Production of Micro-algal Concentrates for Aquaculture
Part 2:
Development and Evaluation of Harvesting, Preservation, Storage and Feeding Technology

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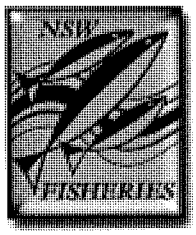

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TABLE OF CONTENTS

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1. NON-TECHNICAL SUMMARY

93/123 & 96/342	Production of micro-algal concentrates for aquaculture Part 2: development, storage and feeding technology

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OBJECTIVES:

1. To investigate and identify appropriate harvesting and concentrating methods for micro-algae species commonly used in marine hatcheries.
2. To determine the best method of storing/preserving the concentrated algae across an array of species that would collectively meet the demands of current and future marine finfish, crustacean and mollusc hatcheries and nurseries in Australasia.
3. To ensure that the concentrated product can be resuspended to a form that can be effectively fed to the larvae and juvenile molluscs, shrimp and other invertebrates of commercial significance.
4. To confirm the quality and demonstrate the practical utility of the concentrated and stored product in controlled laboratory experiments and in commercial trials, ie. the implementation of feeding trials using larvae and juveniles of commercially important aquaculture species, especially bivalves.
5. To assist the Australian aquaculture industry by developing the technology to produce a reliable source of a high quality, reasonably priced micro-algae concentrate. Such products will also have international markets, thus providing a new export opportunity for Australian aquaculture.
6. The targets for this project are to develop the technology to produce nutritionally adequate microalgal concentrates with a shelf life of 6-12 weeks at a price well below current estimated production costs of $200 - $400/kg packed cell weight.

NON TECHNICAL SUMMARY:

This report refers only to work undertaken by staff at NSW Fisheries dealing with technological development of harvesting, preservation, storage and feeding of micro-algae. The results of complementary research to evaluate tubular photo-bio-reactor culture of micro-algae for marine hatcheries is the topic of a separate report by Michael Borowitzka, Murdoch University, W.A.

Micro-algae are essential for commercial rearing of many aquatic animals, especially the larvae and juveniles of bivalve molluscs, penaeid prawns and live food organisms such as rotifers used to hatchery rear marine fin-fish and crustaceans. Recent surveys conducted in Australia and overseas have revealed that 30 - 40% (max.70%) of marine hatchery operating costs can be attributed to micro-algal culture. The problem of high costs of individual hatcheries producing their own algae is compounded by the need of scarce expertise, without which *crashes* of algae at critical periods occur quite commonly.

The aims have been to develop reliable cost efficient methods for centralised large-scale production, concentration, storage, preservation, transportation and resuspension of micro-algae over a suite of species that collectively meet the needs of all types of marine hatcheries throughout Australasia. These include penaeid shrimp, finfish and bivalve hatcheries that account for the great bulk of micro-algal production and use.

This report describes trials and tribulations encountered in the development of successful harvesting, preservation and storage techniques. These techniques have had to be customised for each of seven individual species of micro-algae in order to achieve a minimum practical shelf life of 4 to 6 weeks. Pleasing results have been achieved with stored concentrate diets which have yielded growth rates of 80% or greater than those supported by live algae control diets.

2. BACKGROUND

Mass production of micro-algae has been recognised as a major bottle-neck to many forms of marine hatchery and nursery production. This has prompted a search for alternatives to on-site production such as the use of dried micro-algae grown heterotrophically, micro-encapsulated diets, yeasts and preserved algal concentrates (pastes or slurries). However, Coutteau and Sorgeloos (1992) report that commercial marine hatchery operators throughout the world have tried and discarded substitutes for live micro-algae. This is in spite of encouraging experimental results achieved by researchers and developers of such products. The only significant exceptions have been:

- the partial or total substitution of live algae by formulated micro-particulate diets by a limited number of penaeid shrimp hatcheries in Australia and elsewhere (Yashiro et al., 1985; Amjad and Jones, 1992).
- the use of algal pastes especially of the diatoms *Thalassiosira pseudonana* and *Skeletonema costatum* used as a short term interim food source for remote setting of hatchery produced Pacific oyster (*Crassostrea gigas)* pediveligers by farmers mainly on the coast of USA. Indeed the major seed supply for the 25 000 tonne Pacific oyster industry in this part of the world is dependent upon this method of remote settlement. (Donaldson, 1989; 1991).

Major constraints to reducing high costs and risks of marine hatcheries producing micro-algae is poor practical performance and consequential reluctant commercial adoption of most off the shelf diets developed as substitutes for live micro-algal feeds (Coutteau and Sorgeloos, 1992; Southgate et al., 1992; Nell et al., 1996; Knauer and Southgate, 1999). The former include, freeze or spray dried micro-algae cells, micro-particulate formulated diets, yeast's, micro-encapsulated diets and micro-algae concentrates (pastes or slurries). Micro-algae concentrates have been adopted as partial substitutes with wider adoption being limited by poor shelf life and/or very limited choice of species.

NSW Fisheries has had a long standing interest in nutrition research with particular interest in diet development for larval and post larval bivalves (Nell and O'Connor, 1991; O'Connor and Nell, 1992; Nell et al., 1996). Much of this work has involved the use of micro-algae concentrates, and has been used to the extent of supplying commercial and research facilities that have suffered micro-algae production failures or shortfalls at critical times.

3. NEED

Surveys of the use of micro-algae in Australian aquaculture have been conducted by Brown et al., (1989) and more recently by DOSAQUA on behalf of Western Biotechnology Ltd. (WBL) in 1993 the latter being a precursor to this study. Results of this survey which achieved a 50% response rate (23 respondents) to a comprehensive questionnaire distributed to all commercial and research marine hatcheries in Australia revealed that on average, 30 to 40% (max. 70%) of hatchery costs can be attributed to micro-algae culture. These costs are in general agreement with those of a global survey conducted by Coutteau and Sorgeloos (1992) who reported that micro-algal production in commercial hatcheries at the time averaged about US$40,000 p.a., representing 30% of total seed production costs. This same survey also reported that for six hatcheries with typical algae production levels of 1 to 10 m^{-3} per day, estimated actual cost of micro-algae production was in the range $US300 - 400 kg^{-1} of algae cells (i.e. packed cell weight) but that this cost reduced to $US50 -100 kg^{-1} of algae biomass (Fulks & Main, 1991) in very large hatcheries producing 30 to 110 m^{-3} of microalgal culture per day.

Micro-algae culture requires specialist expertise and is often regarded as a diversion of resources as well as a source of major problems when cultures "crash". The solution to the high costs of micro-algae culture and the associated problems with culture stability, lies in having dedicated production units that can use efficient culture techniques, have appropriately trained staff, a quality management program, and which can achieve economies of scale, thus providing hatcheries with a cheaper, more reliable and consistently high quality source of micro-algae.

The major barrier to the implementation of such a facility is the need to effectively harvest and preserve the algal biomass so that it can be shipped to hatcheries for use. Dried algae have been available in the past (Cell Systems Ltd. England), however these heterotrophically grown algae were nutritionally inadequate for many species and their use has caused problems with fouling of the water (Laing & Verdugo, 1991; Numaguchi & Nell, 1991; Knauer and Southgate, 1999 and market survey conducted by WBL). The price of this product was also very high. Wet algal concentrates (pastes) appear to be a much better option. Several Australian hatcheries have had some limited experience with algal concentrates and algal concentrates have been used in a number of research facilities (Nell and O'Connor, 1991; O'Connor and Nell, 1992). However, further work was essential to extend the storage life of the algal concentrates and to develop appropriate methods of supplying these concentrates to the target species.

Major cost savings and reduced risks through centralised production and distribution of micro-algae have been constrained by an inability to concentrate very low cell densities of cultures to high density, pastes or slurries that:

- can be reconstituted into low density suspensions of single, neutrally or very slightly negatively buoyant cells in seawater under low turbulence culture conditions used to rear delicate marine larvae
- have nutritional quality equal or close to that of the original live cells
- can be efficiently filtered from suspension, ingested, digested and assimilated by the animals being cultured.
- feasible storage for minimum practical periods of 4 to 6 weeks under storage temperature regimens that fall within the normal operating capability of domestic or industrial freezers and chillers (refrigerators) available to hatcheries throughout Australasia.

Once a suitable preservation method has been developed, appropriate packaging and shipping methods for the algae concentrates also have to be available so that the aquaculturalists receive a cost-effective and nutritionally adequate product.

An additional but understated cost of individual hatcheries having to produce their own micro-algae is the difficulty of reliably maintaining levels of supply that consistently match highly variable demand. This problem is compounded by the fact that the culturing of micro-algae often requires a high level of specialist expertise without which failures (*crashes*) of cultures at critical times occur quite commonly. Such problems appear to be particularly acute under difficult culture conditions experienced by shrimp *(Penaeus monodon)* hatcheries and to a lesser extent, pearl oyster *(Pinctada maxima* and *P. margaritifera)* hatcheries in tropical Australia. Shrimp hatcheries need to produce large amounts of micro-algae, mainly *Chaetoceros muelleri* and *Tetraselmis spp.* over brief but critical periods of five to seven days that encompass protozoeal and early mysis larval stages, within a total hatchery production cycle spanning 25 to 35 days. The annual hatchery production period can stretch to 9 or 10 months but is mainly confined to a very distinct seasonal peak demand for post-larvae from August to December (C. Robinson, pers comm., June 1995).

There are additional benefits to direct economic benefits of centralised production of and subsequent use of micro- algae concentrates. Chemical and microbial loads associated with direct feeding of algal cultures have been found deleterious to some mollusc (Watson et al., 1986) and crustacean (Zein-Eldin in Griffith et al., 1973) larvae. Elimination of growth media via the use of micro-algal concentrates may therefore explain reports of enhanced larval growth and survival of some species when fed such concentrates (Nell & O'Connor, 1991). Accordingly, wider use of concentrates could facilitate production of species for which efficient output of consistently high quality post-larvae or juveniles has thus far proven elusive. Such species include the commercial scallop, *Pecten fumatus* (Heasman & Nell, unpublished report); the jumbo tiger prawn, *Penaeus monodon*; the Sydney rock oyster, *Saccostrea commercialis* (Nell et al., 1991) and the silverlip pearl oyster, *Pinctada maxima* (R. Rose - pers. comm. 1992).

Initiation of the present study was largely as a response to:

- frequent requests made by commercial hatcheries to Port Stephens Research Centre (PSRC) of NSW Fisheries for emergency supplies of micro-algae to offset failures in their own production;
- a keen interest in off-the-shelf micro-algal pastes expressed by many respondents to the DOSAQUA questionnaire;
- the presumption that such demand would, in all probability, extend to many of the 4 000 to 5 000 mainly small to moderate scale penaeid prawn hatcheries found throughout Southeast Asia (Anon., 1994) thereby generating sufficient demand to prompt the establishment of a specialist micro-algae concentrate enterprise in Australia.

4. OBJECTIVES

1. To investigate and identify appropriate harvesting and concentrating methods for micro-algae species commonly used in marine hatcheries.
2. To determine the best method of storing/preserving the concentrated algae across an array of species that would collectively meet the demands of current and future marine finfish, crustacean and mollusc hatcheries and nurseries in Australasia.
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5. RESEARCH METHODS, RESULTS AND DISCUSSION

5.1. Selection of Micro-Algal Species

Eleven species of micro-algae were selected using the following criteria:

- Patterns of usage revealed by surveys of experimental and commercial marine hatcheries conducted by CSIRO (Brown et al., 1989; Jeffrey et al., 1990) by DOSAQUA in 1993 and by Coutteau and Sorgeloos (1992). Results of these surveys identified that nine of these eleven species collectively account for the vast bulk (more than 90%) of all production and use in Australia and elsewhere.
- A demonstrated ability to retain nutritional quality through concentration and protracted storage (Laing et al., 1990; Brown, 1995; Montaini et al., 1995).
- The likely suitability of the particular micro-algae for cost effective mass production particularly in tubular bio-reactors as evaluated in a companion study conducted by Professor Michael Borowitzka at Murdoch University in Western Australia (refer to Part 1 of this report).
- Nutritional qualities of the micro-algae as demonstrated in previous experimental investigations including those conducted at the PSRC on Sydney Rock Oyster larvae (Nell et al., 1991) and spat (O'Connor et al., 1992).

The eleven species were:

Two **golden brown flagellates** (Prymesiophyceae):

Isochryis sp. (Tahitian Strain) (Parke) CSIRO Culture Number CS-177. This is one of the most widely used algal species by mollusc hatcheries throughout the world, and *Pavlova lutheri,* (Droop) Green, arguably the best performing single species for marine bivalve larvi-culture yet identified in our laboratory (Nell and O'Connor, 1991; O'Connor and Heasman, 1997). However, for both of these species, satisfactory methods of harvesting and preserving are complicated by the fragility of cell walls.

Two **green flagellates** (Prasinophyceae):

Tetraselmis chuii (Butcher) *and T. suecica* (Kylin) Butcher.
These tough walled easily propagated and highly productive species are used by most mollusc, finfish and penaeid hatcheries for direct feeding of larvae, or to produce intermediate live foods such as rotifers and other zooplankton. They have however been reported to be poorly digested by larval invertebrates.

Five species of diatom (Bacillariophyceae):

Diatom cells are characterised by an outer silica frustule and thus were not expected to be particularly vulnerable to high shear forces that can be applied by high speed centrifugation. Accordingly they were not expected to exhibit poor shelf life but instead proved to be highly species specific on both counts. The five diatoms used included:

(i) Chaetoceros muelleri (Lemmermann) is the most common species used globally by penaeid prawn hatcheries (Simon, 1978; Chu, 1989; Chu and Lui, 1990).

(ii) Chaetoceros calcitrans (Paulsen) Takano has been shown to support good growth and survival over a wide array of larval and juvenile bivalves including several species of clams, oysters and scallops (Brown et al., 1989; Nell and O'Connor, 1991; O'Connor et al., 1992; O'Connor and Heasman, 1997). *C. calcitrans* has also been shown to support excellent growth and survival of Sydney rock oyster larvae after being centrifuged and stored for 1 to 2 weeks (Nell and O'Connor, 1991).

(iii) Skeletonema costatum (Greville) Cleve is used widely for the hatchery rearing of penaeid prawn larvae and postlarvae (Kuban et al., 1985; Smith et al., 1993) and bivalve larvae and spat being identified as the best monospecific diet for Sydney rock oyster spat by O'Connor et al. (1992).

(iv)Thalassiosira pseudonana (Hustedt) Hasle et Heimdal supports good growth and survival over a wide array of larval and juvenile bivalves including oysters (Thompson and Harrison, 1992; Thompson et al., 1996), clams (Hetrick, 1995) and scallops (Thompson et al., 1985).

(v)Phaeodactylum tricornutum (Greville) Cleve has generally performed poorly as a fresh diet for larval and juvenile bivalves (Nell and O'Connor, 1991), this resilient, highly productive species is well suited to production in tubular photo-bioreactors (Charismada and Borowitzka, 1994) and has a high EPA content (Borowitzka pers. comm., 1996). The nutritional quality of *P. tricornutum* was shown to be enhanced following centrifugation (Nell and O'Connor, 1991).

One **Cryptomonad** - *Rhodomonas(*formerly *Chroomonas)salina* (Wislouch) Hill et Wetherbee - This particular species is high in EPA, DHA, and other PUFA's (Brown et al., 1989) and has been found to perform comparatively well as a monospecific diet for juvenile *Ostrea edulis* (Laing and Millican, 1986).

One **Eustigmatophyte** (Yellow-green) - *Nanochloropsis oculata* (Japanese Chlorella) this species is arguably the most extensively used species for the production of rotifers used as live food for the larvae of tropical marine finfish.

5.2. An alternative to Bio-Assay for Evaluating Nutritional Quality of Stored Micro-Algae Concentrates.

Introduction

While bio-assay techniques were used extensively in this study to assess the impact of harvest, preservation and storage techniques for micro-algal concentrates, their use did present a number of technical and practical constraints. They are costly, especially in terms of time labour and requisite facilities to the extent that numbers of experimental treatments and replicates are frequently restricted. Use of direct bio-assay to assess the nutritional value of micro-algae concentrates as food for mollusc spat or larvae entails ripe mature brood stock that may have highly restricted seasonal availability and experimental durations of one to several weeks to establish treatment differences.

As a result of the difficulties inherent in bioassay trials, an array of indirect methods for assessing the retained quality of stored micro-algae concentrates were evaluated:

- gross appearance and odour of concentrates prior to resuspension;
- microscopic examination of resuspended cells for evidence of physical damage to or degradation of cell walls and of intracellular structures and materials;
- use of specialist stains such as Evan's Blue to establish the integrity or otherwise of cell membranes.

Biochemical assay of essential labile nutrients such as HUFA's and Vitamins C, B1 and E, were not conducted due to prohibitive costs.

Evan's Blue Staining
Evan's Blue has been used as a dead-cell stain for various species of marine microalgae (Reynolds et al. 1978; Walsh, 1983; Gallagher, 1984; Molina Grima et al., 1994; Amsler and Raymond, 1995). Its diagnostic action is that it stains the organic matter within dead cells a deep blue. By contrast, it is repelled by live cells with a functional cell membrane (Molina Grima et al., 1994).

Materials and Methods

A series of comparisons were made between the staining response of healthy, log phase cultures of *P. lutheri*, T. *Isochrysis* and *C. calcitrans* and the responses of stored concentrates of each of these species.

Algal pastes were produced using a *Sharples* supercentrifuge and were stored at 2°C in a refrigerated cabinet for periods of up to nine months. When required, a small sample of stored paste was resuspended in seawater to approximate culture densities equivalent to those normally observed for logarithmic phase cultures of the respective species (7×10^6 - 20×10^6 cells mL^{-1}).

For staining, a 20 mL sample of each fresh or stored algal suspension was treated with 1 mL of 1% (w/v) stock solution of Evan's Blue. The samples were allowed to stand at room temperature for a minimum of thirty minutes before microscopic examination. A subsample of each stained suspension was then inspected at 250 x magnification using an Improved Neubauer Haemocytometer (*Superior Co.*, Berlin, Germany).

Results

None of the cells in any of the live cultures tested took up any stain, even when exposed to the stain for durations exceeding12 h. Similarly, day-old pastes of T. *Isochrysis* and *S. costatum* showed little to no uptake of the stain. However, in contrast, resuspended cells from nine month old stored paste exhibited a minimum of 80% of the cells stained within 35 minutes (Table 5.2.1).

Table 5.2.1. Percentage of algal cells stained using Evan's Blue on two commonly produced algal species.

Percentage of Cells Stained Using Evan's Blue			
	Live Culture	**Day Old Paste**	**9 Month Old Paste**
Pavlova lutheri	0%	-	>80%
Tahitian Isochrysis	0%	<5%	>93%
Skeletonema costatum	0%	0%	>99%

Discussion

Widespread use was made of staining response to Evan's Blue during screening trials to refine optimum methods of harvesting, preserving and storing concentrated micro-algae pastes and slurries subsequently assessed in bioassay experiments.

5.3. Micro-Algal Harvest and Concentration

General Introduction

For the most part development of harvesting techniques for micro-algae has been focused on removal of algal biomass from waste water (Golueke and Oswald, 1965; Dodd, 1979; Benemann et al., 1980; Moraine et al., 1980; Koopman et al., 1987). Nevertheless much of this research has acknowledged the potential to apply these techniques to production of micro-algae concentrates for use in marine hatcheries (De La Noue and De pauw, 1988; Millamena et al., 1990; Kennedy et al., 1996). Harvest techniques can be divided into four categories namely:

1 specific gravity based harvesting
2 filtration
3 chemical flocculation
4 electro-flocculation

Harvesting of micro-algae for use in marine hatcheries and nurseries, has been largely restricted to the use of industrial centrifuges, based on specific gravity principles. This limitation has arisen from the specific requirements of hatcheries. First, some of the most commonly used micro-algae species are susceptible to physical damage, especially the delicate prymnesiophyte species *Pavlova lutheri* and *Isochrysis* sp. (Tahitian strain) which form the basis of many mollusc larval diets. Susceptibility to cell damage is generally less acute in the diatoms and of little apparent significance to tough cellulose walled chlorophyte species. Second, there is frequently a requirement for the resuspension of algal cells so that they may be consumed by the various filter feeding organisms being cultured.

At the commencement of this project, the routine method of concentrating micro-algae in our laboratory was use of a *Elecrem*®, 220V cream separator. This method had a number of known shortcomings, namely:

- Harvest efficiencies using this apparatus were highly species specific, in some cases being too low to be considered practical. (O'Connor and Nell, 1992).
- The throughput capacity of the cream separator was too low for large scale applications.
- Cell viability after separation was unsatisfactory for some species. Microscopic examination and staining of harvested cells with Evan's Blue revealed that with some species, a significant proportion of cells sustained overt physical damaged during centrifugation. In the worst case of *Isochrysis* sp., up to 13% of cells immediately resuspended after concentration were found to be damaged. (Table 5.3.1.1).

Preliminary comparative harvesting trials were conducted in collaboration with *Alfa Laval, Australia Ptd.Ltd.* in Sydney in early 1994 to identify a more satisfactory type of centifugation harvesting. A range of different types of industrial high speed centrifuges were evaluated across a small but representative array of species including *Pavlova lutheri,* (to evaluate the degree of cell damage to delicate walled prymnesiophyte species), and *Chaetoceros calcitrans* which had previously proven to be the species least efficiently harvested by the cream separator. A super "high speed" centrifuge yielded the best apparent results combining the highest rates of cell harvest efficiency (>95%) and lowest apparent rates of cell wall damage to *P. lutheri* of all types of centrifuge tested.

On the basis of these results a second hand, commercial scale Sharples "super-centrifuge" (Type MV 35 32Y 22 KY32) that operates at 13,000g with a throughput capacity of up to 2000 L/hour,

was purchased and then modified so that effluent/natant was channelled through one rather than two outlets and a modified accelerator plate fitted to reduce shear forces.
Initial trials conducted were undertaken to identify the most appropriate type of pump to deliver micro-algae suspensions to the centrifuge. Three types of pumps evaluated were:

- a 220 watt open impeller submersible centrifugal pump (*DAB, Nova,* Model 300).
- a variable speed positive displacement helical impeller "mono pump" (*Monopump England, Manchester Ltd* Model No. SJ301 PR).
- a "½" diaphragm pump (*ARO Corporation*, Ohio USA, Model No.66606X-X).

These were evaluated for damage to micro-algae by microscopic examination of cells before and after pumping. The mono pump was tested at various pumping speeds/rates and did not inflict any obvious physical visual damage to cells. The centrifugal pump however, caused cells to clump and obvious structural damage was demonstrated in about 5% of cells. The effect of the diaphragm pump on cell damage was evaluated by microscopic examination coupled with Evan's blue staining with results indicating no apparent cell damage.

During the full course of this study it became progressively more apparent that although relatively little or no detectable physical damage was sustained by micro-algae cells during super centrifugation, any degree of overt damage, however slight, inevitably resulted in substantially reduced viability and nutritional quality following short to moderate term storage. Alternative harvesting methods to high speed centrifugation were therefore investigated particularly during the latter half of this study.

5.3.1 Harvesting by sedimentation

Introduction

As an initial step in the evaluation of alternative harvesting techniques to high speed centrifugation, sedimentation, a simple non-mechanical method of harvesting algae was trialed. Sedimentation which is commonly used to harvest filamentous, chain and colony forming algae, was anticipated as the least likely of described harvesting methods to cause chemical or physical damage to micro-algae cells.

Difficulties in sedimentation were expected with smaller motile species. To this end, sedimentation was coupled with refrigeration and darkness to attempt to increase harvest efficiency.

Materials and Methods

Samples from cultures of seven micro-algae species (listed in Table 5.3.1.1) were collected during logarithmic growth phase and the cell density of each determined using an "*Improved Neubauer*" haemocytometer (*Superior Co.,* Berlin, Germany) at 200x magnification. A litre of each culture was decanted into a 1 L Schött bottle and stored in a chest freezer (*Fisher and Paykel P/L*, model H-360X) fitted with a *Thermo-Eye* (Type PLE - Saginomiya P/L, Japan) digital temperature controller that maintained the samples at -1 ± 0.5°C. Each sample was visually assessed every 8 - 14 h until sedimentation had apparently stabilised. Stabilisation was assumed to have occurred when the apparent volume of settled cells did not differ from one sampling occasion to the next.

The clear supernatant fraction was decanted off so as to minimize disturbance to the settled algal cells. The volume of the residual slurry was then measured to the nearest ± 5 mL using a graduated

measuring cylinder. The cell density of the slurry was again determined using an *"Improved Neubauer"* haemocytometer (Superior Co., Berlin, Germany) at 200x magnification. In some cases slurries required dilution to permit accurate counts. This was achieved using 1µm filtered sterile sea water as the diluent.

Percentage harvest efficiency was simply estimated by dividing the number of cells in the settled fraction by the number of cells in the original one litre culture sample viz.,

$$\text{Harvest efficiency} = \frac{\text{Cells in settled fraction}}{\text{Original cell numbers in sample}} \times 100$$

Results

For all algal species tested some degree of sedimentation was apparent within 24 h, the upper layers of each culture increasing in clarity and a layer of settled cells forming at the base of each culture bottle. Sedimentation rate was generally slow and the time taken for the sedimentation layer to stabilize varied greatly between species. Sedimentation rate was most rapid in cultures of large strain *S. costatum* and most protracted in cultures of *Chaetoceros* spp. and small strain *S. costatum*. Neither the times taken for the stabilisation of the sediment fraction, nor the harvest efficiencies were significantly correlated with the cell dry weights of the respective species ($r = 0.10$ and $r=0.22$, respectively; $P>0.05$). Moreover there was no significant correlation between sedimentation times and harvest efficiencies ($r= 0.01$).

Table 5.3.1.1. Duration and efficiency of cell sedimentation for seven species of micro-algae

Species of micro-algae	Cell dry weight *& (dimensions)	Initial density (cells/mL)	Time to harvest	Volume of settled Slurry	Density of cells within slurry (cells/mL)	Concentration factor	Estimated harvest efficiency
Tetraselmis chuii	96 (13.8x9.1)	$0.20x10^6$	95h	53mL	$3.12x10^6$	15.6x	83%
Skeletonema costatum small strain	11.5 ?	$3.66x10^6$	162h	55mL	$55.75x10^6$	15.2x	84%
Skeletonema costatum large strain	50 ?	$0.29x10^6$	18h	64mL	$2.92x10^6$	10.1x	64%
Chaetoceros muelleri	20 (7.4x5.4)	$1.88x10^6$	162h	119mL	$3.76x10^6$	2.0x	24%
Chaetoceros calcitrans	15 (4.0x3.1)	$8.25x10^6$	162h	292mL	$28.96x10^6$	3.5x	102%
Pavlova lutheri	23 (6.9x4.9)	$2.14x10^6$	68h	61mL	$25x10^6$	11.7x	71%
Isochrysis sp.(Tahitian)	19 (8.5x5.4)	$4.44x10^6$	92h	293mL	$12.83x10^6$	2.9x	85%

* With the exception of small strain *S. costatum*, cell dry weights (pg) and dimensions (µm) are from Nell and O'Connor (1991) and O'Connor et al. (1992).

Discussion

A general observation made prior to this study was that micro-algae cells of most commonly cultured species will settle from suspension once strong aeration to the cultures is stopped. Observed rates of settlement under normal culture conditions of high ambient light intensity and temperatures of 22 to 24°C however appeared far too slow for practical harvesting purposes. Thus temperature reductions and darkness were introduced in an attempt to accelerate the process. An

absence of light and reduced temperature did appear to accelerate rates of sedimentation in most if not all the species of micro-algae but not sufficiently so in most cases to constitute a practical alternative to centrifugation.

It was envisaged that sedimentation would constitute a useful precursor to low shear force methods of centrifugation or alternative methods of de-watering provided complete or near complete settlement of cells could be achieved within 12-18 h. In most cases, the times taken for sedimentation to stabilise was 3-4 days which is only marginally faster than the time taken by inoculated cultures to reach harvest densities!

Of the seven types of algae evaluated, only four, *T. chuii, S. costatum* (small strain), *S. costatum* (large strain), and *P. lutheri* could be harvested with a useful slurry cell density at least 10 times that of the original culture. However only in the case of large strain *S. costatum* was sedimentation sufficiently fast (18 h) to be incorporated into routine harvesting schedules. Alas even the benefit of relatively rapid sedimentation of *S. costatum* was negated by the fact that only 64% of cells settled within 18 h thereby representing a loss of over a third of the available algae biomass.

There were no apparent patterns to rates and efficiencies of sedimentation that would allow predictions to be made with respect to other species of micro-algae. While *S. costatum* and *C. muelleri* had the fastest and slowest rates of sedimentation rates respectively, they were neither the largest nor smallest respectively of the species tested. Although cell size, and density clearly effect rates of sedimentation, these results together with those in later centrifugation trials (Table 5.3.2.1), show that other factors are involved. Motility for instance could prolong suspension, although *P. lutheri* , a small flagellate, had the second fastest sedimentation rate. Other factors that could influence the speed and extent of sedimentation are species morphology, such as the spines in *C. muelleri* and internal biochemical changes, such as gas or lipid content, to promote buoyancy in the absence of light.

5.3.2 Centrifugation

Introduction

Of the various methods of harvesting micro-algae from aqueous suspensions developed over the past three or four decades, centrifugation is considered the most direct (Becker, 1994) and has been the most popular for aquaculture feed applications (Griffith et al., 1973; Watson et al., 1986; Stewart et al., 1987; Donaldson, 1989; Nell and O'Connor, 1991). Centrifuges increase the gravitational force experienced by algae (Wheaton, 1977) such that their settling rates increase proportionally to the centrifugal force applied. These methods are considered simple and reliable, while providing a product free of added flocculants or other chemicals (Becker, 1994).

