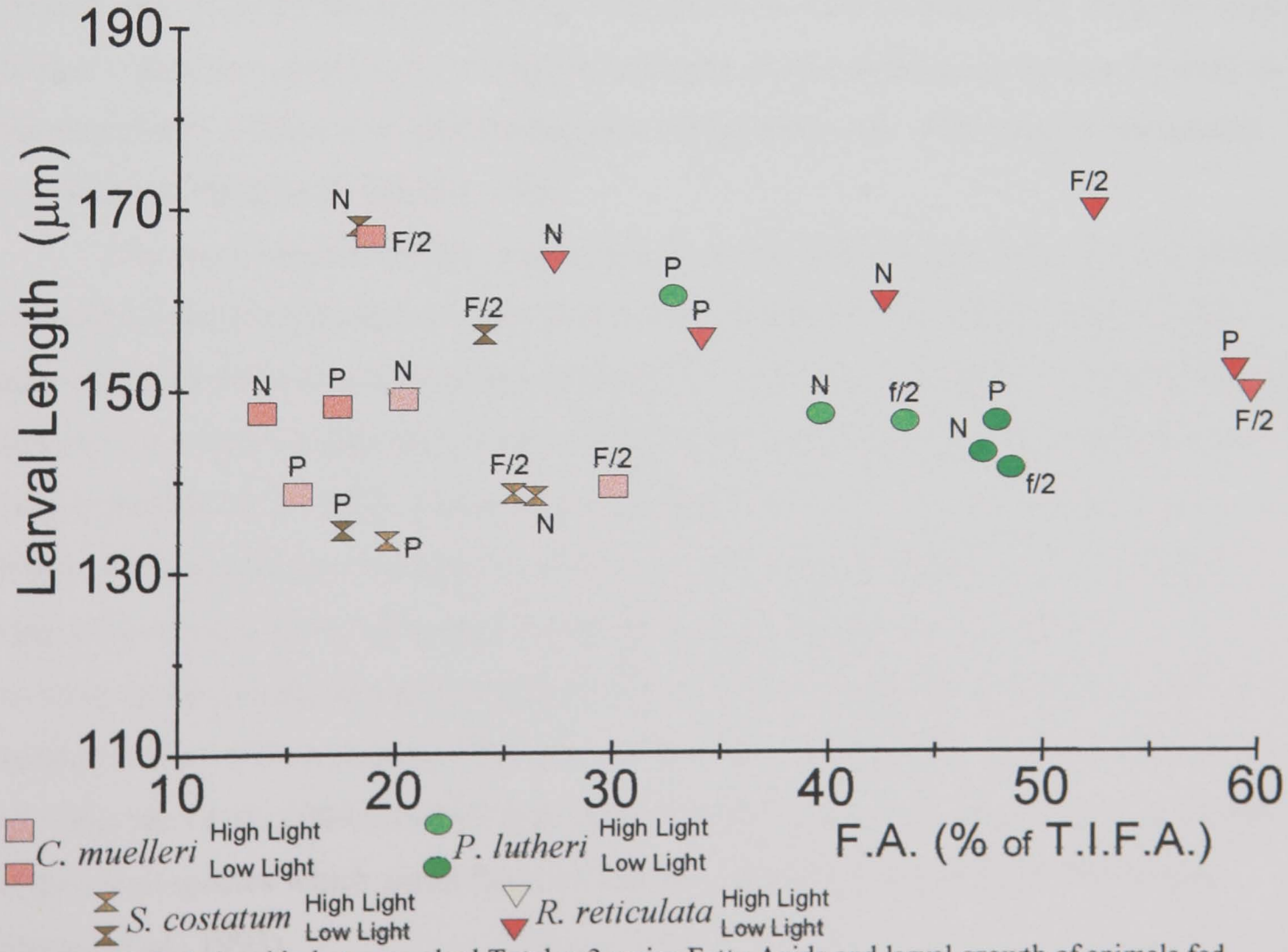

Figure 4.2.17. Relationship between algal Total ω:3 series Fatty Acids and larval growth of animals fed on the corresponding algal species. Results shown are of the mean values of both feeding trials.

There is a general tendency of the FA of the grazer to resemble that of the food (Ackman *et al.*, 1964; Chuecas & Riley, 1969a). In some cases, as reported when the variation of the fatty acid profile among different algal species was presented, this was observed when the two diatoms, *S. costatum* and *C. muelleri*, were fed to the larvae. Closer examination of the results concerning the larvae fed on the other two phytoplakton species tested here, *R. reticulata* and *P. lutheri*, and their corresponding FA profile reveals that this was not observed. However, direct comparisons of the FA profiles of the different batches of larvae, cannot be made since it has been shown that there is inherent variation of the FA profile of the larvae which largely depends on the lipid content of the eggs. The reasons for this variation have been explained in the introductory section of this chapter when an attempt was made to use some larval FA as indices of larval growth.

On many occasions in the past the nutritional value of algal species used as food for various larval stages has been dealt with on the basis of their essential fatty acid content (**EFA**), especially the polyunsaturated ω:3 series family, such as the 20:5ω3 and 22:6ω3 (Webb & Chu, 1983; Ben-Amotz *et al.*, 1985; Su *et al.*, 1988, Yongmanitchai & Ward, 1991; Nelson *et al.*, 1992; López Alonso *et al.*, 1993). Since the discovery that certain long-chain polyunsaturated fatty acids (**PUFA**'s) are essential fatty acids for many marine organisms, particularly during larval stages, considerable research has focused on the availability of EFA's in both the hatchery (Watanabe *et al.*, 1983) and in the natural environment (Sargent & Whittle, 1981).

The nutritional suitability of phytoplankton for bivalves has been assessed, at least partially by the phytoplankton's fatty acid profile (Webb & Chu, 1983). Many studies have screened different algal species for their EFA content, most of them with the hint that these species with a higher EFA content should be a better suited food for many marine larvae (DeMort *et al.*, 1972; Chu & Dupuy, 1980; Nelson *et al.*, 1989; Volkman *et al.*, 1989). Many studies have concentrated around improving the cellular content of these fatty acids in algal cells, most often by manipulating algal cultures in different environmental conditions such as temperature, nutrient stress (Rhee & Gotham, 1981a,b), irradiance level (Blanchemain & Grizeau, 1996), harvest phase (Brown *et al.*, 1996b) or pH (Sánchez *et al.*, 1994); another approach commonly used is screening various strains of the same species which might demonstrate the desired biochemical profile (López Alonso *et al.*, 1993).

Results, in the present study, show that for *P. lutheri* & *S. costatum* the above statement cannot be readily substantiated. The only fatty acid that could be positively related with the growth of larvae of *M. edulis* is the 16:0. The same finding was produced by Thompson *et al.* (1994a) for *Patinopecten yessoensis* larvae. Therefore there should not be much doubt about the nutritional superiority that the increased amounts of 16:0 give to the nutritional value of *S. costatum* cells. But the same cannot be positively concluded about *P. lutheri*, since the sum of its Saturated fatty acids was also positively correlated with larval growth. Thompson *et al.* (1994a), also treat together the sum of 14:0 and the 16:0 FA as positively correlated with larval growth. Although it is true that these two fatty acids are the dominant saturated fatty acids in marine phytoplankton (Ackman & Tocher, 1968), since in fact only the 18:0 was not added to the Total Saturated FA sum and there was only a negligible amount of 20:0 FA (usually below detection levels or present in minute quantities), it could be that the nutritional significance of the 16:0 fatty acid is mostly (or partly, depending which way you look at it!) explained by its saturated nature. The way this biological relationship takes place remains to be investigated. At any rate, the conclusion about the link between the proportion of dietary saturated FA's (in some cases, especially the 16:0) of *P. lutheri* and *S. costatum* and mussel larvae growth holds true.

Further to this, and again in accordance with various publications by Thompson and coworkers (Thompson & Harrison 1992l; Thompson *et al.*, 1994a, 1996), increased amounts of the polyunsaturated fatty acids resulted in inferior larval growth. The overall negative correlation of the PUFA and the ω:3 series of FA's with growth are also similar to those obtained by Dickey-Collas and Geffen (1992) for plaice larvae, which imply that while small amounts of these FA may be essential, large amounts may be deleterious.

Although the results obtained here do indicate the existence of various relationships between dietary FA and larval growth caution must be exercised when drawing conclusions. In the light of the non-existence of such relationships between the same algal FA's of the other species tested here, the relationships found before, should not be overgeneralized, as has been the tendency before in the past. The present study adds to the often overlooked fact that these relationships are mostly (if not totally) species specific.

4.3 Combined effects of algal biochemical components on larval size

The performance as a diet of each single algal species has been extensively examined in the corresponding chapters presenting the results for all the algae used in this report (ch. 3.2 – 3.5). Results were then dealt collectively under the perspective of the effect of each environmental condition (ch. 3.6) and finally a correlation analysis of algal biochemical components and larval growth was presented in ch. 4.2.

However in all cases, since multivariate regression analysis could not be used for reasons of statistical consistency, the studied algal biochemical components were correlated singly with larval growth, using correlation analysis. But as has been stressed at various points in this thesis, it is logical to assume that the overall nutritional value of any algal species should rely on the combination of a number of biochemical components. In this chapter a graphical presentational approach is adopted to try to explain the effect displayed by *M. edulis* larvae in relation to the simultaneous change of the major biochemical components in the algae.

If one tries to relate two parameters with each other, an x – y plot would suffice, and that was indeed the method applied before. To study the effect of three parameters a three dimensional graph can be employed. To further enhance the number of simultaneously examined parameters a fourth variable can be superimposed in the three dimensional graph data, in form of a colourmap key, which would indicate the range of values of the fourth variable. Since the analysed biochemical components for any of the algal species were more than four (protein, dry weight, carbohydrate, chlorophylls and around 20 identified fatty acids) this method will still not fully describe all of the analysed parameters. However, since only few of the components have been shown to have a important nutritional role the three dimensional analysis can be limited to them.

The important dietary components have been shown by numerous studies tabulated from the literature to be: carbohydrate, protein, and fatty acids. Therefore these will be examined in greater detail.

In all of the three dimensional graphs the Z – axis is always the final length achieved by the larvae by the end of the feeding trial. Then the Y – axis is the protein concentration of the diets in each of six conditions studied. The X – axis is always a fatty acid or a group of fatty acids, and finally carbohydrate concentration is displayed in the graph by means of a colourmap key; each data point assumes a different color

depending on the concentration of carbohydrate and the range of values are displayed in form of a colourmap legend.

In most cases in order to facilitate the reader when making comparisons and also for making easier to comprehend the different displayed pattern of the data, a smaller two dimensional graph (x – y plot), which has been presented in corresponding sections of the previous sub – chapter (4.2) is also included with the three dimensional graph.

Displaying more than two parameters simultaneously at any one time requires essentially a more abstract way of explaining the observed patterns. This can be difficult for the reader. On top of that, the different performance among repetitions of the same experiment can confuse the reader even more. To make things simpler, as far as the performance of the larvae is concerned, which as explained is displayed on the Z – axis of the graphs, only the trials that were explained to be fully representative of the overall trends are included in the analysis.

For example, in the second *P. lutheri* trial, one of the three larval beakers fed on *P. lutheri* under low light and phosphorus limitation did not perform similarly as the other two. Although in the corresponding chapter (ch. 3.5) both trials were rigorously examined and statistical tests applied, for demonstrative reasons it is easier to concentrate on the results from the first trial as more representative of the overall performance.

Similarly as explained when the results from the two diatoms, *S. costatum* and *C. muelleri*, were presented (ch. 3.2 and 3.3), the high and low light trial were carried with different batches of larvae every time. However, in the first trial of algae grown under high light conditions, the larvae grew significantly less than in the repetition trial. However, this was not attributed to the diet itself, since the control diets also grew significantly less than the repetition control diet, thus indicating that the larvae of the first trial were, as a whole, of inferior quality. Therefore the second trial is used here.

During both feeding trials of *R. reticulata*, the results obtained by the larvae followed the same trends between diets grown under different light and nutrient conditions. The repetition trial was used for the present analysis.

Of the four tested species and numerous protein, carbohydrate and fatty acid combinations only few of them were found to be further explained by the use of three dimensional graphs. Nevertheless, as shown in the previous section (ch. 4.2), in few

cases the obtained response of larval growth was found to be sufficiently explained by the variation of one single algal component. These cases will also be included here so that the reader will be able to create a better idea of how a significant two – variable correlation is represented in three – dimensional space. Other cases, that were before unable to be resolved by the two – variable analysis, will be demonstrated to be better explained when taking into consideration more variables. But, still, not all cases can be explained this way.

To facilitate the reader, an example of random relationship between algal carbohydrate, protein and fatty acid content and larval growth is presented first. Then, the cases where previous correlation between algal components and larval growth was established will be presented. In these cases a thumbnail size graph will also be included in the presentation to ease the transition from the two dimensional surface to the three dimensional space. Finally, the cases that were presented as random relationships before but can now be better explained with the use of more variables will be shown. In all cases the data points are named **a**, **b**, **c**, **d**, **e** and **f**, each one the High Light f/2 medium cells (**HL f/2**), High Light Phosphorus limited cells (**HL P**), High Light Nitrogen limited cells (**HL N**), Low Light f/2 medium cells (**LL f/2**), Low Light Phosphorus limited cells (**LL P**) and Low Light nitrogen limited cells (**LL N**) correspondingly. The naming of the data points is consistent among all graphs.

Fig. 4.3.1 shows an example of apparently random relationship between algal protein, carbohydrate and the total omega : 3 Fatty Acids of *R. reticulata* and larval growth.

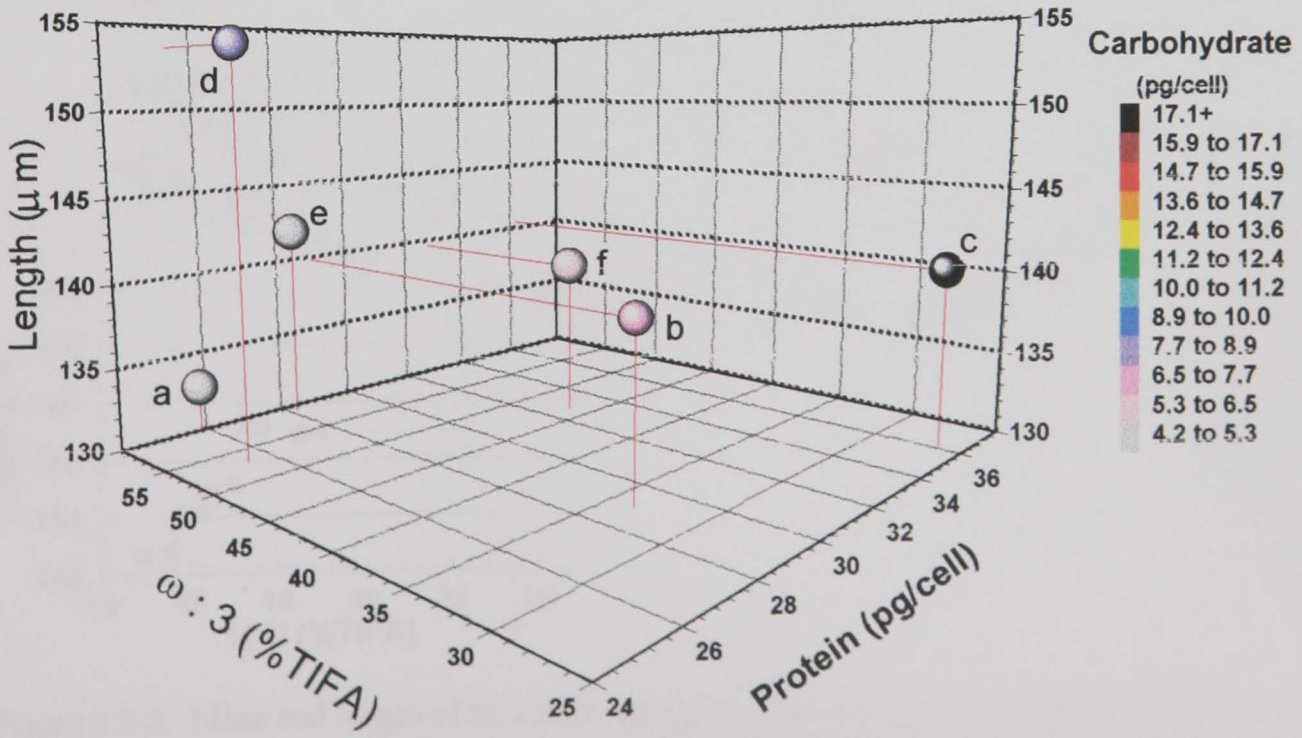

Figure 4.3.1. Mean end length of *M. edulis* larvae, in relation to protein, carbohydrate content and proportion of the Total omega : 3 proportion (expressed as percentage of Total Identifiable Fatty Acids, % **TIFA**) of *R. reticulata*, in the repetition trial. **a= HL f/2**, **b= HL P**, c= **HL N**, **d= LL f/2**, **e= LL P** and **f= LL N**.

In this case, none of the displayed parameters can be meaningfully shown to be correlated with larval growth, neither with a linear nor with a threshold – like model. To explain this in more detail, some larvae fed on *R. reticulata* that has similar protein content and almost identical percentage of the sum of ω: 3 FA, have mean end lengths which are significantly different (data points **a, d** and **e**). Similarly data points with similar carbohydrate content and ω: 3 proportion again produce larvae which assume significantly different length (data points **a** and **e**).

An example were a positive correlation between an algal biochemical component and larval growth was found concerned the *P. lutheri* 16:0 FA. This is now transformed into the graph appearing in Fig. 4.3.2, with protein and carbohydrate content also incorporated.

As mentioned earlier, the x – y plot, concerning the relationship between the 16:0 FA of *P. lutheri* and resulting larval growth is also displayed. The original graph appeared in Fig. 4.2.12 and the presently shown illustration, which shows only the results relative to *P. lutheri*, is a magnified version of that graph. For easier cross – referencing the data points have been named in both graphs.

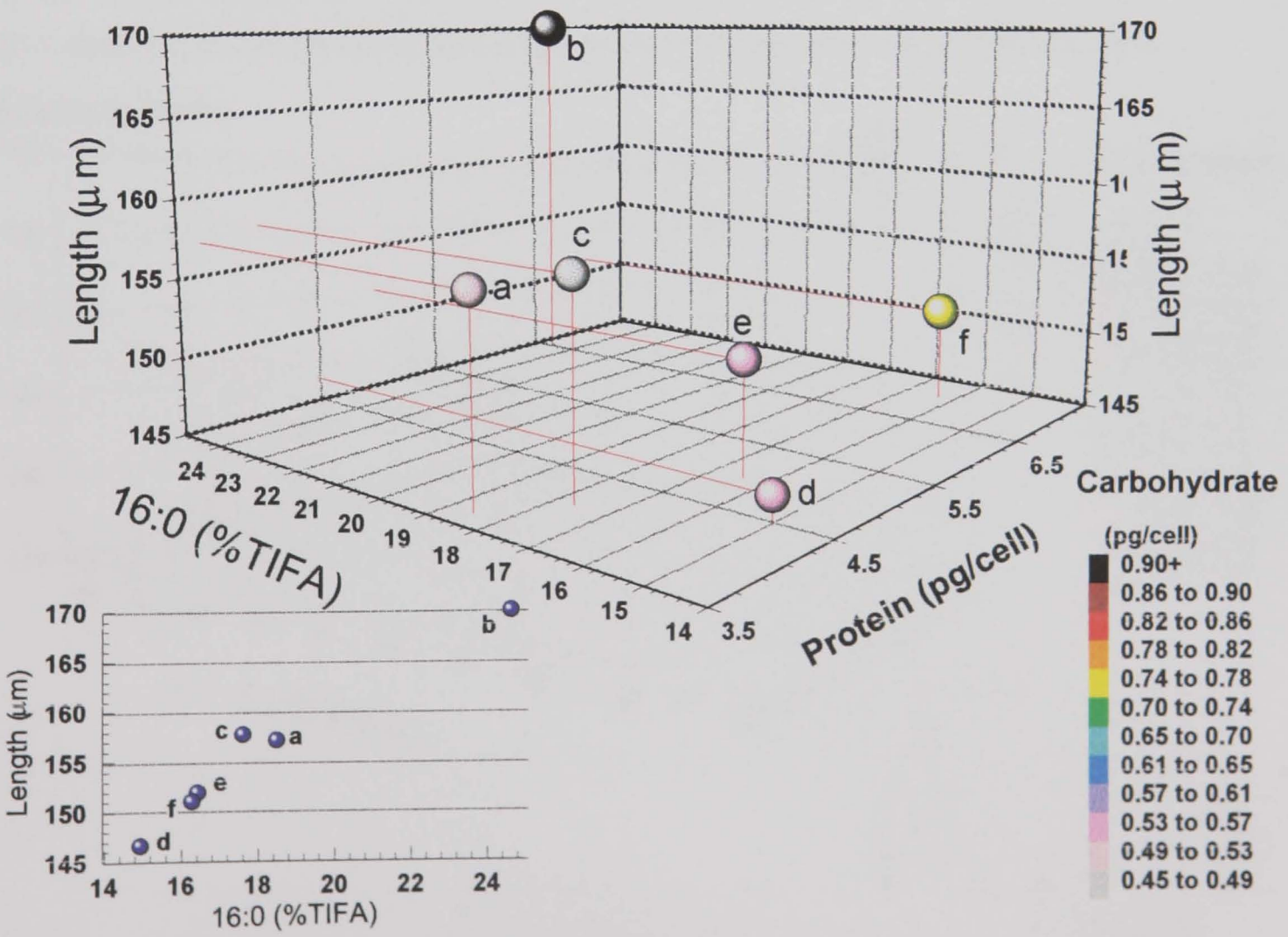

Figure 4.3.2. Mean end length of *M. edulis* larvae, in relation to protein, carbohydrate content and proportion of 16:0 (expressed as percentage of **T**otal **I**dentifiable **F**atty **A**cids, **% TIFA**) of *P. lutheri*, in the first feeding experiment. **a= HL f/2**, **b= HL P**, c= **HL N**, **d= LL f/2**, **e= LL P** and **f= LL N**.

In this case the simple x – y plot explains sufficiently well the covariance between the proportion of 16:0 FA of *P. lutheri* and larval growth, with a correlation factor of 0.89 (A. 4.2.3). Consequently the incorporation of the other algal biochemical components adds very little to the previous conclusion.

Both protein and carbohydrate content seem to have a limited effect on the nutritional value of *P. lutheri*. The different protein concentration of data points **e** and **f** results into larvae of similar end length. The only possible comment that can further be made concerns the carbohydrate content that seems to have a minor negative effect to the nutritional value of two pairs of data points: **e – f** and **c – a**. Both **e** and **c** data points contain less carbohydrate than **f** and **a** correspondingly although the do have similar proportions of 16:0. However, by no means can it be argued that, carbohydrate content is more descriptive of the nutritional value of *P. lutheri* since it is obvious that it is the 16:0 percentage that can be directly and positively correlated with larval growth.

A negative correlation was found between larval growth and sum of ω : 3 fatty acid proportions of *P. lutheri*. The results appeared originally in Fig. 4.2.16. Now the same data, but with the added variable of protein and carbohydrate concentration of this algal species is illustrated in Fig. 4.3.3. Thumbnail image of the relative to *P. lutheri* data is also incorporated and data points and cross referenced with the same alphabetical labels.

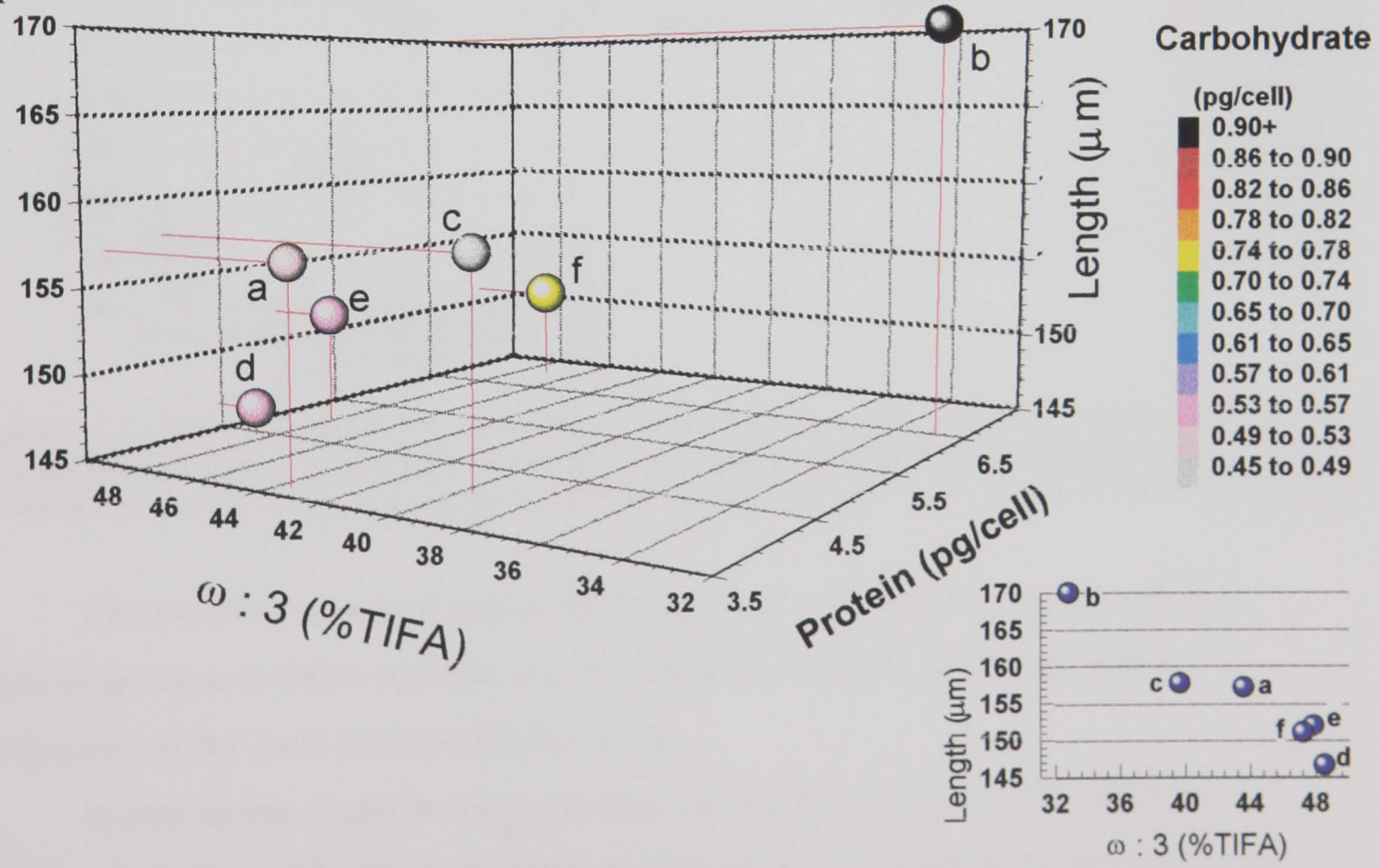

Figure 4.3.3. Mean end length of *M. edulis* larvae, in relation to protein, carbohydrate content and proportion of **ω : 3** (expressed as percentage of **T**otal **I**dentifiable **F**atty **A**cids, **% TIFA**) of *P. lutheri*, in the first feeding experiment. **a= HL f/2, b= HL P**, c= **HL N, d= LL f/2, e= LL P** and **f= LL N**.

As with the previous comments, since the ω : 3 proportion was found to be negatively correlated with larval growth in a significant manner, it, alone, plays a pivotal role in describing the response of the nutritional value, albeit with an inverted manner.

To conclude the description of *P. lutheri*, the last component that was found to be positively correlated with larval growth, proportion of Total **Sa**turated **F**atty **A**cids (**SaFA**), is also presented in Fig. 4.3.4 in both three – dimensional space and as a smaller two – dimensional surface which was rearranged from the previously shown Fig. 4.2.14.

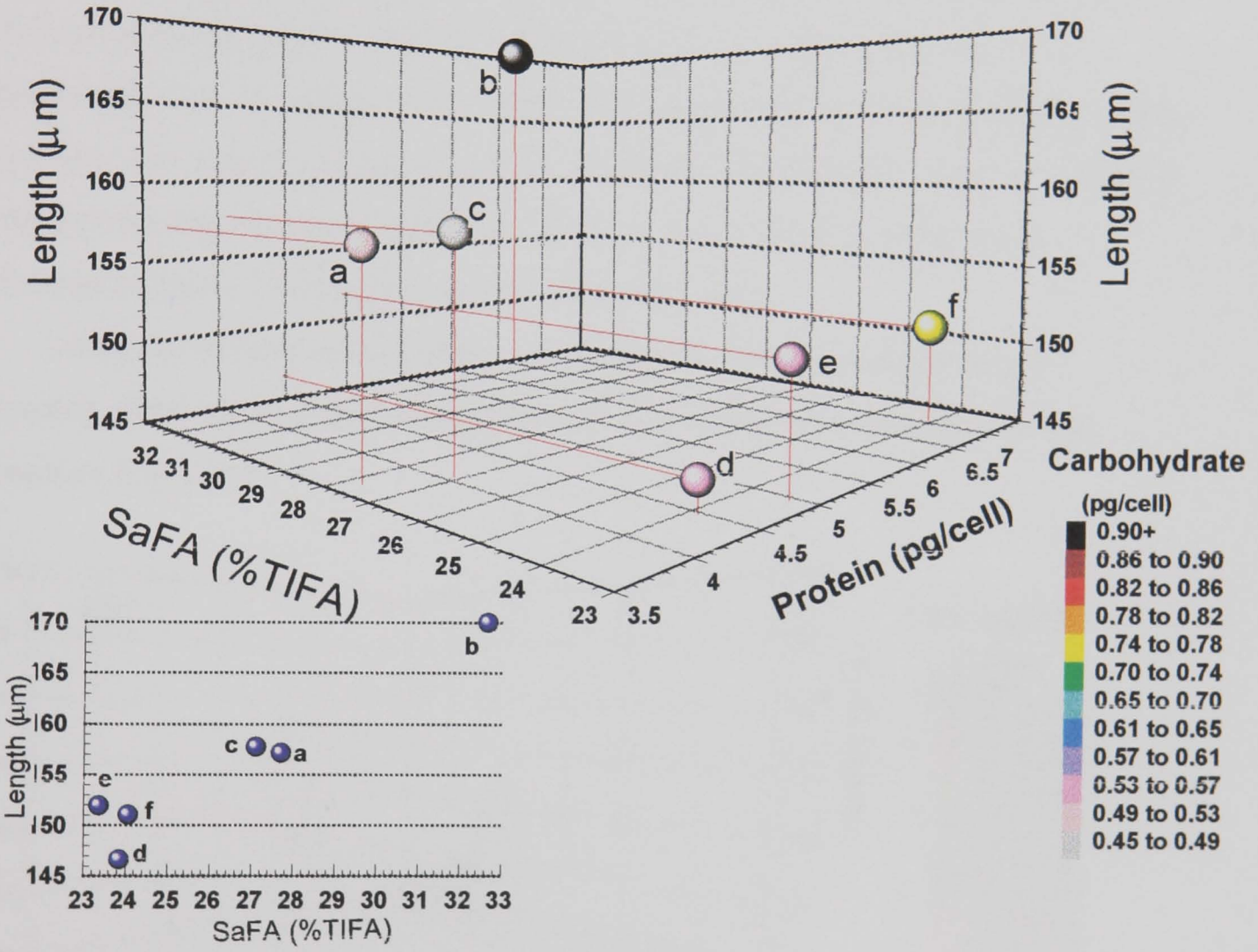

Figure 4.3.4. Mean end length of *M. edulis* larvae, in relation to protein, carbohydrate content and proportion of Total **Sa**turated **F**atty **A**cids, **SaFA** (expressed as percentage of **T**otal **I**dentifiable **F**atty **A**cids, % **TIFA**) of *P. lutheri*, in the first feeding experiment. **a= HL f/2**, **b= HL P**, c= **HL N**, **d= LL f/2**, **e= LL P** and **f= LL N**.