Centrifuges for algal concentration have been divided in to various types by different authors (Wheaton, 1977; Mohn, 1980; Becker, 1993). While the classifications themselves are of limited importance here, the act of classifying centrifuges acknowledges design differences. Differences which result in performance variation in several critical respects, such as harvest efficiency, operational costs and the degree of physical damage to micro-algae cells. For example, Wheaton (1977) lists six types centrifuges that vary in maximum centrifugal force applied from 2×10^3 to 7.5×10^6 g. This variation is indicative of the speed and efficiency with which particles can be removed from suspension but also the potential for damage to the particles removed. In some cases, damage to micro-algal cells may be beneficial in to subsequent nutritional value (O'Connor and Nell, 1992; Sauriau and Baud, 1994), however, where micro-algae cells are to be stored for prolonged periods, cellular damage is considered to be a disadvantage.

The performance of many types of centrifuge can be altered by adjusting variables such as rotation speed, liquid throughput, solids throughput and pool depth (Wheaton, 1977). Again these have the potential to affect micro-algae harvest efficiency and cellular damage.

Given the diversity of centrifuges available, the variety that have been used by previous researchers, their variability in operation and the physical and morphological differences in the micro-algae of interest, it was considered imperative to evaluate several centrifuges for concentrating micro-algae. For this purpose, the previously described cream separator and super-centrifuge, plus a bucket centrifuge (*Hitachi*, Model 05P-21B) were compared in terms of harvest efficiency and cellular damage to nine species of micro-algae representative of those commonly used in mariculture in Australia.

Materials and Methods

An array of nine species of micro-algae, listed in table 5.3.2.1 were concentrated using, a bucket centrifuge, a cream separator and a super-centrifuge. In each case, three different cultures of each species algae were used. The densities of the cultures were determined prior to concentration by microscopic examination at 200x using an *Improved Neubauer* haemocytometer (*Superior Co.*, Berlin, Germany). Each culture was also examined for apparent cell viability using the Evan's Blue staining technique previously described in Section 5.2.

During the centrifugation process the natant (effluent) was collected to evaluate harvest efficiency. At the completion of centrifugation, the resultant concentrate was resuspended in 1 µm nominal filtered sea water with a Sunbeam "Maestro" hand-held food processor, stained with Evan's blue and examined microscopically at 250x to evaluate apparent cell damage as previously described.

Statistical analysis
Harvest efficiency and apparent cell viability data were arcsine $x^{0.5}$ transformed before analysis by two way ANOVA. For viability data, homogeneity was confirmed using Cochran's test, however transformation of harvest efficiency data failed to remove heterogeneity of variances. Means were compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Results

In accordance with previous experience, harvest efficiency differed significantly with both centrifugation method and species concentrated, with a significant interaction between both factors (Table 5.3.2.2). In general, the super-centrifuge harvest efficiency (range of 93.3-100% according to species) exceeded that of the cream separator (range 45.8- 96.0%) which in turn had greater harvest efficiencies than that of the bucket centrifuge (range 5.2 - 66.3%).

Although weak but significant correlation's ($P<0.05$) occurred between cell dry-weight and harvest efficiency for the super centrifuge and cream separator ($r=0.38$ and $r=0.44$, respectively), cell dry-weight was an inconsistent indicator of likely harvest efficiency. The low degree of correlation was due in part to the re-suspension of motile micro-algae cells following concentration. This was particularly marked in the case of *T. chuii* that retained motility through bucket centrifugation, so much so in fact that most cells were observed to have returned to the water column before the supernatant could be decanted.

Degree of apparent cell damage was generally low irrespective of concentration method with significant damage restricted to *C. muelleri* and T. *Isochrysis* (0-12%, Table 5.3.2.2). Never the less, ANOVA showed significant differences with both concentration method and species concentrated, with a significant interaction between both factors (Table 5.3.2.2). Across all species tested, mean cell viability following concentration (as indicated by a negative staining response to

Evan's blue), was greatest following bucket centrifugation (>99%) where only *C. muelleri* suffered significant (5%) detectable damage.

Table 5.3.2.1. Comparison of three types of centrifugation across an array of species of micro-algae on the basis of harvest efficiency and apparent viability following resuspension.

	Apparent Cell Viability (%)			Harvest Efficiency(%)		
Type of Centrifuge	Super-centrifuge	Cream Separator	Bucket Centrifuge	Super-centrifuge	Cream Separator	Bucket Centrifuge
P. lutheri	92±2 ^{bc}	99±1 ^{ab}	100±1 ^{ab}	100±0 ^{a}	79±3 ^{abc}	66±2 ^{bcd}
Isochrysis sp.	88±1 ^{c}	98±0 ^{ab}	100±3 ^{a}	100±0 ^{a}	65±4 ^{bcd}	54±6 ^{de}
C.calcitrans	98±1 ^{ab}	100±0 ^{ab}	100±0 ^{ab}	97±0 ^{ab}	52±3 ^{de}	48±8 ^{de}
S.costatum	100±0 ^{ab}	98±1 ^{ab}	100±0 ^{ab}	98±0 ^{a}	47±8 ^{de}	39±11 ^{e}
T.pseudonana	97±0 ^{ab}	99±0 ^{ab}	100±0 ^{ab}	99±0 ^{a}	76±5 ^{bcd}	57±8 ^{cde}
P.tricornutum	100±0 ^{ab}	99±1 ^{ab}	100±0 ^{ab}	94±3 ^{ab}	65±7 ^{bcd}	56±7 ^{cde}
C.muelleri	88±3 ^{c}	88±8 ^{c}	95±4 ^{abc}	96±1 ^{ab}	46±7 ^{de}	15±3 ^{f}
T.chuii	100±0 ^{ab}	100±0 ^{ab}	100±0 ^{ab}	100±0 ^{a}	96±1 ^{ab}	5±3 ^{f}
N.oculata	100±0 ^{ab}	99±1 ^{ab}	99±1 ^{ab}	95±1 ^{ab}	53±2 ^{de}	65±7 ^{bcd}

Mean percentage ± s.e. apparent cell viability and harvest efficiency data. Means with a common superscript do not differ significantly (SNK, $P<0.05$).
NB. Data for harvest efficiency should be interpreted with care as variances are heterogeneous.

Table 5.3.2.2. ANOVA of a) harvest efficiency and b) cell viability for nine species of micro-algae concentrated using either a super-centrifuge, a cream separator or a bucket centrifuge.

a) **Harvest efficiency**

Factor	SS	df	*F*	*P*
Species	5524.4	8	9.8	<.0001
Method	38288.0	2	271.1	<.0001
Interaction	12739.3	16	11.3	<.0001
Residual	3812.8	54		

b) **Cell viability**

Factor	SS	df	*F*	*P*
Species	659.5	8	6.2	<0.001
Method	225.6	2	8.5	<0.001
Interaction	453.1	16	2.1	0.0193
Residual	713.8	54		

The mean apparent cell viability across all species following concentration in a cream separator and the super centrifuge were approximately 98 and 96% respectively, with *C. muelleri* the most vulnerable species to cell damage (12% of cells damaged in both cases). Cells of the prymnesiophytes *P. lutheri* and T. *Isochrysis* also sustained moderate levels of apparent physical damage following supercentrifugation but ostensibly low cell damage of only 1 and 2% respectively, when harvested using a cream separator. The remaining six micro-algae species tested exhibited very low apparent cell damage (0-3%) regardless of the method of centrifugation, including super-centrifugation at 13 000 G.

Within each centrifugation method, there was no significant correlation ($P>0.05$) between the harvest efficiency and apparent cell viability across the various species tested ($r= 0.22$, 0.18 and 0.23 for super-centrifugation, cream separation and bucket centrifugation, respectively).

Discussion

The relative efficiency of the centrifugation techniques used differed greatly and indicated that the super-centrifuge was far more effective in algal biomass removal than the techniques used previously in our laboratory. Further, as these trials indicated that the super-centrifuge extracted >95% of micro-algae cells from most cultures, there seemed no apparent need to look for more efficient centrifuges capable of exerting greater forces on micro-algae cells. Rather in specific cases, such as more delicate cell wall species such as T. *Isochrysis*, it was concluded that alternative harvesting techniques involving lower applied forces could be explored in an attempt to reduce cellular damage.

As expected, the apparent cellular damage caused during centrifugation was affected by the particular type of centrifugation and was generally greatest in concentrates produced by the cream separator and super-centrifuge, particularly in the case of the diatom, *C. muelleri* and two prymnesiophyte species *P. lutheri* and T. *Isochrysis*. However apparent cellular damage in most cases was very low and at the time considered not sufficient to preclude the use of the super-centrifuge on any species.
Ultimately this decision had to be reversed in relation to those species that sustained >2% apparent damage when harvested by high G force centrifuges such as the cream separator (3000G) and the super-centifuge (13 000G). In all such cases, acceptable retention of nutritional value of stored concentratres (effective shelf-life of ≥ 6 to 8 weeks), as demonstrated by direct bioassay, could not be achieved.

5.3.3 Filtration

Discussion

Despite an almost bewildering array of water filters and filter equipment available, filtration has been largely ignored as a method of harvesting micro-algae for marine hatchery use. In our experience this may be due to the propensity of filters to clog coupled with difficulties in recovering concentrated micro-algae cells from the filters. It is nevertheless acknowledged that specialised filtration systems or adaptations thereof could be successfully developed.

Examples that appear to hold particular promise are:

- Cross flow self cleaning membrane filter of the type developed by *Memtec P/L*. Costs of purchasing and evaluating this type of filtration equipment were unfortunately well beyond the capital resources of this project.
- A reputedly much simpler and cheaper high capacity sea water l filtration system is currently being adapted in the UK for bulk selective harvesting of desirable and non desirable species of micro-algae from sea-water (Dr Alan W.Bunch, Dept. of Bio-sciences, University of Kent - pers. comm., October, 1998).

The following types of filters were actually tested over a wide spectrum of micro-algae:

Filter papers and discs:

- *Gelman Sciences P/L* membrane filter (cellulose triacetate), 47 mm diameter, 0.2 µm pore size
- Schleicher & Schüll No.6 47 mm diameter, glass fibre disc
- Millipore type HA 47 mm diameter, 0.45 µm pore size
- Millipore type SS 47 mm diameter, 3 µm pore size

In each case, the filter paper or disc was placed in a 47mm diameter *Millipore P/L* vacuum assisted filter funnel and a small volume (approx. 200 mL) of micro-algae suspension poured onto the filter surface. Densities of micro-algae cells within the filtrate were determined before attempts were made to re-suspend algae from the filter. Filters used in trials with motile species were initially soaked in filtered seawater to see if the cells would re-suspend themselves. As "auto-suspension" failed, all filters, irrespective of micro-algae species involved, were pre-rinsed with sea-water using a squeeze bottle into a clean beaker before being inverted, repositioned in the filter funnel and finally back-washed under vacuum with sea-water into the same beaker.

Without exception, each filter paper or disc rapidly blocked and recovery of the cells from the filter was found to be impractical.

Depth Filters and polyester felt filter socks:

Log phase cultures of *P. lutheri*, T. *Isochrysis, C. calcitrans* and *T. chuii* were filtered through wound polyester depth filter cartridge elements with rated nominal pore sizes of 1 and 5 µm mounted in a *Filterite, (Memtec P/L)* filter housing. In each case, 50 to 100 L of micro-algae culture was passed through the elements using a 220 watt open impeller submersible centrifugal pump (*DAB, Nova,* Model 300). In no instance did either 1 or 5 µm pore size filter elements retain significant amounts of micro-algae. Indeed there was no obvious reduction in cell concentration of the filtrate of any micro-algae culture when visually compared with corresponding original cultures.

Exactly the same set of negative results were obtained when the above procedures were repeated using polyester felt filter socks *(Filtermation P/L,* Sydney) with rated nominal pore sizes of 1 and 5µm, respectively.

5.3.4 Chemical flocculation

Introduction

In the past, coagulation or flocculation has been one of the primary means of harvesting micro-algae cells from suspension (Barnarbe, 1990). This can be achieved by auto-flocculation through manipulations of nutrient levels, CO_2 and pH (Becker, 1994) and has been experimentally induced (Richmond and Becker, 1990), however, a lack of predictability with auto-flocculation (Becker, 1994) has meant the addition of chemical flocculants is far more common.

Chemicals such as lime and ferric-alum have been widely used for treating wastewater and effluents (Lincoln, 1985; Koopman and Lincoln, 1983; Benemann et al., 1980; Moraine et al., 1980; Dodd, 1979; McGarry, 1970), although, from an aquaculture perspective, these chemicals pose the potential risk of toxic residues if used as a food source. An edible nontoxic alternative is

chitosan, a flocculant used to concentrate various species of micro-algae (Lubian, 1989; Morales et al., 1985; Lavoie and de la Noüe, 1983; and Nigam et al., 1980).

Chitosan is the name for a family of compounds formed from the partial deacetylisation of chitin, a polymer of N-acetyl glucosamine extracted from crustacean exoskeletons (Deshpande, 1986). While the characteristics and performance of chitosan can vary with its manufacture (Bough et al., 1978) it remains insoluble in water, alkalis and alcohol, but dissolves readily in dilute acids (Deshpande, 1986). Cell aggregation using polymeric flocculants is thought to be via cross-linking of algal cells by extended polymer chains (Richmond and Becker, 1990) and their effectiveness is a function of culture conditions. Chitosan in particular is only effective as a flocculant at reduced ionic strengths and thus increasing salinity reduces its effectiveness (Shelef et al., 1984).

The reported lack of toxicity, easy manufacture and low dosage rates for chitosan (Richmond and Becker, 1990) encouraged an assessment of its abilities to concentrate algae, despite its reduced efficiency in salt water. In addition the re-suspension of micro-algae flocs was assessed using a variety of dilute acid solutions.

Materials and Methods

Flocculation:
Samples of each of seven species of micro-algae listed in Table 5.3.4.1 were poured into 1 L glass beakers and placed on a magnetic stirrer. The pH of each culture was determined using a *Metrohm* (Model, 605) pH meter fitted with an intermediate junction *Ionode*® pH electrode. A stock solution of 0.5% (w/v) Chitosan (*Aldrich Chemicals*, Prod. No. 41,941-9), was made up in a 1% acetic acid solution (Lubian, 1989) and was added progressively to each micro-algae suspension. Chitosan concentrations tested ranged from 10 mg/L through to 150 mg/L.

At each of the tested concentrations of chitosan, the culture and flocculating agent were mixed for 1 minute before adjusting the pH to 8.0 with the addition of 1 N sodium hydroxide solution. The culture was then removed from the magnetic stirrer and allowed to stand for several minutes.

The dose rate of chitosan was progressively raised by increments of 10 mg/L until a sedimentary floc formed on the bottom of the beaker. The clear aqueous supernatant was siphoned off and the remaining floc was de-watered on a 75μm pore size polyester mesh screen. The flocculated product was then transferred to a sterile 70 mL plastic screw-cap container (*Disposable Products P/L*) and stored at 2 ± 0.5°C for several days before undertaking resuspension trials.

For each trial, pH changes and the effective dose of chitosan were recorded. Due to the aggregation of the harvested cells within flocs, the effectiveness of chitosan was calculated by deducting density counts of cells within the siphoned fluid from those of the original cultures. These counts were determined by microscopic examination using an "Improved Neubauer" haemocytometer (*Superior Co.*, Berlin, Germany) at 200x magnification.

Resuspension of flocs:
Using flocs of *Chaetoceros calcitrans,* several methods of dissociating aggregated cells were tested. Initially, 10 mL of flocculated material was added to either 100 mL of filtered sea-water, distilled water or to a hyper-saline solution of either 70 or 140 mg/kg. Each suspension was then poured into a beaker, placed on a magnetic stirrer and agitated for several minutes. Each suspension was then acidified to pH 5.0 using 0.1 M hydrochloric acid.

The effectiveness of an anionic surfactant, sodium dodecyl sulfate (SIGMA, L-5750), as an aid to the dispersion of flocs was also evaluated. Sufficient volume of the surfactant was added to bring

the suspension to an effective sodium dodecyl sulfate concentration of 2×10^{-3} M. The suspension was again acidified as previously described using 0.1M hydrochloric acid.

Hayes et al, 1978, noted that the choice of solvent for chitosan influenced its solubility and hence six "non toxic" organic acid solvents were evaluated at the following concentrations;

Tartaric acid (SIGMA T-0375)	0.5M
Lactic acid (SIGMA L-1893)	0.5M
Dichloroacetic acid (SIGMA D-6399)	0.2M
Citric acid (BDH Prod. 10081)	0.5M
Glycolic acid (SIGMA G-1884)	0.5M
Acetic acid (Ajax UN No. 2789)	1.0% w/v

Six samples each of *P. lutheri*, *C. calcitrans* and *S. costatum* were collected and the pH of each was adjusted to 6.5 using 0.5N HCl. Chitosan solutions were then made using each of the six listed acids. In each case, sufficient acid was added to permit enough chitosan to be dissolved to form a 0.5% w/v solution. Each chitosan solution was then used at a rate of 80 mg chitosan l^{-1} to flocculate cells within samples of each of the three micro-algae species. The micro-algae samples were allowed to stand until a floc formed and settled on the bottom of the vessels. Micro-algae flocs were harvested by siphoning off the supernatant and the resultant slurry stored at 2 ± 0.5°C in sealed 70mL plastic screw cap specimen jars *(Disposable Products P/L)* for several days.

Two sub-samples of each of the 18 flocculated slurries were then each added to 100 mL of 1µm filtered seawater before one of the samples was re-acidified to a pH of 5.0 using the same acid used as the chitosan solvent for that particular sample. The remaining sample from each pair was re-acidified using 0.1 N HCl. The number of cells successfully resuspended was then assessed using 'Improved Neubauer' haemocytometer slide (*Superior Co.*, Berlin, Germany) and a light microscope at 200x. Results were recorded as a percentage of the original number of flocculated cells.

Results

Flocculation:
Flocculation efficiency was enhanced with the manipulation of pH. The best results obtained for each micro-algae species are summarised in Table 5.3.4.1. Optimal chitosan dosages ranged from 40 mg/L for *Tetraselmis chuii,* T. *Isochrysis* sp and *Thalassiosira pseudonana,* to as high as 150 mg/L for *Chaetoceros muelleri* with no apparent consistency within algal taxonomic groups. For example within the Bacillariophyta (diatoms), optimum chitosan concentration ranged from 40mg/L for *Thalassiosira pseudonana* up to 150mg/L for *C.muelleri.*

Table 5.3.4.1.

Species	Chitosan Dosage	Culture pH	pH after chitosan addition	Estimated cell recovery
Chaetoceros muelleri	150mg/L	8.06	5.03	95%
Chaetoceros calcitrans	80mg/L	7.29	5.27	80%
Skeletonema costatum	80 mg/L	8.66	5.42	70%
Thalassiosira pseudonana	40mg/L	8.29	6.31	90%
Tetraselmis chuii	40mg/L	7.69	6.03	80%
Pavlova lutheri	80mg/L	7.28	5.30	80%
Tahitian *Isochrysis*	40 mg/L	7.43	6.26	90%

Re-suspension of Flocs:
Attempts to re-suspend flocs by agitation alone were unsuccessful. Single cells could not in fact be dissociated from the clumps until suspensions were acidified to a pH of 5.0 using 0.1 N HCl. Even then approximately 50% of cells remained clumped.

The attempted suspension of flocs in distilled water produced no tangible benefit to re-suspension of flocs while use of hyper-saline solutions (70 and 140g/l) had little effect beyond causing the flocs to float. Acidifying these solutions with HCl did not increase the numbers of fully dissociated cells beyond levels observed in normal sea-water (35g/l).

All six alternative organic acid solvents used to prepare chitosan solutions, successfully flocculated all species of micro-algae tested with no observed differences between solvents. However when the same acid solvents used to prepare chitosan solutions were used to re-acidify resultant flocculated cells, the percentage of cells dissociating from flocs varied greatly (Table 5.3.4.2). Among the tested acids, citric and hydrochloric acid appeared most suitable for re-suspending flocs irrespective of algal species, although tartaric acid provided excellent results with *S. costatum* flocs.

Table 5.3.4.2. The percentage of single re-suspended cells for three micro-algae species flocculated and re-suspended using various combinations of Chitosan solvents and re-acidification solutions

	Algal species		
Chitosan solvent / Re-acidifiction solutions	***Chaetoceros calcitrans***	***Pavlova lutheri***	***Skeletonema costatum***
Citric acid/Citric acid	69.7	65.4	89.0
Citric acid /HCL	50.5	68.6	81.1
Acetic acid /Acetic acid	67.5	45.0	72.0
Acetic acid /HCl	50.6	49.1	85.4
Tartaric acid /Tartaric acid	59.7	51.9	92.2
Tartaric acid / HCl	53.9	64.5	86.4
Glycolic acid/Glycolic acid	47.3	62.3	78.1
Glycolic acid / HCl	46.0	64.2	80.7
Lactic acid/ Lactic acid	42.8	60.7	35.1
Lactic acid / HCl	47.1	59.7	73.0
Dichloroacetic acid/Dichloroacetic acid	40.8	65.2	76.8
Dichloroacetic acid / HCl	38.6	67.6	72.3

Discussion

Chemical flocculation methods are normally used for either clarifying drinking water or for extracting particular micro-algae products such as beta carotene and highly unsaturated fatty acids. Such processes require effective flocculation of the micro-algae, but the retention of cell wall and membrane integrity is of less importance than in aquaculture feed applications. More importantly, water clarification or chemical extraction does not require the re-suspension of clumped cells. Chitosan was found to be extremely effective in flocculating marine micro-algae despite the saline environment. However subsequent dissociation and efficient re-suspension of single cells from the flocs proved difficult.

In case of the diatom, *S. costatum*, chitosan flocculation and re-suspension efficiency was high using tartaric acid. However we had previously demonstrated that this particular species is harvested with 100% efficiency by super-centrifugation without any appreciable cell damage (Table 5.3.2.1). On the other hand, as a substantial proportion (8%) of *P. lutheri* cells sustain

damage during super-centrifugation chitosan flocculation does merit further consideration in relation to this and other species susceptible to damage during centrifugation.

The simplicity, effectiveness and low cost of chitosan flocculation does suggest its possible useful application to harvesting of micro-algae to be fed to deposit feeders such as abalone, turban shell, trochus, marine bait worms and beche de mer.

5.3.5 Electroflocculation

Introduction

Electroflocculation has been proposed as an alternative method to chemical flocculation of micro-algae cells for clarifying waste water (Kumar et al., 1981; Sridhar et al., 1988). This process does not require chemical additives and thus avoids potential problems of re-suspending cells, chemical toxicity and reduced palatability already discussed.

Passage of a mild electric current through a suspension of micro-algae results in hydrolysis. Fine bubbles of hydrogen and oxygen produced adhere to the micro-algae cells to form buoyant flocs that can be skimmed from the culture surface (Richmond and Becker, 1986). In fresh water, low voltage electric fields (3-9 V cm^{-1}) have be used to clear *Chlorella* and *Scenedesmus* from the water column (Kumar et al., 1981). However, in salt water the electro-flocculation may produce high levels of chlorine. In trials with *Isochrysis galbana*, Shelef et al. (1986) found chlorine levels of up to 100 mg L^{-1}. Although the authors noted that during short exposures this had no apparent adverse effect, chlorine is generally considered toxic to algae and to mollusc larvae. Regardless, electro-flocculation was evaluated as a potential harvesting method with the view that should it be suitable if the problem of chlorine generation could be addressed, possibly using neutralising agents if necessary.

Materials and Methods

The algal species trialed were *Pavlova lutheri, Isochrysis sp.* Tahitian and *Chaetoceros calcitrans.* 400 mL of algal culture from 500 L bag cultures at late exponential growth phase were placed into a 500 mL glass beaker fitted with a pair of 12 x 3 cm electrodes 1 cm apart (Kumer et al., 1981). Both aluminium and stainless steel electrodes were trialed. A voltage transformer was used to supply 3-9 V cm^{-1} AC current and in combination with a rectifier, to supply a similar voltage of DC current.

The % efficiency of the flocculation process was established by comparing the number of algal cells in the subnatant that was siphoned out during harvest to the number of algal cells in the original 400mL of culture. The algal cell densities of the initial cultures and of the subnatants were established microscopically at x250 using an "Improved Neubauer" haemocytometer (Superior Co., Berlin).

During the electroflocculation process, 10 mL samples were regularly removed from the beaker and tested for the presence of chlorine using a Lovibond 2000 Comparator Mk.11.

Harvesting of the flocs was achieved by siphoning away the subnatant i.e. relatively clear water under the flocs. De-watering of the harvested flocs was attempted by filtering onto a range of screens from 20 - 75 μm. Harvested flocs were stored in 150 mL sample jars at 2^0C. For re-suspension, 10mL samples of stored flocs were added to 100 mL of filtered seawater and homogenised for up to 10 sec with a blender (Sunbeam Food Processor JM035).

Results

DC current was much more effective than AC current with regard to voltage required, time taken for flocs to form as well as the buoyancy and harvestability of the resultant flocs. No chlorine generation was experienced.

For all algal species, 3V DC current was sufficient with both aluminium and stainless steel electrodes to produce buoyant flocs containing 80 - 90 % of the algal cells within 20 min. However, unlike the results of previous researchers working with freshwater algal cultures (Kumar et al., 1981; Richmond and Becker, 1986) the buoyant flocs created with our saltwater cultures were not a thin layer of algal cells on the surface but a very thick band of algal cells embedded within a matrix material occupying 25 - 30 % of the culture volume. Removal of the subnatant by siphoning resulted in a harvested product that was only three to four times more concentrated than the original cultures. Dewatering by filtering proved to be both difficult and inefficient because of fine screen blockage and/or cell losses through coarse screens.

Algal cells in the resuspended harvested flocs were badly clumped and remained bound within the matrix material.

Table 5.3.5.1. Voltages, current type and treatment times applied for electro-flocculation trials.

Algal Species	Voltage	AC/DC	Time Exposed	Resultant Flocs.
Pavlova lutheri	3V	AC	15 minutes	Nil
Pavlova lutheri	7V	AC	20 minutes	floating floc
Chaetoceros calcitrans	5V	AC	25 minutes	many cells (approx ½) in floating floc
Pavlova lutheri	5V	DC	5 minutes	floating floc
Pavlova lutheri	2V	DC	not recorded	floating floc

Discussion

Electroflocculation appears to be much more effective for clarifying waste water and/or producing algal concentrates in fresh water than in salt water. Cell densities within harvested flocs resulting from the electro-flocculation of salt water algal cultures were impractically low. Because of the high percentage of clumped algal cells and the fact that the cells are bound within a matrix material even after re-suspension, the product could not be classified as a suitable feed for mariculture.

6. SCREENING TRIALS USING INDIRECT CRITERIA TO EVALUATE PRESERVATION AND STORAGE TECHNIQUES

Introduction

Stored microalgal concentrates are susceptible to rapid loss of nutritive value if not stored under appropriate conditions. A key to centralised production and supply of micro-algae for use by aquaculture hatchery and nursery production is extended shelf-life of stored concentrated products. A major prerequisite to extended shelf-life is maintenance of cell wall and/or membrane integrity, and consequently, the cell contents and their chemical integrity.

For the purpose of this study, a minimum practical shelf-life requirement of 4-6 weeks was assumed. Such a period spans the duration of hatchery and preliminary nursery rearing cycles for most marine invertebrates species including all commercially important species of penaeid prawns (Fast and Lester, 1992) and bivalve molluscs. Most commercially important examples of the latter are edible oysters, including *Crassostrea spp.*(Chew, 1991), *Saccostrea spp.*(Frankish et al., 1991) and *Ostrea spp.* (Hickman et al., 1988); pearl oysters (Alagarswami, et al., 1989; Rose and Baker, 1994); clams (Manzi and Castagna, 1989); scallops (Bourne et al., 1989; Heasman et al.,1995) and mussels (Lutz and Kennish, 1992).

Extension to the shelf life of micro-algae concentrates was investigated using one or a combination of three techniques used by the food industry. The first was the use of non-toxic preservatives (food additives) that can be classified by their mode of action into the following categories:

- *Anti-oxidants* - prolong shelf life of food by preventing oxidation which causes rancidity and colour changes. These are particularly useful in retaining the organic chemical integrity of essential lipids, especially HUFA's and vitamins (especially C, B1 and E).
- *Food acids* - help induce and maintain constant low pH (<5) to inhibit autolysis and microbial decomposition.
- *Other preservatives* - additional additives that help protect against spoilage especially that caused by micro-organisms. Common examples are high osmolarity levels of salt and sugar inhibitory to autolysis and microbial decomposition.
- *Cryo-protectants* - agents that prevent damage to cell membranes caused by the formation of intracellular ice crystals thereby enhancing the benefits of sub-zero storage temperatures.
- *Vitamins* - make up for losses in processing and storage and are added to certain foods to supplement dietary intake, Vitamins C and E also double as anti-oxidants.

The second and simplest technique evaluated was low temperature storage. Reduced temperature slows both metabolic processes whilst cells remain alive (viable) and post-mortem changes including oxidative denaturation of essential vitamins and highly unsaturated fatty acids (HUFA's), autolysis and microbial degradation. Concerning the latter, all micro-organisms have definite minimum, maximum and optimal growth temperatures (Stanier et al., 1977). Reducing temperature of concentrated algae was therefore seen as an obvious and simple potential method of retarding microbial degradation of stored concentrates.

The third technique evaluated was lower density slurries created either by less extreme forms of centrifugation or by diluting high density super-centrifuged pastes with sterile seawater. The slurries were then stored under an array of physio-chemical conditions thought to affect retention of cell viability and/or post-mortem retention of physio-chemical integrity of the cells. The latter included alternative types of atmosphere (Montaini et al., 1995), storage in either light or in darkness and use of chilled storage, alone or in combination with one another.

Three additives initially selected and evaluated for enhancing the shelf life of micro-algae concentrates were glycerol, ascorbic acid (Vitamin C) and citric acid.

Glycerol has been widely demonstrated as an effective cryo-protectant for marine micro-algae (Brown, 1972; Aujero and Millamena, 1979; Fenwick and Day, 1992; Day and Fenwick, 1993; Molina Grima et al., 1994) and was selected ahead of dimethyl sulphoxide (DMSO) because of the potential toxicity of the latter to both micro-algae (Canavate and Lubian, 1994) and to invertebrate larvae including those of molluscs (Chao et al., 1994) and crustaceans (Fisher et al., 1996).

Ascorbic acid was the first choice anti-oxidant because of its widespread use in the food industry, low toxicity, dual action as a food acid and potential benefit as a dietary vitamin supplement (Vitamin C). Citric acid is a non toxic naturally occurring food acid widely used in the food industry that was included in some treatments to maintain a constant low pH in stored micro-algal concentrates.

Because of the large array of micro-algae species included in this study and the need to test a wide variety of alternative food additives, storage methods and combinations thereof across these species, it was necessary to supplement direct bio-assay experiments with a series of screening experiments. The latter had the advantage of being fast and very easy to implement by virtue of simple indirect criteria used to assess retained quality of stored concentrates.

Simple indirect criteria used to evaluate retained quality of stored concentrates included gross visual appearance, odour, pH, plus the ease and persistence of cell re-suspension. These criteria were augmented by microscopic examination for evidence of cell degradation, clumping and retention of viability as indicated by staining response of cells to Evan's blue.

For ease of interpretation and continuity, a progression of screening trials using indirect criteria to evaluate the efficacy of alternative preservation and storage techniques are reported separately from a complimentary series of bio-assay experiments.

6.1. Screening Trial 1: Preliminary Evaluation of Three Additives

Aims

This screening trial was designed to evaluate three initially selected additives, the anti-oxidant ascorbic acid (Vitamin C), citric acid induced low pH and the cryo-protectant glycerol for enhancing the shelf-life of chill stored super-centrifuged microalgal pastes. The diatom *Skeletonema costatum* was chosen as an initial test species because of its widespread use for hatchery rearing of bivalve molluscs and penaeid shrimp and its high apparent resistance to cell wall damage during super-centrifugation.

Materials and Methods

S. costatum was batch cultured in cylindrical 1000 L tanks using inocula. In all cases, culture solutions comprised oceanic salinity (34-35g/l) seawater stored and settled for a minimum of one week, filtered through a 1um nominal depth filter, chlorinated (10 mg/L for ≥12 h), neutralised and fertilised with f/2 beta growth medium (Guillard, 1983). Cultures were maintained at 23°C under a 16:8 h light:dark regime. Illumination was generated by 58 watt 'Coolwhite' fluorescent tube lights that provided a surface intensity of approximately 4,000 lux.

Cells were concentrated using a *Sharples* Super-centrifuge operating at 13,000 g. The algal culture was supplied to the centrifuge via a 1/2" diaphragm pump (ARO Corporation, Ohio USA, Model No.66606X-X) at approximately 25L/minute. The concentrated cells were harvested from the centrifuge bowl using a spatula. A minimum of three different cultures were used of each species in order to combat batch variability. After harvest the micro-algae concentrates from individual cultures were combined and blended to achieve an homogeneous mix. Sub samples of concentrate were weighed and additives included either singly or in combination at the following rates:

- glycerol (SIGMA G-7893) at 10% (w/w)
- ascorbic acid (Vitamin C) (SIGMA A-7506) at 1% (w/w)
- citric acid (BDH Prod No. 10081) until pH was lowered to the range 4 - 4.5

The eight resultant treatments comprised:

- a raw paste control containing no additives
- three treatments each containing a single additive only
- all three possible combinations of two additives
- a combination of all three additives

The additives were blended into the concentrates to ensure homogeneous dispersion. The concentrates were transferred into 70mL screw top containers (Disposable Products P/L) and stored at temperatures of 2 ± 0.5°C in a chest freezer (Fisher and Paykel P/L, (New Zealand) model H-360X) fitted with "Thermo-Eye" digital temperature controllers (Type PLE - Saginomiya P/L, Tokyo, Japan).

Retained quality of stored concentrates was evaluated at successive intervals of 2 weeks over a total period of 10 weeks using a scoring regime specified in Table 6.1.1. The gross appearance including obvious contamination by yeast, bacteria and fungi, and colour change was assessed as were offensive odours. A sample was drawn from each replicate using a 1.0 mL syringe, the process being performed in a laminar flow cabinet to reduce extraneous contamination.

Table 6.1.1. Criteria used to assess retained quality of stored pastes.

Degree of Contamination	Odour
0 = no apparent contamination 1 = few small isolated colonies representing< 5% of surface 2 = moderate number of isolated colonies 3 = moderate to high contamination 4 = majority (> 50%)of surface covered 5 = entire surface covered in microbial growth	0 = normal fresh odour 1 = different, but not offensive 2 = offensive

A small sample of micro-algae paste was re-suspended in filtered sea water at the rate of approximately 0.1mL of paste / 100mL sea-water using a "Maestro" (Sunbeam Australia P/L) hand held food processor at the low speed setting. A 20.0mL aliquot of micro-algae re-suspension was then transferred to a sterile container and inoculated with 1mL of 1% (w/v) Evan's blue (Sigma Chemicals P/L, Cat. No. E-2129) stain and left to incubate at room temperature for a minimum of 30 minutes (modified from Grima et al., 1994). Cell wall integrity was assessed using a light microscope at 250x and an 'Improved Neubauer' haemocytometer (Superior Co., Berlin, Germany). Cells that did not take up the stain were considered viable, and those that stained blue, non-viable.

A second smaller sample was also drawn for measurement of pH using a 'Manutec' Soil pH test kit.

It was anticipated that results of this trial would identify the individual and combined effects of additives on shelf-life of pastes thereby revealing possible complimentary and antagonistic effects.

Results

The best result, as indicated by retention of cell viability (Fig 6.1.1) after ten weeks of chilled storage, was conferred by the combination of all additives. Use of both Vitamin C and of citric acid alone or in combination also enhanced the retention of cell viability above that of the raw paste.

A positive response to the inclusion of glycerol was only apparent when it was combined with both Vitamin C and citric acid.

Results relating to degree of microbial contamination of the surface of stored pastes (Fig 6.1.2) and to offensive odours (Fig 6.1.3) varied from that of cell viability in that none of the three additives alone improved retained quality above that of the raw paste control. However best results were again obtained with the inclusion of all three additives.

The pH of raw paste (Fig. 6.1.4) rose above 10 within a fortnight of storage and remained so for the full 10 week duration of the trial. While the inclusion of either citric acid or Vitamin C singly or in combination with glycerol did maintain pH at lower levels (range 5 to 8) over 10 weeks of storage, only the combination of Vitamin C and Citric acid (with or without glycerol) was successful in maintaining pH within the targeted range of 4 to 4.5.

Discussion

The encouraging results of this first screening trial demonstrated that all three classes of common food additives tested were beneficial especially when used in combination on super-centrifuged pastes of the diatom *S. costatum*. Indeed *S. costatum* paste protected by the combination of Vitamin C, citric acid induced low pH and glycerol when chill stored at 2 ± 0.5°C retained >90% cell viability and remained essentially free of microbial contamination and associated offensive odours for up to 10 weeks.

Fig 6.1.1 Effects of additives on cell viability in supercentrifuged pastes of *S. costatum*

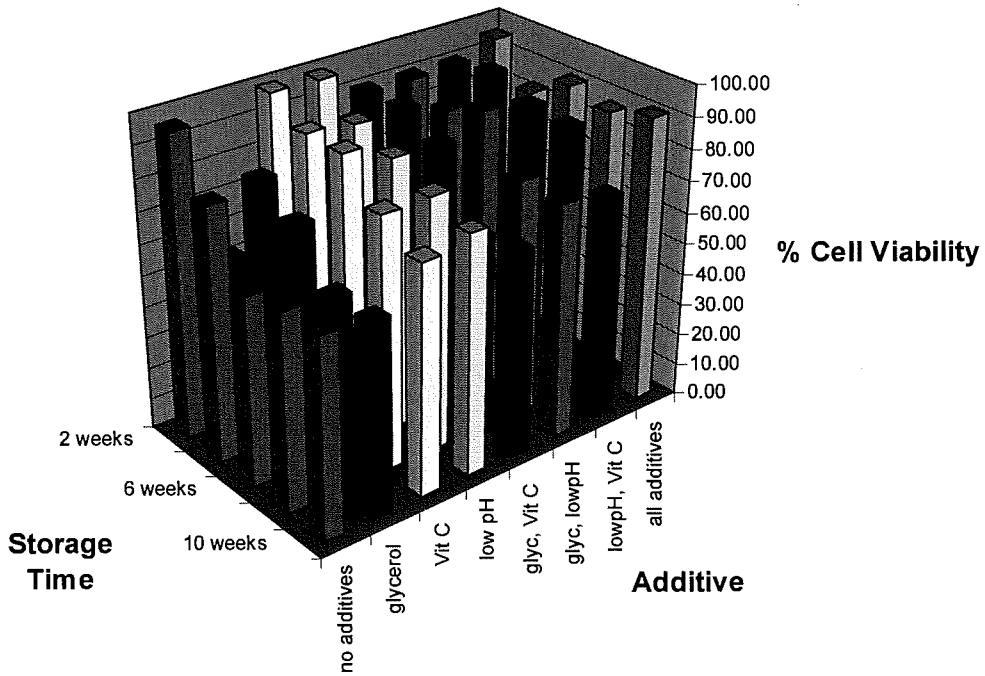


Fig 6.1.2 Effects of additives on contamination of supercentrifuged *S.costatum* pastes

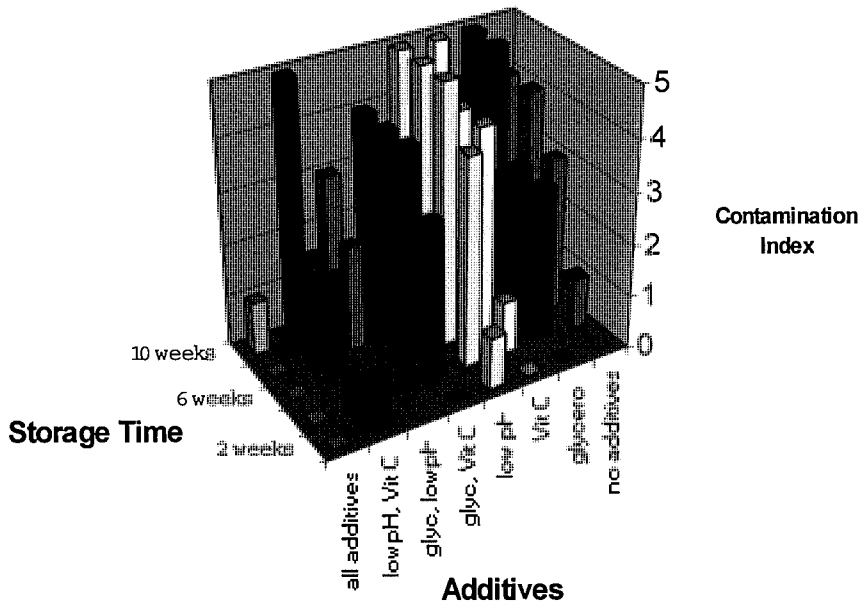


Fig 6.1.3 Effect of additives on off odours in supercentrifuged pastes of *S. costatum*

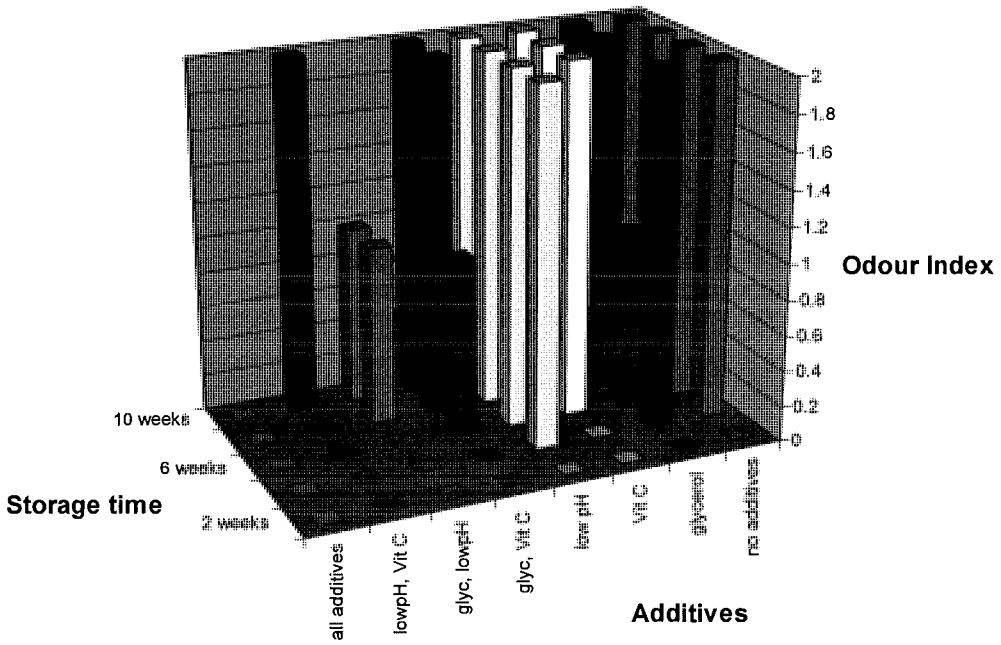
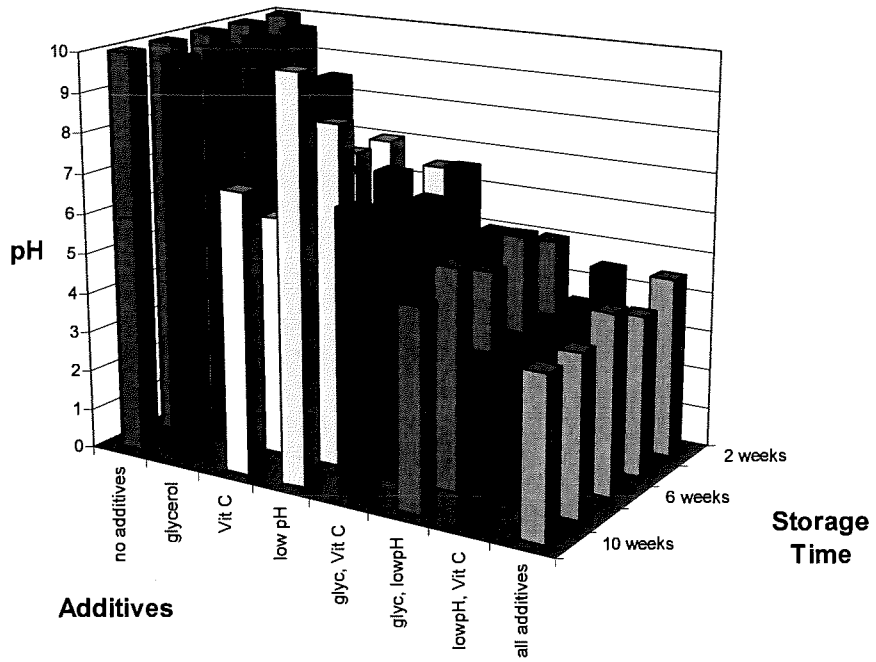


Fig 6.1.4 Effects of additives on pH of supercentrifuged pastes of *S. costatum*



6.2. Screening Trial 2: Evaluation of Low Temperature Storage in Combination with Additives

Aims

This very large screening trial was prompted by encouraging results of Screening Trial 1. It was designed to expand and extend evaluation of the same suite of three initially selected food additives, the anti-oxidant ascorbic acid (Vitamin C); citric acid to induced low pH and glycerol as a cryo-protectant. The basis of expansion and extension was incorporation of four chilled storage temperature regimes including a sub-zero regime and an increase in species of micro-algae tested from one to four.

Materials and Methods

The four species of micro-algae tested included *Skeletonema costatum*, the sole species in Screening Trial 1. *S. costatum* was retained as a reference species to check whether or not quality assessment criteria and results obtained in Screening Trial 1 were reliable and repeatable. The second selected species, another diatom *Chaetoceros muelleri,* was chosen in recognition of its key nutrition role in *Penaeus monodon* hatcheries throughout the Indo west Pacific. *C. muelleri* was also included because it contrasts with *S. costatum* in its high susceptibility to physical damage during super-centrifugation (see Section 5.3.2). The third and fourth species used, the naked flagellates, Tahitian *Isochrysis* (T. *Iso*) and *Pavlova lutheri,* were also selected to determine whether shelf-life prolonging properties of the additives in relation to *S.costatum* as demonstrated in Screening Trial 1 could be extended to more fragile species prone to damage imposed by high speed centrifugation.

Four storage temperatures trialed were 4 ± 6°C (provided by a general purpose domestic upright refrigerator subject to routine daily use); 2.0 ± 0.5 ° C (consistent with the temperature used in Screening Trial 1); 0.0 ± 0.5 ° C and -2.0 ± 0.5 ° C. All three lower temperature storage conditions were provided by individual chest freezers (Fisher and Paykel P/L, New Zealand, model H-360X) each fitted with a "Thermo-Eye" (Type PLE - Saginomiya P/L, Japan) digital temperature controller.

The four micro-algae species used were grown either in batch culture in 500 L bags (T. *Iso* and *P. lutheri.*) or cylindrical 1000 L tanks (*C. muelleri* and *S. costatum*) from inocula. All other culture techniques were the same as described for Screening Trial 1.

Cells were concentrated using a "Sharples P/L, Super-centrifuge" by the same techniques described for Screening Trial 1 with a minimum of three different cultures again being used of each species in order to combat batch variability.

Twenty-six treatments (Table 6.2.1) were run for each of the first three species of micro-algae. *(C. muelleri* and *S. costatum* and *T. Iso*). They comprised a 4 x 5 factorial combination of four storage temperatures (4 ± 6°C ; 2.0 ± 0.5°C ; 0.0 ± 0.5°C and -2.0 ± 0.5°C) and 5 additive types (a raw paste control containing no additives; 3 types of paste containing a single additive only and fifth type containing all three additives). The balance of six treatments comprised supplementary 2 x 3 factorial array of two temperatures (2.0 ± 0.5°C and -2.0 ± 0.5°C) and all three possible binary combinations of the three additives. The latter were included in order to help discern possible complimentary or antagonistic interaction between the three additives. Techniques used to prepare treatments with one or more of the three additives were the same as described for Screening Trial 1.

Table 6.2.1.

Treatment No.	Treatment	Treatment No.	Treatment
1	glycerol, low pH, Vit C @ 4°C	14	glycerol @ 2°C
2	glycerol, low pH, Vit C @ 2°C	15	glycerol @ 0° C
3	glycerol, low pH, Vit C @ 0°C	16	glycerol @ -2°C
4	glycerol, low pH, Vit C @ -2°C	17	no additives @ 4°C
5	glycerol, lowpH @ 4°C	18	no additives @ 2°C
6	glycerol, lowpH @ 2°C	19	no additives @ 0°C
7	glycerol, lowpH @ 0°C	20	No additives @ - 2°C
8	glycerol, lowpH @ -2°C	21	low pH, Vit C @ 4°C
9	glycerol, Vit C @ 4°C	22	low pH, Vit C @ 2°C
10	glycerol, Vit C @ 2°C	23	low pH @ 4°C
11	glycerol, Vit C @ 0°C	24	low pH @ 2°C
12	glycerol, Vit C @ -2°C	25	Vit C @ 4°C
13	glycerol @ 4°C	26	Vit C @ 2°C

Nine storage treatments used for *P. lutheri* comprised eight additive types (a raw super-centrifuged paste control containing no additives; three types of paste containing a single additive only; three types comprising each of the possible binary combinations of additives and an eighth type containing all three additives). All eight treatments just described were stored at 2.0 ± 0.5°C while the ninth treatment comprising paste with all three additives was stored at the lower temperature of -2.0 ± 0.5°C.

As in Screening Trial 1, all treatments were sealed in 70 mL screw cap containers during storage. These were examined macroscopically and at 250x at successive intervals of 2 weeks for a total period of 6 weeks in the case of *C. muelleri,* 8 weeks for *T. Iso* and 10 weeks for *S. costatum.* Supplementary sampling for retained cell viability and degree of contamination was also applied after 15 weeks of storage to a select array of eight *S. costatum* treatments held at 2.0 ± 0.5°C. By contrast, the nine *P. lutheri* treatments were only sampled on a single occasion after 5 weeks of storage.

Methods used to assess retained quality or stored pastes included all those applied in Screening Trial 1(odour, obvious contamination by spoilage micro-organisms and an estimate of cell viability based on staining response to Evan's blue). Additional quality indicators used were an evaluation of cellular breakdown, degree of cell clumping and the speed and efficiency of cell re-suspension. The latter was determined by the degree of cell clumping and amounts of broken down cellular material present in re-suspensions. Scoring criteria for these various assessments of retained quality are provided in Table 6.2.2.

Table 6.2.2. Gross macroscopic quality assessment criteria.

Odour	**The degree of cell clumping**
	This was assessed by counting the number of cell clumps in a fixed grid area of a haemocytometer. A clump was defined as >3 cells.
0 = normal (like fresh paste)	Closely associated with each other viz.
1 = different but not offensive	small clump = 3 - 5 cells
2 = offensive	medium clump = 5 - 10 cells
	large clump = > 10 cells

Contamination	Degree of broken down cellular material
0 = normal	0 = very low
1 = few small isolated colonies representing < 5% of surface	1 = low
2 = moderate no. of isolated colonies	2 = low - moderate
3 = moderate - high contamination	3 = moderate
4 = majority of surface covered > 50%	4 = moderate - high
5 = entire surface covered in microbial growth	5 = high
	6 = very high

Ease of re-suspension	
This was evaluated according to the length of time required for the paste to be fully re-suspended.	
1 = 2 seconds	
2 = 3 - 5 seconds	
3 = 5 - 10 seconds	
4 = 10 seconds or more	

On all sampling occasions, a small sample of micro-algae paste was re-suspended in filtered sea-water at the rate of approximately 0.1mL of paste/100mL seawater using a "*Maestro*" (Sunbeam Australia P/L) hand held food processor at the low speed setting. A 20.0mL aliquot of micro-algae re-suspension was then transferred to a sterile container and inoculated with 1mL of 1% (w/v) Evan's blue (SIGMA Chemicals P/L E-2129) stain and left to incubate at room temperature for a minimum of 30 minutes (modified from Grima et al., 1994). Cell wall integrity was assessed using a light microscope at 250x and an 'Improved Neubauer' haemocytometer ("Superior Co.", Berlin, Germany). Cells that did not take up the stain were considered viable, and those that stained blue, non-viable.

Results

For convenience results for each of the three micro-algae species are discussed under two topics namely, effects of alternative chilled storage temperatures and effects of additives.

S.costatum - effects of storage temperature:
Greatest protraction of shelf life of raw *S.costatum* pastes, as indicated by retention of cell viability (Figs 6.2.1 and 6.2.2) and contamination (Fig 6.2.3) was unexpectedly provided at a chilled storage temperature of +2.0 ± 0.5°C rather than at lower test temperatures of 0.0 ± 0.5°C or -2.0 ± 0.5° C. As expected, the pastes exhibiting poorest shelf life were those stored in the refrigerator at the highest and most variable temperature of 4 ± 6°C. As discussed in detail below, inclusion of additives extended shelf life of *S. costatum* paste across all four storage temperatures but with best results occurring at -2.0 ± 0.5° C and +2.0 ± 0.5°C, intermediate results at 0.0 ± 0.5°C and poorest results again for paste stored at 4 ± 6 ° C in a domestic refrigerator (Fig 6.2.2).

S.costatum - effects of additives:
Results obtained were essentially identical to those obtained in Screening Trial 1. The best extension of shelf life, as indicated by retention of *S. costatum* cell viability over protracted periods of chilled storage, was again conferred by the combination of all three additives (Fig 6.2.5). Incorporation of Vitamin C and citric acid alone or in combination also enhanced the retention of cell viability above that of the raw paste but inclusion of glycerol did not. Positive response to the inclusion of glycerol was only apparent when combined with either Vitamin C and/or citric acid induced low pH.

Equivalent results relating to two other indicators of quality namely, odour and degree of microbial contamination of *S.costatum* pastes, varied from that of cell viability (Fig 6.2.4) in that while the three additives in combination improved these indicators of retained quality, none of them alone improved retained quality above that of the raw paste control. As in Screening Trial 1, inclusion of all three additives effectively suppressed all contamination and development of offensive odours. The only exception was a high level of contamination that developed in pastes stored in a domestic refrigerator at the highest and most variable test temperature of 4 ± 6°C.

An example of results relating to other supplementary indicators of retained quality, namely ease of re-suspension and degree of cell clumping after 10 weeks of storage, are provided in Table 6.2.3. These further support the contention that the combination of all three additives confers excellent retention of the quality of stored super-centrifuged paste of *S. costatum* when held at stable temperatures of +2 to -2 °C for periods of at least 10 weeks.

The pH of raw *S. costatum* paste (Fig.6.2.6) rose above 10 within a fortnight of storage and remained so for the full 10 week duration of the trial. While the inclusion of either citric acid or Vitamin C, alone or in combination with glycerol did maintain pH at lower levels (range 5 to 8) over the 10 weeks of storage, only the combination of Vitamin C and citric acid (with or without glycerol) was successful in maintaining pH within the targeted range of 4 to 4.5.

Table 6.2.3.

Skeletonema costatum (10 weeks)					Degree of clumping (no. of clumps)			
	Treatment	Contamination	Odour	Ease of Re-suspension	small	medium	large	Degree of broken down cells
1	glycerol, low pH, Vit C @4°C	3	2	1	1	0	0	1
2	glycerol, low pH, Vit C @ 2°C	0	0	1	0	0	0	1
3	glycerol, low pH, Vit C @ 0°C	0	0	1	0	0	0	1
4	glycerol, low pH, Vit C @-2°C	0	0	1	2	0	0	1
5	glycerol, lowpH @ 4°C	3	2	2	0	0	0	1
6	glycerol, lowpH @ 2°C	2	1	1	0	0	0	1
7	glycerol, lowpH @ 0°C	2	0	1	0	0	0	1
8	glycerol, lowpH @ -2°C	0	0	1	0	0	0	1
9	glycerol, Vit C @ 4°C	3	2	1	0	0	0	2
10	glycerol, Vit C @ 2°C	4	2	1	2	0	0	1
11	glycerol, Vit C @ 0°C	4	1	1	0	0	0	1
12	glycerol, Vit C @ -2°C	0	0	1	0	0	0	1
13	glycerol @ 4°C	3	2	1	0	0	0	2
14	glycerol @ 2°C	5	2	1	0	0	0	1
15	glycerol @ 0°C	4	2	2	0	0	0	1
16	glycerol @ -2°C	3	0	1	1	0	0	1
17	low pH, Vit C @ 4°C	5	2	1	0	0	0	2
18	low pH, Vit C @ 2°C	2	1	1	0	0	0	1
19	low pH @ 4°C	4	2	2	0	0	0	2
20	low pH @ 2°C	5	2	2	0	0	0	1
21	Vit C @ 4°C	4	2	2	3	0	0	2
22	Vit C @ 2°C	4	2	2	0	0	0	1
23	no additives @ 4°C	5	2	2	5	0	0	2
24	no additives @ 2°C	4	2	2	3	0	0	1
25	no additives @ 0°C	4	2	2	3	0	0	1
26	no additives @ -2°C	1	1	2	2	0	0	2

T. Iso - Effects of storage temperature:
No significant protraction of shelf life of raw T. *Iso* pastes, as indicated by retention of cell viability (Fig 6.2.7), was provided at any of the four chilled storage temperatures. As further discussed below, inclusion of all three additives provided best improvements in shelf life of T. *Iso* pastes paste across all four chilled storage temperatures (Fig. 6.2.8). Nevertheless none of the chilled storage regimes was able to maintain satisfactory levels of cell viability (above 80%) beyond 2 weeks of storage.

T. Iso - Effects of additives:
As indicated by cell viability data for super-centrifuged pastes of T. *Iso* stored at +2±0.5°C (Fig 6.2.9), the combination of all three additives provided the best, albeit inadequate, improvement to the very poor base shelf life of raw paste of this fragile walled species.

T. *Iso* paste treatments held at 4.0, 2.0 or 0.0 ± 0.5°C, exhibited moderate to high levels of microbial contamination and had developed a putrid odour in after six weeks storage (Table 6.2.4).

However, T. *Iso* paste stored at the lowest temperature of -2 °0 ± 0.5 °C did not develop offensive odours nor exhibited microbial contamination even after eight weeks of storage.

Table 6.2.4a. Other indicator of retained quality in super-centrifuged pastes of *T.Iso* after 6 weeks of storage

Treatment	Contamination	Odour	Ease of Re-suspension	Degree of clumping (no. of clumps)			Degree of broken down cells
				small	med	lge	
Glycerol, low pH, Vit C @ 4°C	5	1	1	50	3	0	3
Glycerol, low pH, Vit C @ 2°C	1	0	1	31	2	0	3
Glycerol, low pH, Vit C @ 0°C	0	0	2	20	2	0	3
Glycerol,low pH, Vit C @ -2°C	0	0	1	10	0	0	3
Glycerol, low pH, @ 4°C	5	2	1	44	2	0	3
Glycerol, low pH @ 2°C	1	0	1	38	1	3	3
Glycerol, low pH @ O°C	1	0	2	52	0	0	3
Glycerol, low pH @ -2°C	0	0	2	17	0	0	3
Glycerol, Vit C @ 4°C	5	1	2	36	3	0	3
Glycerol, Vit c @ 2°C	1	0	1	26	1	0	3
Glycerol, Vit C @ 0°C	0	0	2	24	0	0	3
Glycerol, Vit C @ -2°C	0	0	2	18	0	0	3
Glycerol @ 4°C	5	2	2	74	2	0	3
Glycerol @ 2°C	2	0	2	39	2	1	3
Glycerol @ 0°C	1	0	2	47	0	0	3
Glycerol @ -2°C	0	0	2	44	0	0	3
Low pH, Vit C @ 4°C	5	2	2	118	8	5	3
Low pH, Vit C @ 2°C	2	0	2	51	2	1	3
Low pH @ 4°C	5	2	3	21	0	0	3
Low pH @ 2°C	1	0	2	31	0	0	3
Vit C @ 4°C	5	2	3	53	1	0	3
Vit C @ 2°C	2	1	3	37	3	1	3
No additives @ 4°C	5	2	3	73	5	0	4
No additives @ 2°C	3	1	2	91	3	4	3
No additives @ 0°C	2	1	3	52	10	2	3
No additives @ -2°C	0	0	2	54	0	0	4

Only the combinations of citric acid and Vitamin C with or without glycerol were consistently successful in reducing and maintaining the pH of *T. Iso* pastes within the targeted range of 4 to 4.5 (Fig 6.2.10). Raw paste exhibited pH in the range of 7 to 8.5. All other combinations of additives resulted in pH's in the intermediate range of 6 to 7 regardless of storage temperature or duration.