The previous comments about the proportion of 16:0 and larval growth also apply in the case of **SaFA** and that is to be expected since the 16:0 is the major component of the Total Saturated Fatty Acids.

In conclusion, it can be observed that the nutritional value of *P. lutheri* is sufficiently explained by the its proportion of various FA components, particularly in that larval size is increased with increased proportion of 16:0 and SaFA and it is

decreased with the increase in the proportion of PUFA. Protein concentration does not affect this nutritional value. But, some minor variation between fatty acid proportions and larval growth can be further explained by the carbohydrate content of *P. lutheri* in these cases. Where for a given fatty acid proportion larval growth was expected to be lower, the slightly inferior growth may have derived from the increased carbohydrate content. This type of characteristic relationship can be described as "one major factor", the FA proportion and a "substantial minor factor", the carbohydrate content. The role of the minor factor is to slightly "modify" the nutritional value of the species.

Of the four algal species tested, *P. lutheri* was an exception in that a relative straightforward and uncomplicated relationship was found in the previous analysis (ch. 4.2) using the fatty acid content of this species as the main indicator of its nutritional value for *M. edulis* larvae. From that perspective multivariate analysis adds little to what was already shown. However, for some other species, where two variant analysis proved insufficient, further elucidation was provided by using three dimensional analysis. These cases will be presented now.

Using the techniques described previously, the results concerning the relationship of the biochemical components of *S. costatum* and larval growth are presented in Fig. 4.3.5.

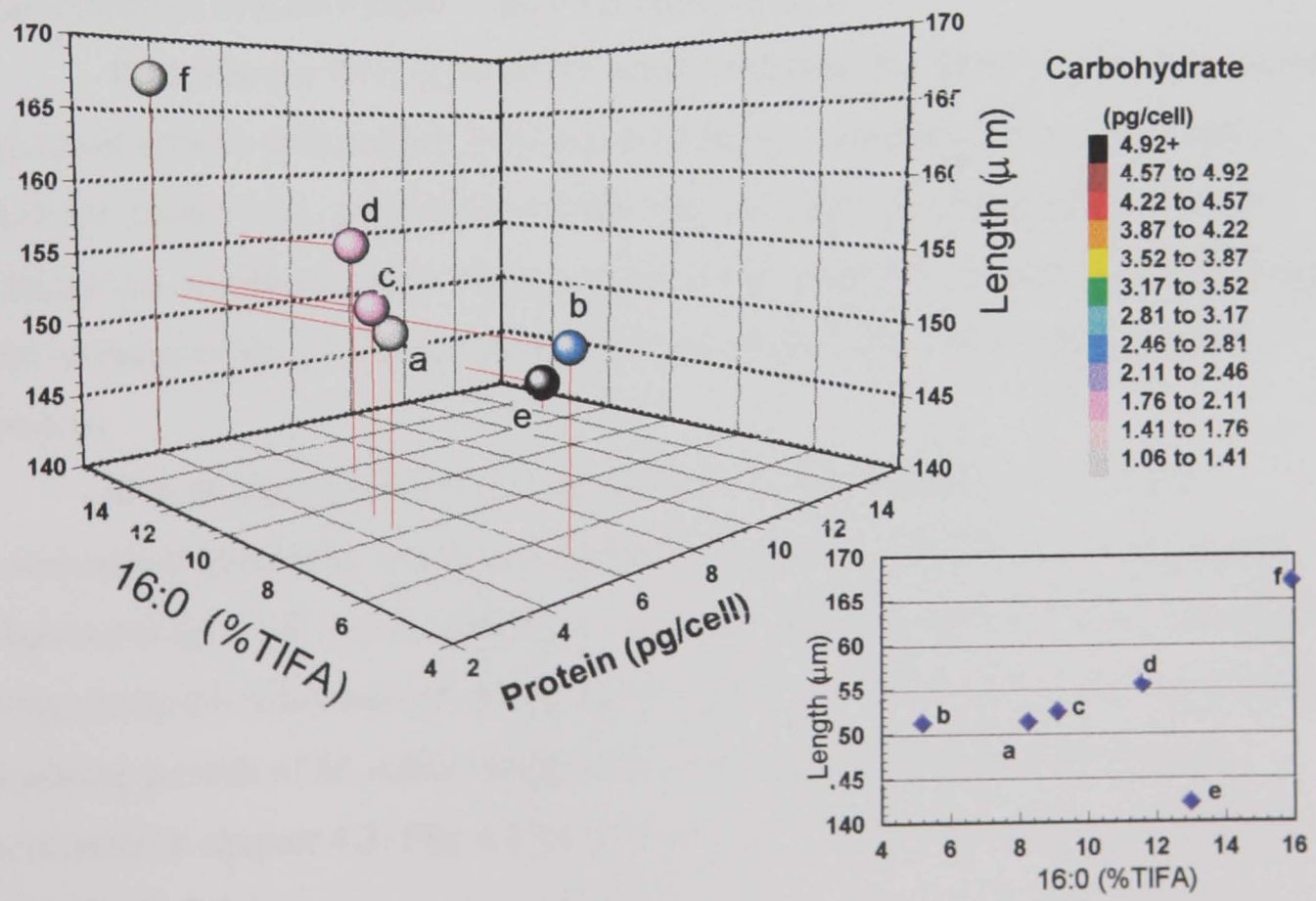

Figure 4.3.5. Mean end length of *M. edulis* larvae, in relation to protein, carbohydrate content and proportion of 16:0 (expressed as percentage of Total Identifiable Fatty Acids, % **TIFA**) of *S. costatum*, in the repetition feeding experiment. **a= HL f/2**, **b= HL P**, c= **HL N**, **d= LL f/2**, **e= LL P** and **f= LL N**.

The graph describes the variation of the end length of *M. edulis* larvae and the protein and carbohydrate content and the proportion of 16:0 fatty acid of the algal diet.

Previously, the data appeared to be in random order, as already established in Fig. 4.2.12 and 4.2.13. However, the overall variation may be explained in relation to the combined effect of two variables, the effect of one of which may be of a major adjusting nature. If a positive relationship between the proportion of **16:0** and larval growth is assumed, as advocated in the previous chapter (ch. 4.3) then, there are basically two major outliers in this group: data points **b** and **e**. In **b**, despite a smaller proportion of 16:0 of the diet, the larvae grow to similar lengths as after feeding on diets with greater proportion of 16:0, as for example points **d** and **a**. The other major outlier of this group is data point **e**, which despite its increased proportion of 16:0 produces larvae of substantially less length. However both points **b** and **e** have increased amounts of both protein and carbohydrate, particularly point **e**. It follows that the overall pattern can be explained on the basis that increased content of protein and/or carbohydrate in the diet may result in inferior nutritional value. In this manner, the poor performance of point **e** can be attributed to increased protein and/or carbohydrate content despite high proportion of 16:0.

From the position of point **b** it can be inferred that the positive correlationship of larval growth with dietary proportion of 16:0 is demonstrated in a threshold manner. In this way, proportions of that fatty acid up to a certain value will not change the nutritional value of the species while when that threshold is exceeded then the increase of the 16:0 proportion will be correlated with an increase in larval growth.

Examining the **PUFA** proportion of *S. costatum* along with its other biochemical properties and the corresponding larval growth, the resulting graphs shown in Fig. 4.3.6, which appears on the next page, are presented. The original data concerning the relationship between the proportion of PUFA of *S. costatum* and the resulting growth of *M. edulis* larvae fed on this algal species were previously presented in chapter 4.2, Fig. 4.2.12. Protein and carbohydrate data were tabulated from Table 3.2.1.

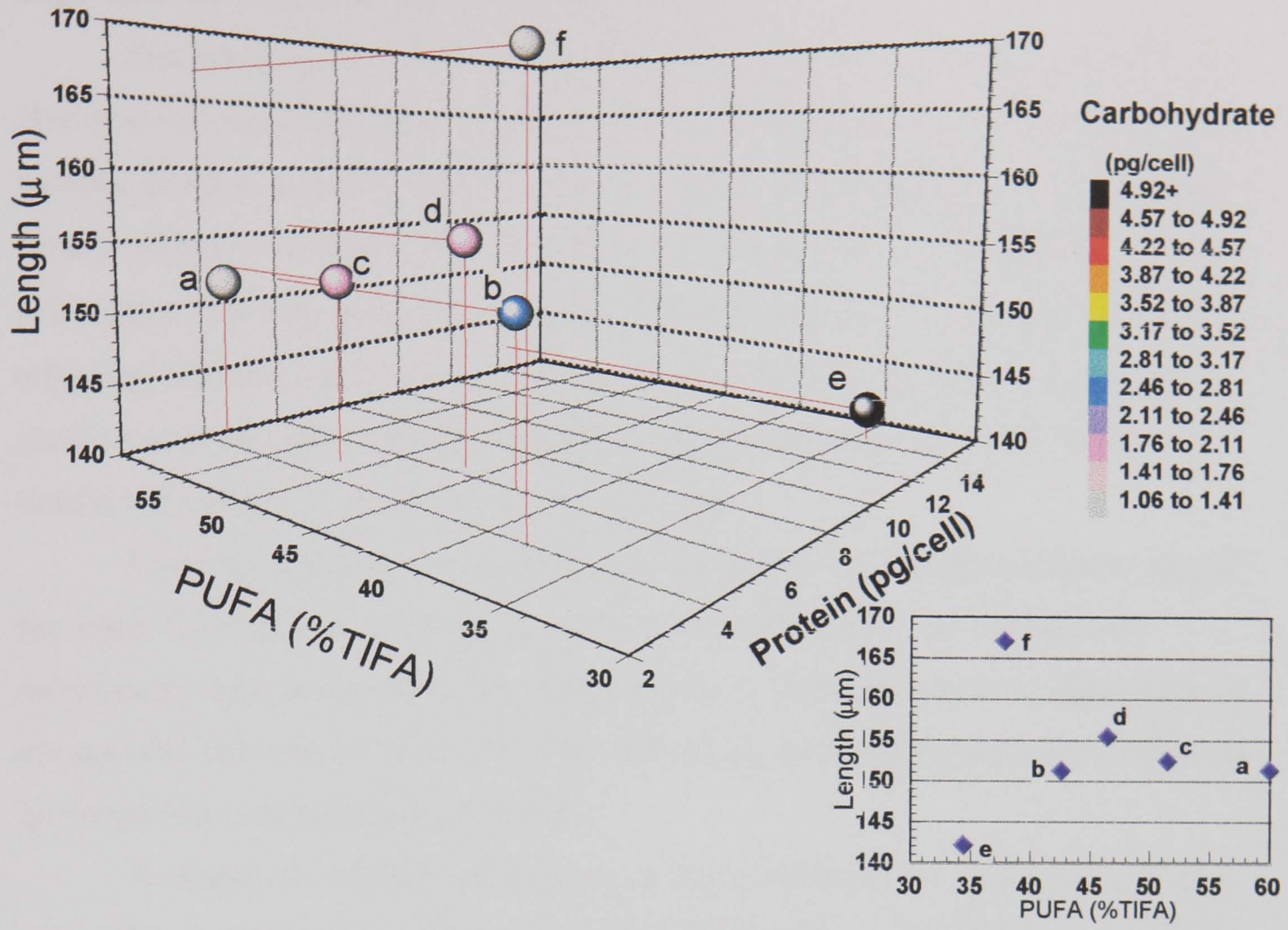

Figure 4.3.6. Mean end length of *M. edulis* larvae, in relation to protein, carbohydrate content and proportion of total PUFA (expressed as percentage of Total Identifiable Fatty Acids, % **TIFA**) of *S. costatum*, in the repetition feeding experiment. **a= HL f/2**, **b= HL P**, c= **HL N**, **d= LL f/2**, **e= LL P** and **f= LL N**.

In the case of algal **PUFA** proportion, the assumed relationship is of negative type. Again the same outlying points **e** and **b** can be put into perspective when examining their protein content. A negative threshold relationship between proportion of **PUFA** and larval growth can be demonstrated while increased protein and carbohydrate levels again negatively influence the length of *M. edulis* larvae. However although a positive relationship is easier to explain biochemically or energetically a negative biochemical relationship is far more difficult to explain. It is obscure how increased levels of protein and/or carbohydrate can have an adverse effect on larval growth. This type of relationship could be explained for other biochemical components on evidence of toxicity effects, but the exact way that proteins can exercise such an effect remains unexplained. This analysis cannot conclusively prove this relationship, basically due to the limited amount of data points

examined every time (which were only six). However it can potentially be used when larger data sets of similar type become available.

Summarizing the results obtained from this type of multivariate analysis it is observed that algal biochemical components can be correlated with larval growth in a number of ways, possibly with all kinds of combinations. **SaFA** or 16:0 proportions were positively correlated with larval growth with or without a threshold, **PUFA** or **ω:3** proportions were negatively correlated with larval growth. Carbohydrate was adjusting the nutritional value of *P. lutheri* that was related to the major factor in a positive manner, while both protein and carbohydrate content was adjusting the nutritional value of *S. costatum* in a negative way.

From the absence of any legitimate explanation of the displayed behaviour in the other two algal species tested, it can be inferred that the algal biochemical components may change their role from positive to random to negative depending on the species. This way it follows that the relationship between dietary components and larval growth is mainly species specific

A simple two variable analysis as can be investigated by correlation analysis, may suffice to explain the change in the nutritional value of the species. It would then be concluded that the change of one of the algal biochemical components would be the major factor determining the nutritional value of the species. However this simplistic approach that has too often be adopted in the analysis of similar results in the literature may obscure the potential significance of other factors that may have interacted between themselves to produce a different outcome.

This four–way variable analysis, overcomes the problem of statistical suitability of the data for statistical multivariable regression analysis and can visualize trends within and between these factors. In this respect it may prove to become an important tool in analysing relationships between various dietary components.

5. Conclusions

All four algal species tested respond to the changes in culturing conditions by altering their growth rate and their biochemical composition and hence their nutritional value.

S. costatum is an adequate food supply and becomes a significantly better food for *M. edulis* larvae under low light conditions and especially under nitrogen limitation. *C. muelleri* is again an adequate food supply. It improves its nutritional value under low light conditions too, but both nitrogen limitation and no nutrient limitation are equally superior conditions to culture this species. *R. reticulata* is a very good food for *M. edulis* larvae, but under the tested experimental conditions, its nutritional value was not significantly altered. Small improvements occurred under low light and no limitation conditions. *P. lutheri* proved to be an excellent food source for *M. edulis* larvae and it was the only of the four species tested that improved its nutritional value under high light conditions and more particularly under phosphorus stress.

Biochemical analysis of the larvae indicated that the proportions of 20:5ω3 and **PUFA** can be used as a positive growth index while **SaFA**, and more specifically both the 15:0 and 16:0 fatty acids were found to be negatively correlated with larval growth. The possibly bacterial origin of the 15:0 suggested that there was a negative effect on larval growth of the bacterial load of the cultures.

Traditional, two factor correlation analysis of dietary components indicated that only in a per species basis, some algal components could be correlated with larval growth. In *S. costatum* and *P. lutheri* the proportion of **SaFA** and especially the16:0 was positively correlated with larval growth, while the proportion of ω:3 **PUFA** was negatively correlated with the growth of *M. edulis* larvae. Dietary components of the other algal species could not be correlated with larval growth.

Besides the previous method, a novel three dimensional graphical approach was adopted in order to analyze the combined correlationship of three algal components simultaneously, with larval growth. The basis of that is that the larvae do not consume specific components separately but feed on the algal cell which contain a variety of components simultaneously.

With this approach a more complex but more elucidating relationship between algal biochemical components and larval growth emerged. The variance on larval growth could be explained by the complementary or counteractive effect, with or without a threshold level of principally, the dietary fatty acids and secondly, carbohydrate and to a lesser extend protein content. It also indicated that there is an interaction factor between all these parameters and that in essence, the relationship among dietary components can be either independent, in some cases or threshold - type dependant, and this combined effect is provisionally expressed in larval growth.

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Appendix

Table A.2.1. Chemical composition of Conway medium. Stock solution 3, vitamins, should be kept in the fridge. Stock solution 3, silicate, is added only when the cultured species is a diatom.

Stock 1, salts		Dosage 1ml l^{-1} seawater
	EDTA Disodium salt	90 gms
	H_3BO_3 crystals	67.2 gms
	$NaNO_3$	200 gms
	$NaH_2PO_4 \cdot 2H_2O$	40 gms
	$MnCl_2 \cdot 4H_2O$	0.72 gms
	$FeCl_3$ (anhydrous)	2.6 gms
	Distilled water (autoclaved)	2 litres
	Trace metal solution	2 mls
	Trace metal solution contains :	
	$ZnCl_2$	2.1 gms
	$CoCl_2 \cdot 6H_2O$	2.0 gms
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.9 gms
	$CuSO_4 \cdot 5H_2O$	2.0 gms
	Distilled water	100 mls
	HCl	sufficient to disolve salts and give a clear solution
Stock 2, vitamins		Dosage 0.1ml l^{-1} seawater
	Thiamine Hydrochloride B_1	100 mg
	Cyanocobalamin B_{12}	10 mg
	Distilled water (autoclaved)	200 mls
Stock 3, silicate		Dosage 1ml l^{-1} seawater
	$Na_2SiO_3 \cdot 5H_2O$	4 gms
	Distilled water (autoclaved)	100 mls

Table A.3.1.1. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, between unfed larvae and unfed larvae that were cultured with excess bacterial populations. Differences are not significant.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Diets	2	121.46	121.46	60.73	2.50	0.084
Beakers	3	166.50	166.50	55.50	2.28	0.079
Error	354	8612.24	8612.24	24.33		
Total	359	8900.20				

Table A.3.1.2. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, fed on the control diet and the same diet but with excess bacterial populations included in these larval cultures. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Diets	2	3344.2	3344.2	1672.1	10.71	**0.000***
Beakers	3	6710.3	6710.3	2236.8	14.33	**0.000***
Error	354	55266.4	55266.4	156.1		
Total	359	65320.8				

Table A.3.1.3. One – Way Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, between unfed larvae and larvae that were reared with Algal Extra Cellular Products. The length of the corresponding D- stage larval batches were also included in the analysis. * indicate significant differences.

Source	DF	SS	MS	F	p
Diets	3	8582.0	2860.7	117.31	**0.000***
Error	476	11607.1	24.4		
Total	479	20189.2			

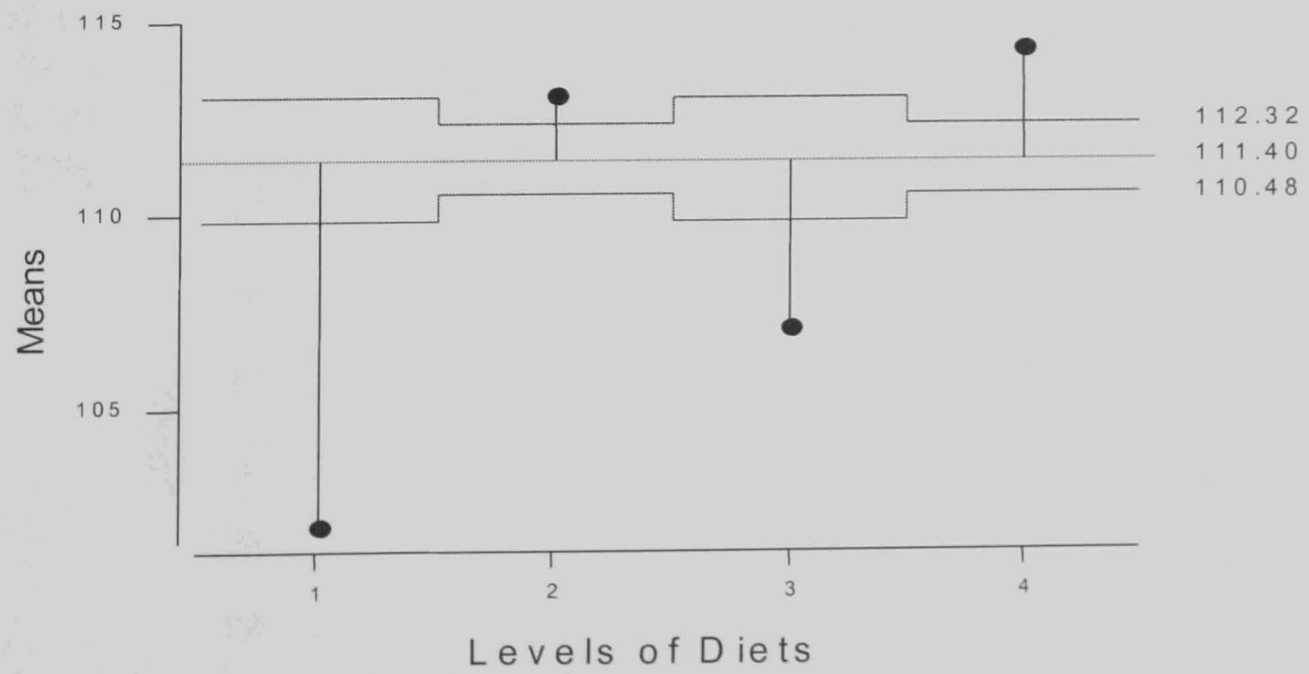

Figure A.3.1.4. One – way Analysis of means between d-stage larvae, unfed larvae and larvae reared with Algal Extra Cellular Products (**AECP**). 1 & 3 =Mean D- stage lengths. 2= Unfed larvae and 4=Larvae with AECP.

Table A.3.2.1. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *S. costatum* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	4937.0	4937.0	2468.5	31.46	**0.000***
Diets	9	99913.6	99913.6	11101.5	141.37	**0.000***
Error	708	55599.3	55599.3	78.5		
Total	719	160449.9				

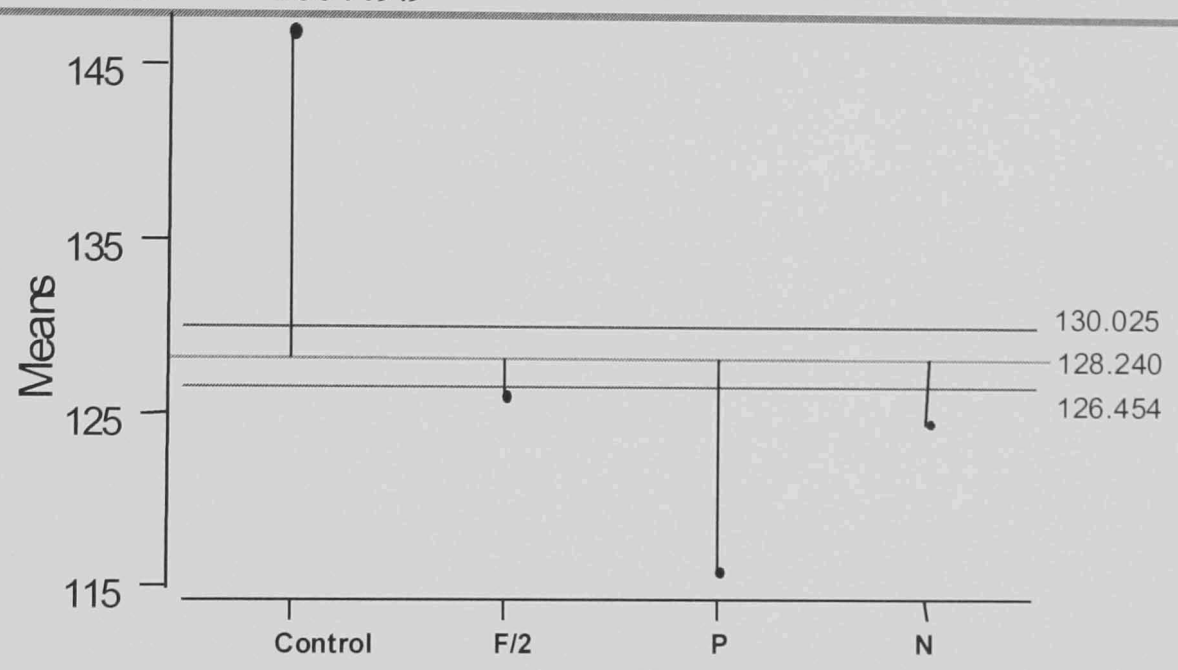

Figure A.3.2.2. One-Way Analysis of Means for the 1st *S. costatum*, High light condition feeding trial.

Table A.3.2.3. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *S. costatum* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	864.2	864.2	432.1	2.20	**0.111**
Diets	9	4597.4	4597.4	510.8	2.60	**0.006***
Error	708	138981.1	138981.1	196.3		
Total	719	144442.7				

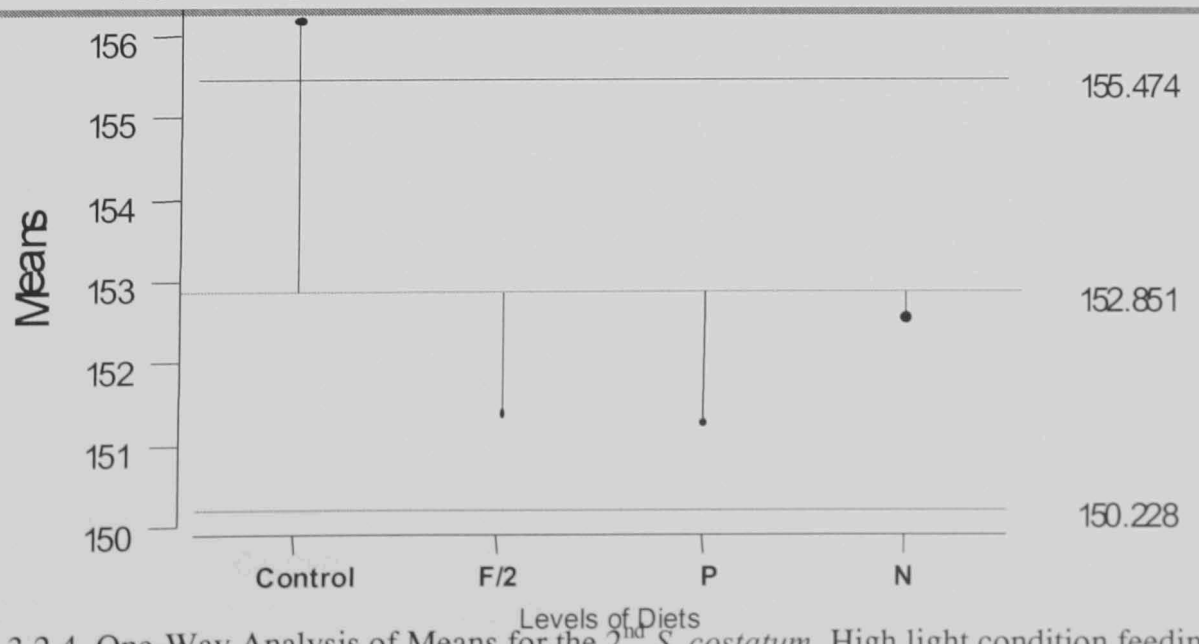

Figure A.3.2.4. One-Way Analysis of Means for the 2nd *S. costatum*, High light condition feeding trial.

Table A.3.2.5. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *S. costatum* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	3811	3811	1905	18.67	**0.000***
Diets	9	172262	172262	19140	188.48	**0.000***
Error	708	71896	71896	102		
Total	719	247969				

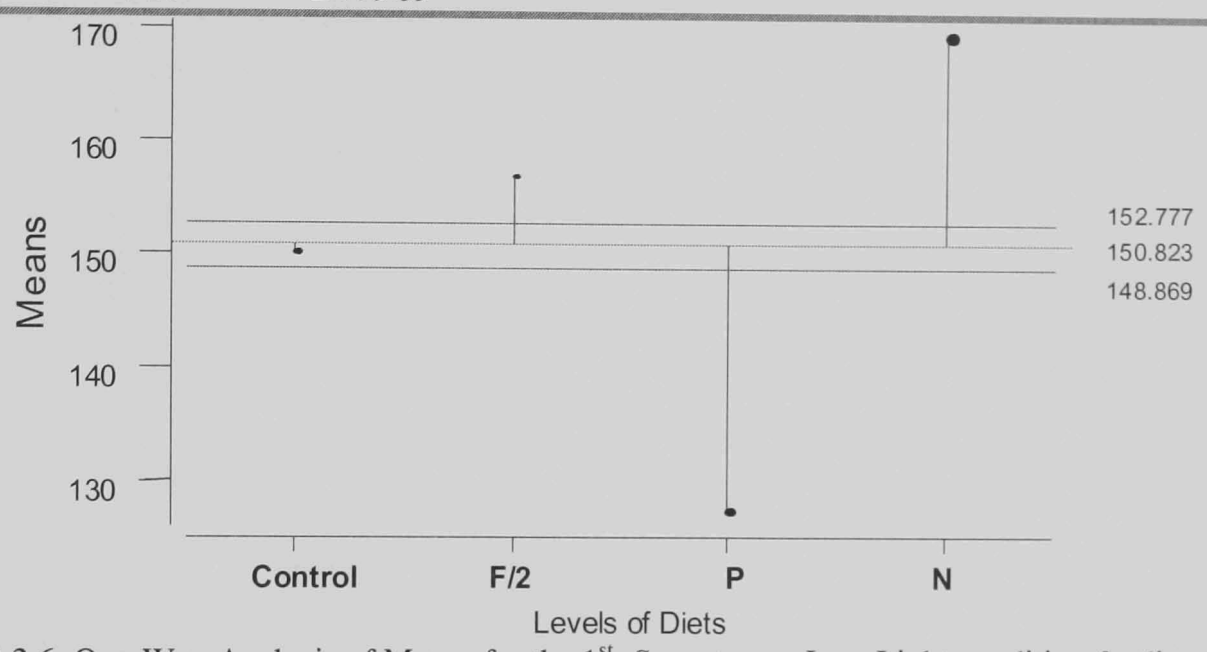

Figure A.3.2.6. One-Way Analysis of Means for the 1st *S. costatum*, Low Light condition feeding trial.

Table A.3.2.7. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *S. costatum* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	3614.8	3614.8	1807.4	13.31	**0.000***
Diets	9	64762.4	64762.4	7195.8	53.00	**0.000***
Error	708	96123.6	96123.6	135.8		
Total	719	164500.8				

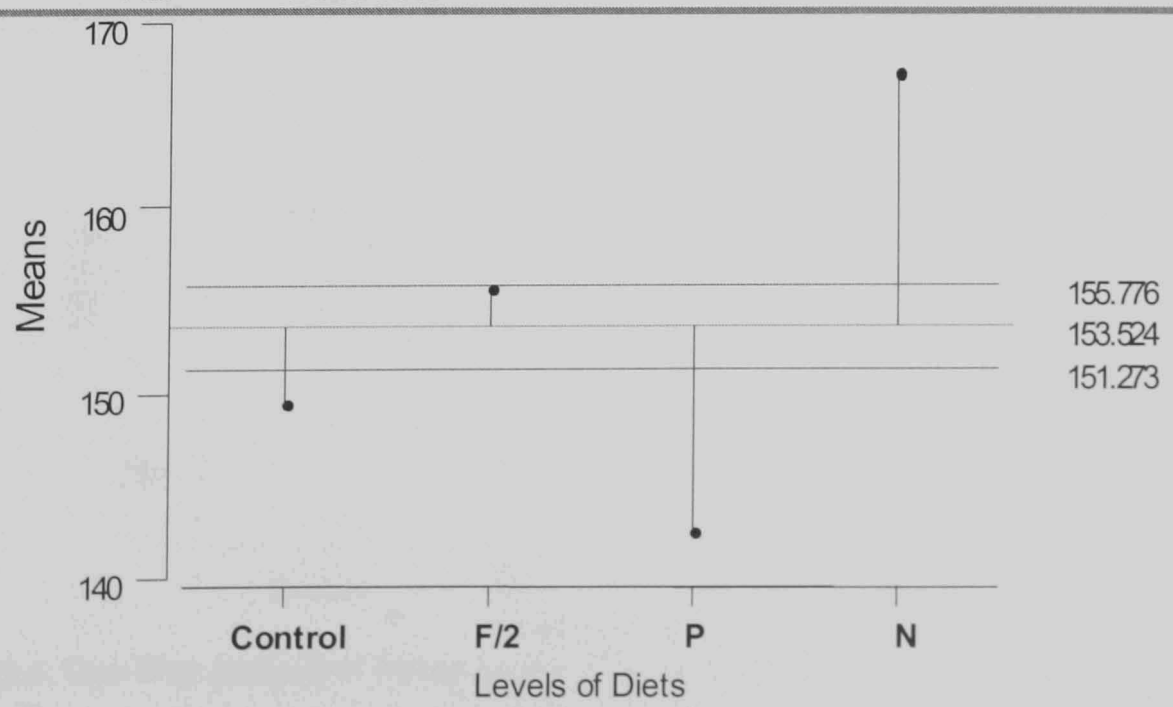

Figure A.3.2.8. One-Way Analysis of Means for the 2nd *S. costatum*, Low Light condition feeding trial.

Table A.3.3.1. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *C. muelleri* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	191.0	191.0	95.5	1.14	**0.322**
Diets	9	42263.8	42263.8	4696.0	55.86	**0.000***
Error	708	59520.1	59520.1	84.1		
Total	719	101974.9				

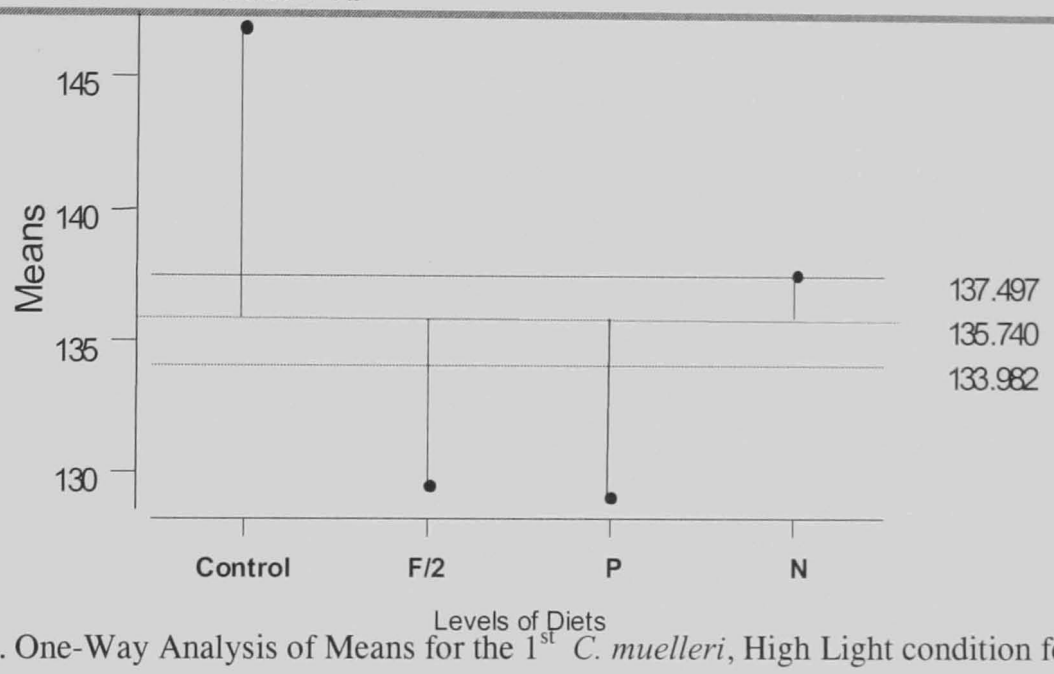

Figure A.3.3.2. One-Way Analysis of Means for the 1st *C. muelleri*, High Light condition feeding trial.

Table A.3.3.3. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *C. muelleri* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	848.8	848.8	424.4	2.54	**0.080**
Diets	9	18995.6	18995.6	2110.6	12.61	**0.000***
Error	708	118506.9	118506.9	167.4		
Total	719	138351.2				

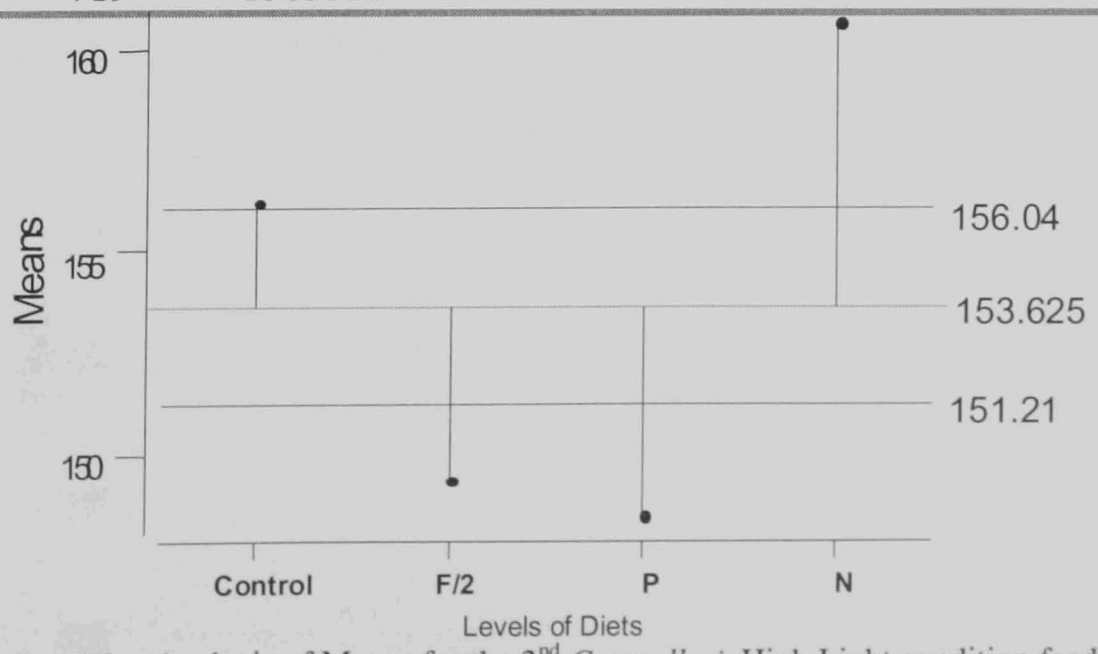

Figure A.3.3.4. One-Way Analysis of Means for the 2nd *C. muelleri*, High Light condition feeding trial.

Table A.3.3.5. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *C. muelleri* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	1664.8	1664.8	832.4	6.12	**0.002***
Diets	9	31274.9	31274.9	3475.0	25.57	**0.000***
Error	708	96228.6	96228.6	135.9		
Total	719	129168.3				

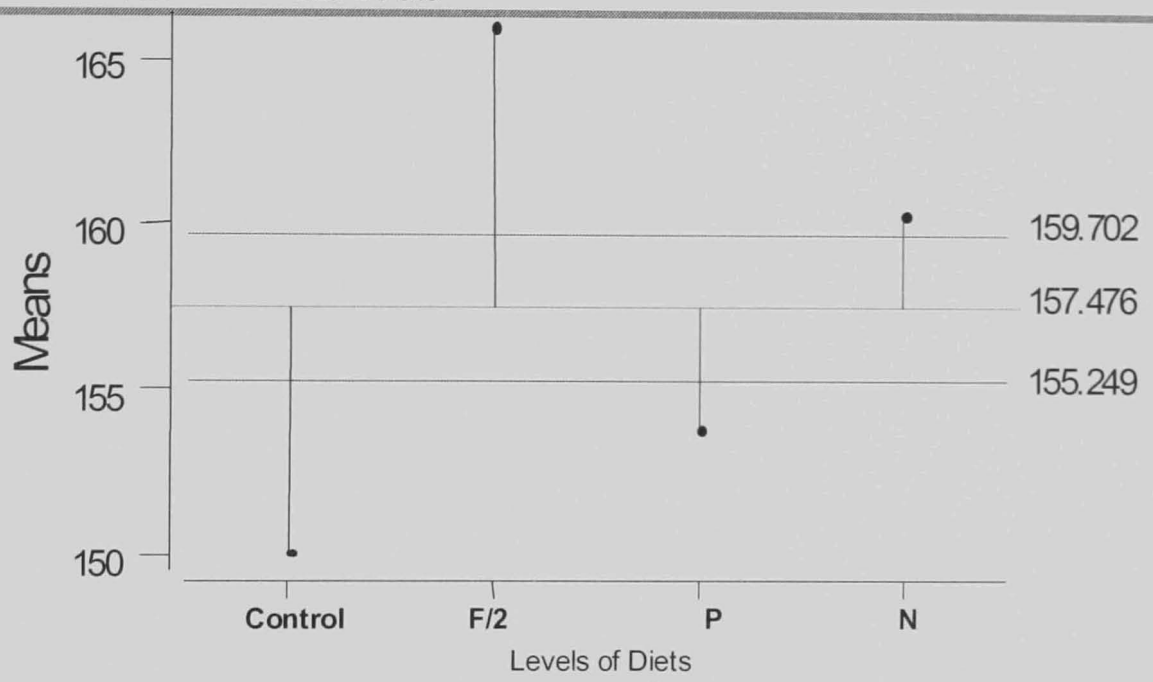

Figure A.3.3.6. One-Way Analysis of Means for the 1st *C. muelleri*, Low Light condition feeding trial.

Table A.3.3.7. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *C. muelleri* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	1362	1362	681	5.27	**0.005***
Diets	9	119430	119430	13270	102.80	**0.000***
Error	708	91396	91396	129		
Total	719	212188				

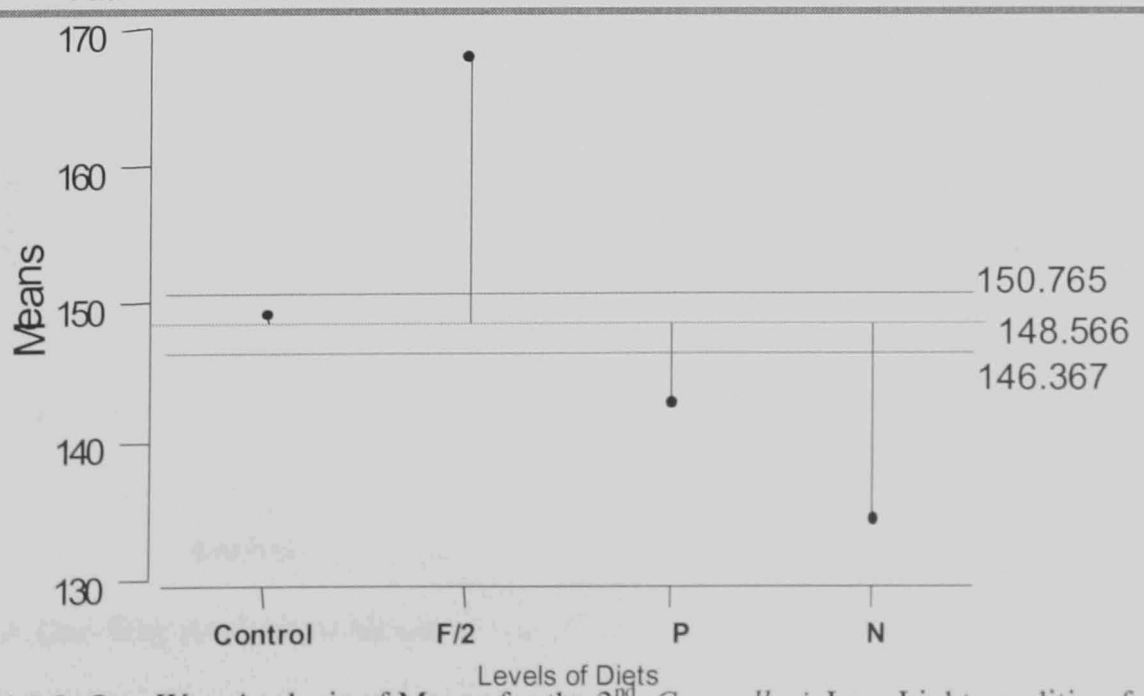

Figure A.3.3.8. One-Way Analysis of Means for the 2nd *C. muelleri*, Low Light condition feeding trial.

Table A.3.4.1. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *R. reticulata* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	809	809	405	0.65	**0.524**
Diets	9	138114	138114	15346	24.55	**0.000***
Error	708	442646	442646	625		
Total	719	581570				

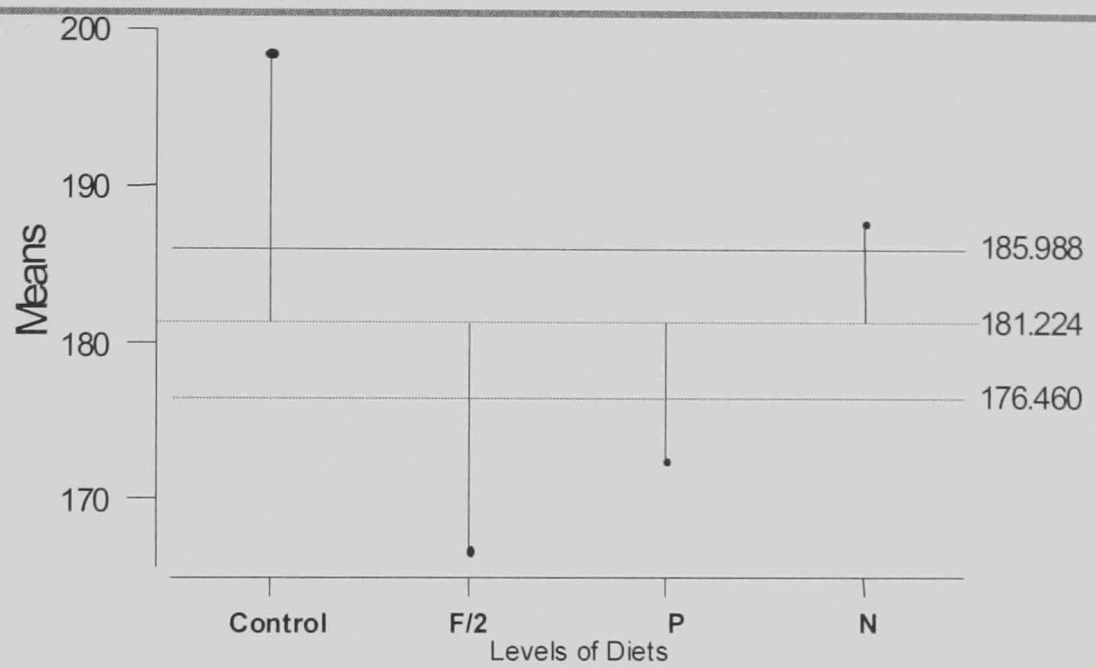

Figure A.3.4.2. One-Way Analysis of Means for the 1st *R. reticulata*, High Light condition feeding trial.

Table A.3.4.3. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *R. reticulata* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	351.2	351.2	175.6	0.48	**0.620**
Diets	9	25485.0	25485.0	2831.7	7.71	**0.000***
Error	708	260117.3	260117.3	367.4		
Total	719	285953.5				

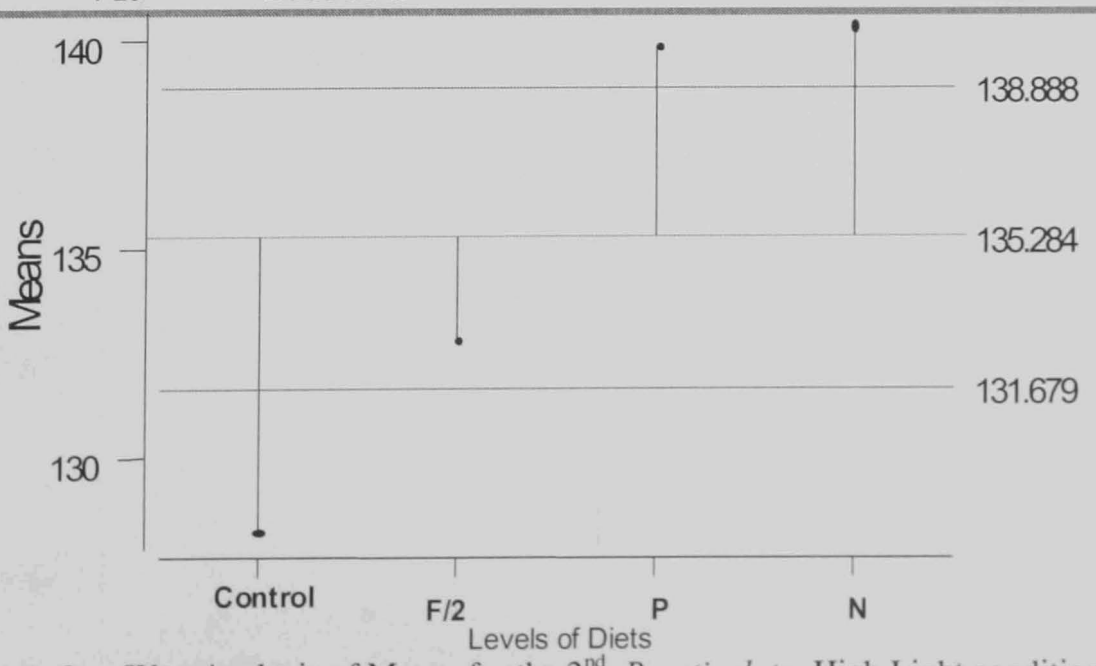

Figure A.3.4.4. One-Way Analysis of Means for the 2nd *R. reticulata*, High Light condition feeding trial.

Table A.3.4.5. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *R. reticulata* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	9468	9468	4734	8.11	**0.000***
Diets	9	163467	163467	18163	31.12	**0.000***
Error	708	413260	413260	584		
Total	719	586195				

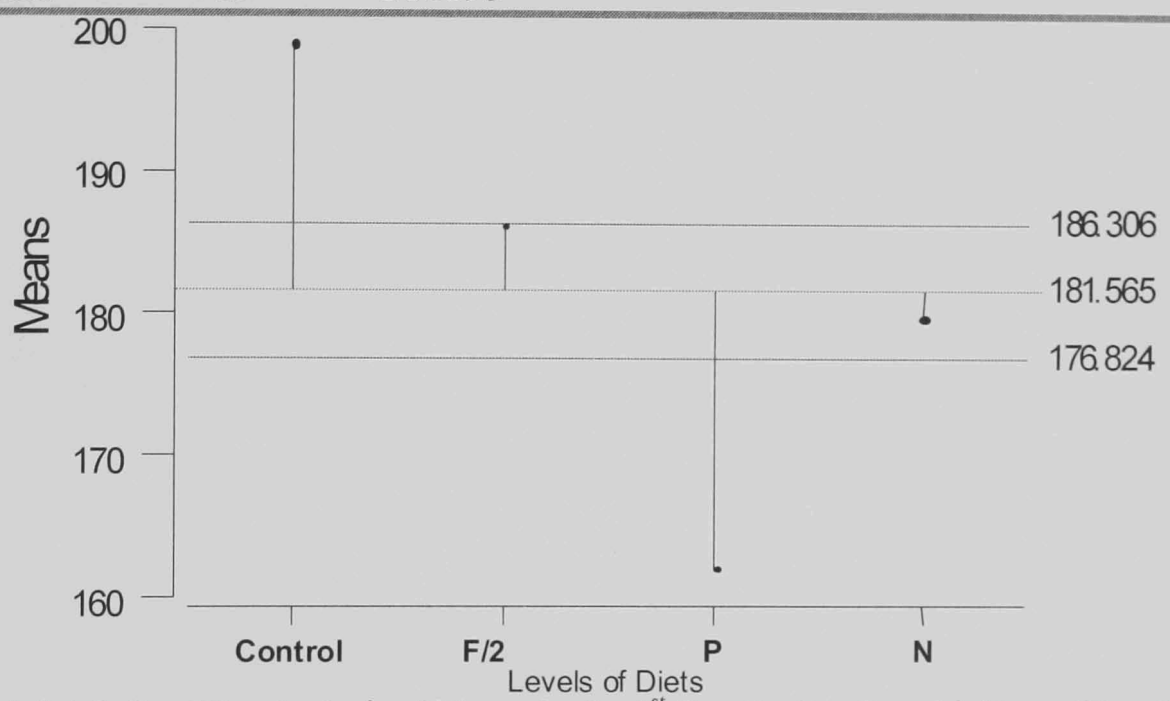

Figure A.3.4.6. One-Way Analysis of Means for the 1st *R. reticulata*, Low Light condition feeding trial.

Table A.3.4.7. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *R. reticulata* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	2718.5	2718.5	1359.3	3.62	**0.027***
Diets	9	80873.2	80873.2	8985.9	23.91	**0.000***
Error	708	266120.3	266120.3	375.9		
Total	719	349712.0				

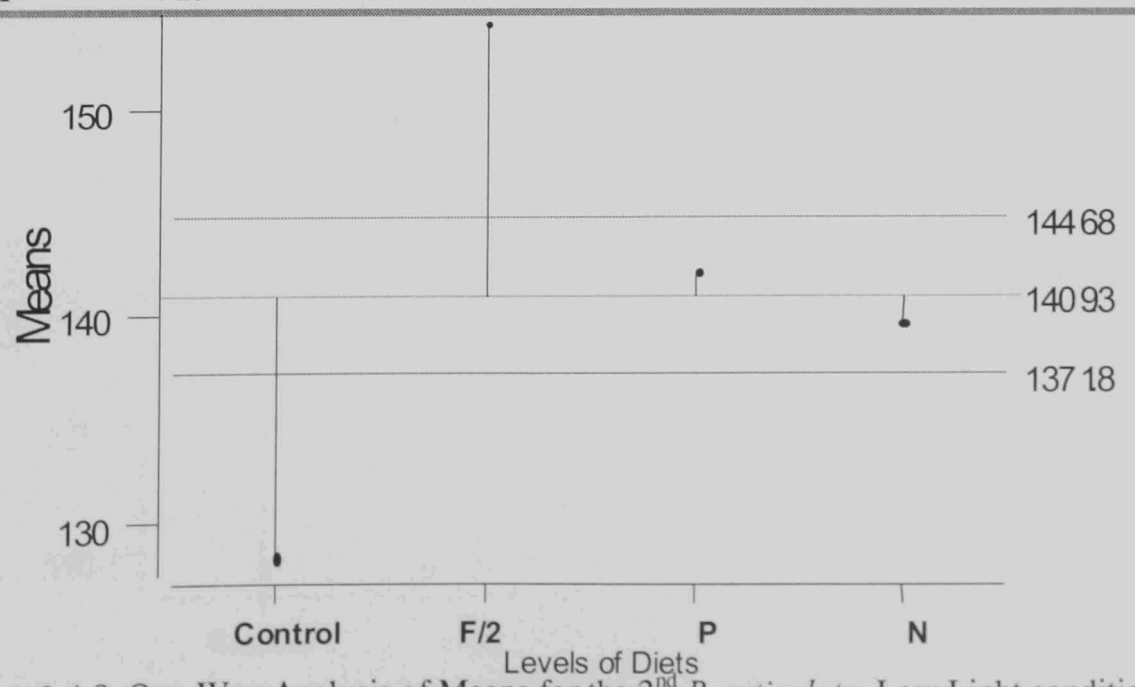

Figure A.3.4.8. One-Way Analysis of Means for the 2nd *R. reticulata*, Low Light condition feeding trial.

Table A.3.5.1. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *P. lutheri* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	1112	1112	556	3.25	**0.040***
Diets	9	206229	206229	22914	134	**0.000***
Error	708	121390	121390	171		
Total	719	328730				

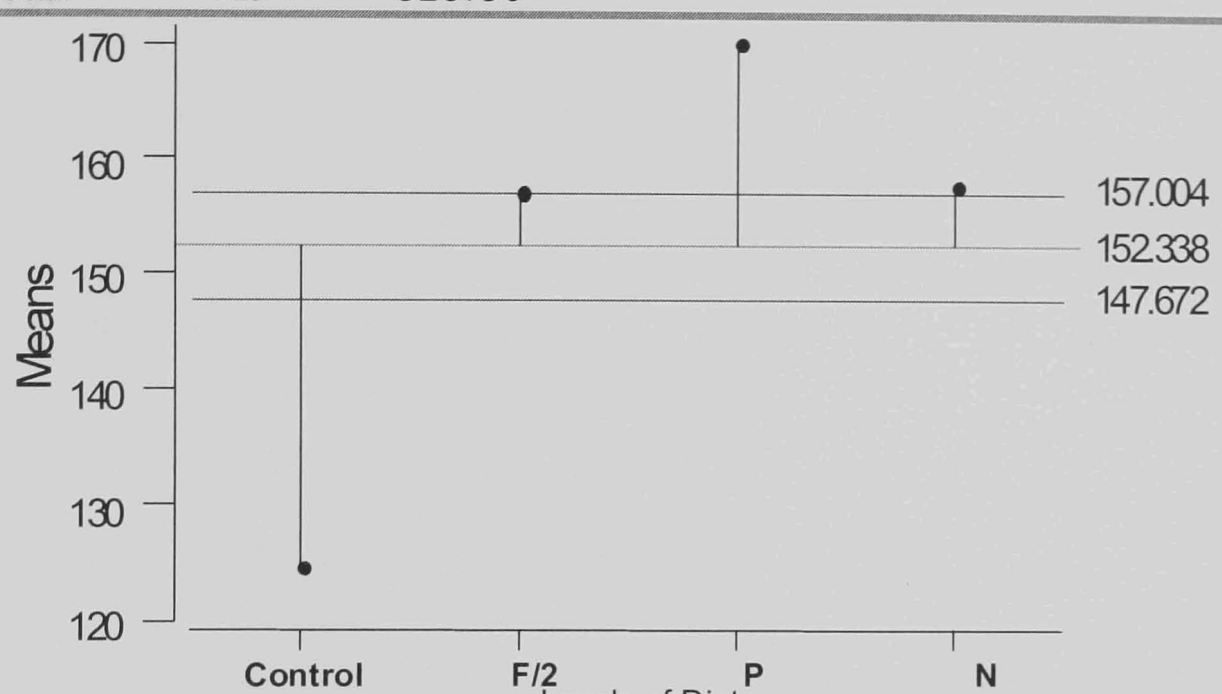

Figure A.3.5.2. One-Way Analysis of Means for the 1st *P. lutheri*, High Light condition feeding trial.

Table A.3.5.3. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *P. lutheri* which was cultured at High Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	2327	2327	1163	5.73	**0.003***
Diets	9	181462	181462	20162	99.32	**0.000***
Error	708	143536	143536	203		
Total	719	327325				

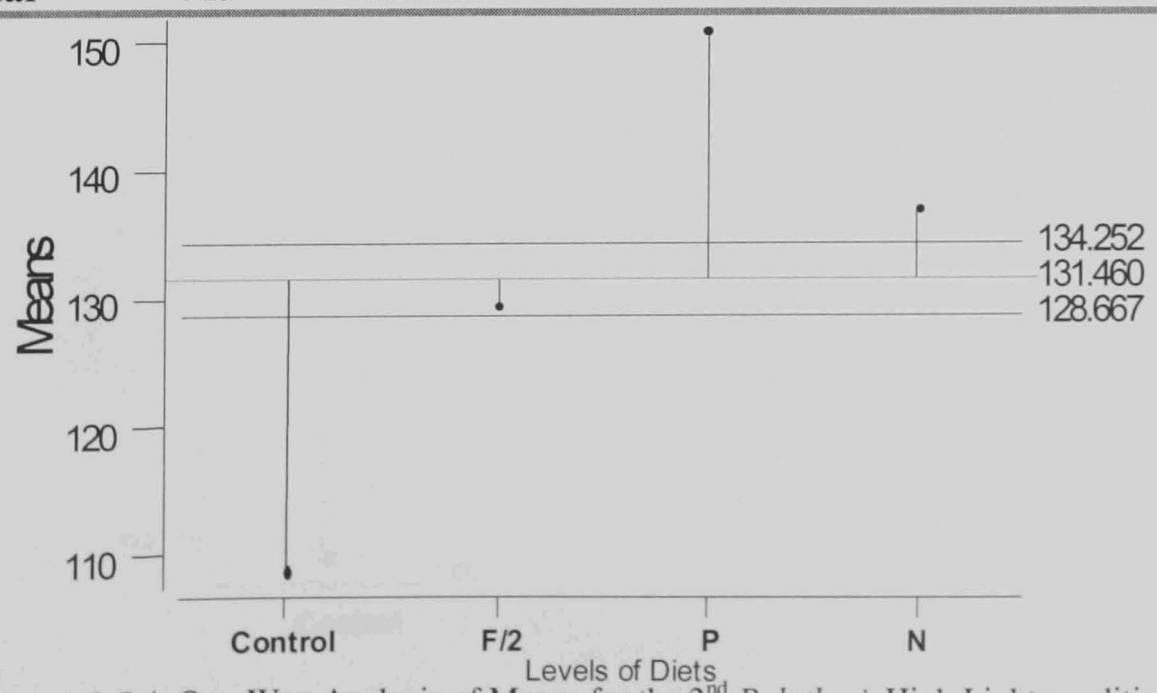

Figure A.3.5.4. One-Way Analysis of Means for the 2nd *P. lutheri*, High Light condition feeding trial.