C.muelleri - Effects of storage temperature and additives:
Shelf life of raw super-centrifuged *C. muelleri* pastes, as indicated by retention of cell viability, (Fig. 6.2.11) was less than 2 weeks. Even after this brief period, retained a cell viability within the best paste treatment (raw pastes stored at +2±0.5°C) was only14.5%. All other treatments exhibited 0 to 7.5% (mainly 0%) cell viability after only 2 weeks of storage. Similarly a very high proportion of decomposing cells were observed after only one week's storage.

Unlike T. *Iso*, poor shelf life of raw super-centrifuged pastes of *C. muelleri* could not be improved by either varying chilled storage temperature (Fig 6.2.11), nor inclusion of any of the common food additives trialed as indicated by data for pastes stored at +2±0.5°C (Fig 6.2.12).

This was in spite of the fact that some combinations (Table 6.2.4) the additives were successful in maintaining the pastes in outwardly good condition. For example, pH was maintained within the targeted range of 4.0 to 4.5 by the addition of citric acid plus vitamin C with or without glycerol at all temperatures tested. Likewise, contamination and development of offensive odours was prevented for up to 6 weeks by storing at -2.0±0.5 °C with or without additives and also at 0±0.5°C and at +2.0±0.5 °C when all additives were used in combination.

Table 6.2.4b. Condition of *C. muelleri* paste after 6 weeks of storage.

Treatment	Contamination	Odour	Ease of Re-suspension	pH	Degree of clumping (no. of clumps)			Degree of broken down cells
					small	medium	large	
Glycerol, low pH, Vit C @ 4°C	3	2	1	4	0	0	0	6
Glycerol, low pH, Vit C @ 2°C	0	0	1	4.5	0	0	0	6
Glycerol, low pH, Vit C @ 0°C	0	0	1	4	0	0	0	6
Glycerol, low pH, Vit C@ -2°C	0	0	1	4	0	0	0	6
Glycerol, lowpH @ 4°C	5	0	1	5.5	0	0	0	6
Glycerol, lowpH @ 2°C	1	0	1	4.5	0	0	0	6
Glycerol, lowpH @ 0°C	1	0	1	5	0	0	0	6
Glycerol, lowpH @ -2°C	0	0	1	5	0	0	0	6
Glycerol, Vit C @ 4°C	5	2	1	6.5	1	0	0	6
Glycerol, Vit C @ 2°C	1	0	1	5	0	0	0	6
Glycerol, Vit C @ 0°C	0	0	1	4.5	0	0	0	6
Glycerol, Vit C @ -2°C	0	0	1	5.5	0	0	0	6
Glycerol @ 4°C	5	2	1	8.5	2	1	0	6
Glycerol @ 2°C	2	1	1	8.5	4	0	0	6
Glycerol @ 0°C	2	1	1	8.5	3	0	0	6
Glycerol @ -2°C	2	0	1	7	3	0	0	6
low pH, Vit C @ 4°C	4	2	1	5	0	0	0	5
low pH, Vit C @ 2°C	1	1	1	4.5	0	0	0	5
low pH @ 4°C	3	2	1	6	1	0	0	6
low pH @ 2°C	2	1	1	4.5	0	0	0	6
Vit C @ 4°C	5	2	1	5.5	3	0	0	6
Vit C @ 2°C	2	1	1	5	0	0	0	6
no additives @ 4°C	5	2	1	7	2	0	0	6
no additives @ 2°C	3	1	1	8.5	4	0	0	6
no additives @ 0°C	3	1	1	8.5	1	1	0	6
no additives @ -2°C	1	1	1	7.5	9	1	0	6

Pavlova lutheri - Effects of storage temperature and additives:
Results of the nine treatments presented in Table 6.2.5 demonstrated that 5 weeks of chilled storage of super-centrifuged pastes of *P. Lutheri* with or without additives, maintained them within a favourable pH range of 4.0 to 5.0 and in excellent outward condition (as indicated by a total lack of offensive odours and contamination and relative ease of re-suspension). On the other hand, relative proportions of broken down cell walls and degree of cell clumping remained universally high in pastes regardless of the inclusion of one or a combination of additives.

These results suggest that cell damage sustained by this fragile species is sustained during the process of super-centrifugation itself. The results also showed that the damage done can be prevented from getting worse with protracted chilled storage but that subsequent cell-death cannot be significantly retarded let alone reversed by chilled storage, regardless of food preservatives or specific chilled temperature used.

Table 6.2.5. Condition of *P. lutheri* paste after 5 weeks of storage.

Treatment	Contamination	Smell	Ease of Re-suspension	pH	% viable cells	Degree of clumping			Degree of broken down cells
						small	med	lge	
no additives @ 2°C	0	0	2	4.5	3.4	13	0	0	3
glycerol @ 2°C	0	0	2	4.5	5.5	22	0	0	4
low pH @ 2°C	0	0	2	5	0.9	67	13	0	3
Vit C @ 2°C	0	0	2	4.5	1.5	86	7	0	3
glycerol, lowpH @ 2°C	0	0	2	4.5	1.1	31	3	0	3
glycerol, Vit C @ 2°C	0	0	2	4.5	1.1	17	0	0	3
lowpH, Vit C @ 2°C	0	0	2	4.5	7.4	76	16	0	2
glycerol, low pH, Vit C @ 2°C	0	0	1	4	5.1	41	2	0	2
glycerol, low pH, Vit C @ -2°C	0	0	1	4.5	3.8	25	3	0	2

Summary and Conclusions

Results of the trial as summarised in Table 6.2.6, illustrate a wide spread in the keeping qualities of the four species of micro-algae as measured by a range of indirect quality criteria and widely differing responses to the use of additives. To illustrate this point the best performing species *S. costatum* suffered negligible detectable physical damage during high speed centrifugation, retained ≥85% cell viability for up to 15 weeks and responded positively to the inclusion of all three types of common food additives. At the opposite extreme, pastes of a second diatom species *C. muelleri* that sustained high levels of physical damage during centrifugation, retained very low cell viability only after 2 weeks of storage and was negatively influenced by the inclusion of one or a combination of additives. The only consistent result across the five species were that:

- best retention of quality occurs at holding temperatures of either or -2.0± 0.5° C.
- storage in a domestic refrigerator at the higher and more variable temperature range of +4.0 ± 4.0° C seriously reduced retained quality and effective shelf life.
- the consequences of physical damage sustained during high speed centrifugation could not be reversed by cold storage nor the inclusion of additives.

Table 6.2.6. Brief summary of results.

Species of algae	Influence of Temperature	Best combination of additives	Best cell viability retention performance of all treatments tested	Comments
S.costatum	+2° C≥ -2°C>0°>+4C°	All additives	85% after 15 weeks	
P. lutheri	+2° C≥ -2°C>0°>+4C°	No substantial differences with or without one or a combination of additives		High proportion of damaged cells immediately following high speed centrifugation but all other indirect quality criteria remained high for up to 5 weeks
T. *Iso*	-2° C≥ +2°C>0°>+4C°	All additives	40% after 6-8 weeks	
C muelleri	-2° C≥ +2°C>0°>+4C°	No additives	14% after 2 weeks	High proportion of damaged cells immediately following high speed centrifugation but all other quality criteria remained high for 6 weeks

Fig 6.2.1 Effect of Storage Temperature on cell Viability of raw *S.costatum* paste

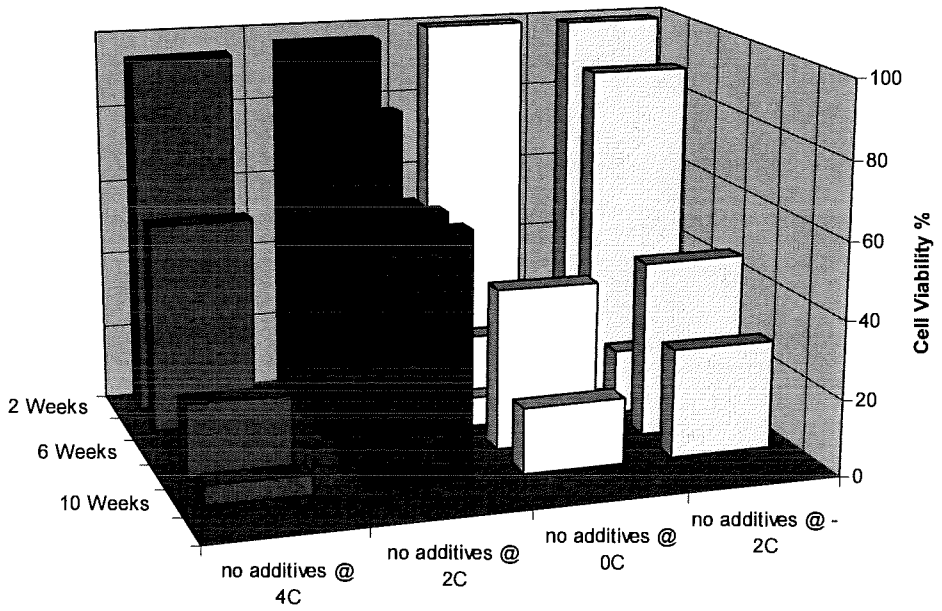


Fig 6.2.2 Effect of Storage Temperature of Viability of *S. costatum* with All Additives

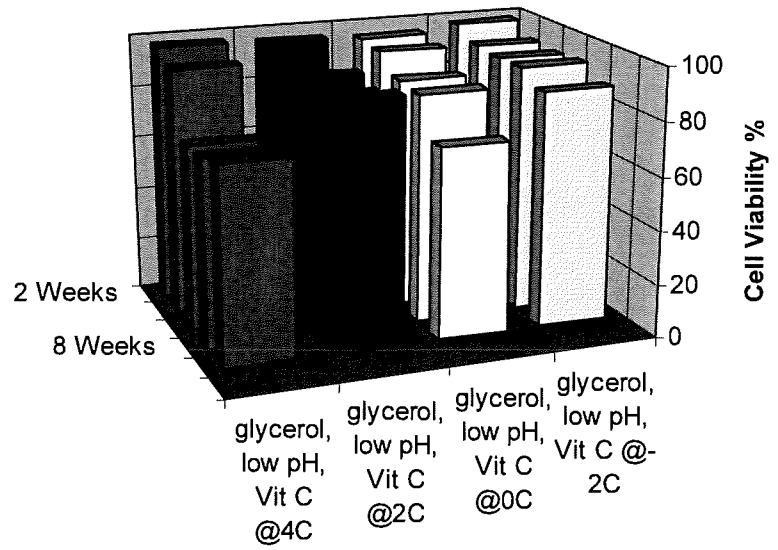


Fig 6.2.3 Effect of temperature on contamination of chill stored paste of *S. costatum* with all additives

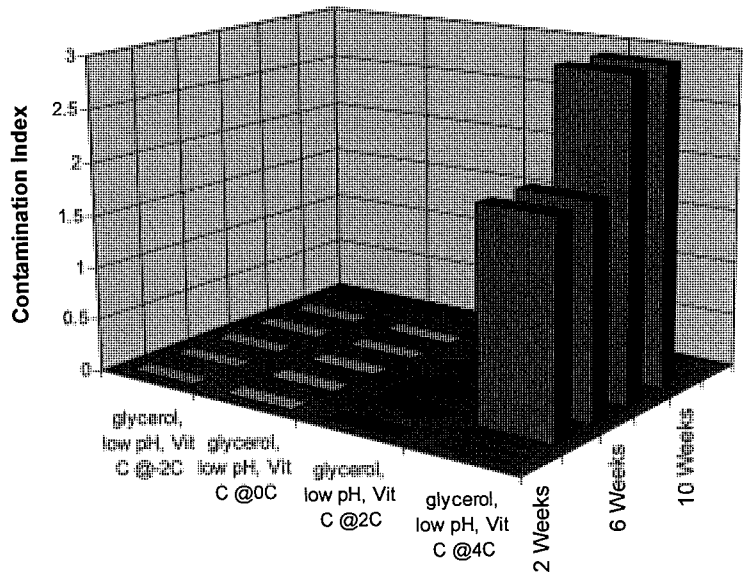


Fig 6.2.4 Effect of temperature on contamination of raw chill stored *S. coststum* paste

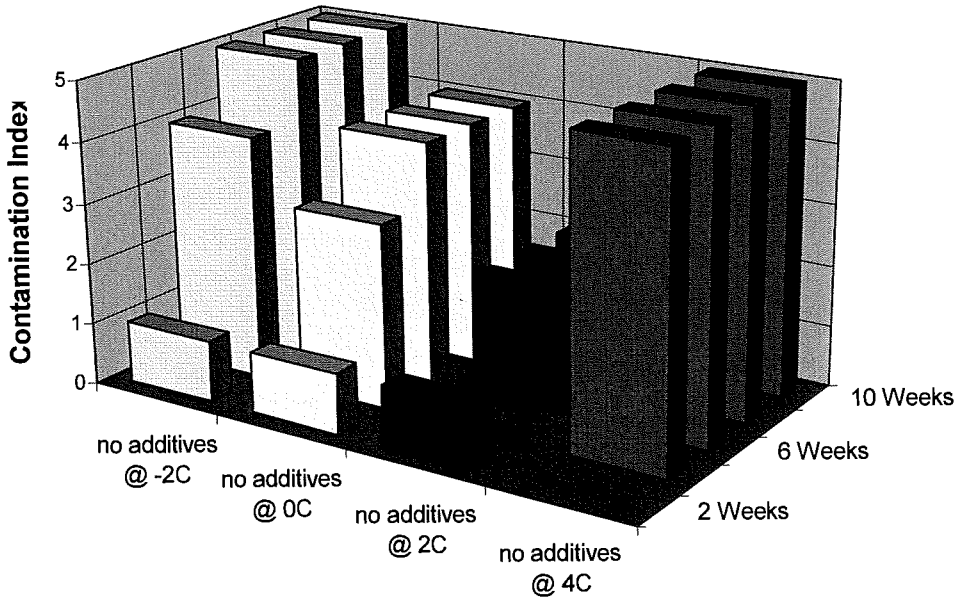


Fig 6.2.5 Effect of Additives on cell viability of *S.costatum* paste

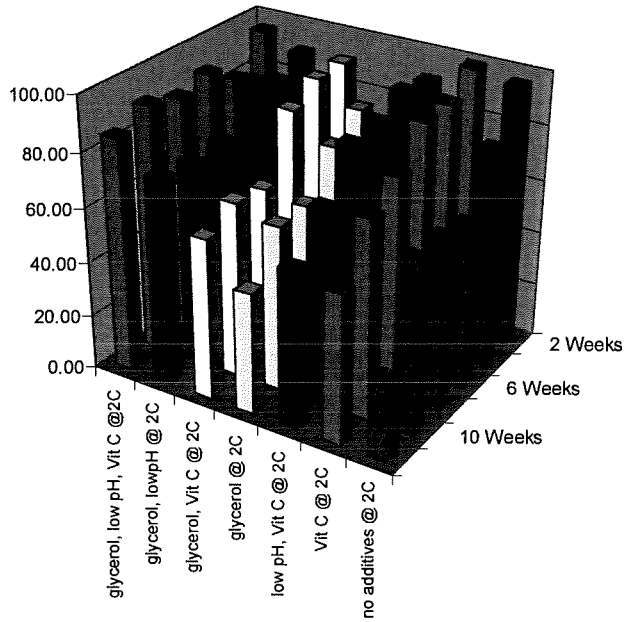


Fig 6.2.6 Effect of additives on pH of *S. costatum* paste

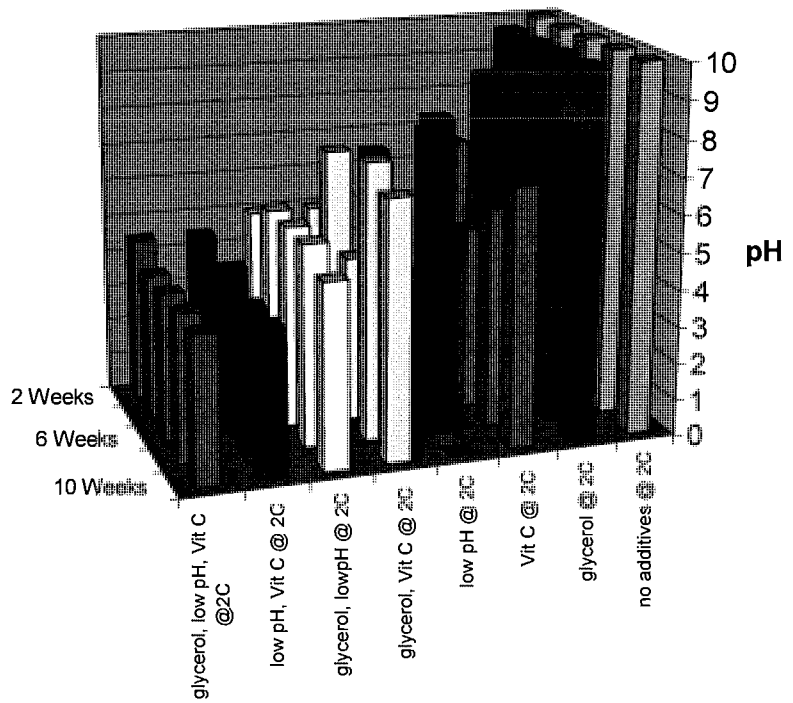
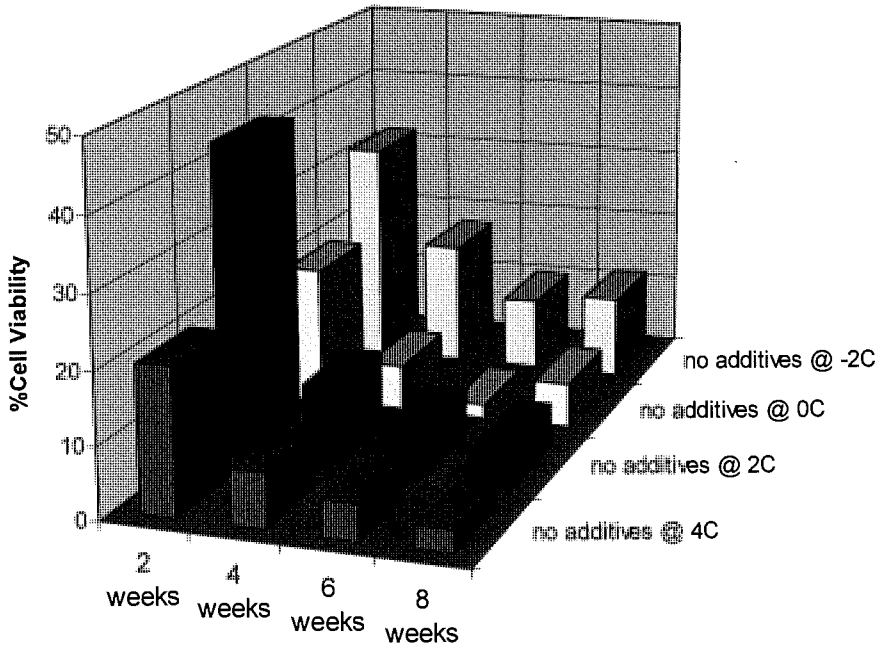


Fig 6.2.7 Effect of storage temperature on the cell viability of raw *T. Iso* paste



6.2.8 Effect of chilled storage temperature on cell viability of T. *Iso* pastes with additives

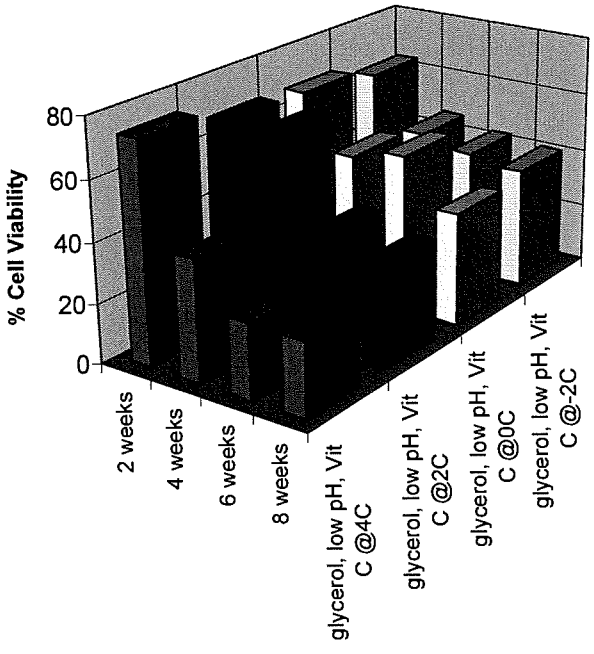


Fig 6.2.9 Effects of additives on cell viability of chill stored T *Iso*. Paste

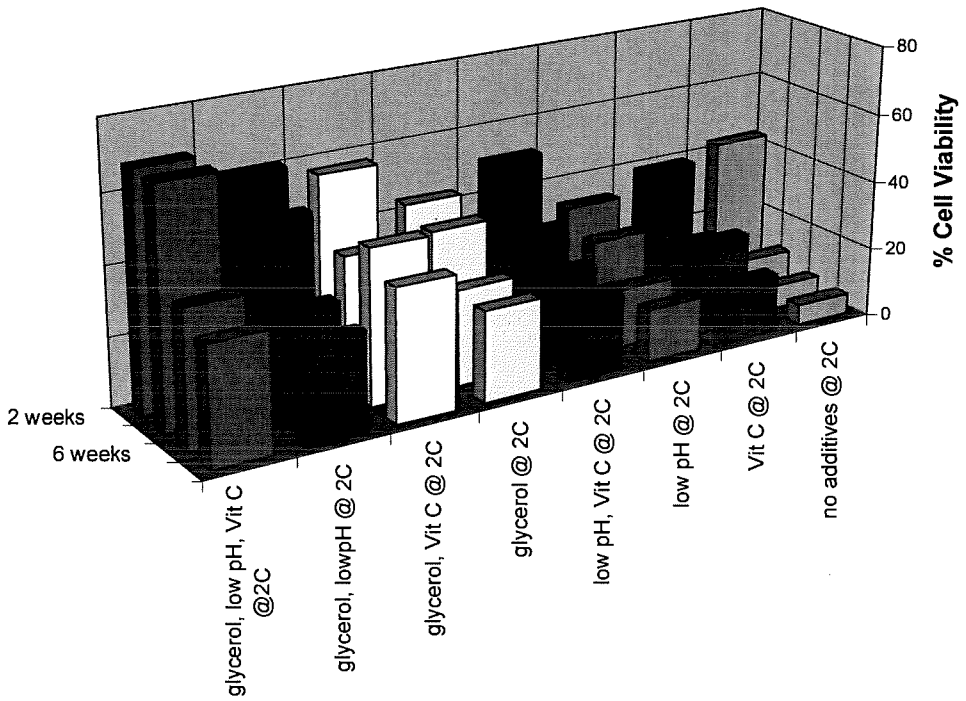


Fig 6.2.10 Effect of additives on pH of T.*Iso* paste of stored *at +2C*

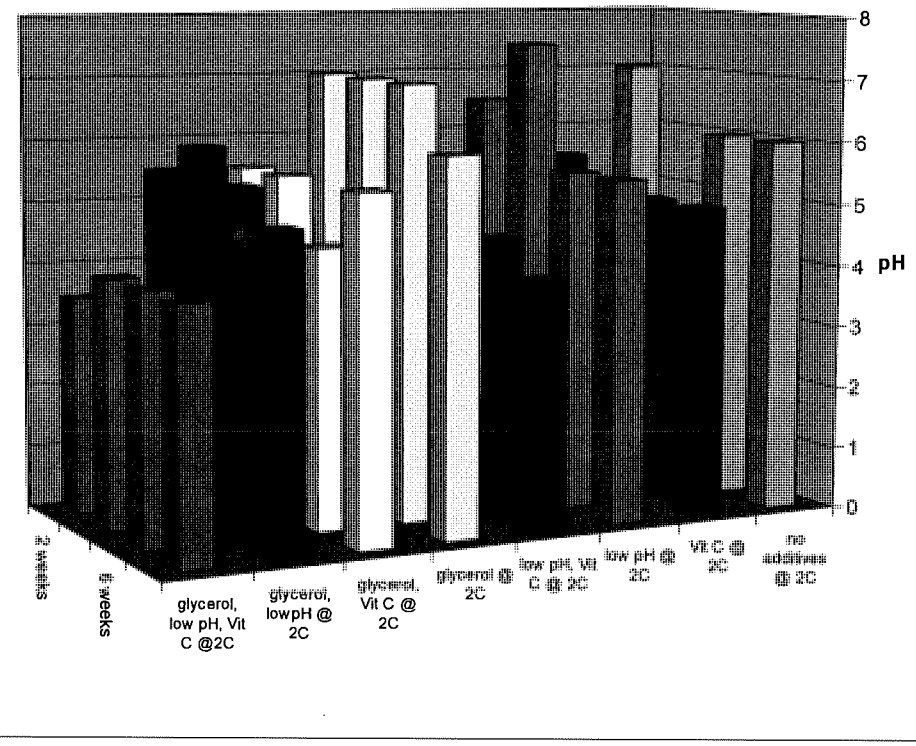


Fig 6.2.11 Effect of storage temperature and additives on cell viability in *C. muelleri* pastes

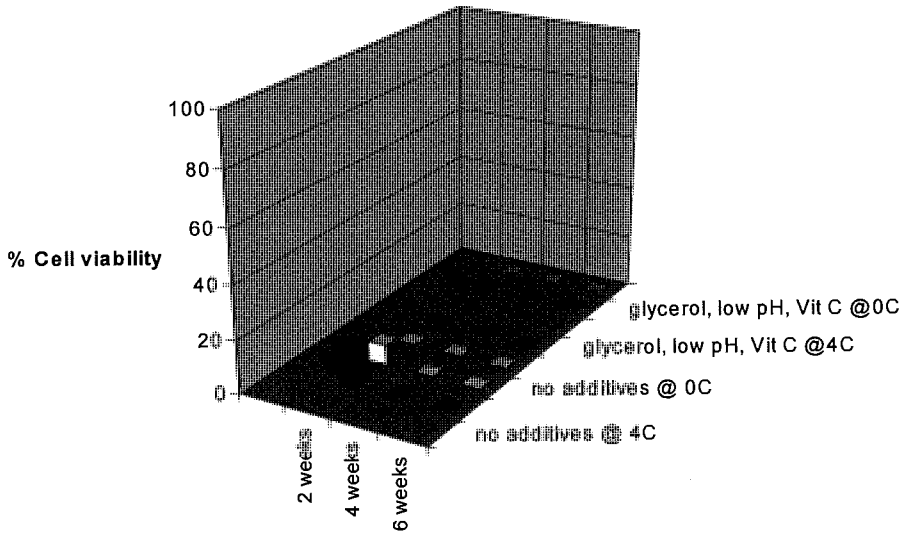
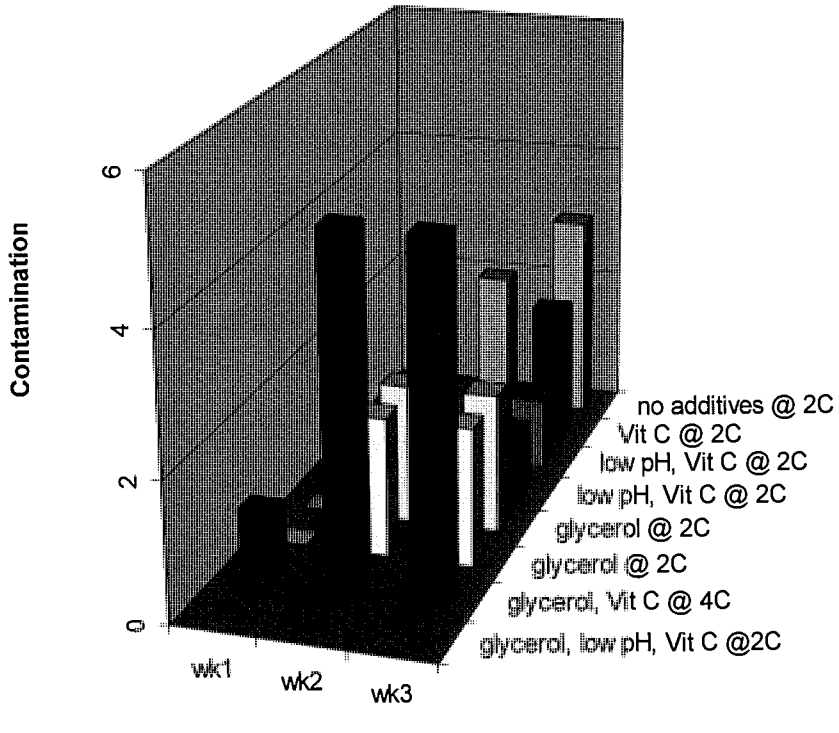


Fig 6.2.12 Effect of additives on *C. muelleri* paste



6.3. Screening Trial 3: Evaluation of Additional Additives

Introduction

This screening trial was designed to compliment Screening Trials 1 and 2 and an earlier bio-assay experiment (see Bio-assay Experiment 1, Section 7.1). In both the former Screening Trials 1 and 2 all three common food additives (Vitamin C; citric acid and glycerol) initially selected for evaluation, were found to be ineffectual in extending the very poor shelf-life of chilled super-centrifuged pastes of more fragile species of micro-algae. The latter had been shown to include the diatom *Chaetoceros muelleri* and two prymnesiophytes, *Pavlova lutheri* and T. *Iso,* all of which are of major national and global importance to marine aquaculture.