Table A.3.5.5. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *P. lutheri* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 1st feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	460.2	460.2	230.1	2.23	**0.108**
Diets	9	94214.7	94214.7	10468.3	101.54	**0.000***
Error	708	72993.0	72993.0	103.1		
Total	719	167667.8				

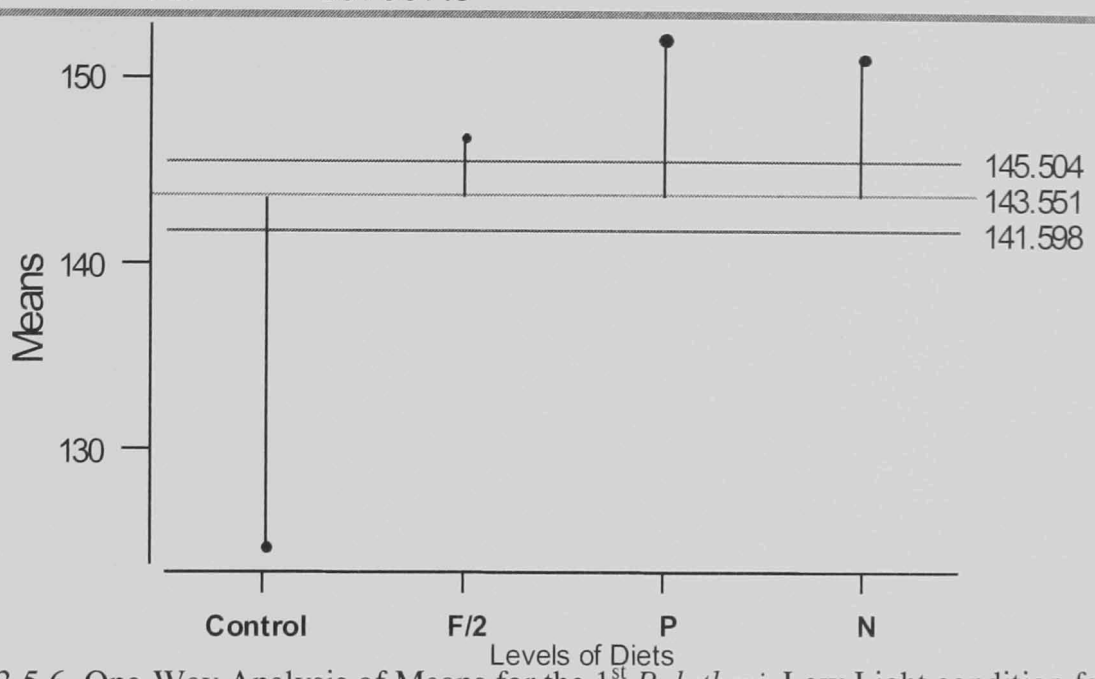

Figure A.3.5.6. One-Way Analysis of Means for the 1st *P. lutheri*, Low Light condition feeding trial.

Table A.3.5.7. Nested Analysis of Variance between results of the end – lengths achieved by *M. edulis* larvae, after being fed for two weeks on *P. lutheri* which was cultured at Low Light and three different nutrient conditions. The fourth diet was the control. Results analysed concern the 2nd feeding trial. * indicate significant differences.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Beakers	2	115	115	58	0.34	**0.710**
Diets	9	118365	118365	13152	78.29	**0.000***
Error	708	118771	118771	168		
Total	719	237251				

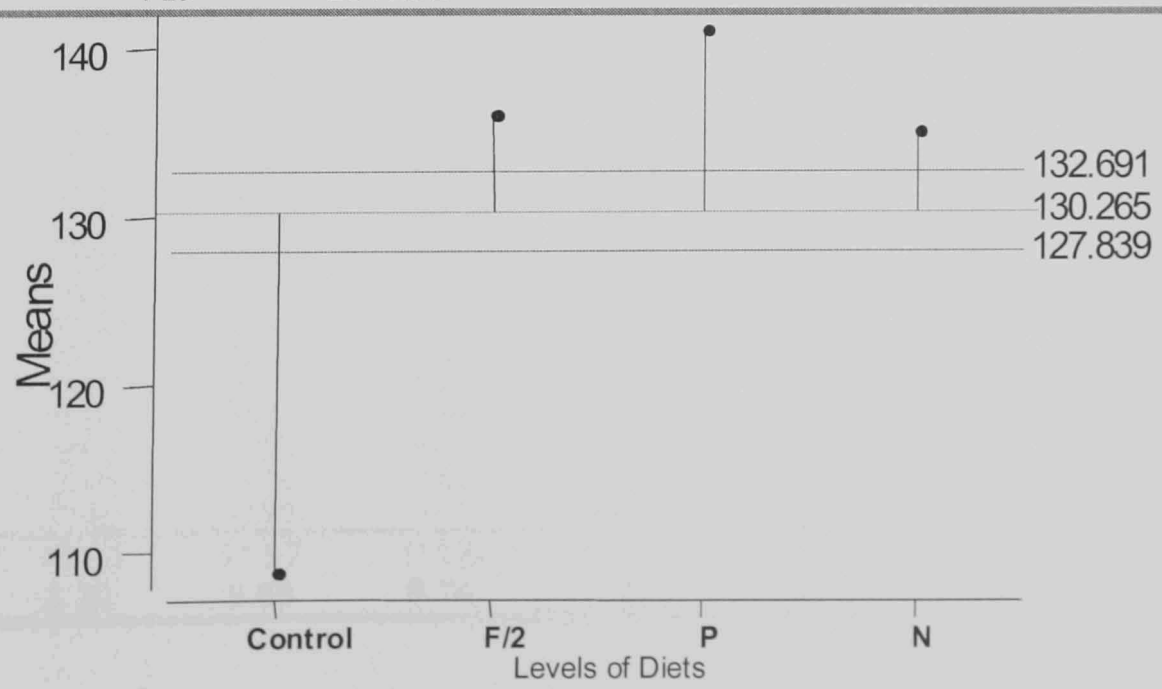

Figure A.3.5.8. One-Way Analysis of Means for the 2nd *P. lutheri*, Low Light condition feeding trial.

Table A.UnfedBacter. Length (in μm) of D-stage larvae and their length after two weeks with no food and another batch of D-stage larvae with their final length after two weeks of no feeding but when reared in seawater, this contains natural bacterial populations (U+Bact). Numbers at the end of the label indicate the number of each individual culture (from the triplicates).

D-stage	Unfed 1	Unfed 2	Unfed 3	D-stage	U+Bact 1	U+Bact 2	U+Bact 3
98.7	114.2	112.0	121.6	112.8	100	110	102.5
104.4	112.7	109.3	115.6	108	105	112.5	110
101.2	112.8	119.7	114.9	115.2	112.5	112.5	110
100.6	108.0	122.3	112.5	103.2	112.5	115	112.5
97.8	115.5	109.6	114.3	105.6	115	117.5	115
106.0	111.9	118.3	113.9	108	112.5	107.5	112.5
98.0	109.6	108.6	112.0	100.8	112.5	115	115
96.9	112.2	107.7	117.0	98.4	115	115	115
105.5	115.3	110.8	112.2	100.8	117.5	117.5	115
102.0	124.2	98.8	92.4	105.6	117.5	115	115
100.3	115.2	98.8	123.6	98.4	115	112.5	110
101.6	116.5	117.9	115.3	100.8	115	112.5	112.5
100.7	117.6	113.2	117.7	110.4	115	115	112.5
104.7	112.9	113.5	116.8	96	115	115	112.5
99.5	114.8	107.8	119.3	96	117.5	120	117.5
100.8	115.6	112.8	110.7	100.8	107.5	102.5	112.5
107.1	114.7	115.6	104.4	100.8	110	110	112.5
101.0	110.7	119.8	114.9	98.4	115	112.5	112.5
103.4	117.6	116.2	117.9	98.4	117.5	115	112.5
103.7	109.0	113.6	116.1	98.4	117.5	117.5	115
88.8	109.6	112.2	118.9	98.4	110	105	112.5
92.2	117.6	117.0	117.0	100.8	110	112.5	112.5
107.4	111.4	110.8	109.0	96	115	112.5	112.5
94.3	113.6	114.8	118.6	98.4	115	117.5	117.5
97.5	108.0	115.8	116.0	100.8	117.5	120	117.5
104.8	117.0	111.9	113.5	96	112.5	112.5	107.5
101.2	113.8	114.8	119.9	96	112.5	115	110
111.4	106.1	114.8	118.6	96	115	115	112.5
104.3	120.1	105.2	114.8	100.8	115	115	115
102.3	117.9	124.3	115.8	108	117.5	115	115
99.4	113.7	114.3	112.0	96	112.5	112.5	105
99.7	109.9	125.7	109.0	96	112.5	112.5	107.5
96.0	115.3	111.9	120.1	98.4	115	112.5	112.5
101.0	116.8	117.2	113.5	100.8	117.5	115	115
95.3	114.9	124.1	112.8	96	120	115	117.5
96.0	104.4	99.1	104.0	96	105	107.5	105
105.7	117.0	103.3	118.0	103.2	112.5	110	112.5
104.7	112.2	115.6	117.9	105.6	112.5	110	112.5
105.7	101.4	118.8	112.0	96	117.5	112.5	122.5
103.6	106.4	106.3	109.0	96	120	115	112.5
95.1	123.5	108.0	112.0	96	115	110	110
110.9	122.8	100.9	107.5	96	115	112.5	110
107.7	120.6	103.6	113.5	96	115	112.5	112.5
106.8	113.2	103.4	104.4	98.4	115	115	115
106.6	115.2	103.9	115.3	100.8	115	117.5	115
100.0	119.9	115.6	115.5	103.2	112.5	102.5	105
95.1	103.9	100.4	113.0	96	115	115	112.5
109.6	114.5	109.0	113.8	98.4	117.5	115	112.5
101.2	114.2	111.9	114.5	100.8	117.5	117.5	112.5
98.2	95.4	121.1	117.9	96	122.5	117.5	115
103.2	107.8	105.2	117.9	96	107.5	110	107.5
105.6	119.3	117.6	117.6	98.4	112.5	110	112.5
99.6	112.2	106.5	114.9	98.4	115	112.5	115
104.5	117.6	111.9	114.9	100.8	115	115	117.5
110.1	128.3	96.2	119.8	96	117.5	120	120
115.2	117.7	108.0	116.1	96	107.5	110	112.5
100.1	102.5	115.6	109.9	96	110	112.5	112.5
95.7	115.3	106.2	110.7	108	112.5	115	115
111.9	117.6	112.0	109.3	96	115	115	115
91.6	113.0	107.8	116.1	100.8	115	120	117.5
Mean	101.9	113.7	111.5	114.1	100.0	114.0	113.5	112.9
SD	5.25	5.68	6.74	4.94	4.61	3.85	3.71	3.59

Table.A.ControlBacter. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet, in filtered seawater and on the control diet but in seawater that contains natural bacterial populations. C+Bact=Control diet with natural bacteria populations. Numbers at the end indicate the number of the triplicate culture.

	D-stage	Control 1	Control 2	Control 3	C+Bact 1	C+Bact 2	C+Bact 3
	112.8	152.5	137.5	142.5	137.5	125	132.5
	108	157.5	140	145	137.5	132.5	135
	115.2	160	150	147.5	145	142.5	140
	103.2	167.5	155	160	152.5	150	155
	105.6	157.5	157.5	162.5	175	155	162.5
	108	165	150	137.5	140	137.5	127.5
	100.8	167.5	155	142.5	150	142.5	127.5
	98.4	140	155	157.5	155	157.5	137.5
	100.8	152.5	160	162.5	160	127.5	140
	105.6	152.5	175	165	187.5	137.5	142.5
	98.4	155	145	147.5	127.5	165	132.5
	100.8	155	147.5	140	132.5	137.5	130
	110.4	165	150	155	132.5	147.5	135
	96	150	157.5	160	137.5	150	155
	96	152.5	175	167.5	150	167.5	160
	100.8	160	137.5	130	152.5	202.5	127.5
	100.8	177.5	137.5	132.5	155	132.5	127.5
	98.4	162.5	150	140	157.5	137.5	137.5
	98.4	165	150	155	160	140	140
	98.4	167.5	160	165	162.5	152.5	150
	98.4	172.5	145	145	130	152.5	130
	100.8	152.5	150	145	132.5	127.5	135
	96	155	155	147.5	137.5	130	140
	98.4	162.5	162.5	157.5	190	137.5	147.5
	100.8	182.5	167.5	162.5	137.5	140	162.5
	96	147.5	137.5	150	150	150	132.5
	96	140	145	150	152.5	132.5	137.5
	96	155	150	152.5	140	147.5	155
	100.8	155	155	157.5	145	167.5	155
	108	157.5	160	160	157.5	187.5	162.5
	96	155	142.5	140	137.5	142.5	125
	96	155	152.5	152.5	142.5	147.5	140
	98.4	157.5	155	152.5	147.5	150	145
	100.8	160	157.5	157.5	152.5	152.5	155
	96	165	142.5	162.5	162.5	180	157.5
	96	155	150	125	132.5	135	127.5
	103.2	157.5	155	145	137.5	147.5	132.5
	105.6	165	162.5	152.5	142.5	162.5	137.5
	96	167.5	175	157.5	145	155	157.5
	96	170	137.5	165	165	187.5	162.5
	96	135	155	132.5	127.5	150	127.5
	96	137.5	155	152.5	135	150	130
	96	160	165	155	140	165	137.5
	98.4	162.5	165	157.5	162.5	167.5	157.5
	100.8	165	165	165	135	177.5	167.5
	103.2	150	147.5	142.5	137.5	132.5	125
	96	152.5	150	145	140	137.5	125
	98.4	152.5	157.5	152.5	145	155	130
	100.8	160	160	155	165	167.5	132.5
	96	165	162.5	162.5	132.5	172.5	155
	96	155	140	137.5	135	140	127.5
	98.4	162.5	155	140	137.5	150	130
	98.4	162.5	157.5	145	145	152.5	132.5
	100.8	165	162.5	147.5	162.5	162.5	150
	96	172.5	162.5	155	187.5	175	152.5
	96	155	150	157.5	132.5	132.5	130
	96	157.5	155	145	137.5	137.5	135
	108	162.5	160	155	142.5	140	147.5
	96	165	162.5	165	152.5	155	152.5
	100.8	170	167.5	165	180	167.5	172.5
Mean		100.0	159.0	154.3	151.4	148.0	151.0	141.8
SD		4.61	8.87	9.34	9.93	14.91	16.46	12.78

Table.A. UnfedECP. Length of D-stage larvae and their length after two weeks with no food and another batch of D- stage larvae with their final length after two week of no feeding but with Algal Extra Cellular Products added (U+ECP). Numbers at the end of the label indicate the number of the individual culture (from the triplicates).

D-stage	Unfed 1	Unfed 2	Unfed 3	D-stage	U+ECP 1	U+ECP 2	U+ECP 3
100	117.5	102.5	115	100	115	115	112.5
105	117.5	105	115	105	115	115	112.5
105	120	112.5	117.5	105	117.5	115	112.5
107.5	125	112.5	120	107.5	120	117.5	115
112.5	112.5	117.5	122.5	110	120	117.5	120
102.5	115	107.5	110	107.5	110	110	107.5
102.5	115	110	117.5	112.5	110	112.5	112.5
107.5	117.5	115	117.5	112.5	112.5	115	112.5
112.5	122.5	120	120	115	112.5	117.5	115
112.5	115	120	127.5	115	115	120	115
97.5	110	107.5	112.5	102.5	112.5	107.5	110
107.5	110	107.5	112.5	105	112.5	112.5	112.5
112.5	112.5	117.5	120	105	115	112.5	112.5
112.5	115	117.5	122.5	107.5	120	112.5	115
112.5	122.5	117.5	125	110	120	115	117.5
100	115	107.5	112.5	102.5	105	105	120
102.5	117.5	115	115	102.5	112.5	112.5	112.5
105	120	117.5	115	105	112.5	112.5	112.5
112.5	122.5	117.5	117.5	105	115	112.5	112.5
107.5	125	117.5	117.5	112.5	117.5	115	115
95	107.5	107.5	110	105	112.5	102.5	117.5
102.5	115	115	110	107.5	112.5	115	110
105	117.5	115	112.5	107.5	112.5	117.5	115
105	120	117.5	120	107.5	115	120	115
107.5	122.5	117.5	122.5	107.5	125	120	117.5
102.5	115	105	112.5	105	107.5	110	110
107.5	115	110	112.5	107.5	112.5	112.5	112.5
110	117.5	112.5	117.5	107.5	115	112.5	112.5
110	120	122.5	117.5	110	115	115	115
107.5	120	122.5	127.5	110	120	117.5	120
105	107.5	112.5	120	102.5	107.5	112.5	112.5
107.5	115	112.5	120	105	112.5	112.5	112.5
110	117.5	117.5	122.5	107.5	112.5	115	112.5
110	117.5	122.5	125	107.5	117.5	120	112.5
112.5	117.5	122.5	127.5	112.5	120	120	117.5
97.5	107.5	107.5	102.5	100	110	102.5	112.5
102.5	112.5	112.5	107.5	105	112.5	112.5	115
105	115	112.5	115	107.5	112.5	112.5	115
105	115	117.5	115	107.5	120	117.5	117.5
107.5	117.5	120	125	107.5	122.5	120	120
97.5	100	110	117.5	102.5	110	105	112.5
100	107.5	112.5	125	105	112.5	112.5	112.5
102.5	110	117.5	127.5	107.5	112.5	112.5	115
102.5	112.5	117.5	127.5	107.5	112.5	115	117.5
105	112.5	120	130	107.5	120	120	117.5
107.5	117.5	105	105	97.5	112.5	110	112.5
115	117.5	107.5	117.5	105	120	112.5	115
117.5	120	112.5	125	107.5	120	115	115
120	120	112.5	130	107.5	122.5	120	117.5
125	122.5	122.5	112.5	112.5	122.5	122.5	117.5
100	102.5	112.5	115	105	107.5	112.5	105
112.5	112.5	112.5	115	105	110	112.5	110
112.5	117.5	115	117.5	105	112.5	112.5	115
115	122.5	122.5	115	110	112.5	115	115
102.5	125	125	107.5	110	117.5	120	117.5
102.5	112.5	107.5	115	105	112.5	112.5	102.5
105	117.5	115	120	107.5	112.5	112.5	112.5
107.5	120	117.5	120	110	112.5	112.5	115
95	122.5	120	122.5	110	112.5	115	117.5
117.5	115	125	125	112.5	120	117.5	117.5
Mean	106.9	116.1	114.6	118.1	107.1	114.7	114.1	114.1
SD	6.03	5.21	5.47	6.18	3.51	4.29	4.20	3.35

Table. A.1. *S. costatum* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet or on *S. costatum*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
112.8	137.5	125	132.5	100	110	102.5	120	117.5	125
108	137.5	132.5	135	105	112.5	110	130	125	127.5
115.2	145	142.5	140	112.5	112.5	110	132.5	125	130
103.2	152.5	150	155	112.5	115	112.5	135	125	132.5
105.6	175	155	162.5	115	117.5	115	125	130	120
108	140	137.5	127.5	112.5	107.5	112.5	125	117.5	122.5
100.8	150	142.5	127.5	112.5	115	115	125	122.5	122.5
98.4	155	157.5	137.5	115	115	115	125	127.5	125
100.8	160	127.5	140	117.5	117.5	115	127.5	130	130
105.6	187.5	137.5	142.5	117.5	115	115	135	132.5	137.5
98.4	127.5	165	132.5	115	112.5	110	125	117.5	142.5
100.8	132.5	137.5	130	115	112.5	112.5	127.5	125	117.5
110.4	132.5	147.5	135	115	115	112.5	127.5	125	117.5
96	137.5	150	155	115	115	112.5	130	125	120
96	150	167.5	160	117.5	120	117.5	130	135	122.5
100.8	152.5	202.5	127.5	107.5	102.5	112.5	120	127.5	122.5
100.8	155	132.5	127.5	110	110	112.5	127.5	122.5	125
98.4	157.5	137.5	137.5	115	112.5	112.5	127.5	122.5	125
98.4	160	140	140	117.5	115	112.5	130	125	132.5
98.4	162.5	152.5	150	117.5	117.5	115	135	125	127.5
98.4	130	152.5	130	110	105	112.5	120	132.5	120
100.8	132.5	127.5	135	110	112.5	112.5	125	132.5	120
96	137.5	130	140	115	112.5	112.5	125	135	122.5
98.4	190	137.5	147.5	115	117.5	117.5	130	120	125
100.8	137.5	140	162.5	117.5	120	117.5	137.5	120	122.5
96	150	150	132.5	112.5	112.5	107.5	122.5	122.5	125
96	152.5	132.5	137.5	112.5	115	110	125	122.5	127.5
96	140	147.5	155	115	115	112.5	125	125	127.5
100.8	145	167.5	155	115	115	115	130	127.5	130
108	157.5	187.5	162.5	117.5	115	115	132.5	132.5	137.5
96	137.5	142.5	125	112.5	112.5	105	122.5	115	117.5
96	142.5	147.5	140	112.5	112.5	107.5	122.5	115	125
98.4	147.5	150	145	115	112.5	112.5	125	117.5	125
100.8	152.5	152.5	155	117.5	115	115	125	117.5	130
96	162.5	180	157.5	120	115	117.5	127.5	122.5	130
96	132.5	135	127.5	105	107.5	105	132.5	117.5	122.5
103.2	137.5	147.5	132.5	112.5	110	112.5	120	122.5	122.5
105.6	142.5	162.5	137.5	112.5	110	112.5	120	125	122.5
96	145	155	157.5	117.5	112.5	122.5	125	130	130
96	165	187.5	162.5	120	115	112.5	132.5	135	137.5
96	127.5	150	127.5	115	110	110	140	122.5	115
96	135	150	130	115	112.5	110	117.5	125	115
96	140	165	137.5	115	112.5	112.5	120	125	132.5
98.4	162.5	167.5	157.5	115	115	115	125	130	135
100.8	135	177.5	167.5	115	117.5	115	130	122.5	142.5
103.2	137.5	132.5	125	112.5	102.5	105	132.5	125	117.5
96	140	137.5	125	115	115	112.5	140	125	117.5
98.4	145	155	130	117.5	115	112.5	120	130	117.5
100.8	165	167.5	132.5	117.5	117.5	112.5	122.5	137.5	120
96	132.5	172.5	155	122.5	117.5	115	122.5	117.5	122.5
96	135	140	127.5	107.5	110	107.5	125	122.5	130
98.4	137.5	150	130	112.5	110	112.5	125	125	115
98.4	145	152.5	132.5	115	112.5	115	132.5	127.5	117.5
100.8	162.5	162.5	150	115	115	117.5	117.5	132.5	117.5
96	187.5	175	152.5	117.5	120	120	120	120	125
96	132.5	132.5	130	107.5	110	112.5	122.5	125	125
96	137.5	137.5	135	110	112.5	112.5	125	125	130
108	142.5	140	147.5	112.5	115	115	130	125	132.5
96	152.5	155	152.5	115	115	115	135	132.5	132.5
100.8	180	167.5	172.5	115	120	117.5	137.5	125	132.5
Mean	100.0	148.0	151.0	141.8	114.0	113.5	112.9	127.1	125.2	125.6
SD	4.61	14.91	16.46	12.78	3.85	3.71	3.59	5.52	5.28	6.66

Table. A.1. *S. costatum*. HL. **(Continued)**

P 1	P 2	P 3	N 1	N 2	N 3
112.5	112.5	112.5	120	127.5	112.5
112.5	115	115	120	137.5	115
112.5	117.5	117.5	122.5	140	117.5
115	117.5	117.5	120	157.5	117.5
117.5	117.5	125	125	130	122.5
115	112.5	112.5	130	132.5	112.5
115	112.5	112.5	137.5	137.5	112.5
117.5	112.5	115	137.5	140	115
117.5	112.5	117.5	117.5	127.5	117.5
120	115	117.5	120	137.5	125
112.5	112.5	112.5	130	140	112.5
115	112.5	115	142.5	145	115
115	112.5	115	117.5	112.5	112.5
115	117.5	115	117.5	125	115
117.5	117.5	117.5	120	127.5	115
112.5	112.5	112.5	120	132.5	117.5
115	112.5	112.5	125	117.5	112.5
117.5	117.5	112.5	132.5	120	115
117.5	117.5	115	132.5	127.5	115
122.5	120	117.5	120	130	117.5
112.5	112.5	112.5	120	137.5	117.5
112.5	112.5	112.5	125	127.5	117.5
115	115	115	125	130	120
117.5	115	117.5	125	132.5	112.5
117.5	117.5	117.5	132.5	142.5	115
112.5	112.5	112.5	145	150	115
112.5	115	112.5	120	125	117.5
115	115	117.5	120	125	120
115	117.5	117.5	127.5	130	117.5
115	117.5	120	132.5	137.5	115
115	117.5	112.5	120	122.5	112.5
117.5	117.5	112.5	122.5	132.5	112.5
120	117.5	112.5	125	137.5	112.5
120	117.5	112.5	125	165	115
122.5	117.5	117.5	125	115	117.5
115	112.5	112.5	125	122.5	112.5
115	115	112.5	125	132.5	112.5
117.5	117.5	115	125	137.5	115
117.5	117.5	117.5	130	117.5	115
120	120	120	132.5	125	117.5
112.5	112.5	112.5	125	127.5	112.5
115	112.5	115	125	127.5	112.5
117.5	115	115	130	140	112.5
122.5	117.5	115	130	120	115
125	117.5	117.5	132.5	125	115
112.5	112.5	112.5	137.5	125	115
115	117.5	115	117.5	132.5	117.5
115	117.5	115	122.5	137.5	117.5
117.5	117.5	117.5	125	112.5	117.5
120	120	120	130	120	120
122.5	112.5	110	130	122.5	120
115	115	112.5	130	125	112.5
117.5	117.5	115	132.5	125	115
117.5	120	117.5	137.5	127.5	115
117.5	120	117.5	120	122.5	117.5
120	112.5	112.5	120	125	117.5
112.5	112.5	115	122.5	135	117.5
117.5	112.5	115	122.5	137.5	117.5
117.5	112.5	115	130	140	117.5
120	115	115	132.5	135	117.5
Mean 116.5	115.5	115.1	126.5	130.9	115.8
SD 3.03	2.56	2.68	6.41	9.80	2.72

Table. A.2.*S. costatum*.HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and on *S. costatum*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and nitrogen limited medium (N). Numbers at the end are the numbers of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
100	120	142.5	155	115	115	112.5	145	130	137.5
105	135	162.5	150	115	115	112.5	155	150	150
105	170	170	152.5	117.5	115	112.5	157.5	155	152.5
107.5	127.5	177.5	162.5	120	117.5	115	162.5	157.5	157.5
110	135	132.5	167.5	120	117.5	120	140	162.5	160
107.5	137.5	135	162.5	110	110	107.5	157.5	132.5	135
112.5	140	137.5	162.5	110	112.5	112.5	167.5	147.5	150
112.5	142.5	137.5	165	112.5	115	112.5	130	155	157.5
115	125	165	162.5	112.5	117.5	115	147.5	160	162.5
115	130	132.5	157.5	115	120	115	150	167.5	172.5
102.5	137.5	132.5	172.5	112.5	107.5	110	150	125	147.5
105	162.5	135	137.5	112.5	112.5	112.5	167.5	125	140
105	125	140	157.5	115	112.5	112.5	140	137.5	152.5
107.5	135	132.5	165	120	112.5	115	142.5	157.5	162.5
110	185	137.5	165	120	115	117.5	150	160	165
102.5	130	150	157.5	105	105	120	150	137.5	152.5
102.5	130	152.5	160	112.5	112.5	112.5	152.5	145	155
105	137.5	127.5	167.5	112.5	112.5	112.5	155	150	155
105	145	140	135	115	112.5	112.5	162.5	162.5	157.5
112.5	185	147.5	140	117.5	115	115	125	167.5	127.5
105	205	150	155	112.5	102.5	117.5	127.5	145	132.5
107.5	132.5	132.5	160	112.5	115	110	132.5	157.5	147.5
107.5	162.5	147.5	152.5	112.5	117.5	115	150	160	152.5
107.5	215	212.5	160	115	120	115	150	162.5	157.5
107.5	202.5	150	172.5	125	120	117.5	162.5	150	150
105	167.5	145	162.5	107.5	110	110	127.5	142.5	157.5
107.5	190	205	165	112.5	112.5	112.5	145	147.5	162.5
107.5	137.5	142.5	167.5	115	112.5	112.5	152.5	150	165
110	142.5	135	170	115	115	115	162.5	150	167.5
110	165	155	175	120	117.5	120	175	155	152.5
102.5	140	127.5	157.5	107.5	112.5	112.5	137.5	140	140
105	175	157.5	160	112.5	112.5	112.5	142.5	152.5	145
107.5	130	145	162.5	112.5	115	112.5	152.5	152.5	147.5
107.5	190	132.5	172.5	117.5	120	112.5	167.5	155	150
112.5	125	140	147.5	120	120	117.5	127.5	170	157.5
100	137.5	187.5	155	110	102.5	112.5	150	137.5	152.5
105	152.5	187.5	160	112.5	112.5	115	157.5	140	155
107.5	162.5	195	150	112.5	112.5	115	162.5	150	155
107.5	167.5	130	167.5	120	117.5	117.5	162.5	152.5	155
107.5	125	135	177.5	122.5	120	120	140	155	160
102.5	125	150	137.5	110	105	112.5	140	132.5	142.5
105	180	190	145	112.5	112.5	112.5	145	145	147.5
107.5	212.5	140	157.5	112.5	112.5	115	152.5	152.5	150
107.5	150	157.5	162.5	112.5	115	117.5	152.5	155	155
107.5	157.5	185	162.5	120	120	117.5	157.5	167.5	162.5
97.5	192.5	132.5	165	112.5	110	112.5	170	132.5	147.5
105	192.5	132.5	172.5	120	112.5	115	170	145	142.5
107.5	137.5	140	147.5	120	115	115	135	152.5	157.5
107.5	155	177.5	157.5	122.5	120	117.5	150	152.5	172.5
112.5	187.5	197.5	167.5	122.5	122.5	117.5	160	162.5	142.5
105	167.5	202.5	147.5	107.5	112.5	105	167.5	137.5	150
105	167.5	132.5	160	110	112.5	110	140	140	152.5
105	190	140	162.5	112.5	112.5	115	147.5	160	155
110	140	182.5	142.5	112.5	115	115	150	170	155
110	190	170	150	117.5	120	117.5	160	145	147.5
105	197.5	140	160	112.5	112.5	102.5	150	150	130
107.5	132.5	202.5	175	112.5	112.5	112.5	157.5	150	147.5
110	137.5	127.5	155	112.5	112.5	115	160	152.5	155
110	175	140	135	112.5	115	117.5	162.5	155	165
112.5	137.5	145	175	120	117.5	117.5	165	147.5	162.5
Mean	107.1	156.4	153.1	159.0	114.7	114.1	114.1	151.4	150.3	152.5
SD	3.51	26.21	23.36	10.27	4.29	4.20	3.35	11.86	10.66	9.38

Table. A.2. *S. costatum*. HL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
127.5	147.5	130	137.5	150	137.5
130	160	140	137.5	155	150
142.5	162.5	155	140	160	155
125	130	150	150	165	162.5
140	135	170	157.5	147.5	162.5
142.5	150	140	132.5	150	127.5
147.5	162.5	157.5	135	155	132.5
150	132.5	165	137.5	155	150
152.5	135	175	152.5	157.5	150
152.5	170	132.5	157.5	157.5	155
152.5	127.5	125	135	145	142.5
157.5	132.5	150	140	145	145
150	137.5	160	145	155	147.5
152.5	135	175	152.5	155	150
160	157.5	135	155	162.5	167.5
162.5	162.5	155	142.5	137.5	147.5
165	137.5	162.5	147.5	142.5	150
147.5	142.5	170	147.5	150	150
157.5	162.5	137.5	150	155	152.5
160	140	150	167.5	167.5	152.5
172.5	165	165	152.5	155	137.5
147.5	175	165	155	155	142.5
152.5	125	140	155	162.5	150
157.5	132.5	150	157.5	167.5	150
177.5	147.5	150	170	175	152.5
162.5	160	157.5	152.5	147.5	142.5
165	140	162.5	155	150	150
157.5	140	145	160	152.5	152.5
160	150	162.5	162.5	157.5	155
170	152.5	165	167.5	162.5	160
142.5	135	132.5	145	165	137.5
152.5	137.5	137.5	147.5	150	147.5
155	155	167.5	157.5	155	150
162.5	162.5	167.5	162.5	155	155
162.5	140	170	162.5	157.5	162.5
127.5	137.5	132.5	142.5	137.5	147.5
137.5	147.5	137.5	147.5	152.5	150
162.5	147.5	155	150	155	152.5
147.5	150	157.5	152.5	155	155
157.5	140	147.5	155	162.5	157.5
162.5	142.5	150	145	140	152.5
165	145	155	145	145	155
142.5	147.5	162.5	145	155	160
150	160	137.5	147.5	165	165
162.5	140	155	150	170	165
165	152.5	167.5	167.5	142.5	150
167.5	157.5	167.5	137.5	142.5	157.5
137.5	132.5	142.5	142.5	152.5	160
145	142.5	147.5	150	167.5	162.5
150	145	147.5	150	175	162.5
170	150	157.5	152.5	145	147.5
142.5	142.5	150	147.5	145	152.5
152.5	152.5	155	150	152.5	152.5
155	157.5	160	150	155	157.5
162.5	162.5	165	155	160	157.5
137.5	152.5	140	162.5	145	145
150	152.5	142.5	152.5	150	147.5
162.5	152.5	140	155	155	157.5
147.5	155	155	157.5	155	162.5
152.5	175	165	162.5	157.5	167.5
Mean 153.1	148.0	152.8	150.9	154.5	152.2
SD 11.35	11.77	12.41	8.72	8.45	8.19

Table. A.1. *S. costatum*. LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *S. costatum* cells cultured at Low Light conditions. f/2=f/2 medium, P=Phosphorus limited medium and N=Nitrogen limited medium. Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
100	135	142.5	150	117.5	102.5	115	142.5	145	150
105	137.5	142.5	155	117.5	105	115	147.5	147.5	150
105	145	145	157.5	120	112.5	117.5	147.5	165	160
107.5	150	150	160	125	112.5	120	150	167.5	162.5
112.5	160	150	165	112.5	117.5	122.5	155	175	157.5
102.5	147.5	140	142.5	115	107.5	110	140	150	152.5
102.5	142.5	145	145	115	110	117.5	150	150	155
107.5	150	147.5	147.5	117.5	115	117.5	150	155	162.5
112.5	150	150	162.5	122.5	120	120	162.5	165	167.5
112.5	162.5	150	152.5	115	120	127.5	170	167.5	177.5
97.5	142.5	132.5	145	110	107.5	112.5	147.5	150	147.5
107.5	142.5	140	147.5	110	107.5	112.5	152.5	152.5	150
112.5	147.5	142.5	150	112.5	117.5	120	152.5	157.5	152.5
112.5	152.5	147.5	150	115	117.5	122.5	170	170	165
112.5	160	150	152.5	122.5	117.5	125	140	160	145
100	137.5	135	150	115	107.5	112.5	150	152.5	162.5
102.5	140	137.5	150	117.5	115	115	150	155	140
105	142.5	142.5	152.5	120	117.5	115	170	160	152.5
112.5	150	145	157.5	122.5	117.5	117.5	172.5	165	157.5
107.5	150	147.5	157.5	125	117.5	117.5	182.5	185	167.5
95	142.5	150	145	107.5	107.5	110	142.5	180	155
102.5	145	140	150	115	115	110	145	150	165
105	145	142.5	152.5	117.5	115	112.5	152.5	150	167.5
105	150	150	155	120	117.5	120	152.5	160	167.5
107.5	152.5	152.5	160	122.5	117.5	122.5	157.5	167.5	140
102.5	142.5	142.5	145	115	105	112.5	142.5	152.5	147.5
107.5	142.5	145	160	115	110	112.5	150	162.5	150
110	147.5	152.5	160	117.5	112.5	117.5	152.5	165	152.5
110	152.5	155	167.5	120	122.5	117.5	157.5	167.5	152.5
107.5	160	152.5	187.5	120	122.5	127.5	175	152.5	160
105	140	145	147.5	107.5	112.5	120	147.5	152.5	145
107.5	140	147.5	165	115	112.5	120	150	155	150
110	142.5	147.5	175	117.5	117.5	122.5	152.5	157.5	150
110	145	155	182.5	117.5	122.5	125	152.5	177.5	150
112.5	155	160	150	117.5	122.5	127.5	160	180	162.5
97.5	140	137.5	150	107.5	107.5	102.5	142.5	142.5	142.5
102.5	142.5	140	150	112.5	112.5	107.5	147.5	155	157.5
105	142.5	145	152.5	115	112.5	115	150	155	165
105	142.5	147.5	152.5	115	117.5	115	170	165	172.5
107.5	152.5	150	155	117.5	120	125	150	167.5	162.5
97.5	150	142.5	145	100	110	117.5	152.5	142.5	152.5
100	150	145	147.5	107.5	112.5	125	152.5	145	157.5
102.5	150	147.5	150	110	117.5	127.5	155	150	160
102.5	152.5	150	160	112.5	117.5	127.5	160	182.5	170
105	160	155	165	112.5	120	130	170	162.5	180
107.5	140	145	150	117.5	105	105	145	140	152.5
115	142.5	152.5	152.5	117.5	107.5	117.5	150	150	155
117.5	145	157.5	157.5	120	112.5	125	150	155	157.5
120	150	175	160	120	112.5	130	162.5	165	165
125	150	155	162.5	122.5	122.5	112.5	152.5	172.5	172.5
100	142.5	145	145	102.5	112.5	115	152.5	150	155
112.5	150	145	155	112.5	112.5	115	155	150	160
112.5	150	152.5	155	117.5	115	117.5	167.5	152.5	160
115	155	157.5	157.5	122.5	122.5	115	175	152.5	162.5
102.5	160	165	162.5	125	125	107.5	177.5	172.5	162.5
102.5	132.5	137.5	147.5	112.5	107.5	115	145	142.5	152.5
105	137.5	140	150	117.5	115	120	150	142.5	152.5
107.5	142.5	142.5	155	120	117.5	120	150	147.5	160
95	145	147.5	157.5	122.5	120	122.5	155	152.5	165
117.5	150	160	162.5	115	125	125	165	155	140
Mean	106.9	147.0	147.6	155.2	116.1	114.6	118.1	154.9	158.2	157.4
SD	6.03	6.67	7.31	8.62	5.21	5.47	6.18	9.98	10.84	8.86

Table. A.1 *S. costatum*. LL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
117.5	122.5	120	142.5	180	145
120	122.5	130	165	192.5	175
125	125	130	167.5	150	187.5
127.5	132.5	135	167.5	162.5	187.5
127.5	135	137.5	175	175	190
117.5	117.5	122.5	142.5	175	160
120	120	125	152.5	175	177.5
120	125	125	157.5	177.5	185
122.5	130	127.5	162.5	177.5	200
140	140	130	175	155	152.5
117.5	122.5	122.5	150	175	155
122.5	125	127.5	155	182.5	165
130	125	132.5	155	192.5	175
135	127.5	135	162.5	155	147.5
140	132.5	137.5	175	157.5	175
120	115	120	152.5	160	207.5
122.5	120	125	157.5	175	162.5
122.5	120	127.5	152.5	172.5	152.5
125	130	130	162.5	172.5	160
125	140	137.5	165	175	170
115	120	117.5	155	192.5	162.5
122.5	130	122.5	157.5	157.5	182.5
125	132.5	122.5	157.5	162.5	182.5
130	132.5	132.5	160	172.5	205
140	135	137.5	177.5	177.5	165
112.5	112.5	120	157.5	157.5	210
120	127.5	127.5	185	162.5	145
120	130	132.5	157.5	175	170
122.5	137.5	137.5	170	190	152.5
125	137.5	137.5	172.5	167.5	167.5
142.5	120	115	147.5	165	185
115	127.5	125	155	185	182.5
117.5	127.5	127.5	165	187.5	190
120	127.5	130	175	157.5	212.5
127.5	130	135	182.5	170	162.5
130	127.5	125	160	155	175
112.5	127.5	127.5	162.5	177.5	145
117.5	132.5	132.5	170	200	165
117.5	132.5	137.5	187.5	167.5	150
122.5	137.5	140	165	175	157.5
137.5	127.5	127.5	162.5	192.5	162.5
122.5	132.5	127.5	175	165	150
122.5	132.5	130	162.5	177.5	212.5
127.5	137.5	132.5	162.5	187.5	155
130	137.5	140	162.5	180	160
135	120	122.5	165	157.5	167.5
117.5	122.5	130	180	157.5	145
117.5	125	130	160	180	160
117.5	125	135	165	187.5	172.5
125	127.5	140	165	200	160
127.5	125	120	167.5	160	207.5
120	125	125	192.5	170	140
120	130	125	147.5	175	145
125	132.5	130	165	185	152.5
130	132.5	132.5	172.5	187.5	162.5
125	115	122.5	167.5	172.5	147.5
125	120	127.5	162.5	177.5	175
127.5	127.5	127.5	165	182.5	167.5
130	132.5	130	167.5	187.5	167.5
130	135	132.5	160	185	175
Mean 124.4	127.9	129.1	164.0	174.3	169.7
SD 6.87	6.41	5.98	10.17	12.21	18.81

Table. A.2. *S. costatum*. LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *S. costatum* cells cultured at Low Light conditions. f/2=f/2 medium, P=Phosphorus limited medium and N=Nitrogen limited medium. Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
105	140	147.5	137.5	115	110	117.5	150	140	142.5
107.5	140	147.5	142.5	117.5	115	120	170	160	150
110	150	142.5	157.5	120	117.5	122.5	185	160	155
107.5	152.5	147.5	135	125	117.5	125	142.5	160	167.5
117.5	147.5	140	137.5	125	120	127.5	155	162.5	167.5
107.5	207.5	142.5	140	102.5	107.5	117.5	140	132.5	167.5
112.5	137.5	165	142.5	115	115	120	145	150	142.5
112.5	137.5	142.5	152.5	117.5	120	130	160	157.5	157.5
115	142.5	135	155	122.5	120	117.5	142.5	162.5	160
117.5	142.5	140	142.5	117.5	125	120	155	150	130
107.5	150	157.5	142.5	117.5	110	127.5	162.5	162.5	147.5
112.5	162.5	142.5	150	120	117.5	117.5	140	142.5	150
112.5	140	150	137.5	125	117.5	132.5	150	152.5	137.5
115	142.5	155	150	125	122.5	112.5	150	160	155
102.5	190	140	157.5	105	125	122.5	157.5	137.5	165
107.5	145	142.5	145	115	112.5	122.5	150	157.5	132.5
107.5	150	155	147.5	117.5	115	125	150	165	145
110	155	137.5	150	117.5	120	125	162.5	155	157.5
112.5	230	150	152.5	120	125	130	167.5	160	162.5
112.5	150	167.5	137.5	112.5	130	117.5	160	142.5	155
112.5	137.5	135	140	115	112.5	120	137.5	137.5	157.5
115	140	140	150	115	117.5	122.5	140	142.5	157.5
107.5	157.5	150	165	117.5	120	130	155	150	155
110	145	160	147.5	120	125	117.5	145	167.5	140
117.5	145	137.5	160	122.5	125	120	165	165	150
100	150	162.5	167.5	120	115	117.5	137.5	150	150
105	165	142.5	145	115	115	117.5	145	160	147.5
107.5	145	137.5	147.5	122.5	120	122.5	157.5	192.5	157.5
110	147.5	150	157.5	127.5	120	127.5	162.5	155	160
115	157.5	155	145	122.5	120	130	160	170	162.5
105	137.5	145	130	112.5	117.5	105	132.5	157.5	160
112.5	142.5	147.5	137.5	117.5	117.5	115	137.5	167.5	165
112.5	150	180	150	117.5	120	117.5	147.5	170	170
115	137.5	142.5	155	122.5	120	125	150	182.5	155
115	140	142.5	157.5	115	122.5	130	152.5	137.5	165
105	142.5	147.5	142.5	117.5	112.5	117.5	140	165	145
112.5	145	155	145	117.5	115	120	140	182.5	155
115	157.5	137.5	147.5	120	117.5	120	162.5	152.5	155
112.5	140	137.5	155	112.5	117.5	122.5	142.5	155	157.5
112.5	140	157.5	145	117.5	122.5	125	140	165	157.5
115	157.5	140	147.5	117.5	120	110	135	145	152.5
117.5	137.5	142.5	160	120	120	115	142.5	150	155
120	167.5	155	140	122.5	122.5	117.5	155	147.5	167.5
102.5	137.5	142.5	162.5	117.5	122.5	120	180	152.5	167.5
105	145	147.5	190	120	120	122.5	152.5	155	162.5
110	155	167.5	140	120	120	112.5	145	142.5	155
117.5	167.5	142.5	142.5	125	117.5	117.5	152.5	157.5	165
105	130	145	142.5	115	120	120	165	162.5	142.5
112.5	142.5	147.5	140	120	125	122.5	170	190	172.5
112.5	142.5	160	142.5	120	122.5	125	160	157.5	160
115	142.5	157.5	160	122.5	105	115	150	172.5	150
105	145	140	140	117.5	120	117.5	162.5	165	142.5
112.5	162.5	145	155	120	125	117.5	167.5	165	155
112.5	140	155	157.5	122.5	117.5	120	170	157.5	160
115	165	135	162.5	127.5	110	125	175	165	162.5
115	137.5	142.5	147.5	117.5	120	110	140	150	150
105	142.5	145	157.5	120	122.5	112.5	140	155	155
110	150	142.5	167.5	120	125	115	147.5	165	142.5
115	157.5	152.5	150	120	117.5	117.5	167.5	150	172.5
107.5	150	155	160	122.5	117.5	120	187.5	155	165
Mean	111.0	150.2	147.8	149.5	118.8	118.8	120.4	153.5	157.6	155.4
SD	4.45	16.41	9.21	10.17	4.48	4.71	5.52	12.61	12.01	9.50

Table. A.2. *S. costatum*. LL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
140	132.5	127.5	162.5	160	162.5
142.5	150	142.5	167.5	172.5	162.5
142.5	152.5	150	172.5	187.5	165
142.5	155	157.5	160	175	165
142.5	165	140	167.5	182.5	175
125	135	160	162.5	185	165
127.5	142.5	162.5	177.5	157.5	167.5
125	147.5	137.5	157.5	160	172.5
130	152.5	142.5	162.5	165	177.5
140	152.5	145	155	167.5	182.5
127.5	155	130	162.5	180	165
132.5	155	137.5	167.5	172.5	180
150	132.5	147.5	160	182.5	180
150	137.5	155	162.5	187.5	162.5
155	142.5	127.5	167.5	150	185
125	145	157.5	142.5	155	187.5
130	155	160	145	165	165
135	135	167.5	170	165	175
150	160	125	147.5	157.5	192.5
150	172.5	140	150	192.5	167.5
125	140	152.5	157.5	170	165
137.5	160	125	167.5	182.5	165
145	140	152.5	145	160	175
147.5	140	165	157.5	155	175
125	162.5	130	177.5	167.5	180
130	140	145	150	170	157.5
137.5	140	155	155	172.5	165
152.5	150	145	160	145	177.5
130	162.5	145	170	150	157.5
165	150	157.5	165	157.5	177.5
125	137.5	142.5	165	152.5	177.5
137.5	142.5	142.5	187.5	170	180
140	142.5	142.5	182.5	170	185
140	135	147.5	152.5	172.5	200
150	142.5	160	162.5	180	175
125	145	137.5	162.5	157.5	180
127.5	150	142.5	172.5	162.5	187.5
132.5	137.5	147.5	165	167.5	190
150	155	145	167.5	167.5	162.5
152.5	175	157.5	150	175	167.5
120	127.5	125	160	152.5	187.5
125	132.5	125	162.5	165	175
127.5	132.5	127.5	165	175	175
120	152.5	137.5	150	177.5	187.5
120	130	150	152.5	155	150
125	140	135	167.5	162.5	167.5
142.5	137.5	137.5	175	165	197.5
130	162.5	140	152.5	167.5	162.5
135	142.5	147.5	152.5	155	162.5
140	152.5	130	157.5	157.5	180
150	137.5	130	167.5	175	157.5
122.5	162.5	132.5	150	180	172.5
132.5	187.5	142.5	162.5	145	180
137.5	125	150	165	150	150
150	125	155	167.5	157.5	175
125	135	165	152.5	175	180
130	157.5	125	152.5	155	190
132.5	135	137.5	152.5	157.5	157.5
137.5	160	142.5	172.5	160	165
137.5	127.5	167.5	172.5	167.5	175
Mean 136.3	146.4	144.2	161.7	166.3	173.4
SD 10.44	12.86	11.85	9.42	11.16	11.09

Table. A.1. *C. muelleri* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *C. muelleri*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
112.8	137.5	125	132.5	100	110	102.5	127.5	122.5	122.5
108	137.5	132.5	135	105	112.5	110	130	130	125
115.2	145	142.5	140	112.5	112.5	110	132.5	125	125
103.2	152.5	150	155	112.5	115	112.5	137.5	130	125
105.6	175	155	162.5	115	117.5	115	140	130	127.5
108	140	137.5	127.5	112.5	107.5	112.5	125	130	130
100.8	150	142.5	127.5	112.5	115	115	127.5	125	125
98.4	155	157.5	137.5	115	115	115	130	130	127.5
100.8	160	127.5	140	117.5	117.5	115	132.5	130	130
105.6	187.5	137.5	142.5	117.5	115	115	137.5	130	130
98.4	127.5	165	132.5	115	112.5	110	145	140	132.5
100.8	132.5	137.5	130	115	112.5	112.5	125	125	132.5
110.4	132.5	147.5	135	115	115	112.5	132.5	130	135
96	137.5	150	155	115	115	112.5	132.5	137.5	120
96	150	167.5	160	117.5	120	117.5	140	140	125
100.8	152.5	202.5	127.5	107.5	102.5	112.5	130	145	127.5
100.8	155	132.5	127.5	110	110	112.5	125	122.5	130
98.4	157.5	137.5	137.5	115	112.5	112.5	127.5	125	130
98.4	160	140	140	117.5	115	112.5	130	127.5	135
98.4	162.5	152.5	150	117.5	117.5	115	132.5	132.5	135
98.4	130	152.5	130	110	105	112.5	135	132.5	117.5
100.8	132.5	127.5	135	110	112.5	112.5	137.5	125	117.5
96	137.5	130	140	115	112.5	112.5	120	125	125
98.4	190	137.5	147.5	115	117.5	117.5	127.5	127.5	125
100.8	137.5	140	162.5	117.5	120	117.5	140	135	127.5
96	150	150	132.5	112.5	112.5	107.5	145	142.5	135
96	152.5	132.5	137.5	112.5	115	110	125	120	122.5
96	140	147.5	155	115	115	112.5	130	132.5	125
100.8	145	167.5	155	115	115	115	132.5	132.5	132.5
108	157.5	187.5	162.5	117.5	115	115	135	152.5	135
96	137.5	142.5	125	112.5	112.5	105	120	125	120
96	142.5	147.5	140	112.5	112.5	107.5	125	127.5	125
98.4	147.5	150	145	115	112.5	112.5	132.5	130	125
100.8	152.5	152.5	155	117.5	115	115	132.5	132.5	125
96	162.5	180	157.5	120	115	117.5	135	135	130
96	132.5	135	127.5	105	107.5	105	130	140	140
103.2	137.5	147.5	132.5	112.5	110	112.5	130	125	117.5
105.6	142.5	162.5	137.5	112.5	110	112.5	137.5	130	122.5
96	145	155	157.5	117.5	112.5	122.5	142.5	130	127.5
96	165	187.5	162.5	120	115	112.5	122.5	132.5	137.5
96	127.5	150	127.5	115	110	110	127.5	142.5	117.5
96	135	150	130	115	112.5	110	130	122.5	117.5
96	140	165	137.5	115	112.5	112.5	130	125	120
98.4	162.5	167.5	157.5	115	115	115	130	125	125
100.8	135	177.5	167.5	115	117.5	115	122.5	127.5	127.5
103.2	137.5	132.5	125	112.5	102.5	105	125	130	130
96	140	137.5	125	115	115	112.5	125	120	120
98.4	145	155	130	117.5	115	112.5	130	122.5	122.5
100.8	165	167.5	132.5	117.5	117.5	112.5	130	125	125
96	132.5	172.5	155	122.5	117.5	115	125	130	145
96	135	140	127.5	107.5	110	107.5	125	132.5	125
98.4	137.5	150	130	112.5	110	112.5	127.5	120	125
98.4	145	152.5	132.5	115	112.5	115	130	120	135
100.8	162.5	162.5	150	115	115	117.5	135	125	137.5
96	187.5	175	152.5	117.5	120	120	125	125	137.5
96	132.5	132.5	130	107.5	110	112.5	125	125	125
96	137.5	137.5	135	110	112.5	112.5	130	130	127.5
108	142.5	140	147.5	112.5	115	115	132.5	130	130
96	152.5	155	152.5	115	115	115	132.5	132.5	132.5
100.8	180	167.5	172.5	115	120	117.5	140	140	137.5
Mean	100	148.0	151.0	141.8	114.0	113.5	112.9	130.9	129.8	127.8
SD	4.61	14.91	16.46	12.78	3.85	3.71	3.59	5.73	6.58	6.15

Table. A.1.*C. muelleri* HL. (continued)

P 1	P 2	P 3	N 1	N 2	N 3
125	122.5	125	132.5	125	125
125	122.5	125	135	127.5	125
125	122.5	127.5	135	130	142.5
130	125	127.5	137.5	137.5	142.5
132.5	125	137.5	142.5	145	150
140	125	145	130	127.5	150
125	127.5	127.5	135	130	135
125	127.5	127.5	135	132.5	137.5
127.5	130	130	137.5	132.5	137.5
130	137.5	130	150	135	142.5
135	120	130	142.5	130	147.5
125	125	135	127.5	132.5	135
125	127.5	137.5	137.5	137.5	137.5
127.5	130	122.5	140	140	142.5
137.5	130	125	140	145	145
140	135	125	142.5	130	145
142.5	132.5	130	125	132.5	152.5
122.5	125	130	130	135	132.5
125	125	137.5	130	137.5	135
127.5	127.5	117.5	130	150	135
130	127.5	122.5	132.5	135	140
142.5	130	125	135	135	155
125	132.5	125	135	137.5	137.5
125	137.5	130	135	137.5	137.5
127.5	125	132.5	135	140	145
127.5	125	130	135	142.5	145
127.5	130	135	135	147.5	152.5
130	130	135	142.5	130	150
127.5	135	137.5	140	135	140
130	130	140	147.5	150	142.5
122.5	120	130	140	122.5	127.5
125	120	130	140	130	130
125	125	130	140	137.5	142.5
132.5	125	135	142.5	145	145
135	125	137.5	142.5	145	150
142.5	125	120	142.5	125	135
122.5	125	122.5	127.5	127.5	135
122.5	127.5	127.5	135	127.5	137.5
125	127.5	130	135	132.5	137.5
127.5	130	132.5	140	137.5	140
137.5	122.5	125	145	125	145
122.5	122.5	127.5	127.5	127.5	147.5
125	125	127.5	127.5	135	135
127.5	127.5	135	130	137.5	140
130	132.5	137.5	132.5	142.5	140
130	137.5	125	137.5	125	145
132.5	122.5	125	127.5	137.5	150
125	130	127.5	127.5	142.5	152.5
125	130	130	132.5	147.5	132.5
125	132.5	132.5	135	147.5	132.5
127.5	135	125	137.5	137.5	135
130	127.5	127.5	125	140	137.5
140	127.5	130	127.5	140	137.5
117.5	130	135	127.5	145	137.5
120	132.5	135	132.5	155	135
125	135	117.5	135	135	140
125	137.5	130	127.5	137.5	140
130	125	132.5	137.5	137.5	147.5
140	130	132.5	137.5	137.5	160
130	132.5	137.5	142.5	140	142.5
Mean 128.8	128.2	129.9	135.4	136.4	140.9
SD 5.84	4.54	5.50	5.78	7.18	7.19

Table. A.2.*C. muelleri* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *C. muelleri*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
100	120	142.5	155	115	115	112.5	140	137.5	142.5
105	135	162.5	150	115	115	112.5	140	140	147.5
105	170	170	152.5	117.5	115	112.5	145	147.5	147.5
107.5	127.5	177.5	162.5	120	117.5	115	147.5	152.5	152.5
110	135	132.5	167.5	120	117.5	120	150	155	155
107.5	137.5	135	162.5	110	110	107.5	157.5	135	147.5
112.5	140	137.5	162.5	110	112.5	112.5	157.5	140	147.5
112.5	142.5	137.5	165	112.5	115	112.5	157.5	152.5	160
115	125	165	162.5	112.5	117.5	115	160	165	160
115	130	132.5	157.5	115	120	115	160	155	167.5
102.5	137.5	132.5	172.5	112.5	107.5	110	135	140	147.5
105	162.5	135	137.5	112.5	112.5	112.5	142.5	142.5	150
105	125	140	157.5	115	112.5	112.5	145	147.5	150
107.5	135	132.5	165	120	112.5	115	150	150	155
110	185	137.5	165	120	115	117.5	152.5	167.5	162.5
102.5	130	150	157.5	105	105	120	150	137.5	142.5
102.5	130	152.5	160	112.5	112.5	112.5	150	140	145
105	137.5	127.5	167.5	112.5	112.5	112.5	155	150	147.5
105	145	140	135	115	112.5	112.5	160	157.5	147.5
112.5	185	147.5	140	117.5	115	115	160	167.5	150
105	205	150	155	112.5	102.5	117.5	137.5	145	137.5
107.5	132.5	132.5	160	112.5	115	110	140	150	140
107.5	162.5	147.5	152.5	112.5	117.5	115	147.5	150	142.5
107.5	215	212.5	160	115	120	115	147.5	155	147.5
107.5	202.5	150	172.5	125	120	117.5	155	155	150
105	167.5	145	162.5	107.5	110	110	142.5	142.5	145
107.5	190	205	165	112.5	112.5	112.5	147.5	150	145
107.5	137.5	142.5	167.5	115	112.5	112.5	150	152.5	145
110	142.5	135	170	115	115	115	150	157.5	147.5
110	165	155	175	120	117.5	120	155	160	155
102.5	140	127.5	157.5	107.5	112.5	112.5	140	140	137.5
105	175	157.5	160	112.5	112.5	112.5	150	142.5	147.5
107.5	130	145	162.5	112.5	115	112.5	140	147.5	150
107.5	190	132.5	172.5	117.5	120	112.5	152.5	150	152.5
112.5	125	140	147.5	120	120	117.5	160	162.5	162.5
100	137.5	187.5	155	110	102.5	112.5	147.5	140	142.5
105	152.5	187.5	160	112.5	112.5	115	147.5	140	142.5
107.5	162.5	195	150	112.5	112.5	115	150	157.5	147.5
107.5	167.5	130	167.5	120	117.5	117.5	152.5	157.5	150
107.5	125	135	177.5	122.5	120	120	157.5	160	172.5
102.5	125	150	137.5	110	105	112.5	140	135	137.5
105	180	190	145	112.5	112.5	112.5	145	137.5	137.5
107.5	212.5	140	157.5	112.5	112.5	115	147.5	140	145
107.5	150	157.5	162.5	112.5	115	117.5	150	145	145
107.5	157.5	185	162.5	120	120	117.5	160	147.5	152.5
97.5	192.5	132.5	165	112.5	110	112.5	147.5	137.5	142.5
105	192.5	132.5	172.5	120	112.5	115	150	145	142.5
107.5	137.5	140	147.5	120	115	115	155	150	147.5
107.5	155	177.5	157.5	122.5	120	117.5	155	157.5	155
112.5	187.5	197.5	167.5	122.5	122.5	117.5	167.5	162.5	187.5
105	167.5	202.5	147.5	107.5	112.5	105	135	142.5	137.5
105	167.5	132.5	160	110	112.5	110	137.5	142.5	147.5
105	190	140	162.5	112.5	112.5	115	142.5	145	152.5
110	140	182.5	142.5	112.5	115	115	150	150	155
110	190	170	150	117.5	120	117.5	160	155	165
105	197.5	140	160	112.5	112.5	102.5	142.5	137.5	140
107.5	132.5	202.5	175	112.5	112.5	112.5	152.5	140	145
110	137.5	127.5	155	112.5	112.5	115	155	150	145
110	175	140	135	112.5	115	117.5	152.5	150	147.5
112.5	137.5	145	175	120	117.5	117.5	160	152.5	152.5
Mean	107.1	156.4	153.1	159.0	114.7	114.1	114.1	149.8	148.6	149.4
SD	3.51	26.21	23.36	10.27	4.29	4.20	3.35	7.33	8.37	8.90

Table. A.2.*C. muelleri* HL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
137.5	142.5	140	145	145	157.5
140	145	140	150	152.5	167.5
142.5	147.5	140	155	157.5	167.5
145	150	140	160	160	172.5
155	155	165	175	162.5	160
132.5	140	140	150	147.5	165
140	142.5	145	152.5	152.5	180
150	145	147.5	157.5	157.5	152.5
150	157.5	150	167.5	157.5	165
150	160	165	167.5	162.5	167.5
137.5	137.5	140	155	152.5	167.5
145	142.5	147.5	157.5	157.5	162.5
160	150	162.5	157.5	157.5	152.5
177.5	152.5	137.5	160	162.5	157.5
157.5	155	147.5	162.5	175	162.5
140	140	150	150	155	167.5
142.5	142.5	140	157.5	155	145
162.5	142.5	142.5	162.5	162.5	152.5
155	145	147.5	167.5	162.5	157.5
160	157.5	162.5	177.5	170	165
162.5	137.5	142.5	155	150	157.5
137.5	140	150	162.5	155	167.5
150	142.5	157.5	162.5	155	175
152.5	152.5	155	162.5	162.5	162.5
135	160	147.5	177.5	170	165
142.5	137.5	145	150	150	172.5
162.5	137.5	155	150	152.5	155
142.5	142.5	145	150	152.5	155
150	142.5	155	162.5	175	157.5
150	155	147.5	162.5	157.5	160
142.5	137.5	137.5	145	155	152.5
145	137.5	147.5	157.5	160	155
147.5	142.5	150	160	162.5	167.5
150	142.5	150	160	165	175
140	142.5	152.5	167.5	175	150
142.5	135	137.5	140	150	155
147.5	145	137.5	150	155	160
155	145	142.5	172.5	170	152.5
160	155	155	177.5	177.5	157.5
162.5	157.5	157.5	150	157.5	162.5
137.5	137.5	137.5	155	160	165
140	147.5	140	160	167.5	162.5
142.5	157.5	152.5	165	167.5	162.5
150	145	152.5	145	147.5	167.5
160	147.5	167.5	152.5	150	167.5
145	150	142.5	155	150	175
165	137.5	145	165	155	157.5
170	140	145	145	155	162.5
162.5	150	155	155	155	172.5
145	150	157.5	165	167.5	157.5
147.5	140	142.5	170	170	150
157.5	142.5	145	157.5	157.5	155
165	150	147.5	167.5	162.5	160
137.5	152.5	150	185	165	185
140	160	157.5	157.5	167.5	160
142.5	147.5	132.5	160	152.5	180
157.5	152.5	150	165	157.5	170
150	152.5	155	157.5	162.5	150
150	162.5	167.5	167.5	167.5	155
157.5	167.5	145	170	182.5	162.5
Mean 149.7	147.1	148.4	159.7	159.9	162.3
SD 9.66	7.58	8.18	9.14	8.09	8.21