The aim of this trial was to evaluate whether an alternative array of common food additives to Vitamin C, citric acid and glycerol, alone or in combination, could be used to prolong the shelf-life of super-centrifuged pastes of both *Pavlova lutheri* and T. *Iso.*

Materials and Methods

Alternative food additives assessed included three preservatives, common salt (sodium chloride, BDH Prod. code 10241) at 5, 10, 15, and 20% (w/w); propionic acid (SIGMA, P-1386) at 0.5mg/g of paste and potassium sorbate (SIGMA, S-1751) at 0.3mg/g of paste. Two alternative food grade anti-oxidants were also assessed. These were ethoxyquin (SIGMA, E-8260) at 0.125mg/g of paste and butylated hydroxytoluene (B.H.T.) (SIGMA, B-1378) at 0.1 mg/g of paste.

Pastes of *P.lutheri* and T. *Iso.* produced by super-centrifugation as previously described for Screening Trial 1(Section 6.2.1), were variously blended with additives according to a schedule of treatments described in Table 6.3.1 to achieve homogenous mixes. Control (raw) treatments for super-centrifuged pastes of each of the two species contained no additives. All twelve paste treatments were stored in sterile 70ml screw cap containers (Disposable Products P/L) within a chest freezer (Fisher and Paykel P/L, New Zealand, model H-360X) fitted with a "Thermo-Eye" (Type PLE - Saginomiya P/L, Japan) digital temperature controller.

Storage temperature was set at 2.0±0.5°C on the basis of results of comparative storage temperature treatments in Screening Trial 2. Samples of paste were resuspended in sea-water and examined at 250x after progressive storage periods of 1, 2 and 4 weeks. Evan's blue stain was used as previously described (Section 5.2) to quantify apparent cell viability.

Three replicates were run per treatment. Except for the control raw paste treatment, the pH of stored pastes was adjusted to 4 - 4.5 with citric acid to suppress microbial growth. Vitamin C was added to all treatments except those containing alternative anti-oxidants and the raw micro-algae paste control.

Table 6.3.1. Schedule of Treatments.

	Additives
1	No additives (Raw Control)
2	Citric acid induced pH of 4-4.5; Vit. C;
3	Citric acid induced pH of 4-4.5; Vit. C; 5%NaCl
4	Citric acid induced pH of 4-4.5; Vit. C; 10%NaCl
5	Citric acid induced pH of 4-4.5 Vit. C; 15%NaCl
6	Citric acid induced pH of 4-4.5; Vit. C; 20%NaCl
7	Citric acid induced pH of 4-4.5; Vit. C; Propionic acid
8	Citric acid induced pH of 4-4.5; Vit. C; Potassium Sorbate
9	Citric acid induced pH of 4-4.5; B.H.T.; Propionic acid
10	Citric acid induced pH of 4-4.5; B.H.T.; Potassium Sorbate
11	Citric acid induced pH of 4-4.5; Ethoxyquin; Propionic acid
12	Citric acid induced pH of 4-4.5; Ethoxyquin; Potassium sorbate
12	Citric acid induced pH of 4-4.5; Vit. C

Results

Tahitian Isochrysis:
As in Screening Trial 2, a rapid fall in cell viability was exhibited by raw super-centrifuged paste of T. *Iso.* (Table 6.3.2) Cell viability fell from a post-harvest mean±s.e value of 88±1% (see Table 6.2.1) to 40±2% after a week of chilled storage and thence to 13± 4% after 4 weeks.

Effects of additives (Table 6.3.2) were rather inconclusive. None of the new additive treatments trialed were sufficiently effective to maintain a high degree of cell viability. Various combinations of propionic acid, potassium sorbate, BHT and ethoxyquin did nevertheless improve retained cell viability values significantly above those of untreated (raw) paste and paste blended with Citric acid and Vitamin C. Amongst the new additives trialed, the two preservatives, especially propionic acid, appeared to offer best prospects of further improving retained cell viability above that of raw super-centrifuged T. *Iso* paste. The addition of to 5 to 20 % of NaCl did not however improve retention of cell viability over that of the raw paste control and neither of the alternative anti oxidants appeared to be superior to Vitamin C.

Table 6.3.2 Retained % cell viability and pH of chilled super-entrifuged pastes of T.*Iso.*

Treatment	**% Cell viability (mean ± s.e)**		**pH**
	Week 1 (n=3)	**Week 4 (n=2)**	**Week 1**
No additives (Raw Control)	39.5±2.2 a b	13.1± 1.1 a	7
Citric acid; Vit. C	36.4±3.1 a b	11.4 ±.3.4 a	4
Citric acid; Vit. C; 5%NaCl	39.8±2.0 a b	15.8±1.0 a	4
Citric acid; Vit. C; 10%NaCl	38.5±1.0 a b	12.3±3.0 a	4.25
Citric acid; Vit. C; 15%NaCl	32.6±2.0 a b	11.9±4.0 a b	4.5
Citric acid; Vit. C; 20%NaCl	34.4±2.0 a b	13.0±2.4 a	4.5
Citric acid; Vit. C; Propionic acid	36.3±4.5 a b	27.3±4.3 b	4
Citric acid Vit. C; Potassium sorbate	39.1±1.9 a b	18.2±4.6 a b	4
Citric acid; B.H.T.; Propionic acid	29.7±3.3 a	19.1±2.1 a b	4.25
Citric acid; B.H.T.; Potassium sorbate	41.4±2.1 b	25.5±3.1 b	4
Citric acid; Ethoxyquin; Propionic acid	30.7±3.0 a b	24.6±0.4 b	5.25
Citric acid; Ethoxyquin; Potassium sorbate	36.7±1.9 a b	21.1±2.3 a b	4.25

Mean values sharing common letters are not significantly different at $P<0.05$ (SNK)

Pavlova lutheri:
As indicated by results presented in Table 6.3.3, raw super-centrifuged paste of *Pavlova lutheri* exhibited a very rapid initial decline in retained cell viability falling from a post harvest value of 92±2.0% (see Table 5.3.2.1, Section 5.3) to 26.5±9.1% after only one week of chilled storage. Mean cell viability continued to decline thereafter falling to less than 10% after 2 weeks and thence to less than 5% after 4 weeks storage at 2.0±0.5°C.

All of the additive treatments tested on chill stored super-centrifuged pastes of *P. lutheri* reduced cell viability to values below that of the raw paste control after one week of storage at 2.0±0.5°C. In this regard effects of additive treatments with *P lutheri* were opposite to those achieved with pastes of T *Iso* already discussed above.

Table 6.3.3. Retention of cell viability of chilled super-centrifuged pastes of *P .lutheri.*

	Retained % Cell viability		
Treatment	**1 Week**	**2 weeks**	**4 Weeks**
No additives (Raw Control)	26.5 ± 9.1 b	<10%	<5%
Citric acid induced; Vit. C	19.0 ± 4.3 b	<10%	<5%
Citric acid ;Vit. C; 5%NaCl	9.4 ± 2.0 a b	<10%	<10%
Citric acid; Vit. C; 10%NaCl	6.8 ± 2.3 a	<10%	<5%
Citric acid; Vit. C; 15%NaCl	7.9 ± 4.2 a b	<10%	<5%
Citric acid; Vit. C; 20%NaCl	7.9 ± 3.1 a b	<10%	<10%
Citric acid; Vit. C; Proprionic acid	11.2 ± 0.7 b	<10%	<10%
Citric acid Vit. C; Potassium Sorbate	8.0 ± 1.8 a	<10%	<5%
Citric acid; B.H.T.; Proprionic acid	5.6 ± 0.3 a	<10%	<5%
Citric acid; B.H.T.; Potassium Sorbate	7.3 ± 0.3 a	<10%	<10%
Citric acid; Ethoxyquin; Proprionic acid	7.3 ± 0.9 a	<10%	<5%
Citric acid; Ethoxyquin; Potassium sorbate	12.1 ± 2.0a b	<10%	<5%

Mean values sharing common letters are not significantly different at $P<0.05$ (SNK)

6.4. Screening Trial 4: Evaluation of Additives across an Expanded Array of Micro-Algal Species

Introduction

The principle aim of this trial was to evaluate the effectiveness of the same suite of additives evaluated in Screening trials 1 and 2, to extend the shelf life of the super centrifuged pastes of a wider array of species of micro-algae. The additives comprised Vitamin C, citric acid and glycerol, alone or in combination. The species of micro-algae comprised five diatoms, including the two species, *Chaetoceros muelleri* and *Skeletonema costatum,* already demonstrated in Screening Trials 1 and 2 as poor and good shelf life reference species respectively. Diatoms assessed also included three other commercially important species, *Phaeodactylum tricornatum, Thalassiosira psuedonana* and *Chaetoceros calcitrans.* The remaining two species selected were the green flagellates *Tetraselmis chui* and *Nannochloropsis oculata.* The prymnesiophyte species *Pavlova lutheri* and T. *Iso* were omitted in view of comprehensive information already gathered on these species during the course of Screening Trials 1, 2, and 3.

Materials and Methods

All species of micro-algae trialed were produced in 1000 L cultures by techniques described for *S. costatum* in Screening Trial 1. Pastes of each of the seven micro-algae species produced by super-centrifugation comprised a blend of three separate cultures to avoid batch variability as previously applied in Screening Trials 1, 2 and 3. These pastes were blended with all three additives. The combination of all three additives had been shown by results of both Screening Trials 1 and 2 to provide best enhancement of shelf life, (as indicated by cell viability and also by a range of other quality criteria) across three out of four species tested, the exception being *Pavlova lutheri* which as previously stated, was omitted from this trial. As in the three previously reported Screening Trials, glycerol was used at 10% (w/w); Vitamin C (ascorbic acid) at 1% (w/w) and sufficient citric acid added to reduce pH to the range 4 - 4.5. These additives were blended with super-centrifuged pastes in accordance with methods described for Screening Trial 1.

Control raw paste treatments for each of the seven micro-algae species contained no additives. All fourteen treatments were stored in sealed 70ml screw cap containers within a chest freezer (*Fisher and Paykel P/L*, model H-360X) fitted with a *Thermo-Eye* (Type PLE - Saginomiya P/L, Japan) digital temperature controller at 2.0 ± 0.5°C. This temperature had been shown in Screening Trial 2 to be superior to higher and lower chilled storage temperature regimes of -2.0±0.5°C, 0.0±0.5°C and 4.0±6.0°C. Cells of the stored pastes were resuspended in 1 µm filtered seawater and examined at 250x at successive intervals of 2 to 3 weeks for total periods of up to 13 weeks. Evan's blue was used to stain these cells in accordance with methods described in Section 5.2, as a measure of retained viability of cells.

Results

Diatoms

Skeletonema costatum:
Mean ±.s.e. cell viabilities from chill stored super-centrifuged pastes of *Skeletonema costatum,* with and without additives, (Fig.6.4.1a) were equal after 5 weeks having fallen from an initial post harvest level of 100% (see Section 5.3.2) to levels of 85.7±1.4% and 85.4±1.7%, respectively. Thereafter however the two treatments diverged widely. Raw paste continued to exhibit a

progressive decline in mean± .s.e. cell viability falling from 85.4±1.7% to 33±1.7% between weeks 5 and 13. Mean cell viabilities of *S. costatum* paste with all additives were much higher and constant varying only marginally (81.6% to 82.6 %) over the same period. These results were entirely consistent with those of equivalent treatments in Screening Trial 2 in which cell viabilities of raw paste fell progressively from 96% after 2 weeks to about 60% after 10 weeks thence to about 30% after 15weeks at 2.0±0.5°C, while the equivalent set of progressive cell viability values for pastes with all additives were 96%, 91% and 86% respectively.

Chaetoceros calcitrans:
Mean ± .s.e. cell viabilities of super-centrifuged pastes of *C. calcitrans* with and without additives (Fig. 6.4.2a) exhibited progressive declines over 12 weeks of chilled storage. The rate of decline was however greater in the raw paste treatment with mean cell viability falling to significantly lower values than those of the all-additives treatment beyond 8 weeks of storage. Indeed at the time the trial was terminated after 12 weeks of storage, cell viability of paste with all additives was fives times greater (51.6±7.0%) than that of raw paste (10.0±0.0%). Beneficial effects of additives was just as pronounced in the suppression of microbial contamination (Fig 6.4.2b).

Chaetoceros muelleri:
Super-centrifuged paste has exhibited by far the poorest retention of cell viability of the five diatom species tested and in fact of all seven species used in this trial. Indeed cell viability in both the raw and all-additive treatments had fallen to zero by the time of the first sampling after a mere two weeks of chilled storage. These results were generally consistent with those obtained in Screening Trial 2 except that small levels of retained viability after two weeks of storage in Screening Trial, (14.5% in raw paste and 7.5% in paste with additives), did not occur in this trial.

Thalassiosira pseudonana:
Retention of cell viabilty in *T. pseudonana* (Fig. 6.4.3a) was intermediate to that of *C.calcitrans* and *C. muelleri.* While cell viability of raw super-centrifuged paste was totally lost after 4 weeks of chilled storage paste with additives exhibited enhanced cell viability values of 60.2±2.7 % after 4 weeks storage and 10% after 7 weeks of storage. Additives also greatly suppressed microbial contamination that remained at very low levels even after seven weeks of storage (Fig. 6.4.3b).

Phaeodactylum tricornutum:
As with *T. psuedonana* but in contrast to *C muelleri* cell viability of raw chill stored super-centrifuged paste of *P. tricornutum* (Fig 6.4.4a) remained very high for the first 4 weeks of chilled storage but slumped thereafter falling to zero after a further 3 weeks. In stark contrast to the four other diatom species used in this trial, additives had a severe negative rather than positive effect on retained cell viability in *P. tricornutum* paste. Indeed cell viability had fallen to zero by the time of first sampling a mere 2 weeks into the trial. Microbial contamination was however suppressed by the inclusion of additives (Fig. 6.4.4b) as was the case with the three other diatom species.

Green Flagellates

Tetraselmis chui:
Super-centrifuged paste of *T. chui,* with or without additives retained cell viability rates (Fig 6.4.5a) above 90% over the full 12 weeks duration of this experiment. Additives appeared to provide some advantage for the retention of cell viability that remained marginally higher than that of raw paste at and beyond 10 weeks of chilled storage. Contamination of cultures increased progressively over the first 6 to 8 weeks to reach high levels regardless of the presence or absence of additives (Fig. 6.4.5b).

Nannochloropsis oculata:
Raw *N. oculata* paste exhibited near perfect (>99%) retention of cell viability over the full 12 week term of the experiment (Fig 6.4.6). However in contrast with that of *T chui, N. oculata* paste exhibited reduced retention of cell viability when additives were used. Indeed pastes with additives exhibited a slow continuous decline in cell viability from an initial level of 100% to about 68% 12 weeks later. Additives also had a progressive and profound negative influence on contamination levels of *N. oculata* pastes (Fig. 6.4.6b).

Summary and Conclusions

Assuming that a cell viability of say ≥60% represents an acceptable level of retained quality for marine hatchery and nursery use, shelf life of raw pastes of the five diatom species, as summarised in Figs 6.4.1,3,5,7 and 9, were generally brief and followed the sequence *S. costatum* and *C. calcitrans* (8weeks)>>*P. tricornutum* (5weeks) >*T. psuedonana* (4 weeks) >> *C. muelleri* (less than 2 weeks).

Effects of additives were highly species specific in their effect on retained cell viability. At one extreme the shelf life *S. costatum* (also see results of Screening Trial 2) was substantially extended. The shelf life of *T. psuedonana* was also improved but only marginally. No discernible effects occurred with *C. calcitrans* nor with *S. costatum.* At the opposite extreme to *S costatum,* additives had a pronounced negative (shelf life destroying) influence on *P. tricornutum* and a slight negative effect on *C. muelleri* (also see results of Screening Trial 2).

Effects of additives on degree of contamination by bacteria, yeasts and fungi in chill stored pastes, followed very similar trends to their respective cell viability data, with additives reducing contamination by varying degrees in pastes of all species except *N. oculata.* As in Screening Trial 2, cells within poorer keeping pastes exhibited progressively greater variation in shape and size and increasing rates of clumping and development of off odours.

Shelf lives of super-centrifuged pastes of the five diatom species containing the three additives thus followed the sequence: *S. costatum* (>13weeks) >> *C. calcitrans* (10weeks) >> *T. psuedonana* (4 weeks) >> *C. muelleri* and *P. tricornutum* (less than 2 weeks).

Raw super-centifuged pastes of the green flagellates both exhibited excellent shelf life beyond the 12 week duration of this trial, *T, chui* paste retaining cell viabilities above 90% and that of N. oculata above 99%. Again effect of the additives was species specific. Use of additives on *T. chui* paste was positive raising retained cell viability from 91.2 ± 2.2% in fresh paste to 98.8±0.2% after 12 weeks of storage at 2±0.5°C. By contrast, additives had a pronounced negative effect on paste of *N. oculata* reducing retained cell viability from 99.0≥±0.0% in fresh paste to 69.0±3.7 %, a margin of 30%, after 12 weeks of chilled storage.

Fig 6.4.1a Effect of additives on mean+/- s.e. % retained cell viability of high speed centrifuged chill stored paste of *Skeletonema costatum*

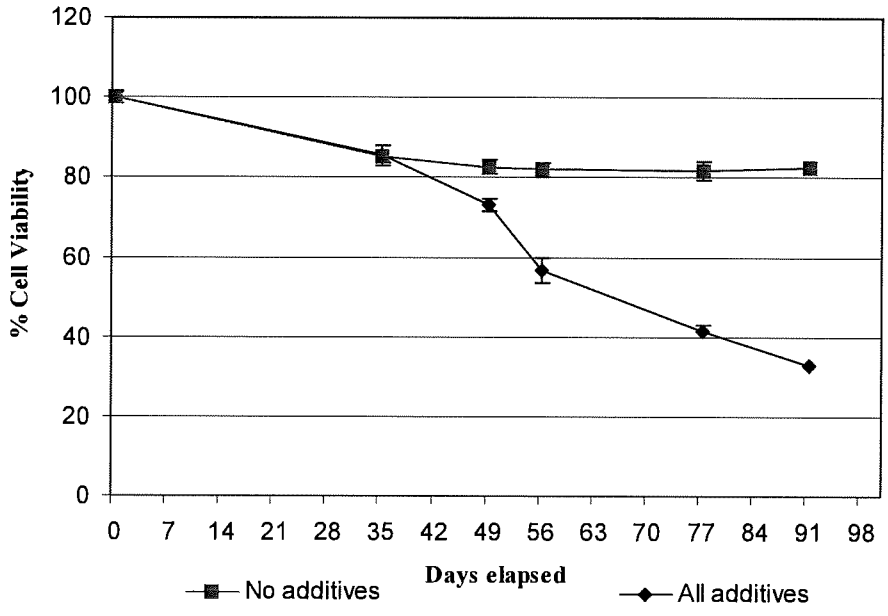


Fig 6.4.1b Effects of additives on microbial contamination of *Skeletonema costatum* paste

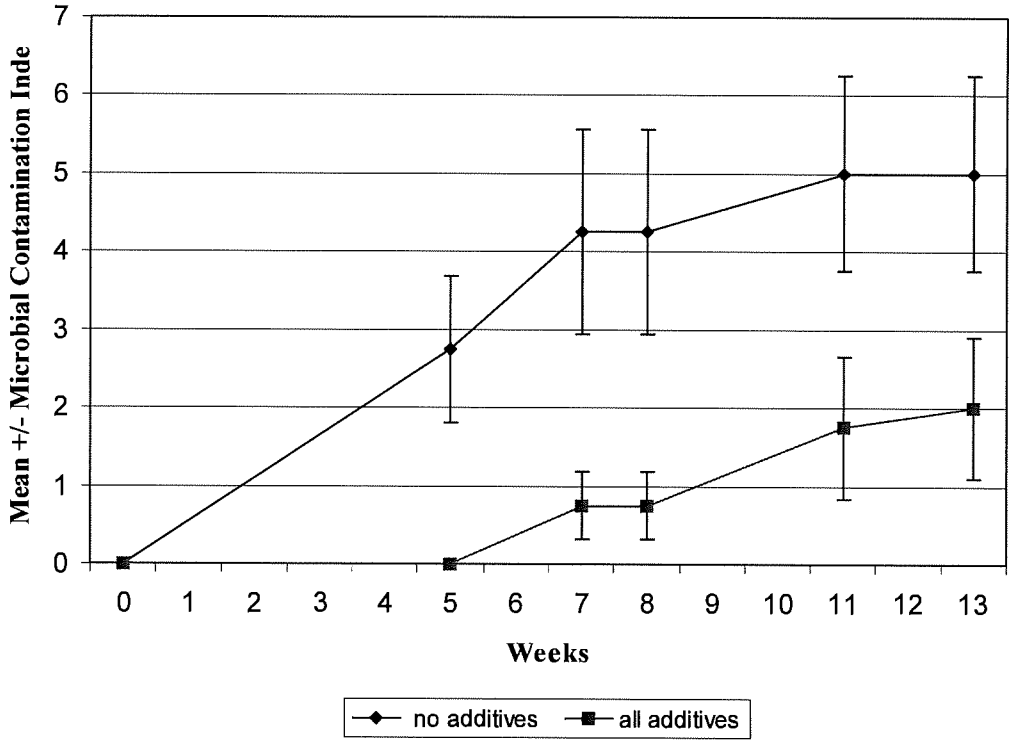


Fig 6.4.2a Effect of additives on mean +/- s.e. % retained cell viability of high speed centrifuged chilled paste of *Chaetoceros calcitrans*

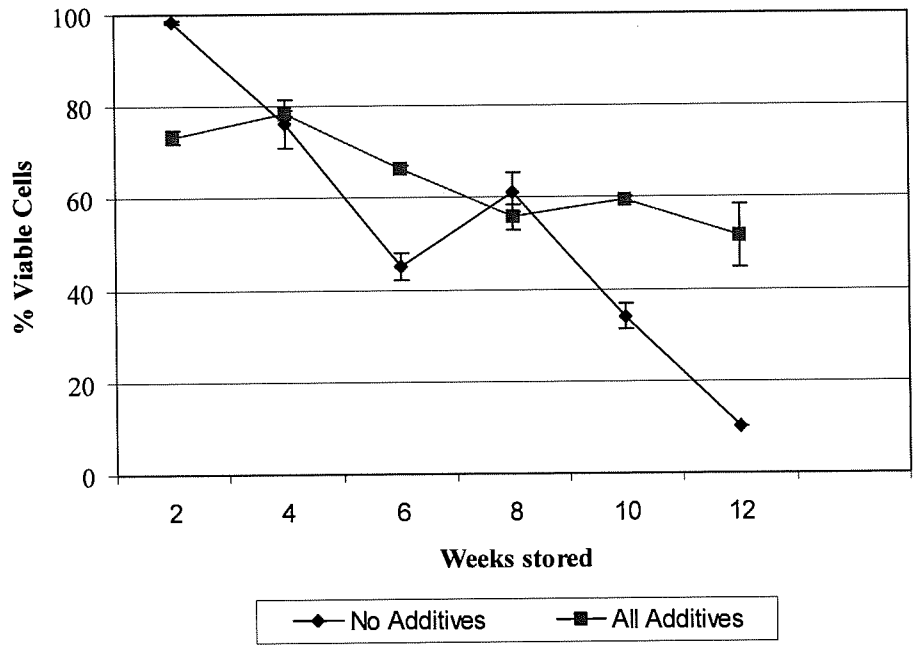


Fig 6.4.2b Effects of additives on microbial contaminaion of *Chaetoceros calcitrans* paste

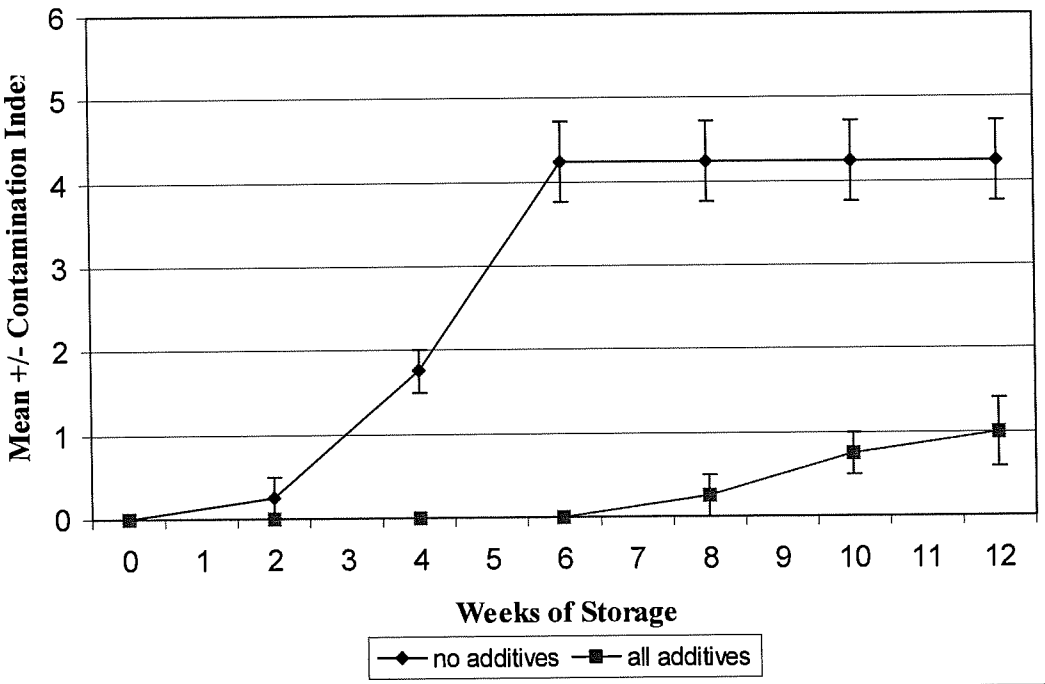


Fig 6.4.3a Effect of additives on mean +/-s.e. % retained cell viability of high speed centrifuged , chill stored paste of *Thallasiosira pseudonana*

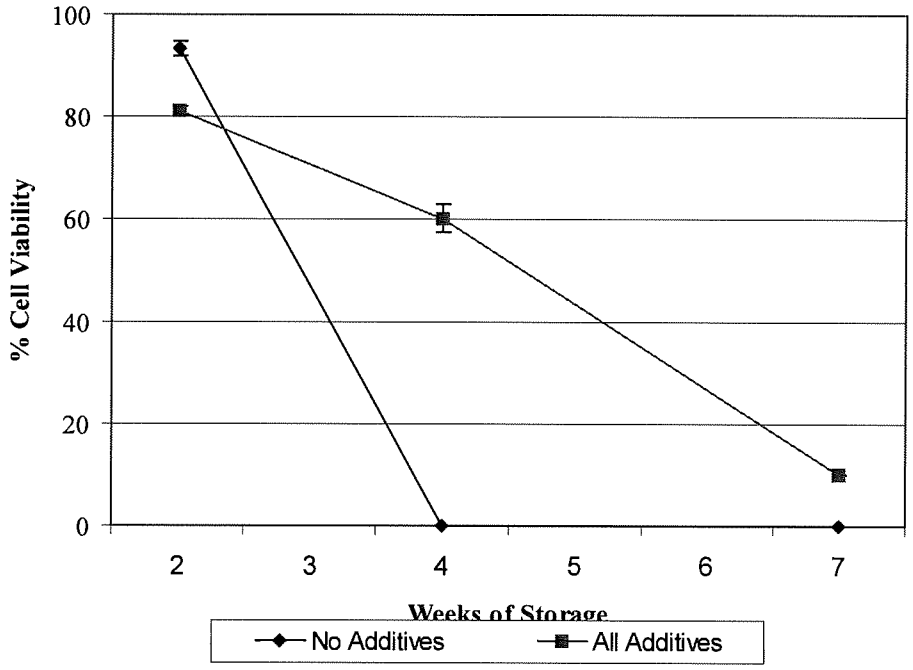


Fig 6.4.3b Effect of additives on microbial contamination of *Thallassiosira pseudonana* paste

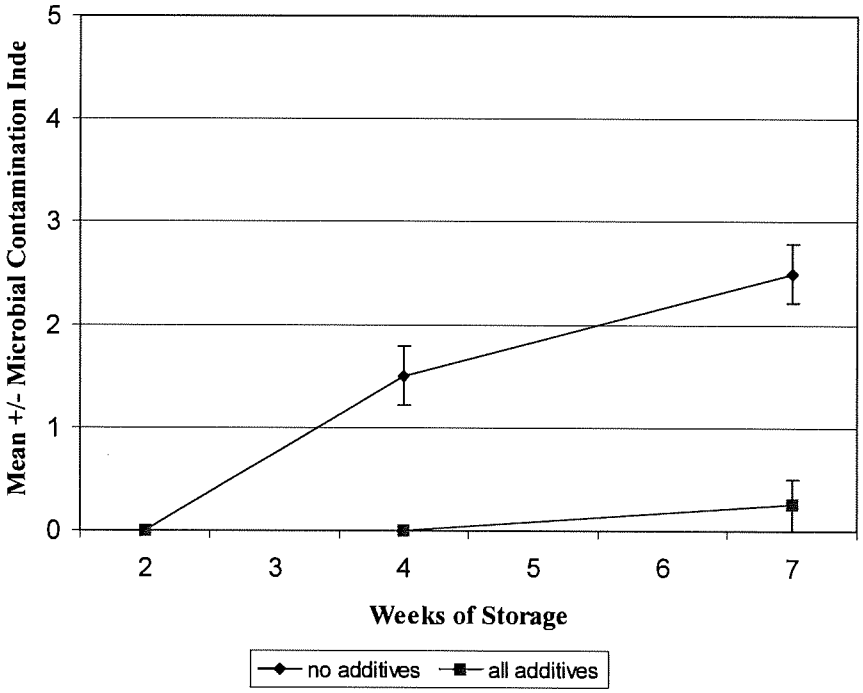


Fig 6.4.4a Effects o additives on mean +/- % retainerd cell viability of high speed centrifuged chill stored paste of *Thallasiosira pseudonana*