Table. A.1.*C. muelleri* LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *C. muelleri*, cultured at Low Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
100	135	142.5	150	117.5	102.5	115	157.5	185	170
105	137.5	142.5	155	117.5	105	115	162.5	140	180
105	145	145	157.5	120	112.5	117.5	162.5	160	182.5
107.5	150	150	160	125	112.5	120	165	162.5	182.5
112.5	160	150	165	112.5	117.5	122.5	145	150	192.5
102.5	147.5	140	142.5	115	107.5	110	175	175	180
102.5	142.5	145	145	115	110	117.5	145	162.5	150
107.5	150	147.5	147.5	117.5	115	117.5	167.5	165	167.5
112.5	150	150	162.5	122.5	120	120	175	155	147.5
112.5	162.5	150	152.5	115	120	127.5	152.5	155	150
97.5	142.5	132.5	145	110	107.5	112.5	160	170	155
107.5	142.5	140	147.5	110	107.5	112.5	165	160	157.5
112.5	147.5	142.5	150	112.5	117.5	120	172.5	187.5	162.5
112.5	152.5	147.5	150	115	117.5	122.5	167.5	152.5	185
112.5	160	150	152.5	122.5	117.5	125	140	162.5	182.5
100	137.5	135	150	115	107.5	112.5	147.5	192.5	157.5
102.5	140	137.5	150	117.5	115	115	157.5	155	162.5
105	142.5	142.5	152.5	120	117.5	115	165	157.5	160
112.5	150	145	157.5	122.5	117.5	117.5	155	182.5	192.5
107.5	150	147.5	157.5	125	117.5	117.5	167.5	167.5	175
95	142.5	150	145	107.5	107.5	110	167.5	192.5	182.5
102.5	145	140	150	115	115	110	175	177.5	195
105	145	142.5	152.5	117.5	115	112.5	175	162.5	165
105	150	150	155	120	117.5	120	175	175	187.5
107.5	152.5	152.5	160	122.5	117.5	122.5	180	175	172.5
102.5	142.5	142.5	145	115	105	112.5	145	162.5	180
107.5	142.5	145	160	115	110	112.5	162.5	162.5	167.5
110	147.5	152.5	160	117.5	112.5	117.5	182.5	157.5	192.5
110	152.5	155	167.5	120	122.5	117.5	162.5	180	140
107.5	160	152.5	187.5	120	122.5	127.5	170	150	177.5
105	140	145	147.5	107.5	112.5	120	192.5	142.5	192.5
107.5	140	147.5	165	115	112.5	120	152.5	162.5	182.5
110	142.5	147.5	175	117.5	117.5	122.5	155	165	177.5
110	145	155	182.5	117.5	122.5	125	160	142.5	155
112.5	155	160	150	117.5	122.5	127.5	170	155	155
97.5	140	137.5	150	107.5	107.5	102.5	147.5	157.5	165
102.5	142.5	140	150	112.5	112.5	107.5	162.5	175	190
105	142.5	145	152.5	115	112.5	115	152.5	152.5	140
105	142.5	147.5	152.5	115	117.5	115	160	162.5	180
107.5	152.5	150	155	117.5	120	125	152.5	185	190
97.5	150	142.5	145	100	110	117.5	162.5	142.5	142.5
100	150	145	147.5	107.5	112.5	125	172.5	150	175
102.5	150	147.5	150	110	117.5	127.5	190	157.5	160
102.5	152.5	150	160	112.5	117.5	127.5	150	167.5	175
105	160	155	165	112.5	120	130	165	150	180
107.5	140	145	150	117.5	105	105	152.5	162.5	152.5
115	142.5	152.5	152.5	117.5	107.5	117.5	175	175	177.5
117.5	145	157.5	157.5	120	112.5	125	180	140	185
120	150	175	160	120	112.5	130	192.5	142.5	145
125	150	155	162.5	122.5	122.5	112.5	160	150	170
100	142.5	145	145	102.5	112.5	115	162.5	152.5	150
112.5	150	145	155	112.5	112.5	115	177.5	165	180
112.5	150	152.5	155	117.5	115	117.5	190	145	207.5
115	155	157.5	157.5	122.5	122.5	115	165	165	177.5
102.5	160	165	162.5	125	125	107.5	172.5	175	152.5
102.5	132.5	137.5	147.5	112.5	107.5	115	185	157.5	157.5
105	137.5	140	150	117.5	115	120	197.5	162.5	177.5
107.5	142.5	142.5	155	120	117.5	120	152.5	167.5	145
95	145	147.5	157.5	122.5	120	122.5	137.5	180	152.5
117.5	150	160	162.5	115	125	125	157.5	165	162.5
Mean	106.9	147.0	147.6	155.2	116.1	114.6	118.1	165.0	162.8	170.1
SD	6.03	6.67	7.31	8.62	5.21	5.47	6.18	13.44	13.00	16.00

Table. A.1.*C. muelleri* LL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
135	145	140	150	145	147.5
157.5	150	155	152.5	167.5	170
140	155	162.5	180	157.5	175
147.5	162.5	145	180	157.5	157.5
150	172.5	147.5	162.5	162.5	160
137.5	150	150	175	155	170
140	152.5	150	190	175	150
150	157.5	152.5	180	147.5	155
140	175	182.5	187.5	152.5	162.5
170	175	145	152.5	147.5	177.5
147.5	137.5	147.5	162.5	152.5	150
147.5	137.5	167.5	187.5	172.5	165
152.5	142.5	145	152.5	145	177.5
147.5	147.5	152.5	175	147.5	145
155	137.5	167.5	182.5	152.5	172.5
137.5	142.5	140	157.5	155	177.5
145	145	150	165	165	152.5
137.5	150	162.5	177.5	167.5	160
140	150	162.5	167.5	152.5	162.5
157.5	152.5	137.5	172.5	147.5	150
140	157.5	150	147.5	155	170
182.5	175	155	165	165	137.5
137.5	155	147.5	167.5	145	175
145	140	165	142.5	155	165
162.5	140	175	145	180	180
150	150	155	180	145	145
150	162.5	155	145	145	155
152.5	157.5	162.5	150	165	157.5
157.5	165	165	165	157.5	147.5
162.5	167.5	170	147.5	165	200
155	170	140	152.5	157.5	142.5
165	177.5	160	162.5	170	150
180	155	167.5	175	172.5	157.5
140	157.5	150	182.5	175	162.5
145	162.5	152.5	160	157.5	150
152.5	167.5	150	162.5	167.5	155
152.5	137.5	150	150	155	162.5
167.5	150	137.5	152.5	140	140
135	152.5	142.5	157.5	142.5	162.5
135	155	145	167.5	145	145
150	160	165	185	152.5	150
165	167.5	155	142.5	150	162.5
132.5	137.5	165	152.5	175	145
142.5	137.5	150	155	142.5	147.5
145	150	152.5	165	150	150
150	147.5	155	165	165	150
155	155	140	155	155	162.5
162.5	175	150	175	157.5	187.5
135	202.5	152.5	150	165	152.5
152.5	142.5	162.5	175	165	165
162.5	162.5	140	152.5	167.5	150
167.5	175	147.5	160	152.5	162.5
177.5	152.5	157.5	175	155	145
147.5	180	150	167.5	162.5	152.5
155	160	155	157.5	152.5	162.5
160	155	140	150	170	150
145	157.5	150	142.5	175	167.5
150	160	162.5	150	157.5	175
175	140	147.5	165	160	175
145	167.5	160	172.5	160	165
Mean 151.3	156.3	153.6	163.3	157.9	159.6
SD 11.83	13.15	9.69	12.97	9.80	12.45

Table. A.2.*C. muelleri* LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *C. muelleri*, cultured at Low Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
105	140	147.5	137.5	115	110	117.5	142.5	162.5	145
107.5	140	147.5	142.5	117.5	115	120	170	180	150
110	150	142.5	157.5	120	117.5	122.5	170	185	167.5
107.5	152.5	147.5	135	125	117.5	125	150	197.5	167.5
117.5	147.5	140	137.5	125	120	127.5	165	167.5	160
107.5	207.5	142.5	140	102.5	107.5	117.5	182.5	167.5	175
112.5	137.5	165	142.5	115	115	120	155	190	182.5
112.5	137.5	142.5	152.5	117.5	120	130	165	182.5	152.5
115	142.5	135	155	122.5	120	117.5	167.5	145	157.5
117.5	142.5	140	142.5	117.5	125	120	172.5	182.5	180
107.5	150	157.5	142.5	117.5	110	127.5	147.5	200	170
112.5	162.5	142.5	150	120	117.5	117.5	150	147.5	172.5
112.5	140	150	137.5	125	117.5	132.5	157.5	172.5	175
115	142.5	155	150	125	122.5	112.5	177.5	172.5	150
102.5	190	140	157.5	105	125	122.5	140	165	152.5
107.5	145	142.5	145	115	112.5	122.5	157.5	175	182.5
107.5	150	155	147.5	117.5	115	125	170	197.5	187.5
110	155	137.5	150	117.5	120	125	202.5	160	162.5
112.5	230	150	152.5	120	125	130	175	157.5	162.5
112.5	150	167.5	137.5	112.5	130	117.5	157.5	177.5	182.5
112.5	137.5	135	140	115	112.5	120	182.5	182.5	167.5
115	140	140	150	115	117.5	122.5	185	140	185
107.5	157.5	150	165	117.5	120	130	145	150	190
110	145	160	147.5	120	125	117.5	152.5	157.5	160
117.5	145	137.5	160	122.5	125	120	152.5	170	167.5
100	150	162.5	167.5	120	115	117.5	147.5	162.5	167.5
105	165	142.5	145	115	115	117.5	165	165	185
107.5	145	137.5	147.5	122.5	120	122.5	192.5	170	167.5
110	147.5	150	157.5	127.5	120	127.5	167.5	172.5	170
115	157.5	155	145	122.5	120	130	180	175	172.5
105	137.5	145	130	112.5	117.5	105	137.5	155	162.5
112.5	142.5	147.5	137.5	117.5	117.5	115	150	170	170
112.5	150	180	150	117.5	120	117.5	165	180	167.5
115	137.5	142.5	155	122.5	120	125	177.5	192.5	182.5
115	140	142.5	157.5	115	122.5	130	152.5	150	150
105	142.5	147.5	142.5	117.5	112.5	117.5	172.5	162.5	150
112.5	145	155	145	117.5	115	120	180	167.5	155
115	157.5	137.5	147.5	120	117.5	120	200	172.5	167.5
112.5	140	137.5	155	112.5	117.5	122.5	155	167.5	152.5
112.5	140	157.5	145	117.5	122.5	125	157.5	167.5	162.5
115	157.5	140	147.5	117.5	120	110	160	192.5	170
117.5	137.5	142.5	160	120	120	115	162.5	192.5	172.5
120	167.5	155	140	122.5	122.5	117.5	152.5	172.5	185
102.5	137.5	142.5	162.5	117.5	122.5	120	167.5	182.5	167.5
105	145	147.5	190	120	120	122.5	167.5	172.5	192.5
110	155	167.5	140	120	120	112.5	170	155	162.5
117.5	167.5	142.5	142.5	125	117.5	117.5	150	165	170
105	130	145	142.5	115	120	120	172.5	185	185
112.5	142.5	147.5	140	120	125	122.5	172.5	162.5	187.5
112.5	142.5	160	142.5	120	122.5	125	175	172.5	145
115	142.5	157.5	160	122.5	105	115	157.5	175	155
105	145	140	140	117.5	120	117.5	162.5	192.5	185
112.5	162.5	145	155	120	125	117.5	162.5	175	145
112.5	140	155	157.5	122.5	117.5	120	152.5	145	162.5
115	165	135	162.5	127.5	110	125	157.5	165	175
115	137.5	142.5	147.5	117.5	120	110	177.5	167.5	187.5
105	142.5	145	157.5	120	122.5	112.5	157.5	157.5	152.5
110	150	142.5	167.5	120	125	115	162.5	175	152.5
115	157.5	152.5	150	120	117.5	117.5	182.5	200	165
107.5	150	155	160	122.5	117.5	120	185	157.5	187.5
Mean	111.0	150.2	147.8	149.5	118.8	118.8	120.4	165.0	171.3	168.2
SD	4.45	16.41	9.21	10.17	4.48	4.71	5.52	14.02	14.22	12.98

Table. A.2.*C. muelleri* LL. (continued)

P 1	P 2	P 3	N 1	N 2	N 3
132.5	132.5	140	142.5	125	132.5
157.5	135	180	145	125	135
162.5	140	142.5	152.5	127.5	142.5
130	137.5	145	130	132.5	125
135	140	142.5	132.5	137.5	137.5
140	145	175	150	125	142.5
147.5	132.5	177.5	130	125	160
130	137.5	127.5	140	132.5	130
137.5	137.5	155	157.5	130	130
140	135	155	130	130	135
142.5	140	145	130	145	137.5
125	142.5	157.5	135	130	130
132.5	142.5	162.5	130	132.5	137.5
150	145	145	130	137.5	137.5
167.5	155	152.5	140	150	147.5
127.5	137.5	152.5	142.5	127.5	130
130	140	157.5	130	130	130
132.5	152.5	137.5	130	132.5	132.5
137.5	127.5	145	140	135	132.5
135	145	157.5	132.5	125	137.5
135	152.5	160	130	130	140
150	135	175	130	130	140
152.5	137.5	175	135	132.5	142.5
132.5	140	192.5	125	132.5	132.5
137.5	137.5	150	130	132.5	137.5
137.5	150	152.5	132.5	140	137.5
147.5	150	137.5	145	130	145
155	130	180	125	125	127.5
132.5	132.5	147.5	125	135	130
135	152.5	140	145	137.5	130
127.5	132.5	130	127.5	127.5	125
130	132.5	140	130	132.5	130
135	140	142.5	135	132.5	132.5
142.5	140	152.5	152.5	137.5	127.5
147.5	132.5	130	130	127.5	130
125	140	137.5	135	127.5	130
130	142.5	137.5	140	130	132.5
137.5	137.5	145	140	137.5	140
137.5	142.5	167.5	142.5	125	132.5
140	135	135	130	125	137.5
130	135	147.5	142.5	135	125
135	140	150	157.5	145	132.5
140	140	140	160	127.5	135
147.5	127.5	140	137.5	130	125
125	135	152.5	145	132.5	125
127.5	137.5	132.5	150	137.5	135
130	127.5	140	135	125	137.5
150	130	142.5	147.5	130	125
130	137.5	155	150	130	125
132.5	137.5	162.5	150	132.5	127.5
140	142.5	140	125	135	130
152.5	142.5	140	125	135	145
130	145	167.5	127.5	132.5	125
130	130	145	132.5	132.5	125
130	140	147.5	125	137.5	130
142.5	142.5	160	132.5	127.5	137.5
132.5	130	137.5	140	130	125
142.5	142.5	142.5	127.5	130	127.5
145	152.5	155	137.5	132.5	130
132.5	157.5	177.5	137.5	137.5	137.5
Mean 138.1	139.4	150.9	137.0	132.0	133.5
SD 9.44	6.96	14.28	9.14	5.25	6.77

Table. A.1.*R. reticulata* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *R. reticulata*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
110.0	219.0	252.6	153.3	149.2	138.7	133.2	148.9	141.7	170.8
112.5	262.6	240.9	169.3	129.7	134.8	127.2	178.0	163.5	148.0
115.0	219.1	191.0	226.9	141.9	132.3	156.8	168.8	173.7	158.2
117.5	223.7	191.8	195.9	159.7	112.7	129.7	168.9	151.8	130.6
102.5	161.2	202.6	190.3	125.5	124.1	156.0	200.8	178.0	164.5
117.5	172.4	174.3	187.7	128.8	127.8	141.9	155.4	183.9	141.7
117.5	181.6	197.4	172.4	120.5	155.1	147.3	136.7	141.5	198.7
122.5	271.2	282.8	209.3	143.2	123.3	126.8	205.7	187.3	162.4
112.5	213.8	153.0	174.6	130.7	128.5	138.7	167.5	192.5	182.5
115.0	190.5	251.9	180.9	147.4	134.2	158.7	173.9	198.1	146.9
117.5	194.2	188.1	169.5	129.2	151.6	146.1	169.3	162.4	177.5
122.5	191.7	157.6	223.0	116.3	126.2	160.7	180.5	200.6	146.9
117.5	200.1	189.7	225.2	132.3	154.4	139.3	181.8	206.1	153.3
117.5	203.5	151.9	155.2	160.4	151.2	128.5	123.9	134.2	169.3
120.0	189.9	192.5	179.8	118.7	126.8	143.2	175.5	176.3	172.7
120.0	194.5	188.9	182.5	150.5	105.0	144.9	189.0	171.8	151.9
107.5	234.3	179.5	287.1	121.7	141.5	155.2	141.5	176.3	206.1
110.0	185.2	206.1	230.1	121.7	134.7	154.8	154.4	164.5	161.3
112.5	177.5	210.3	158.7	127.8	130.5	145.2	123.9	150.6	166.5
112.5	239.4	168.2	171.0	145.5	134.8	140.5	173.9	176.0	128.5
117.5	206.3	154.4	208.1	129.1	167.4	163.0	154.8	176.1	155.2
120.0	159.1	201.5	158.4	173.4	132.3	153.3	196.6	155.2	164.5
120.0	153.0	150.6	167.4	145.0	154.9	164.5	183.0	196.2	151.8
122.5	239.0	163.5	195.3	138.7	120.5	165.8	165.3	165.9	171.8
125.0	195.9	145.8	189.7	160.2	164.3	166.5	183.9	226.5	146.1
117.5	210.3	206.5	182.1	139.6	141.7	131.6	138.7	201.2	131.3
120.0	204.0	168.9	168.9	122.7	124.5	120.4	168.9	164.8	132.3
120.0	187.5	179.7	190.1	127.8	151.0	126.2	157.1	123.5	125.3
115.0	237.2	178.1	256.8	155.1	148.6	132.3	150.6	148.9	140.5
115.0	180.5	134.2	265.4	150.6	107.4	155.2	138.7	162.8	157.3
112.5	218.1	226.9	188.6	116.6	119.4	120.0	184.5	141.5	187.5
120.0	209.3	253.0	261.1	151.0	122.4	165.8	195.0	184.7	165.3
120.0	158.4	187.3	203.5	152.9	120.8	130.7	164.3	183.0	205.0
122.5	199.9	165.9	219.4	150.8	138.7	143.2	179.7	158.4	156.8
122.5	190.3	191.9	195.9	140.5	159.1	157.3	136.9	141.5	154.8
125.0	207.8	208.1	201.4	150.1	144.3	150.5	171.0	151.1	144.0
115.0	220.7	198.1	244.5	106.8	170.3	159.3	120.8	142.5	186.1
115.0	206.5	180.5	191.1	145.2	130.5	139.0	196.2	178.4	158.9
115.0	196.4	191.0	224.6	116.6	148.9	134.2	155.4	159.7	137.9
117.5	174.6	198.7	203.2	139.6	155.2	156.8	150.6	155.9	174.7
120.0	242.5	143.2	226.5	168.2	127.8	151.1	171.8	151.6	215.2
112.5	234.6	196.8	189.7	160.3	154.4	139.3	132.4	200.7	179.7
115.0	139.0	190.3	166.1	139.0	161.3	164.5	158.1	193.5	155.7
117.5	196.4	220.2	255.5	123.3	128.5	153.0	159.7	203.7	173.4
120.0	196.6	191.7	216.9	133.0	122.8	169.8	169.8	193.7	177.4
112.5	210.6	237.9	220.7	121.2	145.8	159.9	130.6	171.8	191.7
117.5	197.6	171.5	221.3	143.2	142.8	146.1	177.9	177.7	187.1
120.0	193.7	212.8	225.1	112.2	130.7	114.0	137.2	187.1	151.1
120.0	145.2	165.4	166.1	140.9	132.8	139.6	137.5	177.9	176.0
112.5	179.9	179.9	275.6	126.2	157.1	130.5	176.3	180.9	167.4
115.0	210.5	214.5	215.0	116.9	130.5	133.0	153.3	174.6	168.9
120.0	191.7	136.9	246.7	135.1	155.4	141.7	169.3	193.5	165.9
120.0	232.3	199.2	163.3	147.3	123.3	134.2	161.7	177.5	191.8
120.0	206.1	151.6	212.1	134.8	117.7	123.9	145.5	165.8	199.5
102.5	180.9	212.1	197.5	139.3	136.9	154.4	159.7	215.6	164.3
115.0	164.8	177.9	235.5	123.3	114.4	161.8	183.0	161.8	168.3
115.0	212.3	240.7	252.3	152.3	151.6	139.3	137.5	179.7	161.7
110.0	186.2	193.6	217.5	122.8	133.0	158.7	142.9	177.9	187.9
115.0	172.7	242.5	169.8	157.6	137.2	163.8	156.8	137.5	208.5
122.5	187.5	180.8	219.0	155.4	139.0	137.9	142.5	153.3	156.8
Mean	116.7	199.4	191.9	204.2	137.8	137.2	145.5	161.9	172.1	165.6
SD	4.67	26.51	31.24	32.35	15.10	15.07	13.88	20.44	21.41	20.78

Table. A.1.*R. reticulata* HL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
194.9	191.8	147.1	185.2	151.1	198.3
170.3	185.2	142.6	168.8	228.0	194.9
171.5	246.3	137.2	233.8	202.5	197.6
151.9	155.1	182.7	193.7	152.9	168.2
131.7	176.1	157.3	193.7	199.2	191.0
168.9	134.2	169.8	216.1	201.2	225.7
165.3	187.0	132.6	197.4	218.6	155.1
154.4	150.5	156.8	178.8	161.8	156.2
153.3	208.5	165.6	177.2	171.2	189.7
161.8	187.7	151.2	148.0	184.6	157.3
161.3	171.0	140.8	167.1	155.9	196.5
157.3	155.0	142.1	150.8	215.2	182.7
146.1	161.8	189.7	156.0	200.9	195.3
188.4	170.3	166.1	260.3	162.0	193.5
169.3	223.7	157.3	239.4	183.6	187.9
207.8	189.0	170.8	213.0	168.2	169.7
145.0	210.6	180.9	222.7	144.0	151.0
161.3	206.1	189.0	204.9	155.7	150.6
187.9	177.2	210.0	190.3	164.9	194.9
174.3	148.0	169.0	235.5	191.7	171.0
145.2	171.5	163.2	151.9	189.2	172.7
181.7	148.0	120.8	219.4	235.7	186.1
181.1	183.2	150.6	184.6	187.1	180.9
169.0	190.6	170.8	206.1	155.0	190.6
179.3	159.1	201.4	260.1	173.4	165.4
176.2	174.6	164.5	200.4	223.5	177.2
179.9	180.5	148.5	161.7	212.7	205.5
146.1	182.6	158.9	187.3	186.1	138.1
142.8	163.5	202.0	162.4	193.3	180.8
170.3	197.5	204.3	148.9	204.3	172.0
153.0	231.8	228.8	210.3	194.1	171.2
203.5	228.8	172.1	205.0	198.9	209.9
184.5	208.1	212.1	197.0	161.8	143.2
192.5	203.2	183.5	200.9	180.6	168.9
187.3	200.9	174.6	208.5	158.4	188.1
158.1	198.1	202.6	170.3	160.7	173.7
183.9	167.3	187.3	202.5	168.9	203.7
157.0	186.1	216.1	171.8	157.0	165.3
158.1	154.4	143.2	213.0	196.2	228.5
162.0	209.3	157.0	186.2	167.5	220.2
174.6	195.9	158.1	267.0	196.4	144.0
146.1	197.4	168.2	228.1	179.9	233.5
173.7	247.9	192.1	162.4	173.7	155.9
170.8	193.7	197.5	187.1	159.3	196.5
172.7	189.2	151.6	176.3	178.1	172.4
139.0	157.3	197.0	160.2	174.6	191.7
156.0	171.7	184.7	224.7	199.9	186.4
156.0	171.7	173.4	238.8	189.0	173.1
183.5	144.9	142.1	226.9	199.3	202.0
146.9	158.1	155.4	217.5	177.9	204.5
172.7	158.1	138.7	221.7	167.9	208.1
138.5	159.3	154.9	237.1	206.5	146.1
195.3	119.0	153.3	210.0	187.0	177.5
177.4	133.5	165.2	191.9	197.5	134.8
192.5	190.6	156.0	194.0	182.3	139.0
175.5	189.2	147.4	192.1	177.2	160.4
173.6	166.1	140.5	206.5	203.2	176.3
163.5	190.3	168.9	185.7	148.9	201.5
152.5	178.8	129.8	177.4	214.7	204.0
152.5	178.9	200.8	188.4	204.0	186.1
Mean 167.5	181.1	168.3	197.9	183.9	181.0
SD 17.02	26.15	23.98	28.40	21.52	22.93

Table. A.2.*R. reticulata* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *R. reticulata*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
107.5	151.8	134.9	164.3	106.8	111.7	116.3	132.0	122.5	177.5
110.0	152.9	137.9	136.2	93.3	106.2	108.1	127.7	148.6	143.8
112.5	111.0	145.1	112.2	85.7	107.3	91.9	140.4	112.9	146.9
112.5	150.5	104.6	128.8	99.9	106.2	93.3	148.0	161.0	107.3
115.0	122.4	109.3	143.8	99.4	108.8	120.5	157.5	146.2	133.2
105.0	107.1	130.7	103.5	104.2	119.7	108.1	107.1	113.9	116.3
110.0	118.5	126.9	110.4	119.9	96.9	113.7	111.4	154.7	116.7
110.0	137.9	132.0	125.8	106.8	95.1	107.3	147.1	117.0	142.9
115.0	130.7	116.7	136.2	92.0	91.3	101.5	114.7	161.3	148.5
105.0	113.2	123.9	145.1	117.0	123.9	96.9	124.3	113.3	140.6
107.5	137.5	130.0	131.1	116.7	113.7	109.5	122.2	90.0	137.9
112.5	148.0	124.5	134.7	106.5	109.9	102.6	102.9	134.9	136.5
110.0	144.3	108.1	126.5	104.2	91.3	120.3	188.6	121.3	128.1
112.5	137.2	134.5	116.7	94.8	111.8	98.0	123.5	125.5	120.3
112.5	105.4	100.1	121.3	117.9	95.1	120.1	133.2	98.7	146.1
105.0	137.9	137.0	115.0	117.5	116.5	119.9	124.3	128.9	127.0
107.5	179.9	114.9	130.0	95.4	119.5	119.7	102.7	115.0	135.6
112.5	139.7	108.8	103.1	118.7	100.6	116.7	118.7	117.9	146.1
115.0	136.8	120.9	121.3	108.1	111.8	115.7	115.7	113.9	112.9
112.5	108.1	116.3	127.0	102.7	106.8	114.1	119.7	133.4	132.8
112.5	144.3	123.9	149.6	104.7	111.4	99.7	125.5	122.4	116.3
117.5	126.5	129.3	142.4	98.6	104.0	116.3	107.3	130.0	148.5
107.5	176.7	118.1	125.5	112.5	112.0	95.8	140.4	106.0	141.6
112.5	116.1	130.2	128.4	86.1	113.2	99.2	130.1	136.2	126.6
112.5	133.8	153.9	117.8	106.8	92.1	106.2	150.8	146.1	137.4
112.5	133.8	109.4	129.1	122.2	116.7	87.7	135.3	153.6	116.1
115.0	154.7	135.6	129.5	109.3	110.3	106.5	127.7	117.9	145.5
115.0	121.2	115.7	131.1	88.8	107.4	89.8	136.8	142.9	112.9
117.5	149.0	114.7	119.1	101.7	98.6	118.7	155.7	150.6	138.3
107.5	109.1	117.8	106.1	113.7	123.3	92.4	110.1	123.9	127.8
107.5	117.9	123.4	130.0	111.8	117.0	123.2	168.1	161.3	142.8
117.5	121.4	107.1	117.8	120.5	119.7	101.7	154.7	149.0	114.1
105.0	157.3	122.6	127.0	115.0	104.9	116.3	151.1	197.9	124.5
107.5	137.6	144.9	107.1	103.4	101.5	87.2	121.2	141.4	137.9
112.5	105.4	140.2	123.9	106.2	96.4	101.0	127.3	123.2	115.4
102.5	136.2	129.3	139.6	126.1	119.7	112.0	98.7	137.5	109.7
107.5	136.8	135.3	117.9	121.3	96.7	126.6	174.7	143.7	199.8
112.5	125.4	126.1	145.1	112.5	104.0	125.4	128.4	161.6	148.5
102.5	176.7	135.5	113.3	98.6	112.5	119.7	139.6	151.9	143.8
107.5	122.1	166.8	121.4	87.7	116.3	112.0	169.2	149.0	110.5
115.0	132.0	123.3	123.5	112.9	115.4	101.7	129.8	130.0	129.8
102.5	152.9	105.4	139.6	120.5	91.3	126.5	164.2	155.0	142.5
110.0	110.8	137.2	115.7	109.1	119.0	104.7	132.0	121.8	133.8
112.5	107.4	149.4	125.5	116.3	108.7	113.2	170.1	147.1	122.6
117.5	121.4	126.2	134.4	108.1	110.3	95.3	141.0	147.1	140.9
102.5	156.9	150.5	146.1	114.7	120.9	109.5	125.6	171.0	116.5
102.5	125.2	130.7	147.2	105.6	100.6	87.9	144.0	125.8	138.9
105.0	129.1	127.1	140.3	93.4	91.1	105.6	109.5	154.5	104.9
115.0	133.0	113.7	114.7	91.3	111.7	110.5	148.5	108.7	155.3
105.0	119.1	157.4	103.8	107.3	117.9	119.5	160.7	140.2	132.1
107.5	111.8	120.9	96.7	104.7	87.9	104.0	140.3	110.5	111.7
112.5	102.7	148.0	124.5	119.5	93.3	104.7	113.9	111.8	134 1
107.5	121.3	148.2	127.3	115.0	112.0	119.9	137.9	136.8	104.9
110.0	106.4	101.3	128.7	115.4	112.5	114.7	144.3	119.3	124.8
115.0	160.7	109.1	147.1	117.8	116.3	104.0	124.5	116.9	138.5
107.5	152.9	130.0	99.2	116.3	112.0	110.3	139.2	107.1	105.6
110.0	112.0	106.2	125.4	114.7	116.3	111.4	124.5	103.5	151.9
105.0	130.4	139.5	119.0	116.9	102.7	96.3	136.8	122.4	131.6
110.0	104.0	108.7	122.2	104.0	104.0	88.8	113.8	116.5	113.9
110.0	148.0	133.6	125.8	99.4	123.2	106.5	133.8	112.0	122.2
Mean 110.1	131.8	126.7	126.0	107.6	108.1	107.8	134.1	132.3	131.9
SD 4.18	19.15	14.84	13.52	10.02	9.50	10.63	19.34	20.29	17.27

Table. A.2.*R. reticulata* HL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
163.8	157.6	146.7	178.8	127.3	165.0
136.2	117.7	126.5	128.1	150.0	139.7
137.5	149.7	172.1	140.1	125.3	131.6
155.1	173.9	121.9	156.9	119.3	130.5
150.8	148.8	135.7	172.4	139.7	153.8
146.9	149.7	165.9	128.3	163.6	137.4
143.7	160.2	121.9	128.5	155.2	130.5
161.8	144.7	119.2	143.2	154.6	143.4
155.1	121.4	107.0	133.9	123.5	143.2
157.5	170.6	126.8	115.3	147.2	171.3
111.3	171.3	123.5	139.9	149.7	159.1
119.9	154.2	127.3	140.4	139.9	124.1
136.2	160.8	151.9	100.2	156.9	201.4
116.9	143.5	113.3	114.3	114.3	195.1
105.4	163.6	143.5	157.0	105.2	214.8
145.3	123.2	114.3	162.9	159.6	167.7
139.7	117.5	135.2	194.5	130.5	131.2
141.9	115.9	147.5	151.4	154.0	140.1
112.8	117.3	197.6	111.6	125.7	160.2
102.9	115.9	113.3	134.3	119.0	148.1
121.3	123.1	129.6	125.0	111.0	192.1
129.3	141.2	140.5	134.5	118.6	127.3
118.6	150.1	155.4	131.7	129.7	139.1
139.6	108.9	139.2	109.8	115.8	149.5
121.3	141.4	154.0	102.5	137.4	133.1
130.9	155.8	133.3	134.5	137.4	143.1
163.1	118.7	136.4	109.1	140.2	134.8
140.3	141.2	118.2	143.3	106.5	142.3
141.9	105.9	116.1	131.2	118.7	120.9
115.0	116.6	116.1	109.8	111.4	137.0
173.8	130.1	151.9	116.7	133.1	132.6
187.5	143.2	153.4	113.3	161.6	145.7
125.7	128.3	196.7	131.7	174.0	131.7
126.8	185.4	117.5	133.1	165.0	143.2
169.6	133.9	123.5	190.9	165.3	168.7
142.4	121.0	120.6	120.1	160.3	130.5
184.1	152.6	163.8	174.5	141.8	139.7
153.5	127.3	120.9	106.5	157.7	136.7
179.9	143.3	162.0	118.7	133.0	133.3
147.2	178.0	201.8	105.8	160.4	162.5
164.2	132.6	184.6	116.6	202.8	129.6
167.0	140.1	150.3	108.3	122.2	145.6
140.5	147.5	136.1	143.2	143.1	153.4
112.7	132.9	165.3	148.5	167.8	150.9
116.6	148.5	128.3	128.3	143.5	133.3
132.9	158.4	122.2	133.3	139.7	127.3
142.3	143.8	138.8	124.8	137.9	139.8
115.8	140.5	114.3	142.4	120.6	143.1
147.7	167.1	150.9	124.1	134.8	166.7
129.5	128.5	116.1	147.9	146.6	132.9
124.8	148.9	136.9	140.1	139.8	132.6
119.3	158.9	161.9	107.0	145.7	134.3
116.1	162.0	146.9	112.7	168.2	126.5
133.0	172.3	114.3	120.9	139.8	122.7
125.4	158.6	130.5	131.6	164.7	177.0
144.9	112.7	145.0	124.8	160.5	158.1
136.2	153.1	120.6	121.9	142.0	146.6
127.9	148.9	131.2	141.4	156.4	133.1
106.8	119.3	186.7	152.2	149.5	143.7
133.1	127.9	118.7	140.2	125.4	152.2
Mean 138.1	142.1	139.4	133.1	141.5	146.4
SD 20.05	19.11	22.89	20.94	19.21	19.58

Table. A.1.*R. reticulata* LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *R. reticulata*, cultured at Low Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
110.0	219.0	252.6	153.3	149.2	138.7	133.2	212.7	181.7	210.3
112.5	262.6	240.9	169.3	129.7	134.8	127.2	196.2	219.2	168.9
115.0	219.1	191.0	226.9	141.9	132.3	156.8	194.9	198.3	178.8
117.5	223.7	191.8	195.9	159.7	112.7	129.7	157.1	177.7	154.4
102.5	161.2	202.6	190.3	125.5	124.1	156.0	206.7	197.6	205.0
117.5	172.4	174.3	187.7	128.8	127.8	141.9	215.6	188.1	168.3
117.5	181.6	197.4	172.4	120.5	155.1	147.3	177.2	182.1	166.1
122.5	271.2	282.8	209.3	143.2	123.3	126.8	204.9	129.8	177.5
112.5	213.8	153.0	174.6	130.7	128.5	138.7	213.8	179.4	164.5
115.0	190.5	251.9	180.9	147.4	134.2	158.7	216.4	140.5	208.1
117.5	194.2	188.1	169.5	129.2	151.6	146.1	184.5	167.4	165.2
122.5	191.7	157.6	223.0	116.3	126.2	160.7	226.5	195.3	174.2
117.5	200.1	189.7	225.2	132.3	154.4	139.3	189.9	155.2	191.8
117.5	203.5	151.9	155.2	160.4	151.2	128.5	202.6	196.4	226.9
120.0	189.9	192.5	179.8	118.7	126.8	143.2	200.8	193.5	209.3
120.0	194.5	188.9	182.5	150.5	105.0	144.9	170.8	197.9	159.6
107.5	234.3	179.5	287.1	121.7	141.5	155.2	181.7	183.9	159.1
110.0	185.2	206.1	230.1	121.7	134.7	154.8	204.0	212.1	155.4
112.5	177.5	210.3	158.7	127.8	130.5	145.2	195.9	151.6	165.3
112.5	239.4	168.2	171.0	145.5	134.8	140.5	210.3	167.9	182.3
117.5	206.3	154.4	208.1	129.1	167.4	163.0	168.2	143.2	170.3
120.0	159.1	201.5	158.4	173.4	132.3	153.3	221.3	204.0	195.9
120.0	153.0	150.6	167.4	145.0	154.9	164.5	193.7	199.9	159.6
122.5	239.0	163.5	195.3	138.7	120.5	165.8	264.9	204.0	144.9
125.0	195.9	145.8	189.7	160.2	164.3	166.5	171.0	187.2	155.0
117.5	210.3	206.5	182.1	139.6	141.7	131.6	199.9	194.9	141.5
120.0	204.0	168.9	168.9	122.7	124.5	120.4	228.5	180.9	138.1
120.0	187.5	179.7	190.1	127.8	151.0	126.2	181.8	186.2	148.9
115.0	237.2	178.1	256.8	155.1	148.6	132.3	210.5	190.5	139.3
115.0	180.5	134.2	265.4	150.6	107.4	155.2	182.7	200.4	155.9
112.5	218.1	226.9	188.6	116.6	119.4	120.0	193.5	177.7	197.5
120.0	209.3	253.0	261.1	151.0	122.4	165.8	202.6	180.5	162.4
120.0	158.4	187.3	203.5	152.9	120.8	130.7	207.1	177.4	200.4
122.5	199.9	165.9	219.4	150.8	138.7	143.2	202.1	155.4	189.9
122.5	190.3	191.9	195.9	140.5	159.1	157.3	200.8	218.3	168.9
125.0	207.8	208.1	201.4	150.1	144.3	150.5	225.4	211.6	156.8
115.0	220.7	198.1	244.5	106.8	170.3	159.3	215.0	155.2	219.7
115.0	206.5	180.5	191.1	145.2	130.5	139.0	206.5	183.2	205.5
115.0	196.4	191.0	224.6	116.6	148.9	134.2	219.4	196.6	175.6
117.5	174.6	198.7	203.2	139.6	155.2	156.8	196.2	164.5	157.6
120.0	242.5	143.2	226.5	168.2	127.8	151.1	188.9	167.9	177.4
112.5	234.6	196.8	189.7	160.3	154.4	139.3	210.3	164.5	207.1
115.0	139.0	190.3	166.1	139.0	161.3	164.5	219.3	176.1	233.4
117.5	196.4	220.2	255.5	123.3	128.5	153.0	232.6	169.3	173.4
120.0	196.6	191.7	216.9	133.0	122.8	169.8	241.3	172.1	212.5
112.5	210.6	237.9	220.7	121.2	145.8	159.9	211.1	173.7	182.3
117.5	197.6	171.5	221.3	143.2	142.8	146.1	147.3	145.0	158.4
120.0	193.7	212.8	225.1	112.2	130.7	114.0	223.9	151.9	195.9
120.0	145.2	165.4	166.1	140.9	132.8	139.6	182.3	200.6	141.5
112.5	179.9	179.9	275.6	126.2	157.1	130.5	213.7	153.0	164.8
115.0	210.5	214.5	215.0	116.9	130.5	133.0	228.8	183.0	137.2
120.0	191.7	136.9	246.7	135.1	155.4	141.7	226.5	139.9	134.2
120.0	232.3	199.2	163.3	147.3	123.3	134.2	208.1	163.3	144.0
120.0	206.1	151.6	212.1	134.8	117.7	123.9	219.3	191.5	152.5
102.5	180.9	212.1	197.5	139.3	136.9	154.4	210.6	172.4	151.8
115.0	164.8	177.9	235.5	123.3	114.4	161.8	222.4	187.7	140.5
115.0	212.3	240.7	252.3	152.3	151.6	139.3	210.6	180.6	154.9
110.0	186.2	193.6	217.5	122.8	133.0	158.7	211.3	210.6	187.5
115.0	172.7	242.5	169.8	157.6	137.2	163.8	232.7	205.8	142.6
122.5	187.5	180.8	219.0	155.4	139.0	137.9	226.5	225.1	134.2
Mean	116.7	199.4	191.9	204.2	137.8	137.2	145.5	205.4	181.0	171.7
SD	4.67	26.51	31.24	32.35	15.10	15.07	13.88	20.60	21.44	25.13

Table. A.1.*R. reticulata* LL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
132.4	155.4	173.7	178.1	194.9	171.0
174.2	129.8	151.6	138.5	182.7	153.0
148.5	140.9	132.8	201.5	212.7	172.1
187.1	171.8	133.5	214.5	168.8	205.3
164.5	132.6	196.2	165.3	214.8	192.5
124.1	142.5	136.9	167.5	181.8	217.9
139.3	174.6	165.8	175.3	191.5	206.5
148.9	228.1	138.5	194.9	184.5	201.2
166.5	147.3	165.8	209.8	164.5	220.2
161.8	173.1	139.9	173.9	159.1	147.8
134.8	213.0	155.4	164.2	151.2	188.6
187.1	151.9	165.3	180.8	160.3	193.7
132.3	187.0	164.3	153.3	196.4	203.9
157.5	145.5	191.9	183.6	175.5	216.3
199.5	166.1	161.3	205.0	187.9	220.5
168.9	181.7	158.2	190.1	173.7	219.0
173.4	155.4	138.5	172.1	175.3	150.6
155.4	173.6	170.4	142.6	155.7	161.8
158.4	139.6	110.3	188.1	150.1	183.6
173.4	184.5	178.8	178.1	182.3	175.5
169.5	176.1	186.1	172.4	196.4	191.0
173.4	144.3	140.9	196.6	168.3	209.9
178.1	148.9	161.2	141.5	151.8	208.3
148.0	182.5	191.8	163.5	169.8	199.9
151.6	162.0	130.6	167.7	187.0	151.6
143.2	166.1	164.9	171.2	223.7	171.7
168.8	174.3	167.4	174.6	178.0	150.8
158.4	150.6	127.1	159.3	210.6	166.1
136.9	165.2	181.8	164.8	176.1	222.8
155.2	150.6	149.2	166.1	145.6	181.8
167.1	198.1	188.6	183.9	161.7	180.8
178.9	185.8	155.1	181.6	159.6	188.9
150.6	161.3	208.1	205.7	197.0	165.3
155.0	158.4	157.0	224.3	207.8	205.3
178.0	148.9	166.1	229.2	180.9	161.4
159.1	164.5	172.1	149.4	152.3	210.6
138.7	133.5	169.3	171.2	146.1	228.3
155.1	141.7	193.0	155.4	189.0	233.8
148.4	182.7	159.6	154.4	154.9	169.0
130.6	153.3	187.3	165.9	191.7	219.4
154.4	184.9	137.2	214.7	155.7	191.7
151.9	189.9	153.3	187.2	143.2	206.3
169.3	151.1	155.1	183.9	210.5	184.1
128.5	135.1	147.3	171.0	164.9	183.9
158.4	160.7	179.5	189.0	161.4	166.1
198.1	165.3	142.8	208.9	173.4	198.1
182.1	158.1	196.6	151.6	201.2	166.5
158.2	136.9	155.7	190.1	147.8	183.9
170.8	140.8	178.1	158.1	144.9	189.7
169.3	176.2	179.4	159.1	169.0	167.1
160.2	170.3	148.9	170.8	152.5	189.0
177.4	168.9	177.2	200.9	150.5	208.5
165.3	167.1	167.1	198.7	128.5	200.3
192.5	181.8	183.7	175.3	177.7	191.7
183.9	157.3	164.9	161.3	132.6	151.6
206.3	158.7	178.4	183.2	182.7	171.2
132.6	143.2	163.8	219.0	149.4	190.5
147.1	175.5	155.4	189.7	139.0	171.5
160.2	172.1	139.6	178.8	137.2	138.7
136.9	161.8	163.5	137.2	182.5	238.8
Mean 160.6	163.3	162.6	178.4	171.9	188.4
SD 18.62	19.50	19.94	21.43	22.37	23.72

Table. A.2.*R. reticulata* LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *R. reticulata*, cultured at Low Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
107.5	151.8	134.9	164.3	106.8	111.7	116.3	161.9	165.9	157.1
110.0	152.9	137.9	136.2	93.3	106.2	108.1	160.3	169.6	120.5
112.5	111.0	145.1	112.2	85.7	107.3	91.9	132.9	152.3	161.6
112.5	150.5	104.6	128.8	99.9	106.2	93.3	184.2	176.7	167.1
115.0	122.4	109.3	143.8	99.4	108.8	120.5	181.9	158.9	166.0
105.0	107.1	130.7	103.5	104.2	119.7	108.1	157.9	162.4	166.4
110.0	118.5	126.9	110.4	119.9	96.9	113.7	143.8	137.4	144.8
110.0	137.9	132.0	125.8	106.8	95.1	107.3	164.5	183.8	164.5
115.0	130.7	116.7	136.2	92.0	91.3	101.5	135.7	140.9	135.7
105.0	113.2	123.9	145.1	117.0	123.9	96.9	161.6	150.9	152.4
107.5	137.5	130.0	131.1	116.7	113.7	109.5	175.5	158.6	139.7
112.5	148.0	124.5	134.7	106.5	109.9	102.6	171.0	194.3	145.6
110.0	144.3	108.1	126.5	104.2	91.3	120.3	162.4	178.8	144.9
112.5	137.2	134.5	116.7	94.8	111.8	98.0	206.0	139.8	173.9
112.5	105.4	100.1	121.3	117.9	95.1	120.1	151.4	176.8	177.0
105.0	137.9	137.0	115.0	117.5	116.5	119.9	162.5	134.4	136.3
107.5	179.9	114.9	130.0	95.4	119.5	119.7	163.6	159.7	126.1
112.5	139.7	108.8	103.1	118.7	100.6	116.7	190.9	149.7	171.2
115.0	136.8	120.9	121.3	108.1	111.8	115.7	171.4	125.0	161.2
112.5	108.1	116.3	127.0	102.7	106.8	114.1	182.7	149.7	126.2
112.5	144.3	123.9	149.6	104.7	111.4	99.7	173.8	149.4	162.5
117.5	126.5	129.3	142.4	98.6	104.0	116.3	172.8	171.5	167.0
107.5	176.7	118.1	125.5	112.5	112.0	95.8	167.8	157.1	111.0
112.5	116.1	130.2	128.4	86.1	113.2	99.2	157.1	138.0	145.6
112.5	133.8	153.9	117.8	106.8	92.1	106.2	160.2	146.4	136.2
112.5	133.8	109.4	129.1	122.2	116.7	87.7	161.6	163.7	156.4
115.0	154.7	135.6	129.5	109.3	110.3	106.5	151.4	158.6	126.8
115.0	121.2	115.7	131.1	88.8	107.4	89.8	116.1	141.8	132.4
117.5	149.0	114.7	119.1	101.7	98.6	118.7	148.0	143.8	178.9
107.5	109.1	117.8	106.1	113.7	123.3	92.4	131.2	175.5	137.0
107.5	117.9	123.4	130.0	111.8	117.0	123.2	144.7	152.3	147.5
117.5	121.4	107.1	117.8	120.5	119.7	101.7	149.5	164.5	143.8
105.0	157.3	122.6	127.0	115.0	104.9	116.3	143.2	188.0	120.1
107.5	137.6	144.9	107.1	103.4	101.5	87.2	154.0	139.2	138.0
112.5	105.4	140.2	123.9	106.2	96.4	101.0	181.8	154.1	138.1
102.5	136.2	129.3	139.6	126.1	119.7	112.0	174.7	186.0	158.9
107.5	136.8	135.3	117.9	121.3	96.7	126.6	186.7	143.8	131.7
112.5	125.4	126.1	145.1	112.5	104.0	125.4	173.2	173.8	115.9
102.5	176.7	135.5	113.3	98.6	112.5	119.7	161.7	142.4	131.2
107.5	122.1	166.8	121.4	87.7	116.3	112.0	154.4	150.1	119.7
115.0	132.0	123.3	123.5	112.9	115.4	101.7	133.9	155.5	137.4
102.5	152.9	105.4	139.6	120.5	91.3	126.5	146.7	158.9	198.2
110.0	110.8	137.2	115.7	109.1	119.0	104.7	169.6	150.9	129.0
112.5	107.4	149.4	125.5	116.3	108.7	113.2	154.5	152.3	116.5
117.5	121.4	126.2	134.4	108.1	110.3	95.3	170.3	165.0	114.3
102.5	156.9	150.5	146.1	114.7	120.9	109.5	157.1	151.4	132.4
102.5	125.2	130.7	147.2	105.6	100.6	87.9	184.3	138.0	140.1
105.0	129.1	127.1	140.3	93.4	91.1	105.6	163.9	157.1	111.0
115.0	133.0	113.7	114.7	91.3	111.7	110.5	163.6	164.7	116.1
105.0	119.1	157.4	103.8	107.3	117.9	119.5	138.4	192.1	119.5
107.5	111.8	120.9	96.7	104.7	87.9	104.0	136.4	171.0	115.3
112.5	102.7	148.0	124.5	119.5	93.3	104.7	137.6	153.8	120.6
107.5	121.3	148.2	127.3	115.0	112.0	119.9	163.1	181.2	137.4
110.0	106.4	101.3	128.7	115.4	112.5	114.7	177.8	141.8	139.1
115.0	160.7	109.1	147.1	117.8	116.3	104.0	150.6	183.5	148.0
107.5	152.9	130.0	99.2	116.3	112.0	110.3	148.6	169.6	163.7
110.0	112.0	106.2	125.4	114.7	116.3	111.4	173.8	183.8	139.7
105.0	130.4	139.5	119.0	116.9	102.7	96.3	179.3	143.8	211.6
110.0	104.0	108.7	122.2	104.0	104.0	88.8	144.6	163.7	120.9
110.0	148.0	133.6	125.8	99.4	123.2	106.5	143.3	130.2	131.5
Mean	110.1	131.8	126.7	126.0	107.6	108.1	107.8	160.4	158.6	143.0
SD	4.18	19.15	14.84	13.52	10.02	9.50	10.63	17.03	16.30	21.53

Table. A.2.*R. reticulata* LL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
187.6	177.9	187.5	140.5	125.0	154.0
140.1	160.2	167.1	123.5	156.9	150.0
111.3	135.0	187.5	134.3	175.9	118.2
111.6	146.7	143.4	115.3	174.1	112.4
159.6	126.5	144.8	152.3	148.5	140.1
142.4	150.1	139.2	123.5	137.1	112.7
133.3	138.4	140.1	161.9	163.7	165.6
125.7	153.4	152.3	167.0	166.0	159.6
129.6	140.5	162.0	135.7	139.9	124.1
134.0	161.6	139.1	117.4	126.2	120.8
138.8	140.4	126.8	167.1	124.1	137.4
138.4	122.9	148.0	167.9	188.7	129.6
140.1	127.8	159.7	102.5	153.5	121.0
135.7	178.3	141.4	151.4	123.1	100.2
127.6	157.9	147.5	133.1	177.8	150.9
123.1	129.6	135.0	110.4	159.4	139.0
139.7	154.7	128.3	181.4	128.8	149.8
110.6	171.3	160.5	156.8	143.1	145.6
121.9	124.4	151.3	132.6	126.8	150.0
123.5	133.9	120.5	163.9	157.3	137.1
140.4	164.0	151.3	108.3	176.7	115.7
116.1	157.4	149.7	137.9	107.8	135.7
115.7	126.1	145.7	177.8	149.7	124.8
116.1	149.7	126.2	138.8	146.4	146.7
126.5	99.1	119.7	175.3	139.9	150.9
122.9	109.9	198.5	114.5	143.8	126.8
106.2	125.7	147.2	119.0	138.8	144.6
123.5	133.3	208.6	109.1	139.1	122.0
126.5	116.1	136.9	107.0	147.2	118.7
132.6	126.1	139.1	109.1	144.9	116.1
153.2	177.5	173.8	165.2	180.1	111.3
124.8	184.5	164.0	121.0	177.9	211.4
153.4	141.8	154.4	166.5	146.6	119.0
138.8	163.1	211.6	167.1	145.6	131.5
119.7	145.6	154.3	197.2	138.8	135.0
130.2	125.0	167.1	138.4	139.7	196.2
138.8	163.1	127.3	143.7	162.6	167.0
141.2	195.2	168.2	136.4	137.9	163.1
113.9	176.8	193.4	150.0	111.6	134.3
143.3	128.6	147.5	146.4	103.2	119.7
131.6	154.3	153.8	119.0	128.1	126.1
109.1	134.4	133.6	118.9	134.8	113.3
136.3	150.9	126.1	122.0	194.3	139.2
136.4	129.0	170.4	122.9	109.1	140.4
174.4	134.1	115.8	106.0	165.9	130.2
127.8	122.9	138.0	125.1	114.3	107.6
146.8	149.5	163.7	172.8	200.0	160.3
135.2	129.9	129.7	156.4	143.7	137.2
121.0	142.4	126.8	105.7	155.8	112.4
112.1	176.5	126.5	140.1	112.9	140.5
153.8	157.9	157.9	146.4	145.6	132.4
115.8	147.5	177.1	158.6	158.9	122.7
121.0	116.6	134.1	147.5	161.1	147.5
185.5	118.1	125.7	123.5	136.3	132.7
144.9	118.1	175.3	120.6	119.5	155.6
163.2	143.5	146.6	144.7	147.2	141.4
137.4	130.5	150.0	126.7	127.8	112.1
128.3	115.7	129.0	131.6	132.7	116.3
137.4	115.7	122.2	110.0	148.0	126.5
140.5	126.5	115.8	111.0	112.5	123.5
Mean 133.6	142.6	149.7	137.9	145.4	135.5
SD 16.99	20.80	22.45	22.94	21.99	20.30

Table. A.1. *P. lutheri* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *P. lutheri*, cultured at High Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
98.7	140.0	115.3	137.8	114.2	112.0	121.6	180.9	178.6	152.0
104.4	123.2	130.0	141.3	112.7	109.3	115.6	157.0	197.3	166.5
101.2	114.2	126.5	131.9	112.8	119.7	114.9	146.1	160.9	143.5
100.6	123.5	114.8	116.2	108.0	122.3	112.5	187.0	135.9	146.9
97.8	114.7	107.5	116.8	115.5	109.6	114.3	180.9	173.0	141.9
106.0	134.9	132.6	115.6	111.9	118.3	113.9	155.1	145.2	178.9
98.0	130.4	129.1	127.9	109.6	108.6	112.0	161.9	152.8	155.1
96.9	124.2	127.0	137.4	112.2	107.7	117.0	148.2	163.7	154.6
105.5	120.9	125.7	137.8	115.3	110.8	112.2	175.2	165.8	161.0
102.0	131.1	126.3	129.1	124.2	98.8	92.4	151.5	172.1	169.6
100.3	124.1	113.7	132.4	115.2	98.8	123.6	160.8	169.5	174.2
101.6	125.8	109.9	127.8	116.5	117.9	115.3	148.1	167.9	152.2
100.7	114.7	106.1	121.8	117.6	113.2	117.7	157.8	154.5	153.3
104.7	112.5	120.4	117.7	112.9	113.5	116.8	164.9	169.7	147.9
99.5	105.8	138.9	127.4	114.8	107.8	119.3	137.4	149.7	155.1
100.8	123.3	136.3	110.8	115.6	112.8	110.7	144.5	159.1	147.7
107.1	121.1	135.7	110.4	114.7	115.6	104.4	164.3	188.8	153.2
101.0	130.9	124.5	130.1	110.7	119.8	114.9	149.1	159.8	157.8
103.4	152.8	141.1	109.6	117.6	116.2	117.9	104.3	149.2	170.4
103.7	122.1	117.7	130.5	109.0	113.6	116.1	161.5	157.8	166.5
88.8	120.8	121.0	116.2	109.6	112.2	118.9	143.5	156.1	139.0
92.2	114.7	135.4	116.0	117.6	117.0	117.0	158.0	172.1	140.6
107.4	130.2	135.4	112.5	111.4	110.8	109.0	142.4	173.9	155.1
94.3	119.8	132.4	122.3	113.6	114.8	118.6	152.7	131.9	166.4
97.5	121.6	128.6	110.8	108.0	115.8	116.0	138.0	170.1	165.5
104.8	133.5	139.7	119.4	117.0	111.9	113.5	152.3	117.1	151.2
101.2	110.7	121.7	130.8	113.8	114.8	119.9	166.4	146.5	168.4
111.4	151.5	130.1	114.9	106.1	114.8	118.6	138.8	187.9	164.3
104.3	127.8	119.8	123.4	120.1	105.2	114.8	141.5	180.7	159.4
102.3	107.5	131.6	122.6	117.9	124.3	115.8	156.2	146.3	166.0
99.4	146.5	137.7	149.1	113.7	114.3	112.0	166.8	157.9	160.1
99.7	110.8	153.0	127.8	109.9	125.7	109.0	165.5	138.0	173.9
96.0	114.6	123.2	116.1	115.3	111.9	120.1	136.3	168.4	149.4
101.0	122.3	140.7	119.3	116.8	117.2	113.5	147.3	142.4	171.8
95.3	120.6	126.3	113.9	114.9	124.1	112.8	156.2	168.4	159.9
96.0	123.7	126.5	123.2	104.4	99.1	104.0	141.5	166.9	174.5
105.7	109.0	152.5	134.5	117.0	103.3	118.0	163.6	165.5	178.3
104.7	113.6	133.5	114.8	112.2	115.6	117.9	151.2	160.6	170.8
105.7	135.0	111.7	122.5	101.4	118.8	112.0	163.9	163.1	160.6
103.6	101.9	112.0	132.6	106.4	106.3	109.0	164.3	160.6	151.7
95.1	125.2	119.8	109.0	123.5	108.0	112.0	146.3	164.2	149.9
110.9	120.5	115.8	136.0	122.8	100.9	107.5	162.9	168.8	141.1
107.7	130.6	143.4	129.8	120.6	103.6	113.5	173.0	151.4	149.6
106.8	101.7	124.3	127.9	113.2	103.4	104.4	152.9	135.9	136.0
106.6	138.5	123.4	120.6	115.2	103.9	115.3	169.6	172.1	135.3
100.0	121.8	138.8	109.9	119.9	115.6	115.5	162.7	138.5	140.7
95.1	116.2	119.9	125.2	103.9	100.4	113.0	179.3	124.1	165.5
109.6	126.2	118.1	123.6	114.5	109.0	113.8	148.1	127.9	175.1
101.2	109.9	120.8	120.6	114.2	111.9	114.5	152.8	123.8	156.5
98.2	117.7	115.6	123.5	95.4	121.1	117.9	155.9	192.9	158.4
103.2	106.2	119.4	128.3	107.8	105.2	117.9	168.5	149.7	169.3
105.6	123.8	113.0	131.6	119.3	117.6	117.6	156.6	157.5	163.6
99.6	124.3	123.5	112.2	112.2	106.5	114.9	138.0	111.9	152.5
104.5	122.1	142.0	114.8	117.6	111.9	114.9	174.4	160.9	167.9
110.1	139.7	127.8	149.6	128.3	96.2	119.8	160.9	113.8	200.8
115.2	127.0	149.7	115.5	117.7	108.0	116.1	157.1	166.9	152.9
100.1	118.3	121.0	111.9	102.5	115.6	109.9	124.1	187.9	167.3
95.7	124.5	121.7	132.4	115.3	106.2	110.7	128.4	158.2	167.3
111.9	123.8	114.8	112.5	117.6	112.0	109.3	124.4	138.6	148.8
91.6	122.1	172.5	135.0	113.0	107.8	116.1	138.2	177.8	170.7
Mean	101.9	122.8	127.3	123.7	113.7	111.5	114.1	154.3	157.8	159.1
SD	5.25	10.71	12.29	9.80	5.68	6.74	4.94	15.11	19.12	12.34

Table. A.1. *P. lutheri* HL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
170.3	154.5	179.1	143.4	166.9	164.2
202.4	170.6	172.6	148.8	171.7	142.9
172.1	154.5	164.6	157.1	160.9	168.7
177.4	180.6	170.6	152.5	154.4	153.4
136.1	160.7	143.9	178.1	153.0	152.0
148.1	142.9	175.9	167.9	144.7	157.9
176.8	183.6	158.8	170.4	158.4	155.1
156.8	187.3	158.4	154.2	146.6	164.2
171.2	167.3	161.7	163.5	154.5	159.9
157.0	166.8	206.6	150.1	149.9	149.2
155.5	166.8	171.2	148.9	162.8	164.6
175.9	183.0	165.1	128.9	154.1	128.9
182.2	166.4	176.5	184.7	160.9	169.4
171.5	164.2	169.4	171.2	166.0	162.1
165.1	181.6	155.1	165.5	146.4	160.9
165.2	166.8	166.4	174.5	146.6	150.3
170.6	186.0	163.1	139.4	157.8	159.0
174.2	200.9	169.9	146.3	153.1	149.2
164.4	158.2	189.2	136.3	167.1	156.3
150.3	165.7	159.1	140.1	174.4	153.8
181.4	176.1	179.1	151.2	160.3	156.0
178.1	170.6	158.2	145.6	167.7	159.5
175.1	144.1	169.8	175.2	142.4	150.2
152.0	169.3	165.1	152.8	154.2	161.9
165.5	193.7	171.9	150.2	147.0	143.5
178.9	179.5	164.7	176.5	155.1	170.8
159.0	171.2	167.3	160.7	145.4	167.1
148.4	163.1	166.6	178.7	176.9	155.1
181.7	163.5	174.4	157.2	145.1	156.6
183.3	168.8	175.3	163.5	165.5	169.2
175.1	198.1	185.1	156.1	147.7	153.4
162.4	171.1	163.9	152.7	154.2	156.7
175.0	192.5	167.6	159.1	156.0	171.6
147.2	163.1	168.4	150.6	146.6	137.7
150.2	174.2	164.3	160.5	147.3	185.4
164.7	168.5	153.6	160.6	156.5	155.1
158.1	172.1	152.3	173.4	154.9	157.1
177.9	166.8	155.1	150.2	187.7	147.9
133.7	176.7	196.4	151.5	148.1	146.9
192.9	174.0	165.1	163.1	161.6	164.1
175.2	174.6	159.1	153.8	170.8	146.5
165.7	155.1	181.8	191.0	155.5	140.7
162.7	188.7	195.6	143.9	160.6	165.8
179.1	195.8	150.2	160.6	158.1	156.6
166.3	191.1	185.0	156.0	146.3	139.4
186.1	156.1	162.7	158.2	181.1	140.2
178.4	172.7	156.1	145.4	152.1	154.7
154.4	154.2	169.6	175.0	159.9	149.3
189.5	169.3	166.3	161.5	150.8	160.9
163.0	189.9	169.7	180.5	143.9	146.1
147.4	166.5	147.7	168.7	168.4	149.7
155.2	191.4	168.1	153.3	155.9	159.8
167.9	169.9	174.4	172.7	149.2	156.2
166.2	186.7	145.4	173.2	154.2	164.6
168.1	178.7	173.3	158.4	162.5	162.4
150.8	174.2	187.9	177.6	163.3	151.9
177.3	194.8	189.5	160.7	157.9	141.3
169.4	161.5	191.8	163.0	154.7	138.0
151.5	184.4	212.5	178.6	148.6	166.0
146.6	180.6	169.8	158.2	181.1	158.2
Mean 166.7	173.4	170.0	160.0	157.4	155.6
SD 13.71	13.09	13.80	12.80	10.05	10.04