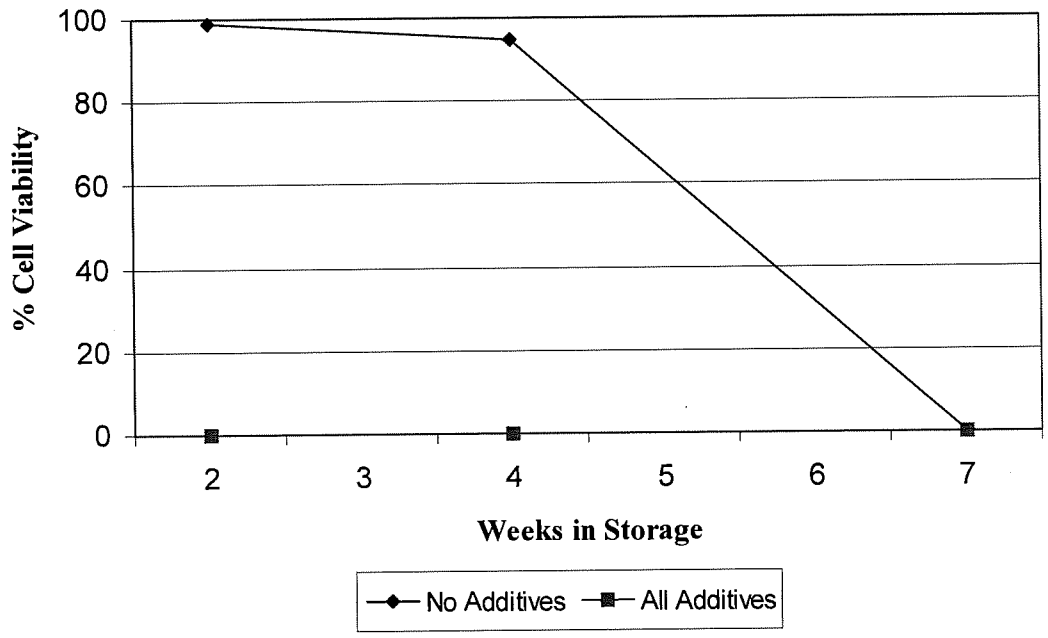


Fig 6.4.4b Effects of Additives on Microbial Contamination of *Phaeodactylum tricornutum* paste

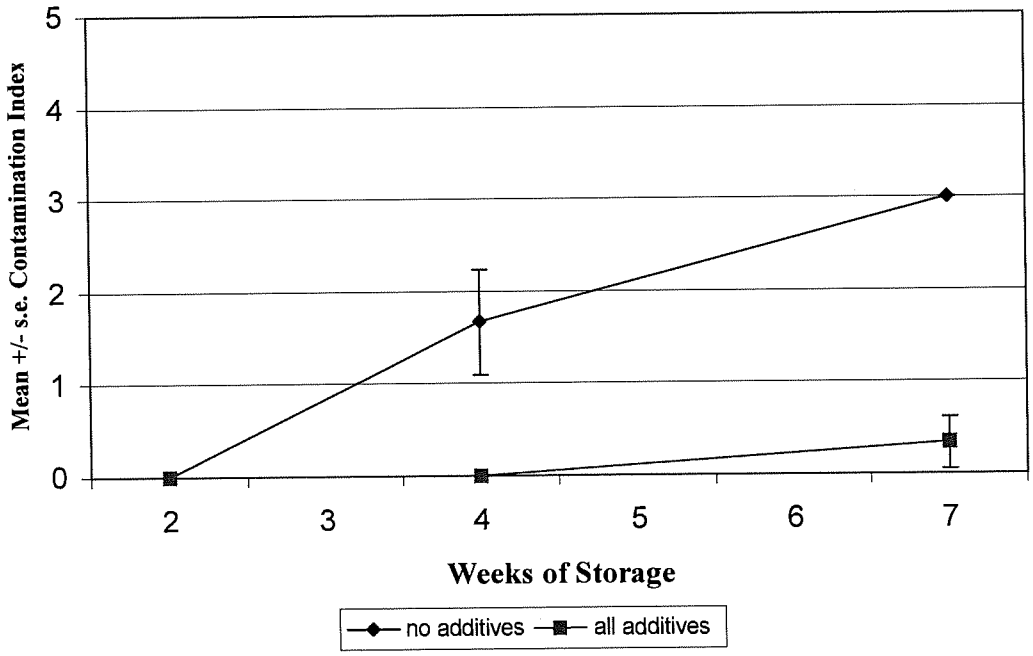


Fig 6.4.5a Effect of additives on mean+/- %retained cell viability of high speed centrifuged chill stored paste of *Tetraselmis chui*

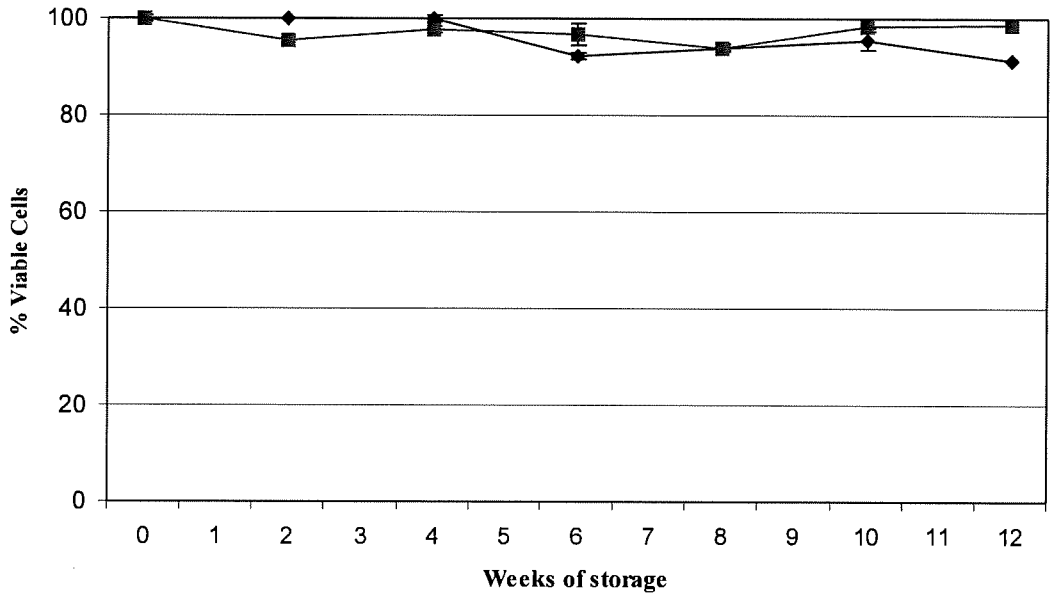


Fig 6.4.5b Effects of additives on microbial contamination of *Tetraselmis chui* paste

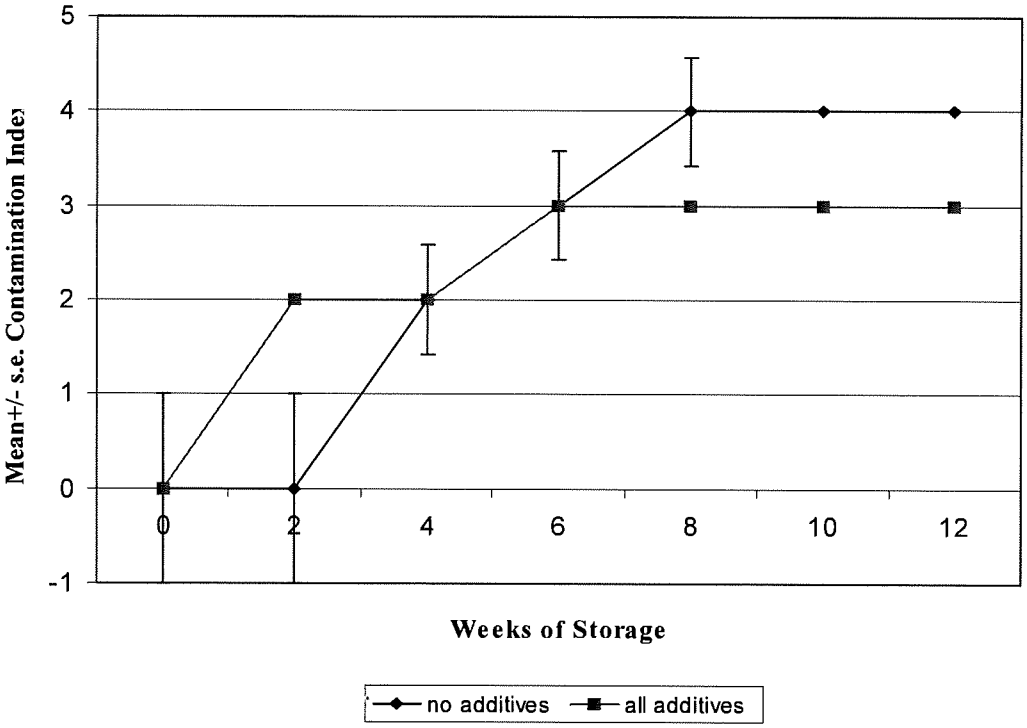


Fig 6.4.6a Effect of additives on mean +/- s.e. cell viability of chill stored paste of *Nannochloropsis oculata*

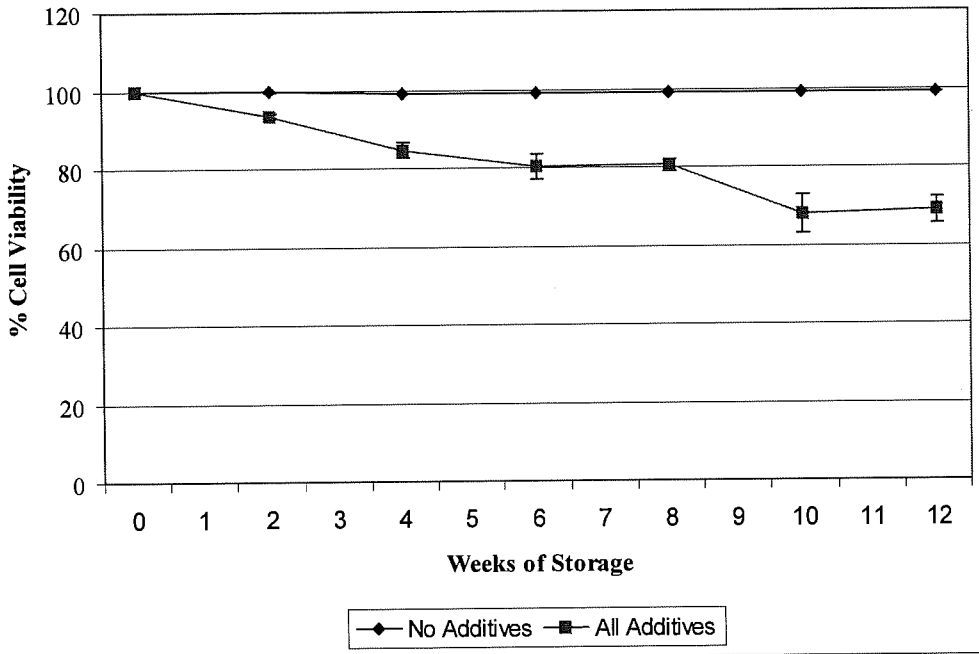
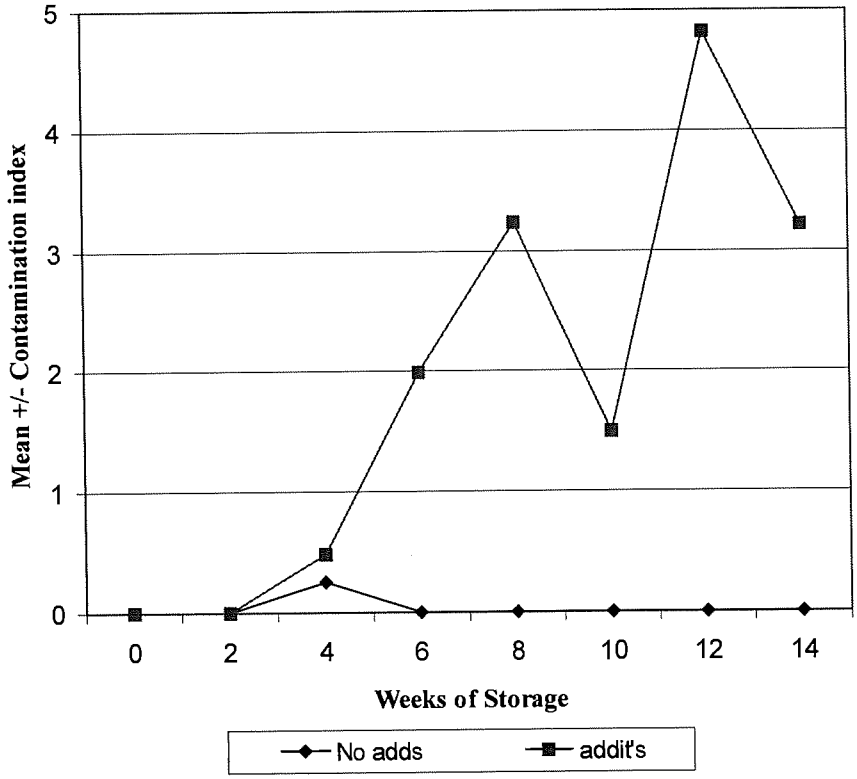


Fig 6.4.6b Effects of additives on microbial contamination of high speed centrifuged paste of *Nannochloropsis oculata*



6.5. Screening Trial 5: Evaluation of Low Density Slurries under Modified Storage Conditions in Extending the Life of Concentrates of *Pavlova lutheri* and Tahitian *Isochrysis* Produced Initially as Pastes by Super-Centrifugation

Introduction and Aims

Montaini et al. 1995., found that provision of free exchange with the atmosphere via a cotton plug and storage in light may extend the length of time algal cells remain viable especially when coupled with cold storage. They also found that survival of *Tetraselmis suecica* cells improves and can be directly correlated with reduced cell concentration.

The aim of this trial was to evaluate the benefits of diluting concentrates of micro-algae initially produced as stiff pastes by high speed super-centrifugation. In addition, this trial aimed to evaluate effects on chilled slurries of alternative storage conditions, namely:

- illuminated as opposed to dark storage
- alternative types of gaseous atmosphere
- the inclusion of two additives Vitamin C and glycerol. Both of these additives had already been shown during Screening Trials 1, 2, 3 & 4 to enhance retention of cell viability and other quality criteria in chilled super-centrifuged pastes in 7 out of 9 species tested.

The micro-algae selected for this experiment comprised the two fragile cell wall prymnesiophyte species, *Pavlova lutheri* and Tahitian *Isochrysis* (T. *Iso*). These species were selected partly because of their strategic importance to bivalve mollusc hatcheries and partly because of the poor interim results achieved in extending very brief shelf lives exhibited by each species when harvested as stiff pastes by high speed super-centrifugation.

Materials and Methods

Micro-algae cultures and super-centrifuged pastes thereof were produced by as previously described for Screening Trial 1 (Section 6.1.1). Control treatments comprised super-centrifuged pastes of each of the two species without additives. Five variables used to formulate treatments are described in Table 6.5.1.

Table 6.5.1 Five variables used to formulate treatments.

A. Concentration of slurries

a) 10g/L *standard
b) 20g/L
c) 40g/L
d) 80g/L
e) super-centrifuged concentrate control
f) algal culture control

B. Atmosphere over Slurry (@10g/L)

a) air/sealed
b) air/open (cotton plug)
c) pure oxygen

C. With or without Glycerol

D. With or without Vitamin C

E. Stored in light or dark

Schedules of 13 treatments used for *P. lutheri* and of 14 treatments used for T. *Iso* are summarised in Tables 6.5.2A&B respectively. In treatments involving additives, pastes or slurries were variously blended with vitamin C at 1% (w/w) and/or glycerol at 10% (w/w) according to methods described for Screening Trial 1.

Table 6.5.2. A & B Schedules of Treatments (x4 replicates of each).

A. Pavlova lutheri
1 Raw paste control (air space cotton plug)
2 Algae culture control (air space cotton plug)
3 Slurry 80g/L (air space cotton plug)
4 Slurry 40g/L (air space cotton plug)
5 Slurry 20g/L (air space cotton plug)
6 Slurry 10g/L (pure oxygen)
7 Slurry 10g/L (air space sealed)
8 Slurry 10g/L (air space cotton plug + Vit C)
9 Slurry 10g/L (air space cotton plug + glycerol)
10 Slurry 10g/L (air space cotton plug, dark storage)
11 Slurry 10g/L (air space cotton plug, light storage)
12 Slurry 10g/L (air space cotton plug + glycerol + Vit C, dark storage)
13 Slurry 10g/L (air space cotton plug + glycerol + Vit C, light storage)
B. Tahitian Isochrysis
1 Paste Control(air space cotton plug)
2 Paste Control + Additives (air space cotton plug)
3 Algae culture control (air space, cotton plug)
4 Slurry 80g/L, (air space, cotton plug)
5 Slurry 40g/L, (air space, cotton plug)
6 Slurry 20g/L, (air space, cotton plug)
7 Slurry 10g/L, (pure oxygen)
8 Slurry 10g/L, (air space sealed)
9 Slurry 10g/L, (air space, cotton plug + Vit C)
10 Slurry 10g/L, (air space, cotton plug + glycerol)
11 Slurry 10g/L, (air space, cotton plug, dark storage)
12 Slurry 10g/L, (air space, cotton plug, light storage)
13 Slurry 10g/L, (air space, cotton plug + glycerol, + Vit C, dark storage)
14 Slurry 10g/L, (air space, cotton plug + glycerol, + Vit C, light storage)

Replicate concentrate samples of approximately 8 ml for each treatment were stored in 20 ml vials set at a slope of at approximately 30 ° to the horizontal in chest freezers. Temperature was maintained at 2± 0.5°C in chest freezers (Fisher and Paykel P/L, New Zealand, model H-360X) fitted with "Thermo-Eye" digital temperature controllers (Type PLE,- Saginomiya P/L, Japan).

Stored samples were evaluated at successive intervals of 1, 2, 5, 7 and 9 weeks in the case of *P. lutheri*. However for T. *Iso* sampling beyond two weeks was suspended when severe loss of cell viability observed in all 14 treatments. On each sampling occasion a sub-sample of slurry or paste was drawn from each replicate using a 1.0ml sterile syringe or a Gilson Pipeman P/L auto-pipette and re-suspended in filtered sea-water using a Sunbeam P/L, "Maestro" hand-held food processor on low speed, the process being performed in a laminar flow cabinet to minimise extraneous contamination. Cell viabilities were determined using the Evan's blue staining technique as previously described for Screening Trial 1 (Section 6.1.1).

Cell condition index criteria used to assess retained quality or stored pastes is presented in Table 6.5.3.

Table 6.5.3. Cell Condition Scoring Criteria.

Score	Description
0	no intact cells apparent
1	severe clumping, very high proportion(>75%) of cell wall breakdown
2	moderate/severe clumping, high proportion (>50%) of cell wall breakdown
3	moderate clumping, moderate (<50%) cell wall damage
4	minor clumping, minor (<10%)cell wall breakdown
5	perfect - normal fresh algae appearance

Results

Pavlova lutheri

Effect of density on storage life of chilled concentrates:
As clearly illustrated by cell viability data presented in Fig 6.5.1, dilution of super-centrifuged pastes of *P. lutheri* with sterile seawater to form slurries had a profound beneficial effect on shelf life. Adopting the previously accepted but arbitrary lower acceptable limit of 60% for retained cell viability (see Screening Trial 4), implied shelf life of chilled concentrates increased progressively with decreasing density from less than 1week for undiluted paste, to 1 week for the 80g/L slurry, to 2 weeks for the 40g/L slurry, 5 weeks for 20g/L slurry and to almost 6 weeks for the 10g/L density slurry.

These results were generally but not entirely consistent with alternative visual quality index data based on microscopic examination of resuspended concentrate presented in Table 6.5.3. Although the visual quality index data also indicated a major benefit of diluting super-centrifuged pastes to slurries, evidence of the superiority of lower slurry densities, so clearly indicated by cell viability data, was not evident.

Table 6.5.4 Effect of density on the visually assessed cell condition of chill stored super-centrifuged concentrates of *Pavlova lutheri*

	Mean Cell Condition Index value				
Treatment	**Week 1**	**Week 2**	**Week 5**	**Week 7**	**Week 9**
Paste control (air space, cotton plug)	3.0	2.0	1.0	0	0
Slurry 80g/L, (air space, cotton plug)	3.0	2.0	2.0	2	1.5
Slurry 40g/L, (air space, cotton plug)	2.0	2.0	2.0	2	1.5
Slurry 20g/L, (air space, cotton plug)	2.0	2.5	2.0	2	1.5
Slurry 10g/L, (air space, cotton plug, dark storage)	2.0	3.0	2.0	2	1.5
Algae culture control (air space, cotton plug)	4.0	4.0	3.0	2	1.5

Effect of atmosphere on storage life of chilled concentrates:
Retained cell viability data presented in Fig 6.5.2 indicate that an atmosphere of oxygen reduced rather than extended the shelf life of low density *P. lutheri* slurries and that shelf life is unaffected whether or not storage containers are sealed with screw cap lids or allowed to "breath" using a porous cotton wool plugs.

These treatment differences were not however evident from cell condition index data presented in Table 6.5.4 based on microscopic examination of resuspended cells.

Table 6.5.5. Effect of atmosphere on the visually assessed cell condition of chill stored super-centrifuged concentrates of *Pavlova lutheri.*

	Week 1	Week 2	Week 5	Week 7	Week 9
Paste Control(air,cotton plug)	3	2	1	0	0
Slurry 10g/L, (pure oxygen,sealed)	2.0	2.5	2.0	2.0	1.5
Slurry 10g/L, (air,sealed)	2.0	2.0	2.0	2.0	1.5
Slurry 10g/L, (air , cotton plug)	2.0	3.0	2.0	2.0	1.5

Effect of additives on shelf life of low density chill stored slurries:
Both glycerol and vitamin C when used individually, increased retained cell viability (Fig 6.5.3). The inclusion of either additive to chill stored 10g/L slurries of *P. lutheri* increased shelf life, as indicated by an increase of about 1 week in retained cell viability by i.e. from about 5 to 6 weeks. However when used in combination these additives had an opposite negative effect.

The same results were not however apparent from alternative condition index data presented in Table 6.5.6 where the inclusion of glycerol and Vitamin C alone or in combination did not tangibly change retained quality of 10g/L slurries above or below that of slurries without either additive.

Table 6.5.6 Quality index values for low density slurries of *P. lutheri* chill stored for 9 weeks

Treatment	Week 1	Week 2	Week 5	Week 7	Week 9
Paste Control (air space, cotton plug)	3	2	1	0	0
10g/LSlurry Control (air space, cotton plug, no adds)	1	3	2	2	1.5
10g/L Slurry (air space, cotton plug + Vit C)	2	3	2	2	1.5
10g/L Slurry (air space, cotton plug + glycerol)	2	3	2	2	1.5
10g/L Slurry (air space, cotton plug; glycerol, Vit C)	2	2.5	2	2	1.5

Effects of light and dark storage on shelf life of low density chill stored slurries:
Storage of *P.lutheri* slurries with or without additives, glycerol and Vitamin C under light significantly enhanced cell viability over equivalent dark stored slurries (Fig 6.5.4). Again assuming that a cell viability of 60% or greater is acceptable for hatchery use, storage of 10g/L slurries of *P. lutheri* under light prolonged the implied shelf life of 10g/l slurries from 6 up to 7 weeks i.e. full week more than under dark storage.

As discussed above, inclusion of both glycerol and Vitamin C actually reduced retention of cell viability of *P. lutheri* slurries whereas use of either additive alone enhances cell viability. It is possible that the combination of only one additive and storage under light would result in prolongation of shelf life beyond 7 weeks. This hypothesis however remains to be tested.

As with other storage factors assessed, alternative assessment of retained quality using microscopic examination (Table 6.5.7) failed to expose the above differences associated with the use of light of dark storage.

Table 6.5.7 Effects of light and additives on the quality of *P. lutheri* cells stored as chilled slurries

	Mean Cell Condition Index value				
Treatment	**Week 1**	**Week 2**	**Week 5**	**Week 7**	**Week 9**
Paste control (air space, cotton plug)	3.00	2.00	1.00	0	0
Slurry 10g/L, (no additives, dark storage)	2.00	3.00	2.00	2	1.50
Slurry 10g/L, (no additives, light storage)	2.00	3.00	2.00	2	1.50
Slurry 10g/L, (both additives, dark storage)	2.00	2.50	2.00	2	1.50
Slurry 10g/L, (both additives, light storage)	2.00	3.00	2.00	2	1.50

<u>*Tahitian Isochrysis*</u>

Effect of density on storage life of chilled concentrates:
In stark contrast to *P. lutheri*, dilution of super-centrifuged pastes of T. *Iso* with sterile sea-water to form lower density slurries had a pronounced detrimental effect rather than a beneficial effect on cell viability and hence implied shelf life (Fig 6.5.5). Moreover decreasing slurry density was not accompanied by a significant change in retention cell viability. T. *Iso* also contrasted with *P. lutheri* in that shelf life of original cultures stored under the same favourable conditions as undiluted paste and slurries exhibited a much lower retention of cell viability than either of the former. As lowered density and all other techniques tested failed to improve the very poor (<1 week) shelf life of super-centrifuged paste, progressive sampling was ceased after week 2.

Effect of atmosphere on storage life of chilled concentrates:
As indicated by cell viability data presented in Fig 6.5.6, an atmosphere of oxygen reduced rather than extended the shelf life of low density T. *Iso* slurries as it did in *P. lutheri.* Also in common with *P lutheri*, cell viability of chilled 10g/L slurries of T. *Iso* were the same whether or not storage containers were sealed with screw cap lids or allowed to "breath" using a porous cotton-wool plug.

Effect of additives on shelf life of low density chill stored slurries:
Neither glycerol nor vitamin C when used individually or in combination significantly altered retained cell viability and hence useable shelf life of 10g/L T. *Iso* slurries (Fig 6.5.7). This again contrasted with shelf life enhancing effects of individual additives on 10g/L slurries *P. lutheri.*

Effects of light and dark storage on shelf life of low density chill stored slurries:
As indicated by results presented in Fig 6.5.8, dark or light storage had no discernible effect retained cell viability and hence shelf life of 10g/L T. *Iso* slurries regardless of the presence or absence of additives glycerol and Vitamin C.

Conclusions and Discussion

Results of this trial summarised in Table 6.5.8 again illustrated the species specific nature of storage factors that optimise the shelf life of micro-algal concentrates. In this case of *P. lutheri,* optimum shelf life (60% cell viability after seven weeks of storage) with high speed centrifuged concentraes was attained with the highest rate of dilution tested, the inclusion of Vitamin C and storage in light. The corresponding best storage conditions for concentrated T. *Iso* (undiluted paste with both additives stored in the dark), could hardly have been more different nor more disappointing (only 45% cell viability after a mere two weeks of storage).

Table 6.5.8. Optimal storage factors for high speed centrifuged concentrates of *P. lutheri* and T. *Iso.*

Treatment Factor	P. lutheri	T. *Iso*
Density of stored concentrate	10g/L>20g/L>40g/L>80g/L	Original undiluted paste far out-performed all slurries that did not vary over the density range of 10 to 80g/L
Additives	None or Glycerol or Vitamin C alone	No effect on diluted slurries but paste greatly enhanced by inclusion of both additives
Atmosphere	Air with cotton plug or sealed	No discernible effect of different atmospheres
Light/Dark	Light	No discernible effect
Best combination of factors and associated performance (in parenthesis)	10g/L; + Vit C; Air with cotton wool plug (60% cell viability after 7 weeks of storage)	Undiluted paste with both additives stored under air with cotton plug (45% cell viability after 2 weeks of storage)

Fig 6.5.1 Effect of cell density on retained viability of *P. lutheri* concentrates

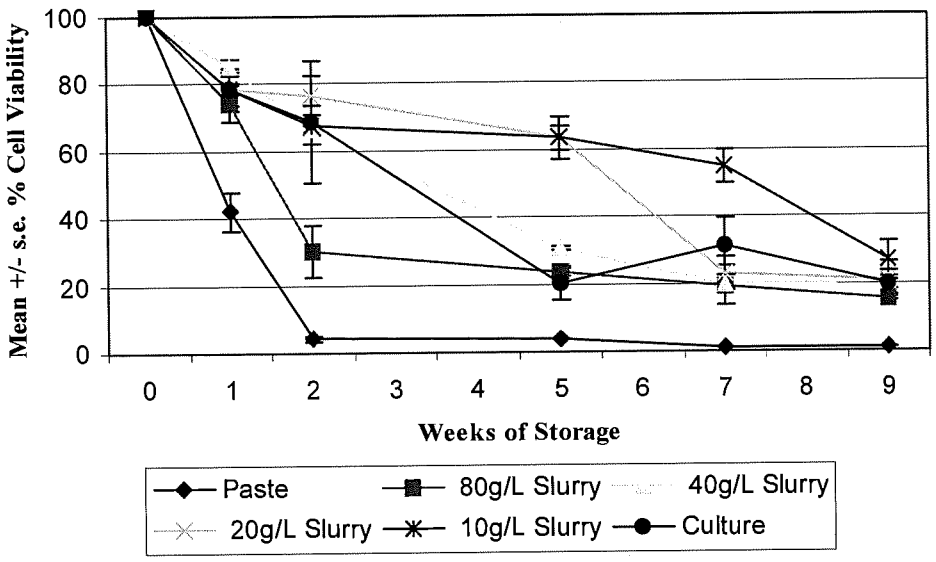


Fig 6.5.2 Effect of atmosphere of retained cell viability in 10g/L chilled slurries of *Pavlova lutheri*

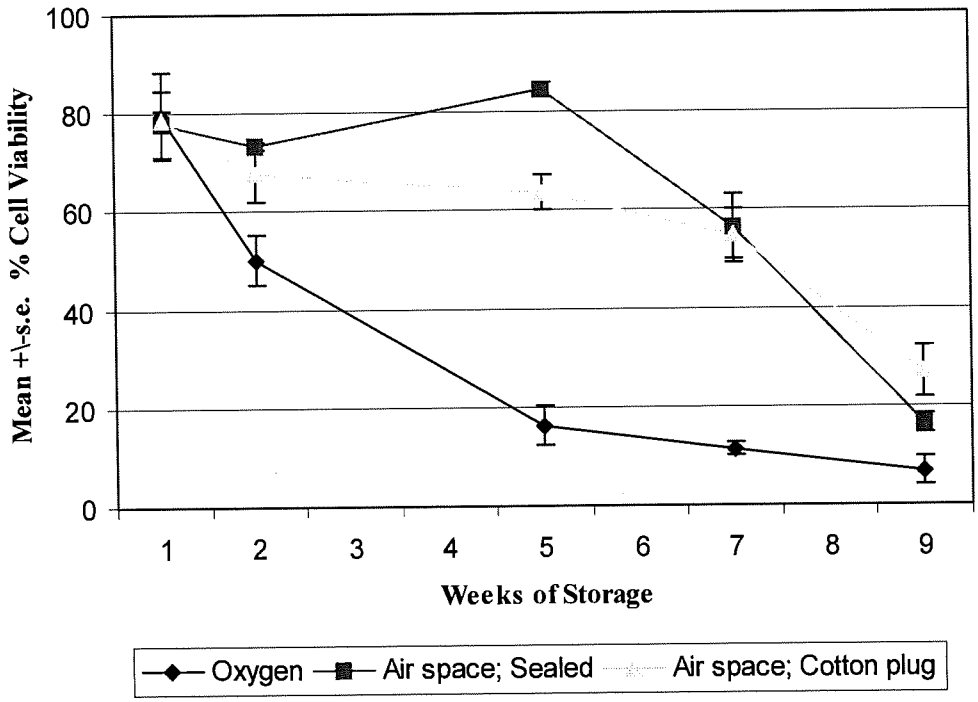


Fig 6.5.3 Effect of additives on retained cell viability of *P. lutheri* paste stored as a chilled 10g/L slurry

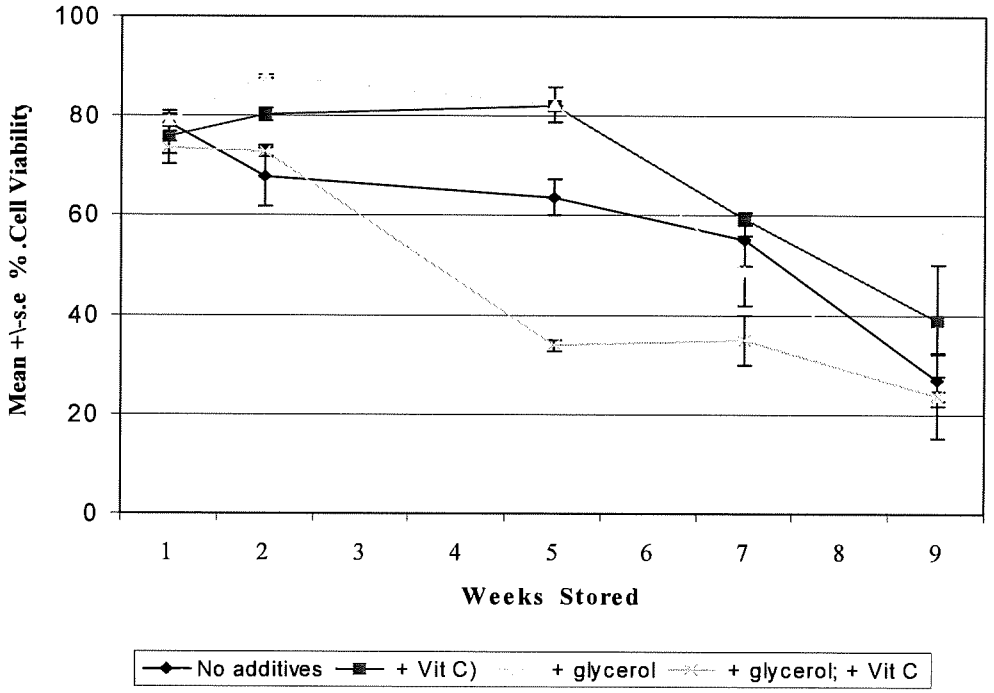


Fig 6.5.4 Effect of light and dark storage of *Pavlova lutheri* paste stored as a chilled 10g/L slurry with and without additives

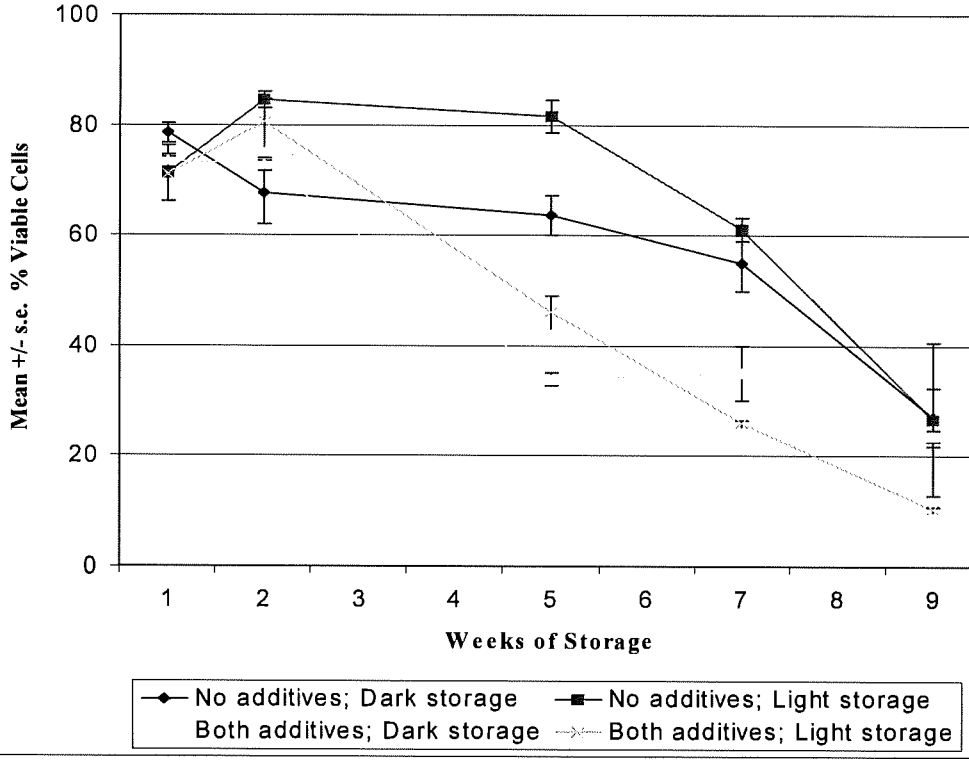


Fig 6.5.5 Effect of cell density on cell viability of chill stored TIso concentrates

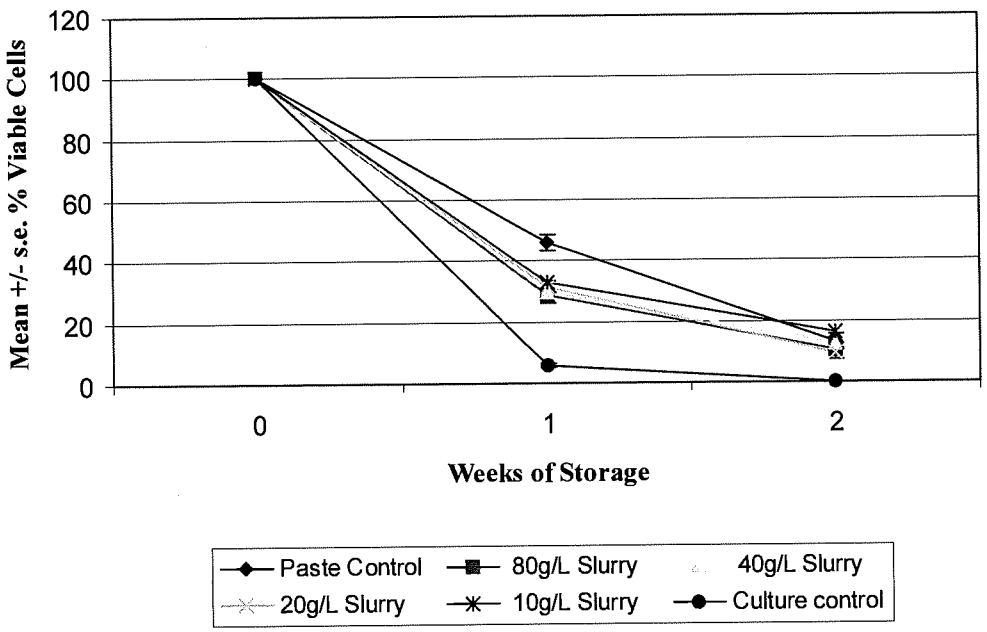


Fig 6.5.6 Effect of storage atmosphere on retained cell viabilty of 10g/L slurries of T.*Iso*

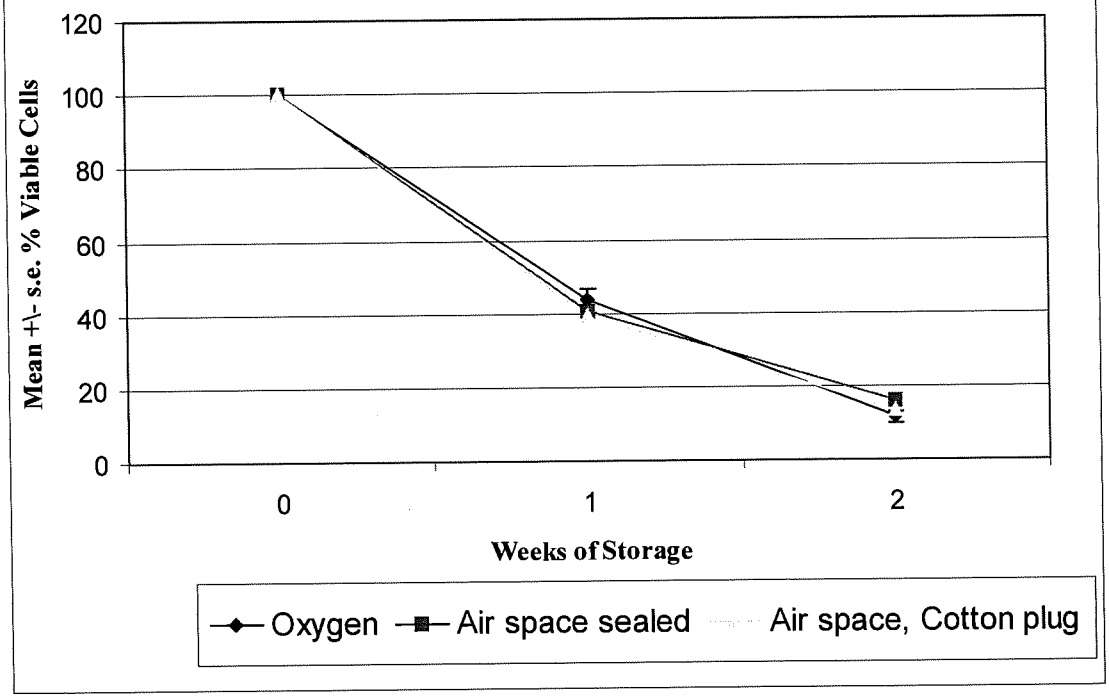


Fig 6.5.7 Effects of additives on cell viability of chill stored 10g/L T *Iso*. concentrates

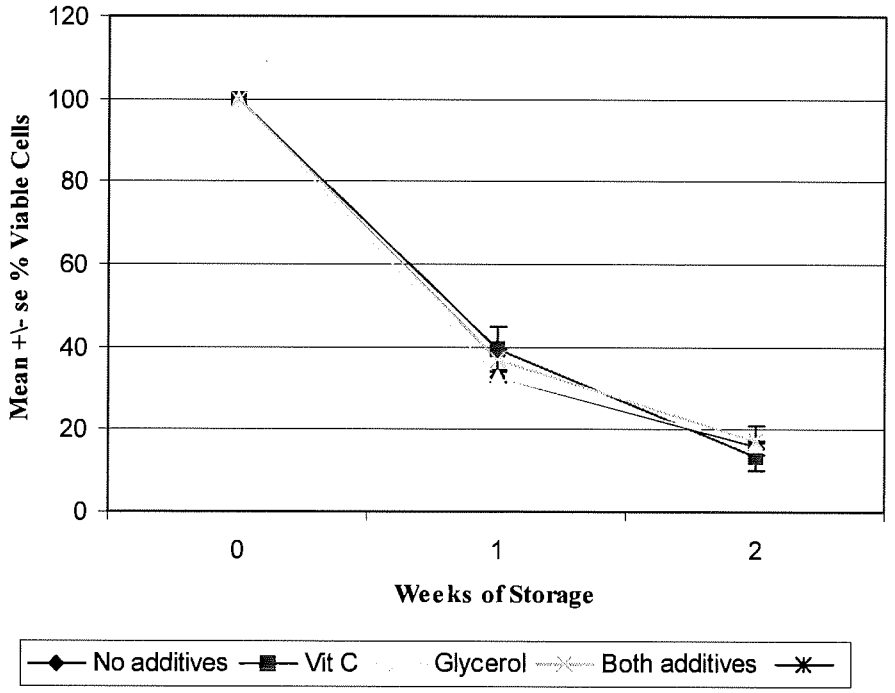
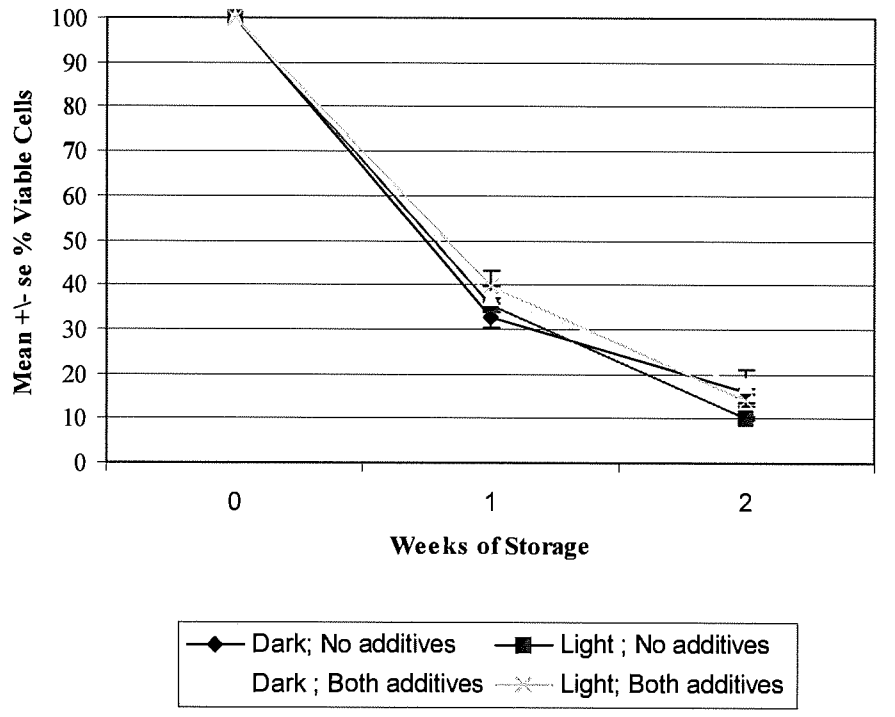


Fig 6.5.8 Effects of light and dark on chill stored 10g/L concentrates of T. *Iso* with and without additives



6.6. Screening Trials 6: Evaluation of Frozen Storage of Micro-Algal Concentrates

Introduction

Freezing and thawing of marine micro-algae results in injury to the cell wall (Ben-Amotz and Gilboa (1980). However, with use of cryo-protectants, chemicals that reduce the inherent cell wall damage, loss of cell viability and hence loss of cell contents, such damage can be overcome. For the purpose of storing marine micro-algae with the intent of feeding resuspended cells to aquatic animals, it is vital that damage to cell walls and membranes are reduced sufficiently to retain the nutrient quality of the cells.

Cryo-preservation has been extensively investigated and applied to micro-algae (Brown, 1972; Aujero and Millamena, 1979; Ben-Amotz and Gilboa, 1980; Fenwick and Day, 1992; Day and Fenwick, 1993; Cañavate and Lubian, 1994; Chao et al., 1994; Molina Grima et al., 1994; Cañavate and Lubian, 1995). An experiment was therefore conducted to evaluate the relative advantages and disadvantages of storing super-centrifuged pastes of nine different micro-algal species at -15°C as an alternative to chilled storage. This particular temperature was selected because it can be provided by cheap, readily available domestic and industrial freezer units. Storage of each species was carried out with and without the use of cryo-protectants at varying inclusion levels.

The selected cryoprotectants, Glycerol and DMSO, (Dimethyl sulphoxide, BDH Prod. 10323), have both been successfully used as cryoprotectants for marine microalgae (Brown, 1972; Aujero and Millamena, 1979; Day and Fenwick, 1993), and have previously been demonstrated to have minimal cytotoxic effects on microalgae (Fenwick and Day, 1992).

Materials and Methods

The nine species utilized included 5 species of diatoms, Skeletonema costatum; Phaeodactylum tricornatum; Thalassiosira psuedonana; Chaetoceros calcitrans; Chaetoceros muelleri, two prymnesiophytes Pavlova lutheri and Isochrysis sp (Tahitian Strain); the golden brown algae; Rhodomonas salina; and the chlorophyte; Tetraselmis chui. The two cryo-protectants, glycerol, and DMSO (Dimethyl sulphoxide, BDH Prod. 10323) used, were tested at three inclusion levels, 0.15, 1.5, and 15% (w/w). Control treatments containing no cryo-protectants were included as were non-frozen treatments stored in 70 ml screw cap containers at +2±0.5 °C.

After the addition of cryo-protectant, all treatments were given an incubation period of approximately 30 minutes at ambient room temperature (approximately 25°C) to allow penetration of the cryo-protectant into the cells. All frozen storage treatments were stored in 70ml screw-cap containers and placed directly into chest freezers set at -15.0±0.5 °C. Sampling was conducted after eleven and again after 32 days of storage. Small sub-samples of algal paste from each treatment was resuspended in ambient temperature sterile seawater and then examined at 200x. Resuspended pastes were evaluated according to a cell condition index scoring scheme provided in Table 6.6.1.

Table 6.6.1. Cell Condition Index Criteria.

Score	Microscopic appearance
5	Excellent indistinguishable from original cultures
4	Good - minor clumping, cell breakdown<10%
3	Satisfactory - moderate clumping, cell breakdown<50%
2	Poor - moderate/severe clumping, cell breakdown>50%
1	Very poor - severe clumping, cell breakdown>75%
0	No intact cells apparent

Results

Frozen super-centrifuged pastes of *Skeletonema costatum* (Fig 6.6.1*)*, *Phaeodactylum tricornutum*(Fig 6.6.2*)* and *Chaetoceros calcitrans*(Fig 6.6.3) all exhibited excellent retention of cell condition with the inclusion of either cryo-protectant at the highest inclusion levels of 15%. By contrast the two other diatom species *Thalassiosira psuedonana* (Fig 6.6.4*)* and *Chaetoceros muelleri* (Fig 6.6.5*)*, both exhibited rapid and progressive loss of cell quality regardless of the type or inclusion level of cryo-protectant. The same was true of both prymnesiophyte species *Pavlova lutheri* (Fig 6.6.6) and T. *Iso.*(Fig 6.6.7) and of *Rhodomonas salina* (Fig 6.6.8). As indicated in Fig 6.6.9, frozen super-centrifuged paste of *T. chui* like those of *S. costatum, C. calcitrans* and *P. tricornutum* retained excellent apparent quality over the 32 day period of the trial in the absence of any cryo-protectant whatsoever but also with glycerol and DMSO at all inclusion levels except the lowest level of 0.15% in the case of glycerol.

Table 6.6.2. Summary of mean cell condition index values after 4 to 5 weeks of frozen storage.

	Prymnesiophytes								
Treatment/StorageTemp	**Phae**	**Skel**	**C.calc**	**Thal**	**C.muell**	**Pav**	**T.Iso**	**Rhod**	**T.chui**
No protectant @ +2°C	5.0	5	5	4	4.0	3.0	3.5	3.0	5.0
No protectant @-15°C	4.5	4	4	2	2.5	1.0	3.0	1.5	5.0
0.15% glycerol @-15°C	5.0	4	4	2	2.5	3.0	3.5	2.0	4.5
1.5% glycerol @-15°C	5.0	4	4	3	3.5	3.0	4.0	2.0	5.0
15% glycerol @-15°C	5.0	5	5	4	3.5	3.5	4.0	2.0	5.0
0.15% DMSO @-15°C	4.0	4	4	2	2.5	2.5	3.0	1.5	5.0
1.5% DMSO @-15°C	5.0	4	4	3	3.5	3.0	4.0	1.5	5.0
15% DMSO @-15°C	5.0	5	5	4	3.5	3.5	3.5	2.5	5.0

Discussion

As indicated by data presented in Table 6.6.2, suitability for frozen storage of super-centrifuged pastes of the 9 species of micro-algae followed the sequence *T. Chui >P. tricornutum, S. costatum* and *C. calcitrans >>T. psuedonana,* T.*Iso, P. lutheri* and *R.. salina.* Only of the first four species retained an acceptable degree of apparent quality. Both cryo-protectants provided protection against damage to frozen cells within super-centrifuged pastes. Best overall results across the full array of nine species tested occurred at the highest inclusion level of 15% for both DMSO and glycerol. However as DMSO is toxic to animals especially aquatic larvae at 10 to 15% w/w, glycerol is clearly the cryo-protectant of choice.

Fig 6.6.1 Cell Condition Index of Treated *S. costatum* paste Over Time.

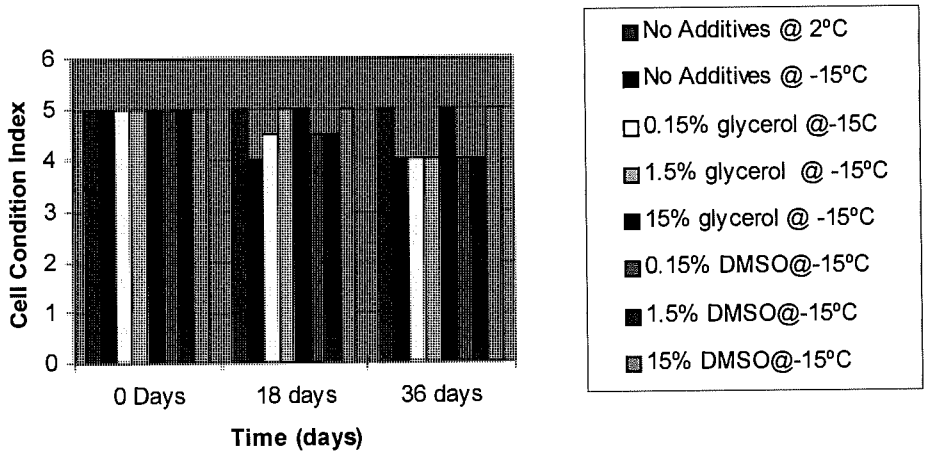


Fig. 6.6.2 Cell Condition Index of Treated *P. tricornatum* Paste Over Time.

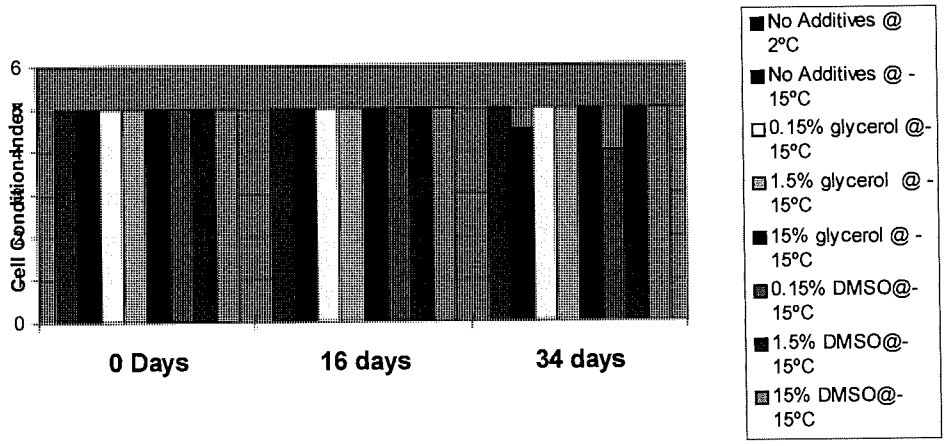


Fig 6.6.3 Cell Condition Index of Treated *C. calcitrans* Paste Over Time.

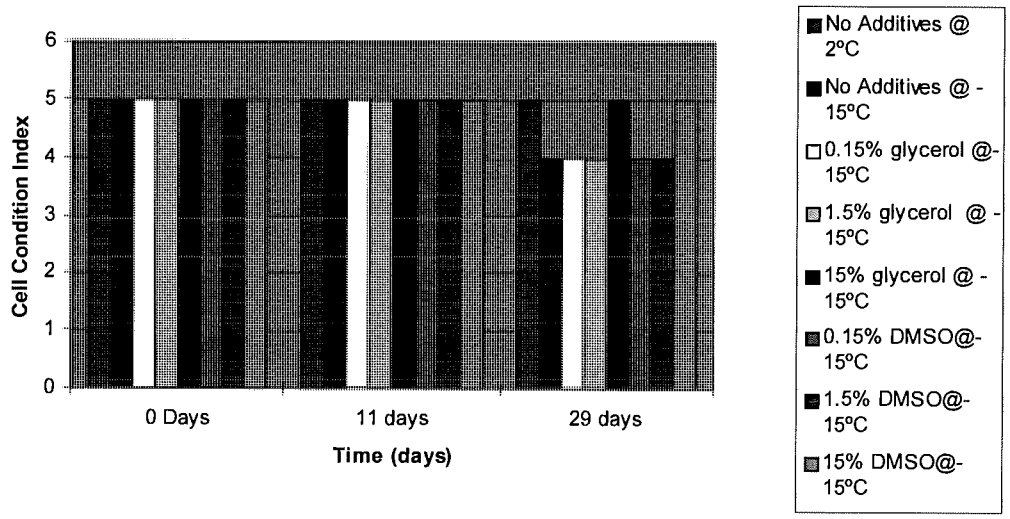


Fig 6.6.4 Cell Condition Index of Treated *Thalassiosira psuedonana* Paste Over Time.

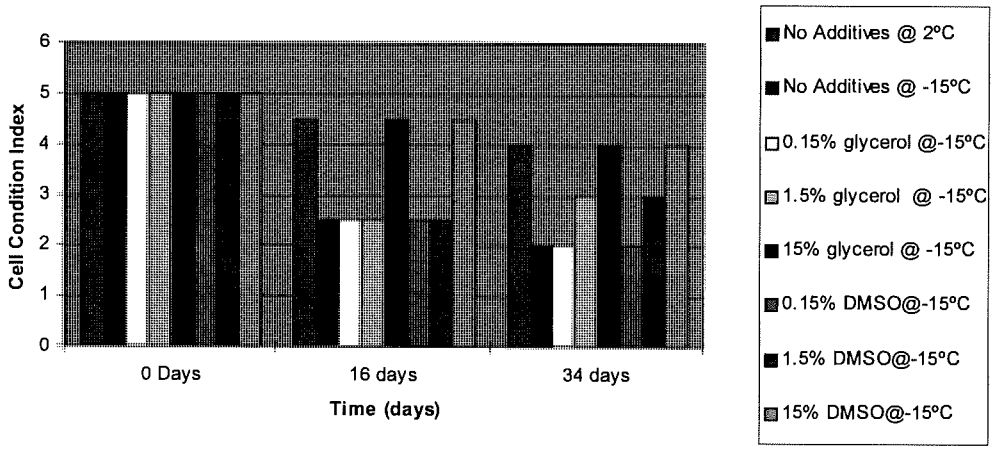


Fig 6.6.5 Cell Condition Index of Treated *C. muelleri* Paste Over Time.