Table. A.2. *P. lutheri* HL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *P. lutheri*, cultured at High Light conditions and f/2 medium (f/2). Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
94.3	120.7	124.2	114.3	123.9	117.3	110.8	146.5	117.7	156.1
96.5	133.5	118.7	107.6	109.1	98.8	86.3	126.3	122.2	137.3
93.7	95.9	136.0	103.4	96.3	99.8	93.0	128.0	139.5	100.6
100.9	118.5	116.1	97.8	112.1	92.7	94.1	107.7	148.2	112.1
90.6	116.2	97.6	110.4	103.5	118.2	97.8	141.3	154.2	110.0
97.9	104.0	105.5	104.4	95.4	76.4	101.8	136.1	125.8	125.8
93.4	108.9	108.7	94.8	94.9	107.7	91.4	130.0	116.6	158.1
93.9	109.2	114.4	100.9	97.8	87.8	92.0	140.1	157.9	123.9
90.3	113.5	94.8	109.1	102.8	97.5	93.5	148.1	142.5	112.3
110.6	90.1	103.7	97.6	93.5	82.8	91.5	161.0	132.2	109.7
96.5	116.3	107.9	96.2	101.1	88.7	104.5	121.8	136.0	122.1
105.6	116.6	106.4	100.5	86.9	93.4	92.4	149.5	122.7	119.9
96.1	106.2	129.2	109.3	97.8	96.8	95.6	160.2	149.8	107.9
113.1	101.4	114.3	95.4	95.5	88.7	95.4	138.2	120.2	112.0
93.8	148.5	119.9	99.1	113.7	108.5	96.4	134.9	133.8	94.9
95.0	130.0	99.5	128.2	90.6	117.1	97.6	104.8	136.4	137.9
99.2	87.2	115.0	118.0	92.6	106.8	95.4	149.8	156.2	132.5
94.3	107.2	101.1	110.4	106.7	88.4	86.1	192.5	161.7	102.6
103.3	109.1	115.0	132.2	87.4	101.9	100.4	153.5	138.5	106.5
102.3	97.6	94.1	127.5	95.1	91.2	100.5	110.5	137.8	138.5
109.9	107.6	98.0	93.1	99.8	95.8	103.5	123.7	151.6	140.0
92.0	105.4	103.0	111.9	112.3	92.7	94.7	140.0	102.4	101.5
88.1	103.5	107.2	109.4	94.9	99.0	91.2	122.6	155.6	125.5
82.1	95.1	116.6	117.7	92.6	85.2	88.7	156.1	124.0	118.3
93.5	112.0	97.5	101.5	109.4	87.3	89.9	125.5	136.1	131.0
107.1	100.3	102.6	96.2	98.0	92.4	94.9	132.2	131.5	134.2
101.3	77.3	97.1	100.0	86.8	103.7	95.6	131.7	122.6	136.4
83.6	88.4	110.4	98.6	87.9	109.9	105.3	136.1	127.5	111.5
98.3	103.5	101.5	94.8	97.6	89.9	109.6	136.1	106.8	135.8
97.6	91.7	105.1	132.1	93.7	90.1	103.5	140.0	134.0	120.2
101.1	142.1	119.4	160.4	109.1	92.6	115.1	155.7	180.6	117.7
107.8	103.7	100.9	105.3	109.9	103.7	95.0	120.7	138.2	121.1
90.3	97.6	116.1	113.7	111.8	103.7	97.5	146.6	113.7	127.9
112.2	94.7	132.2	100.3	95.8	93.8	95.4	137.4	129.8	113.7
92.9	120.7	118.0	117.7	103.5	87.5	102.6	128.6	157.0	124.0
86.2	103.5	127.9	115.4	105.9	92.7	97.1	123.8	165.9	105.8
109.4	101.1	114.9	111.7	89.9	95.4	103.3	130.5	111.8	134.9
104.8	117.1	124.0	93.7	91.2	108.9	100.4	129.4	138.0	121.6
97.7	117.1	90.8	123.3	104.0	82.6	91.9	164.4	126.3	106.5
88.9	109.3	115.7	105.4	107.5	104.0	109.7	158.0	127.5	92.2
95.6	92.7	100.5	124.2	115.7	95.8	107.6	134.9	133.9	106.8
92.6	83.3	92.6	107.2	109.1	87.3	94.7	121.1	123.9	97.1
82.6	104.3	104.8	114.9	112.3	98.0	110.8	128.0	123.5	98.3
89.9	118.0	87.9	126.6	98.0	99.1	98.0	136.1	146.6	130.1
93.1	126.3	107.9	97.6	88.0	121.8	115.8	128.4	133.7	110.9
87.2	112.3	130.6	112.1	87.3	95.1	97.5	138.1	135.4	109.7
96.8	88.4	101.1	126.1	90.6	99.1	118.3	119.5	152.2	97.1
114.4	104.3	99.1	105.4	114.8	92.4	103.5	146.5	134.0	110.8
89.4	100.6	100.1	102.9	84.4	83.7	110.8	137.8	144.5	130.7
86.0	100.9	95.5	96.5	74.8	89.0	109.7	151.3	133.5	99.8
100.9	136.1	98.0	112.1	87.3	97.8	102.7	139.7	138.5	100.4
98.7	101.6	98.0	121.0	89.4	79.6	114.9	125.9	146.4	108.9
84.6	81.6	104.5	101.5	108.3	118.0	97.8	121.1	117.7	94.8
98.6	101.4	103.5	114.8	89.7	75.1	101.1	112.8	134.9	106.5
88.2	126.4	121.0	131.7	88.0	107.6	97.8	131.4	143.5	102.6
94.8	106.8	113.7	132.8	93.5	106.7	96.4	154.7	147.1	106.2
101.7	106.4	115.7	111.6	92.6	99.0	97.6	122.7	146.6	109.1
93.5	105.1	102.9	102.8	85.3	109.2	85.3	143.5	145.1	106.4
93.7	97.8	109.9	109.4	94.0	101.8	86.1	120.7	135.4	107.2
90.2	94.0	110.4	115.8	88.7	92.4	117.8	104.0	134.0	114.8
Mean 96.3	106.8	108.6	110.4	98.2	97.0	99.4	135.6	136.4	116.5
SD 7.72	14.18	10.94	12.77	9.87	10.38	8.14	15.87	14.70	14.90

Table. A.2. *P. lutheri* HL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
138.5	151.6	167.4	154.3	138.5	152.0
155.0	130.9	163.8	137.3	136.1	150.3
143.5	164.4	177.4	138.2	174.5	136.8
148.2	154.7	166.6	127.1	135.2	136.0
137.9	141.7	130.0	149.4	132.3	149.7
129.3	136.4	130.0	135.0	148.3	144.5
121.4	177.9	172.0	152.1	117.7	129.5
147.9	167.2	184.1	132.2	118.8	140.0
143.5	169.4	165.6	136.4	135.1	159.5
147.8	181.5	149.8	150.2	138.1	149.8
149.3	172.2	165.9	146.6	117.1	135.3
156.6	157.9	157.6	142.5	169.6	140.8
145.2	139.5	181.8	140.0	116.6	135.7
163.1	148.8	152.5	128.9	139.5	125.8
178.8	164.5	147.1	159.6	131.9	134.9
146.6	144.0	172.0	113.7	175.2	149.5
147.0	135.1	151.6	144.8	134.9	152.8
128.0	146.2	155.1	137.3	144.2	134.5
166.4	148.5	144.0	115.1	130.7	148.2
158.3	133.1	151.6	124.3	133.5	153.0
154.5	157.0	153.8	117.7	127.1	136.0
144.8	150.5	136.1	119.8	135.0	139.1
141.7	136.1	155.4	136.7	144.3	116.1
130.9	138.2	141.8	124.6	136.7	134.9
137.2	147.8	130.7	122.6	129.0	144.0
137.8	154.6	124.6	109.7	138.8	133.2
149.8	148.9	147.3	122.0	140.1	129.2
137.4	132.5	154.7	130.9	122.8	126.3
122.6	154.7	157.6	110.9	127.4	114.8
176.1	146.4	134.8	128.3	111.6	136.7
138.8	161.4	158.8	155.6	139.9	144.0
144.2	161.6	140.2	146.5	140.8	127.9
133.5	177.0	181.3	129.7	142.8	133.4
143.0	135.8	155.1	141.3	154.0	133.7
188.8	174.9	145.1	130.1	172.4	162.6
158.5	169.4	123.5	138.1	138.6	164.7
159.2	133.5	133.9	127.1	140.7	138.1
150.4	164.4	109.4	119.9	121.9	143.0
146.5	148.2	165.6	142.3	146.4	150.3
147.6	151.5	132.8	139.5	139.3	137.8
134.4	150.2	154.0	162.4	123.6	130.7
152.8	124.2	158.2	152.3	158.4	140.2
121.9	189.0	146.6	124.0	112.8	128.0
167.7	162.4	121.4	138.7	165.8	135.1
151.3	153.4	141.2	149.2	149.2	143.3
132.7	151.6	159.5	132.1	138.1	145.1
150.2	159.1	172.3	139.9	158.0	146.4
147.3	179.1	160.2	143.5	109.2	133.4
152.2	146.2	131.5	152.0	151.1	149.3
138.2	135.1	169.0	130.3	125.0	120.2
161.8	139.2	142.5	147.8	136.0	131.7
154.5	159.1	144.0	126.1	140.8	138.8
156.6	144.1	142.5	119.5	126.4	130.3
149.8	165.9	164.3	131.7	121.1	116.2
184.1	147.8	158.8	132.3	182.8	125.5
147.5	153.2	158.0	140.1	115.5	141.8
133.8	146.6	168.9	111.7	118.2	133.5
156.3	142.1	142.7	119.9	129.2	145.2
144.1	132.3	146.4	118.7	146.6	122.4
144.1	136.4	179.7	128.1	156.7	151.5
Mean 148.0	152.1	152.2	134.3	138.0	138.5
SD 13.78	14.42	16.33	12.76	16.52	10.84

Table. A.1. *P. lutheri* LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *P. lutheri*, cultured at Low Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the first trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
98.7	140.0	115.3	137.8	114.2	112.0	121.6	172.7	125.1	158.4
104.4	123.2	130.0	141.3	112.7	109.3	115.6	157.9	146.1	136.3
101.2	114.2	126.5	131.9	112.8	119.7	114.9	144.1	152.7	139.3
100.6	123.5	114.8	116.2	108.0	122.3	112.5	143.8	151.6	149.7
97.8	114.7	107.5	116.8	115.5	109.6	114.3	142.4	153.8	139.6
106.0	134.9	132.6	115.6	111.9	118.3	113.9	152.3	137.8	148.1
98.0	130.4	129.1	127.9	109.6	108.6	112.0	119.3	144.1	133.9
96.9	124.2	127.0	137.4	112.2	107.7	117.0	140.7	134.7	155.5
105.5	120.9	125.7	137.8	115.3	110.8	112.2	150.2	142.7	138.0
102.0	131.1	126.3	129.1	124.2	98.8	92.4	159.9	146.2	148.9
100.3	124.1	113.7	132.4	115.2	98.8	123.6	138.2	138.2	152.5
101.6	125.8	109.9	127.8	116.5	117.9	115.3	136.4	135.9	147.7
100.7	114.7	106.1	121.8	117.6	113.2	117.7	151.4	146.1	156.6
104.7	112.5	120.4	117.7	112.9	113.5	116.8	136.3	157.8	145.0
99.5	105.8	138.9	127.4	114.8	107.8	119.3	155.9	138.8	153.1
100.8	123.3	136.3	110.8	115.6	112.8	110.7	137.7	152.7	152.2
107.1	121.1	135.7	110.4	114.7	115.6	104.4	137.8	142.4	152.8
101.0	130.9	124.5	130.1	110.7	119.8	114.9	153.4	143.4	136.1
103.4	152.8	141.1	109.6	117.6	116.2	117.9	138.2	149.1	161.3
103.7	122.1	117.7	130.5	109.0	113.6	116.1	155.0	145.1	136.3
88.8	120.8	121.0	116.2	109.6	112.2	118.9	156.1	149.7	134.9
92.2	114.7	135.4	116.0	117.6	117.0	117.0	152.1	141.9	149.3
107.4	130.2	135.4	112.5	111.4	110.8	109.0	162.3	143.9	134.7
94.3	119.8	132.4	122.3	113.6	114.8	118.6	164.8	132.6	132.0
97.5	121.6	128.6	110.8	108.0	115.8	116.0	165.7	140.3	139.9
104.8	133.5	139.7	119.4	117.0	111.9	113.5	146.4	142.5	154.1
101.2	110.7	121.7	130.8	113.8	114.8	119.9	140.6	160.9	143.8
111.4	151.5	130.1	114.9	106.1	114.8	118.6	130.8	137.7	141.1
104.3	127.8	119.8	123.4	120.1	105.2	114.8	138.5	131.4	132.1
102.3	107.5	131.6	122.6	117.9	124.3	115.8	163.5	133.3	133.1
99.4	146.5	137.7	149.1	113.7	114.3	112.0	156.2	160.3	133.3
99.7	110.8	153.0	127.8	109.9	125.7	109.0	139.9	145.4	134.9
96.0	114.6	123.2	116.1	115.3	111.9	120.1	164.9	148.6	152.8
101.0	122.3	140.7	119.3	116.8	117.2	113.5	163.9	151.5	147.9
95.3	120.6	126.3	113.9	114.9	124.1	112.8	150.5	147.5	137.9
96.0	123.7	126.5	123.2	104.4	99.1	104.0	151.8	142.4	119.8
105.7	109.0	152.5	134.5	117.0	103.3	118.0	168.8	148.6	146.1
104.7	113.6	133.5	114.8	112.2	115.6	117.9	155.5	138.8	139.0
105.7	135.0	111.7	122.5	101.4	118.8	112.0	166.0	167.9	159.5
103.6	101.9	112.0	132.6	106.4	106.3	109.0	158.4	134.9	169.9
95.1	125.2	119.8	109.0	123.5	108.0	112.0	153.6	140.8	146.3
110.9	120.5	115.8	136.0	122.8	100.9	107.5	132.0	136.3	138.0
107.7	130.6	143.4	129.8	120.6	103.6	113.5	139.1	139.1	131.4
106.8	101.7	124.3	127.9	113.2	103.4	104.4	145.6	167.9	146.3
106.6	138.5	123.4	120.6	115.2	103.9	115.3	155.9	140.8	150.2
100.0	121.8	138.8	109.9	119.9	115.6	115.5	158.0	160.9	133.3
95.1	116.2	119.9	125.2	103.9	100.4	113.0	152.1	148.7	152.1
109.6	126.2	118.1	123.6	114.5	109.0	113.8	148.2	148.8	130.9
101.2	109.9	120.8	120.6	114.2	111.9	114.5	161.9	137.7	143.2
98.2	117.7	115.6	123.5	95.4	121.1	117.9	148.9	144.7	142.4
103.2	106.2	119.4	128.3	107.8	105.2	117.9	150.3	149.4	134.1
105.6	123.8	113.0	131.6	119.3	117.6	117.6	142.7	155.1	144.1
99.6	124.3	123.5	112.2	112.2	106.5	114.9	151.6	143.7	157.5
104.5	122.1	142.0	114.8	117.6	111.9	114.9	170.4	143.5	144.7
110.1	139.7	127.8	149.6	128.3	96.2	119.8	153.9	140.8	145.2
115.2	127.0	149.7	115.5	117.7	108.0	116.1	150.3	119.6	143.4
100.1	118.3	121.0	111.9	102.5	115.6	109.9	150.6	127.4	139.9
95.7	124.5	121.7	132.4	115.3	106.2	110.7	147.3	136.0	155.0
111.9	123.8	114.8	112.5	117.6	112.0	109.3	160.0	133.1	158.7
91.6	122.1	172.5	135.0	113.0	107.8	116.1	175.2	162.9	152.5
Mean	101.9	122.8	127.3	123.7	113.7	111.5	114.1	151.1	144.4	144.4
SD	5.25	10.71	12.29	9.80	5.68	6.74	4.94	11.15	9.66	9.50

Table. A.1. ***P. lutheri*** **LL.** (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
148.4	160.8	164.3	147.5	150.3	162.4
160.9	161.7	151.5	135.9	149.9	166.0
168.1	155.1	165.5	153.0	145.7	155.3
154.7	143.2	177.4	141.1	150.9	150.2
152.3	158.4	170.6	132.9	145.2	154.1
140.6	137.8	154.2	155.6	143.4	155.1
160.4	157.1	150.2	163.1	148.1	172.2
142.9	145.4	147.7	146.5	141.0	148.9
152.1	159.3	158.8	142.6	153.8	158.0
147.0	144.4	150.2	146.5	160.7	148.4
148.2	154.4	149.9	146.3	144.1	143.4
145.6	159.0	141.1	141.7	147.0	144.8
159.3	160.9	155.2	145.7	157.8	137.1
158.0	147.3	169.3	155.3	152.2	159.1
154.5	158.1	158.3	147.7	148.1	163.6
141.3	162.1	143.5	147.4	143.4	140.1
148.1	140.3	140.1	141.7	142.4	151.7
148.1	143.4	160.9	149.1	145.1	146.4
175.4	177.9	171.6	137.1	150.5	158.0
149.2	157.1	168.9	139.1	165.8	150.9
150.5	169.3	147.0	150.1	153.3	148.6
145.0	147.3	141.5	144.4	165.1	151.0
141.7	134.8	160.0	150.2	151.5	156.1
164.3	117.7	153.8	145.2	154.2	159.1
146.5	146.5	149.3	143.2	164.9	156.2
159.5	128.4	143.4	149.2	148.1	151.8
139.7	155.3	177.8	148.1	160.8	157.8
134.5	152.8	160.4	143.5	152.7	153.8
139.9	163.1	162.7	146.3	145.7	159.5
136.3	168.5	165.2	149.1	151.8	157.8
151.6	165.8	151.0	160.6	160.4	156.8
154.2	149.2	156.7	155.6	140.1	164.7
158.0	154.4	161.3	146.0	146.4	163.1
152.7	153.2	158.7	163.3	137.9	151.9
148.2	159.0	160.9	148.2	145.6	167.9
140.8	137.1	173.3	157.8	158.7	169.6
153.3	131.6	160.1	142.5	163.7	166.9
163.7	148.7	160.7	150.8	155.3	166.4
148.2	166.2	163.7	151.0	142.0	164.1
133.5	137.8	140.0	136.8	139.9	165.5
137.8	163.0	149.7	144.8	162.7	172.3
130.0	140.1	141.3	146.3	159.5	152.5
144.8	165.2	148.1	151.4	152.9	188.6
146.3	144.8	159.7	132.2	146.9	160.3
157.2	154.9	137.8	148.9	152.0	142.4
152.3	161.5	134.9	146.5	142.8	149.4
149.3	158.2	132.9	141.3	146.7	140.6
139.6	147.5	123.8	150.2	136.3	167.9
143.5	155.0	137.3	163.5	152.3	146.6
139.9	169.6	153.3	160.9	154.4	148.6
146.0	164.3	149.9	159.1	153.2	150.3
141.6	158.7	154.9	154.4	145.7	142.7
152.5	148.7	149.4	163.5	136.0	159.0
155.5	154.9	149.7	146.3	139.1	145.7
154.2	150.1	151.5	151.8	146.3	163.1
152.3	145.1	147.9	145.2	159.9	147.0
135.9	154.1	177.8	147.7	160.7	147.7
144.1	165.5	154.2	137.7	134.1	146.6
135.4	156.6	156.9	135.9	140.1	143.5
129.4	153.8	153.6	143.4	146.4	156.6
Mean 148.4	153.0	154.3	147.8	149.9	155.4
SD 9.23	11.07	11.35	7.46	7.90	9.57

Table. A.2. *P. lutheri* LL. Length of D-stage larvae and their corresponding length after two weeks of feeding on the control diet and *P. lutheri*, cultured at Low Light conditions and f/2 medium (f/2), Phosphorus limited medium (P) and Nitrogen limited medium (N). Numbers at the end indicate the number of the triplicate culture. Results are from the second trial.

D-stage	Control 1	Control 2	Control 3	Unfed 1	Unfed 2	Unfed 3	f/2 1	f/2 2	f/2 3
94.3	120.7	124.2	114.3	123.9	117.3	110.8	146.4	132.2	128.1
96.5	133.5	118.7	107.6	109.1	98.8	86.3	134.2	191.9	131.9
93.7	95.9	136.0	103.4	96.3	99.8	93.0	137.9	133.4	134.5
100.9	118.5	116.1	97.8	112.1	92.7	94.1	129.7	135.0	135.4
90.6	116.2	97.6	110.4	103.5	118.2	97.8	150.2	137.2	130.6
97.9	104.0	105.5	104.4	95.4	76.4	101.8	114.8	132.4	139.3
93.4	108.9	108.7	94.8	94.9	107.7	91.4	111.9	137.2	136.1
93.9	109.2	114.4	100.9	97.8	87.8	92.0	134.9	134.9	156.7
90.3	113.5	94.8	109.1	102.8	97.5	93.5	134.0	130.0	130.9
110.6	90.1	103.7	97.6	93.5	82.8	91.5	126.9	117.7	131.0
96.5	116.3	107.9	96.2	101.1	88.7	104.5	123.8	129.0	124.6
105.6	116.6	106.4	100.5	86.9	93.4	92.4	125.3	144.0	109.1
96.1	106.2	129.2	109.3	97.8	96.8	95.6	156.3	123.5	118.1
113.1	101.4	114.3	95.4	95.5	88.7	95.4	131.2	127.4	129.7
93.8	148.5	119.9	99.1	113.7	108.5	96.4	133.4	129.8	130.0
95.0	130.0	99.5	128.2	90.6	117.1	97.6	179.8	119.9	121.1
99.2	87.2	115.0	118.0	92.6	106.8	95.4	157.0	133.5	131.0
94.3	107.2	101.1	110.4	106.7	88.4	86.1	119.8	149.8	145.7
103.3	109.1	115.0	132.2	87.4	101.9	100.4	127.1	142.5	133.9
102.3	97.6	94.1	127.5	95.1	91.2	100.5	154.5	130.7	131.2
109.9	107.6	98.0	93.1	99.8	95.8	103.5	159.4	135.5	145.8
92.0	105.4	103.0	111.9	112.3	92.7	94.7	120.7	136.4	125.0
88.1	103.5	107.2	109.4	94.9	99.0	91.2	138.6	134.8	125.8
82.1	95.1	116.6	117.7	92.6	85.2	88.7	152.8	112.0	134.0
93.5	112.0	97.5	101.5	109.4	87.3	89.9	153.7	114.3	141.1
107.1	100.3	102.6	96.2	98.0	92.4	94.9	148.5	126.0	119.5
101.3	77.3	97.1	100.0	86.8	103.7	95.6	140.3	132.1	145.3
83.6	88.4	110.4	98.6	87.9	109.9	105.3	135.4	125.1	151.3
98.3	103.5	101.5	94.8	97.6	89.9	109.6	138.0	131.4	170.6
97.6	91.7	105.1	132.1	93.7	90.1	103.5	149.2	121.8	131.7
101.1	142.1	119.4	160.4	109.1	92.6	115.1	149.2	133.5	141.8
107.8	103.7	100.9	105.3	109.9	103.7	95.0	161.0	121.1	163.2
90.3	97.6	116.1	113.7	111.8	103.7	97.5	139.1	150.3	152.2
112.2	94.7	132.2	100.3	95.8	93.8	95.4	140.1	134.9	120.6
92.9	120.7	118.0	117.7	103.5	87.5	102.6	144.1	140.9	138.8
86.2	103.5	127.9	115.4	105.9	92.7	97.1	120.7	138.1	138.1
109.4	101.1	114.9	111.7	89.9	95.4	103.3	143.5	118.3	151.3
104.8	117.1	124.0	93.7	91.2	108.9	100.4	130.1	120.6	125.5
97.7	117.1	90.8	123.3	104.0	82.6	91.9	137.2	141.3	126.8
88.9	109.3	115.7	105.4	107.5	104.0	109.7	137.8	121.1	136.4
95.6	92.7	100.5	124.2	115.7	95.8	107.6	140.9	130.2	122.0
92.6	83.3	92.6	107.2	109.1	87.3	94.7	145.3	152.6	142.5
82.6	104.3	104.8	114.9	112.3	98.0	110.8	144.9	136.1	142.5
89.9	118.0	87.9	126.6	98.0	99.1	98.0	146.8	131.7	146.4
93.1	126.3	107.9	97.6	88.0	121.8	115.8	126.3	112.9	142.0
87.2	112.3	130.6	112.1	87.3	95.1	97.5	123.5	127.1	150.3
96.8	88.4	101.1	126.1	90.6	99.1	118.3	124.6	121.8	146.6
114.4	104.3	99.1	105.4	114.8	92.4	103.5	120.7	146.4	133.0
89.4	100.6	100.1	102.9	84.4	83.7	110.8	141.3	126.8	160.8
86.0	100.9	95.5	96.5	74.8	89.0	109.7	144.0	147.6	140.0
100.9	136.1	98.0	112.1	87.3	97.8	102.7	150.8	164.5	149.7
98.7	101.6	98.0	121.0	89.4	79.6	114.9	124.6	116.2	119.1
84.6	81.6	104.5	101.5	108.3	118.0	97.8	135.4	136.0	131.7
98.6	101.4	103.5	114.8	89.7	75.1	101.1	143.5	136.4	118.2
88.2	126.4	121.0	131.7	88.0	107.6	97.8	151.1	138.2	130.7
94.8	106.8	113.7	132.8	93.5	106.7	96.4	140.0	138.0	120.7
101.7	106.4	115.7	111.6	92.6	99.0	97.6	131.9	138.7	128.1
93.5	105.1	102.9	102.8	85.3	109.2	85.3	136.0	149.3	139.9
93.7	97.8	109.9	109.4	94.0	101.8	86.1	134.5	178.6	133.9
90.2	94.0	110.4	115.8	88.7	92.4	117.8	132.3	128.4	119.4
Mean 96.3	106.8	108.6	110.4	98.2	97.0	99.4	138.4	134.3	135.5
SD 7.72	14.18	10.94	12.77	9.87	10.38	8.14	12.59	14.01	12.17

Table. A.2. *P. lutheri* LL. (Continued)

P 1	P 2	P 3	N 1	N 2	N 3
158.4	163.8	150.4	131.0	137.3	160.4
169.0	130.1	148.2	130.3	138.0	137.3
135.4	129.8	124.7	138.0	149.8	149.2
140.2	137.8	146.5	128.0	126.7	131.7
117.8	124.0	138.1	125.3	157.3	134.9
124.2	129.2	155.0	150.2	124.7	135.4
151.1	142.5	152.4	141.8	150.6	114.3
146.9	150.8	144.0	121.8	136.4	128.5
118.0	129.7	132.3	142.0	134.4	118.2
151.5	140.0	126.8	141.2	126.9	130.0
140.4	140.7	146.4	148.2	125.4	119.9
145.5	150.4	135.0	119.4	124.0	125.5
168.0	137.3	147.5	157.6	143.8	122.6
147.8	142.0	140.6	149.2	143.8	115.6
118.8	140.8	138.7	138.0	136.0	155.3
115.4	154.5	146.7	176.8	145.5	129.7
141.6	150.3	126.1	126.4	132.1	115.4
148.9	148.9	137.9	155.7	150.8	130.3
152.6	138.5	160.0	133.2	148.9	155.2
123.7	130.3	142.5	120.6	151.1	164.8
145.7	131.4	142.7	112.5	126.9	125.9
134.9	150.6	136.1	115.7	133.0	126.1
122.1	133.9	139.1	109.3	136.4	107.9
140.0	161.1	129.8	108.0	133.2	147.1
143.5	121.0	123.5	126.6	132.5	138.7
146.6	118.3	126.8	111.6	147.1	146.3
139.1	130.1	129.7	117.1	140.8	119.1
137.1	156.3	154.6	106.2	143.5	123.6
136.0	143.0	150.2	118.9	144.2	134.5
152.0	122.1	152.3	106.2	163.4	121.8
164.4	187.4	161.4	154.2	132.5	137.8
152.1	126.6	137.8	145.7	120.5	152.1
154.2	132.2	137.6	148.2	132.3	130.0
171.2	151.1	136.4	157.0	161.6	136.1
137.2	139.2	127.4	126.2	143.5	133.7
147.6	158.1	120.9	142.5	161.0	142.0
135.3	137.2	144.1	134.0	118.9	128.9
151.1	146.4	144.0	124.2	151.9	132.2
134.9	123.3	142.7	134.4	129.0	139.2
136.8	140.6	138.5	151.1	105.3	112.6
131.0	136.1	138.1	130.9	115.0	144.9
148.2	136.0	158.1	130.7	144.1	128.3
140.9	152.1	140.9	137.8	130.0	138.1
137.9	146.5	144.0	132.5	139.0	130.5
154.6	140.2	117.3	135.3	148.2	124.6
153.5	141.6	134.4	134.0	118.3	129.2
179.7	131.7	131.0	155.6	126.1	148.6
134.9	149.2	154.5	134.5	133.9	152.6
139.7	134.9	134.0	115.1	144.8	139.2
132.2	141.8	147.6	147.5	124.7	137.4
156.3	134.9	147.6	144.2	167.7	123.3
152.8	160.2	133.8	141.2	151.0	134.9
158.1	134.2	130.0	113.0	142.8	125.0
147.9	140.4	127.5	122.1	117.7	134.9
142.8	131.9	137.1	139.9	127.4	133.1
146.7	143.5	132.3	130.0	156.7	160.8
146.4	151.1	145.7	127.1	141.3	135.0
146.6	144.1	128.1	141.8	134.9	153.1
149.8	126.4	107.6	138.9	152.6	144.9
141.2	143.5	136.1	134.0	113.0	108.5
Mean 144.0	140.7	139.0	133.5	137.8	133.9
SD 13.07	12.05	10.75	14.69	13.29	12.99