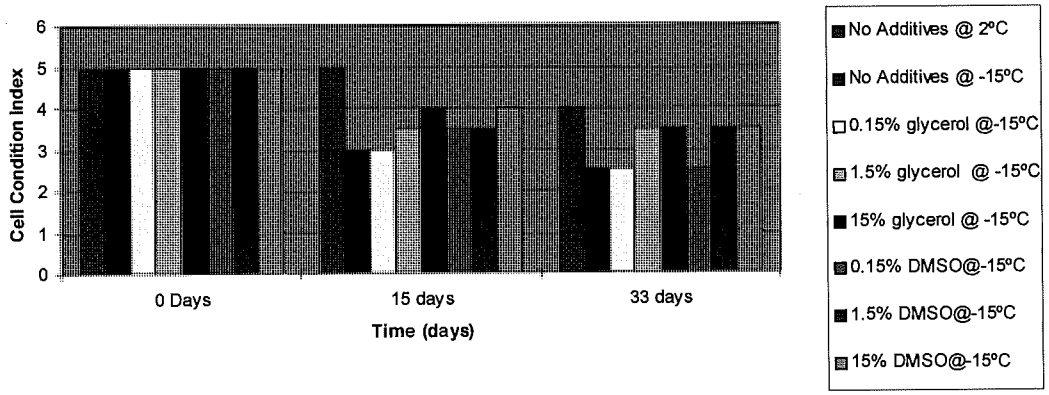


Fig. 6.6.6 Cell Condition Index of Treated *P. lutheri* Paste Over Time

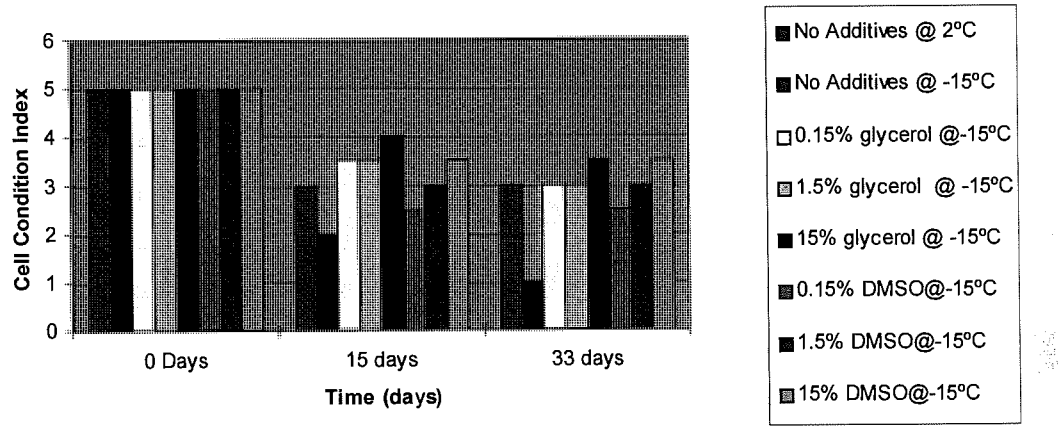


Fig 6.6.7 Cell Condition Index of Treated *Isochrysis* sp. Tahitian Over Time.

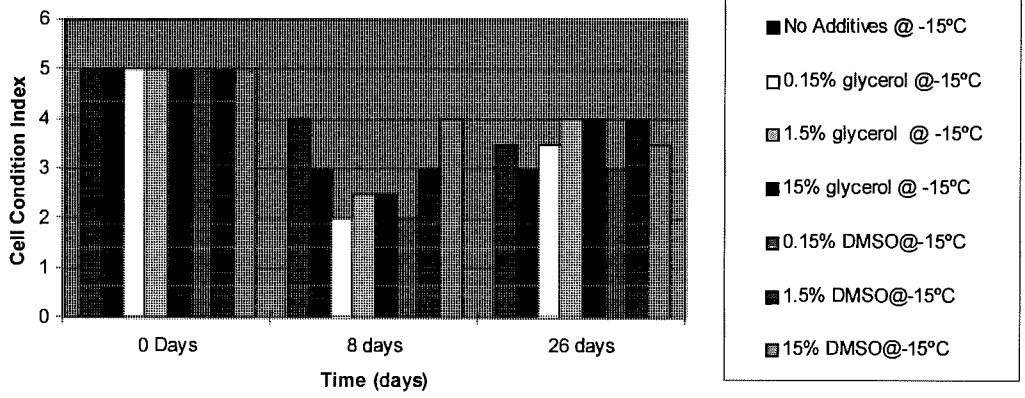


Fig 6.6.8 Cell Condition Index of Treated *Rhodomonas salina* Paste Over Time.

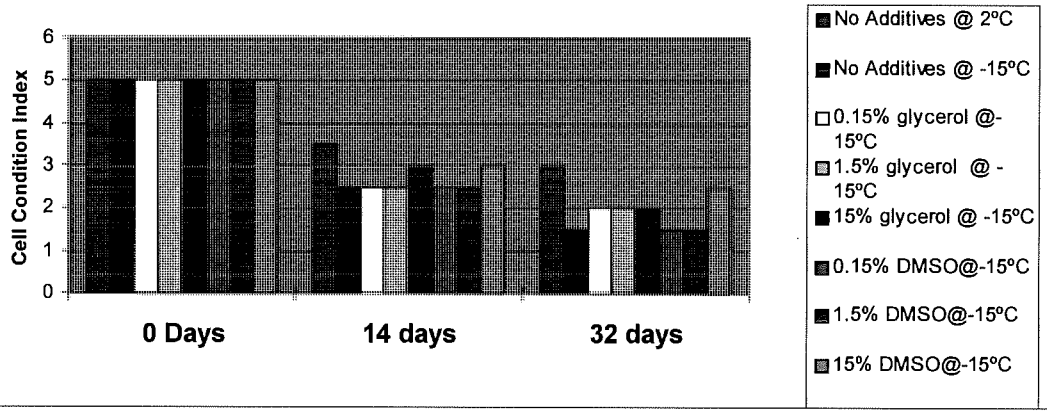
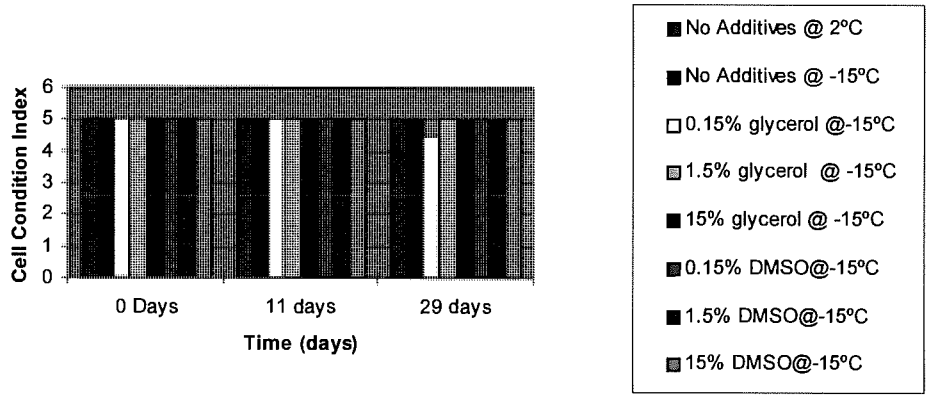


Fig 6.6.9 Cell Condition Index of Treated *T. chui* Paste Over Time.



7. BIO-ASSAY EXPERIMENTS TO EVALUATE ALTERNATIVE HARVEST, PRESERVATION, STORAGE AND FEEDING TECHNIQUES FOR CHILLED MICRO-ALGAL CONCENTRATES

7.1 Bio-Assay Experiment 1: Foundation Experiment to Define the Effect of Chilled Storage Period on Retained Nutritional Quality of a Standard Bivalve Mollusc Larval Diet Comprising Equal Amounts (on a dry weight basis) of *Pavlova lutheri,* Tahitian *Isochrysis* and *Chaetoceros calcitrans* concentrated by Super-Centrifugation

Introduction

Although high-speed centrifugation had previously been shown (see Section 5.3.2) to be the fastest, simplest and most efficient of a range of harvesting techniques evaluated. Results of subsequent screening trials revealed serious problems imposed by this method of harvesting. The problems were apparently linked to physical damage to cells and subsequent loss of quality as indicated by rapid irrevocable loss of cell viability and by other indirect criteria including development of offensive odours and microbial contamination, cell degradation, ease of re-suspension and degree of clumping of resuspended cells.

Of particular concern was the fact that these problems were exhibited by many of the mainstream species of micro-algae used in marine hatcheries. The latter include fragile prymnesiophyte species such as *Pavlova lutheri* and Tahitian *Isochrysis sp.* (*T. iso*) and several species of diatoms especially *Chaetoceros muelleri* and *Thalassiosira pseudonana.*

Nell and O'Connor (1991) found that growth of Sydney rock oyster larvae fed resuspended chill-stored concentrates of mono-specific micro-algal diets, namely *P. lutheri,* T. *Iso, Chaetoceros calcitrans,* produced with a cream separator type high speed centrifuge were reduced by margins of 45%, 32% and 1% respectively below that of larvae fed equivalent live micro-algal diets after only 1 to 2 weeks of storage. By contrast, chill-stored concentrates of some other species (*Tetraselmis suecica* and *Nannochloropsis atomus*) showed little apparent loss of nutritional value while another, namely *Phaeodactylum tricornutum,* exhibited enhanced nutritional value as indicated by growth rates 62% greater than that of live algae fed counterparts.

Aims

This initial bio-assay experiment was conducted to provide baseline quantitative data on the effect of high speed centrifugation and subsequent chilled storage duration on retention of the nutritional value (shelf-life) of a standard optimised ternary bivalve larval diet (O'Connor et al., 1992) comprising equal amounts, on a dry weight basis, of *Pavlova lutheri,* T. *Iso* and *Chaetoceros calcitrans.*

Materials and Methods

Preparation of diets:
Aliquots of 100 to 150 g of paste that had been harvested using a 'Sharples Super Centrifuge' at 13,000 g as previously described in Section 5.3.2, were produced and stored at weekly intervals over a total of 8 weeks prior to commencing feeding trials and for a further 3 weeks during the course of the trial. On each occasion, *P. lutheri* and Tahitian *Isochrysis* cells were each sourced from a minimum of three 500 L log phase bag cultures and *C. calcitrans* cells from a minimum of three 1000 L log phase vat cultures produced by techniques described in Frankish et al. (1991). This procedure was used to combat possible batch variability. After harvesting, the separate batches of paste for each species were contained in sterile 200ml screw-cap jars (Disposable Products - Australia Ltd.). The bottles were in turn stored in a chest freezer (Fisher and Paykel P/L, model H-360X) fitted with a "Thermo-Eye" digital temperature controller (Type PLE - Saginomiya P/L, Japan) operating at 2±0.5°C.

Feeding protocol:
At the commencement of the experiment, 36 miniature upwellers to accommodate 4 replicates of 9 dietary treatments, were individually housed within 8.5L plastic aquaria. The latter, held within a common temperature bath maintained at 25.0±1.0 °C, were stocked with 30 Sydney rock oyster spat. The spat in all treatments were fed to satiation with equal proportions on a dry weight basis of the three micro-algal species. The weekly feed ration was established for each treatment in accordance with methods of O'Connor et al. (1992) based in turn on Epfarnio's 1979 equation, by weighing (live weight after draining and air drying on absorbent paper towelling for 1 hour) spat from 1 replicate of each treatment. Each day small samples of stored pastes were resuspended, counted, and volume to feed/bucket calculated and feed administered. Complete water changes with fresh 1µm filtered seawater preheated to 25°C plus cleaning and disinfection of plastic aquaria was conducted three times a week.

The nine experimental treatments (Table 7.1.1) included a fresh live algae control diet plus eight super-centrifuged raw paste diets stored for varying periods ranging from a minimum of 0 -1 week and thence at weekly increments to a maximum of 7 - 8 weeks.

Measurement of growth as a direct indicator of diet quality:
At stocking, the initial mean (±s.d.) live-weight for individual spat of 12.09±0.06 mg was determined by taking eight representative sub-samples each containing 30 individual spat and air drying before weighing. The corresponding initial mean (±s.d.) (n=8) dry weight of 9.24±0.25mg was then determined by oven drying to a constant weight at 80°C. Finally, mean(±s.d.) initial ash-free dry weight of 0.76±0.03 mg was determined after grinding and combusting the same eight sub-samples for 5 hours in a muffle furnace at 475°C thereby avoiding thermal decomposition of carbonates, especially calcium carbonate within the shell.

The same procedures were used to determine final mean (±s.d) live-weight, dry-weight and ash-free dry-weight for all four replicates in each of the dietary treatments at the conclusion of the 21 day experiment. By that time, spat in the best treatments had exhibited 3 to 4 fold increases in mean live-weight, dry-weight and ash-free dry weight.

Table 7.1.1. Experimental treatments.

Dietary Treatment	Description
1	Fresh live Algae
2	Super-centrifuged paste stored at 2±0.5°C for 0 - 1 week
3	Super-centrifuged paste stored at 2±0.5°C for 1 - 2 weeks
4	Super-centrifuged paste stored at 2±0.5°C for 2 - 3 weeks
5	Super-centrifuged paste stored at 2±0.5°C for 3 - 4 weeks
6	Super-centrifuged paste stored at 2±0.5°C for 4- 5 weeks
7	Super-centrifuged paste stored at 2±0.5°C for 5 - 6 weeks
8	Super-centrifuged paste stored at 2±0.5°C for 6 - 7 weeks
9	Super-centrifuged paste stored at 2±0.5°C for 7 - 8 weeks

Statistical analysis:
For all growth data, homogeneity of variance was confirmed using Cochran's test ANOVA. Means were compared using Student-Newman-Keul's procedures (Winer et al., 1991).

Results

All three growth criteria, namely mean increases in live-weight, dry-weight and ash-free dry-weight (Table 7.1.2), exhibited the same relationship between storage duration of the diet and nutritional value. Growth rates, indistinguishable from those supported by live micro-algae, were exhibited by oyster spat fed on the paste diet stored for up to one week. Storage of super-centrifuged paste beyond one week however resulted in progressively poorer growth rates. The mean increases in live-weight (Fig. 7.1.1) of spat fed 1-2 week old, 2-3 week old, 3-4 week old paste fell to about 70%, 50% and 30%, respectively of their live algae fed counterparts. Live-weight increments of oyster spat fed paste stored for 4-5 weeks or longer exhibited an even lower common growth rate about 20% that of live algae fed spat.

The progressive decline in growth rate with increasing storage time of super-centrifuged paste diets was even more protracted and pronounced from ash-free dry-weight data (Table 7.1.2) with rates declining to about 10% those supported by live algae and fresh (0-1 week old) pastes after 6 to 8 weeks of storage.

Table 7.1.2. Effect of high speed centrifugation and chilled storage duration on the nutritional value of a standard mollusc diet comprising equal amounts of *Pavlova lutheri*, T. *Iso* and *Chaetoceros calcitrans*. Means with a common superscript do not differ significantly (SNK, $P<0.05$).

Diet	Increased Live Weight (mg) mean ± s.d.	Increased Dry Weight (mg) mean± s.d	Increased Ash-free Dry Weight (mg) mean ± s.d
Fresh live Algae	51.92± 4.87 [a]	37.28±3.66 [a]	2.81±0.31
Paste 0-1 wk	49.82±3.15 [a]	36.89±2.33 [a]	2.77±0.25
Paste 1-2 wk	35.71±1.23 [b]	26.06±0.96 [b]	1.81±0.12
Paste 2-3 wk	24.33±5.43 [c]	18.21±3.69 [c]	1.13±0.24
Paste 3-4 wk	15.86±3.17 [d]	11.80±2.68 [d]	0.60±0.18
Paste 4-5 wk	10.55±1.74 [d]	7.50±0.17 [d]	0.37±0.08
Paste 5-6 wk	13.01±0.69 [d]	10.00±0.73 [d]	0.47±0.07
Paste 6-7 wk	9.98±1.38 [d]	7.46±0.99 [d]	0.28±0.09
Paste 7-8 wk	9.82±1.10 [d]	7.51±0.97 [d]	0.26±0.06

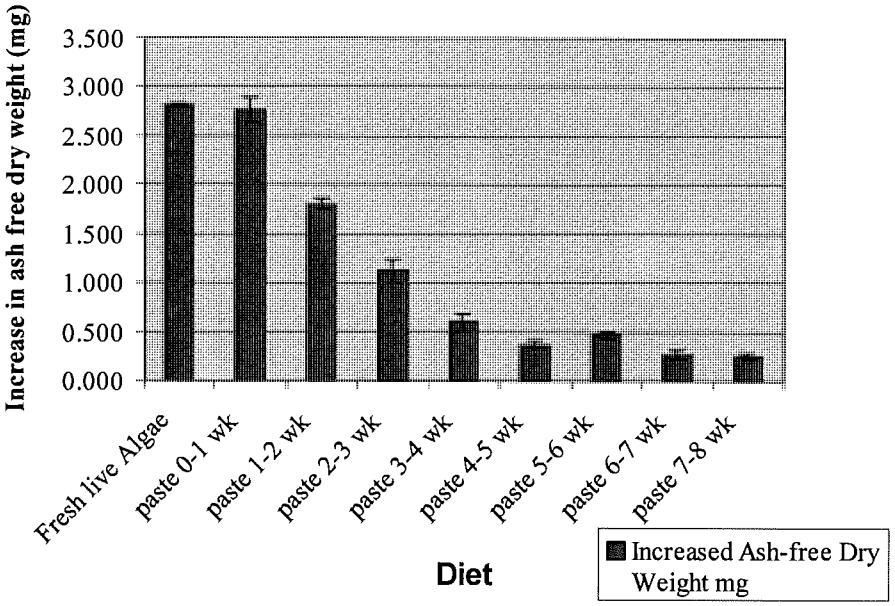
Conclusions and Discussion

The more pronounced differences in the growth performance of diets indicated by ash-free dry-weight over other parameters of growth may be explained by the fact that ash-free dry-weight, unlike live-weight and dry-weight, is not confounded by large fluctuations in water and shell mineral content. Although the latter two parameters do not reflect important elements of growth, they nevertheless constituted the great bulk (about 24% and 70% respectively) of live-weight gains exhibited by Sydney rock oyster spat in this experiment.

In Section 5.3.2, significant damage to cells of *P. lutheri* and T. *Iso*, as indicated by positive staining response to Evan's blue, was shown to be sustained during super-centrifugation. Mean (±s.d.) cell viabilities recorded for the two species immediately following super-centrifugation were 92±2 and 88±1% respectively. As demonstrated in Screening Experiment 2, these values subsequently fell to 27±18% and 40±4% respectively after 1 week of chilled storage and thence to <5% and 13± 2% respectively after 4 weeks of storage.

The progressive and profound decline in the nutritional value of stored super-centrifuged paste of a standard hatchery diet collectively comprising two thirds by dry matter content of *P. lutheri* and T. *Iso* observed in the current experiment was consistent with results of the earlier screening experiments. Accordingly, staining response to Evan's blue as an indicator of cell viability, was also vindicated as a measure of retained nutritional quality of stored micro-algal concentrates.

Fig 7.1.1 Effect of storage time on the nutritional value of a super-centrifuged ternary diet comprising equal amounts of C. calcitrans P. lutheri and T Iso fed to oyster (*Saccostrea glomerata*)spat



7.2. Bio-Assay Experiment 2: Effects of Combinations of Three Types of Common Food Additives on the Shelf Life of *Pavlova lutheri* as indicated by Direct Bio-Assay using Bivalve Larvae

Introduction

Results of Screening Experiments 1 and 2 (Sections 6.1 and 6.2), based on staining response to Evan's blue and on other indirect criteria of retained quality, indicated that effects of three common food additives on the shelf-life of super-centrifuged concentrates of micro-algae, were species specific. The additives, the anti-oxidant Vitamin C, citric acid (to induce low pH of 4.0 to 4.5) and the cryo-protectant glycerol, were beneficial for most micro-algae species tested.

The apparent keeping qualities of raw super-centrifuged concentrates of the prymnesiophytes *Pavlova lutheri,* T. *Iso* and of the diatom *Chaetoceros muelleri* were not however improved by any of the three additives regardless of whether the latter were used alone or in combination. Incidentally, the same three species of micro-algae were those that sustained the highest rates of cell damage during centrifugation also exhibited the poorest keeping qualities. By contrast, some other indicators of retained quality, such as degree of microbial contamination and development of offensive odours, were improved by the inclusion of additives.

Aims

The aim of this experiment was to use growth of larval scallops (*Pecten fumatus*) larvae to verify (or refute) the disappointing results obtained during Screening Experiment 2 in relation to effects of additives on the keeping qualities of centrifuged concentrates of *P. lutheri,* T. *Iso.* and *Chaetoceros muelleri. P. lutheri* was selected as the test species ahead of T. *Iso* or *C. muelleri* because of the former's demonstrated superior performance as a mono-specific diet for rearing *P. funatus* larvae (Heasman et al., 1991).

Materials and Methods

Larvae of the commercial scallop, *P. fumatus,* were fed a range of diets all comprising cells of *P. lutheri* but of varying age, harvest status and method of storage. Methods used to culture and harvest *P. lutheri* were as described for Bio-assay Experiment 1.

Four replicates were used for each of nine experimental treatments. The treatments (Table 7.2.1) included an unprocessed fresh *P. lutheri* control; a second control treatment comprising *P. lutheri* cells resuspended from raw super-centrifuged paste produced daily; 7 chilled paste treatments stored at either 2±0.5°C or -2±0.5°C for 2.5 to 3.5 weeks and incorporating either one or combinations of two or all three of the food additives. Types and rates of additives used were identical to those used in Screening Trial 2 Viz.:

- A cryo-protectant, glycerol (SIGMA G-7893) at 10% (w/w).
- An anti-oxidant, ascorbic acid (=Vitamin C) (SIGMA A-7506) at 1% (w/w).
- A food acid, namely 0.5m citric acid (BDH Prod. No. 10081) which was added and blended with super-centrifuged paste until a pH 4- 4.5 was achieved.

Aliquots of *P. lutheri* concentrates (pastes) harvested by super-centrifugation were weighed and the particular combinations of additives for each experimental treatment combined with the paste and thoroughly blended. The various paste treatments were then stored in sterile 70ml screw cap

plastic bottles (Disposable Products, Australia Ltd) also according to techniques described for Bio-assay Experiment 1.

Table 7.2.1. Experimental treatments.

Treatment Number	Description
1	Fresh *Pavlova lutheri* culture
2	Fresh Paste (spun daily)
3	Aged paste without additives stored @ 2C°C
4	Aged paste with glycerol stored @2°C
5	Aged paste with low pH (+citric acid) and stored @2°C
6	Aged paste with Vit C and stored @2°C
7	Aged paste with low pH (+citric acid) and + Vit C stored @ 2°C
8	Aged paste with all three additives and stored @2°C
9	Aged paste with all three additives and stored @ -2°C

Broodstock scallops were collected from Jervis Bay, transported to Port Stephens Research Centre and held at 15°C in a closed circuit reproductive conditioning system until they were induced to spawn two to three weeks later (Heasman et al., 1996). Two day old larvae with an initial mean (± s.e.) shell length of 106.8±2.7µm (n = 60), were stocked at the rate of 2 larvae/ml into 36 x 8L plastic aquaria. Seawater was aged and settled for a minimum of 7 days, filtered through successive 5 and to 1µm (nominal) wound depth filter cartridges and EDTA added at the rate of 1gm/L prior to use. At each water change, seawater was preheated to an optimum larval rearing temperature of 21±1°C (Heasman et al., 1996a) within a common water bath housing all 36 aquaria. In all treatments, *P.lutheri* cells were fed at a non growth limiting rate of 5000 cells/ml/day. Four replicates of each feeding treatment were randomly allocated to the 36 plastic aquaria.

The larval cultures were gently aerated and complete changes of seawater and vessels were performed every 2 days, the larvae being wet sieved onto 45µm screens. The experiment was terminated after 8 days when the larvae were collected and fixed using 5% formaldehyde in seawater buffered at pH of 8.0.

Daily, samples of stored paste were drawn with a 1ml syringe from each treatment whilst working within a laminar flow cabinet. The sample of micro-algae concentrate was re-suspended into 1µm filtered sea-water at the rate of approximately 0.1ml of paste/ 100 ml of sea-water using a 'Sunbeam Maestro' hand held food processor. The density of cells within re-suspended pastes were calculated from counts made at 200x using a light microscope and an 'Improved Neubauer' haemocytometer (Superior Co., Berlin, Germany). Feed rates were calculated according to methods described in Frankish et al., (1991). The growth of the larvae in each treatment was evaluated by measuring a minimum of 50 larvae from each replicate mounted on a 'Sedgewick Rafter' counting slide. Measurement of individual larvae was made at 200x using an "Olympus," model CH-2, light microscope fitted with a calibrated eye-piece micrometer. Percentage survival was also obtained by counting live and total numbers of larvae in replicate 1.0 ml samples.

Results

P. fumatus larvae fed the P. lutheri control diet of fresh culture exhibited mean (±s.d) increases in shell-length of 44.7±2.1 µm and a mean (± s.d)survival rate of 72.6±4.7% (Table 7.2.2 and Fig. 7.2.1) at the conclusion of the 8 day experiment. These rates of growth and survival were within

the expected and acceptable range for this species (Heasman et al., 1996). Mean (± s.d) increment in shell length of larvae fed freshly centrifuged control paste diet (50.8±4.0μm) exceeded that of the larvae fed the live culture control diet by a statistically significant (SNK, P<0.05) margin of about 10%.

The inclusion of three additives alone or in combination in all seven aged super-centrifuged paste diets failed to enhance growth and survival of P. fumatus larvae. To the contrary, mean (±s.d) growth and survival attained on the raw (no additives) aged paste diet (39.1±2.6μm and 78.2±1.5 % respectively) was significantly (SNK, P<0.05) greater than growth and survival achieved on all other aged paste diets over the eight day duration of trial. Moreover, performance of diets diminished with successive inclusion of additives growth performance of aged paste diets following the sequence: no additives>+low pH>+glycerol>+VitC> low pH and Vit C> all additives @ +2 °C > all additives @ -2 °C. Mean survival data for aged diets followed a similar sequence that also indicated a negative effect of additives namely: no additives> low pH and Vit C >+VitC >low pH > all additives @+2 °C >+glycerol > all additives @ -2 °C.

Table 7.2.2. Effect of additives on nutritional value of chilled super-centrifuged pastes of Pavlova lutheri as indicated by the growth and survival of scallop larvae. Means with a common superscript do not differ significantly (SNK, P<0.05).

Diet Treatment	Mean ± s.d Growth (um)	Mean % Survival ± s.d
Fresh *Pavlova* culture	44.71±2.08^{b}	72.61±4.74^{ab}
Fresh Paste	50.76±3.95^{a}	66.50±3.07^{abcd}
No Additives @ 2°C	39.12±2.57^{bc}	78.16±11.50^{a}
Glycerol @ 2°C	36.07±1.55^{cd}	55.11±5.68^{de}
Low pH @ 2°C	36.12±1.83^{abcd}	60.44±2.93^{bcde}
Vit C @ 2°C	34.66±4.01^{cde}	65.48±10.40^{bcde}
Low pH + Vit C @ 2°C	30.60±1.58^{def}	70.98±13.97^{abc}
All additives @ 2°C	28.92±1.38^{ef}	60.09±7.25^{cde}
All additives @ -2°C	27.97±1.08^{f}	53.90±4.62^{e}

Conclusions and Discussion

The statistically significant (P<0.05) margin of about 10% in growth rate exhibited by larvae fed fresh super-centrifuged concentrate control diet over that of larvae fed the live culture of *P. lutheri* cells was consistent with results achieved by Nell and O'Connor (1991) with veliger larvae of the Sydney rock oyster *Saccostrea glomerata.* Possible explanations for this result are the removal of nutrients and of organic waste products and bacteria from bulk algae cultures during the process of centrifugation or enhanced digestibility associated with damage to *P. lutheri* cells during super-centrifugation.

The observed negative influence of the three common food additives on growth and survival of scallop larvae when used singly and the compounding of this effect when used in combination with super-centrifuged paste of *P. lutheri,* are perplexing. Nevertheless these results were entirely consistent with those obtained in Screening Experiment 2 that employed an array of indirect criteria to evaluate retained quality of stored super-centrifuged pastes of *P. lutheri* and of three other commercially important species of marine micro-algae. Foremost amongst the indirect criteria that indicated a clear negative influence of food additives to super-centrifuged pastes of *P. lutheri*, was retention of apparent cell viability as indicated by staining response to Evan's blue.

A clear inference of these results is that no generalisations or predictions can be made on the likely influence of food additives on keeping qualities of chill stored concentrates of micro-algae produced by high shear force methods of centrifugation. Thus while the results of this experiment did validate the negative influence of food additives on the shelf life of super-centrifuged pastes of *P. lutheri*, the same effects cannot necessarily be expected for concentrates of *P. lutheri* produced by alternative less traumatic procedures. Put another way, it is not possible to ascribe these results either to inherent characteristics of *P. lutheri* cells per sé or to cell damage sustained by them during super-centrifugation. What can be confidently stated is that addition of the food additives trialed, does not improve the poor shelf life of super-centrifuged pastes of *P. lutheri*. A further possible inference of the results of this bio-assay experiment is that they may be valid in relation to super-centrifuged pastes of other micro-algae species used in Screening Trial 2, namely T. *Iso* and *C. muelleri*, that exhibited both apparent susceptibility to cell damage during super-centrifugation and negative apparent responses to the same set of food additives.

7.3. Bio-Assay Experiment 3: Evaluation of Optimal Harvesting Technique for *Pavlova lutheri* (super centrifuge vs bucket centrifuge vs flocculation and stored as slurry)

Introduction and Aims

Results of the preceding Bio-assay Experiment 2 and of Screening Experiments 1 and 2 (Sections 6.1 and 6.2), demonstrated a failure by common food additives to extend the practical shelf life of chilled super-centrifuged paste of the prymnesiophytes *Pavlova lutheri,* T. *Iso.* and of the diatom *Chaetoceros muelleri.* These results also posed the question, does cell damage sustained by fragile micro-algal cells during high speed centrifugation undermine benefits of any subsequent method of preservation or storage? An additional bio-assay experiment was therefore devised to address this question and to clarify results of Screening Experiment 7 on whether or not poor nutritional value of aged *Pavlova lutheri* pastes is due in part to a very rapid deterioration of cells once removed from cold storage and added to hatchery rearing tanks. Towards these ends an evaluation was made of various combinations of alternative harvesting and cold storage techniques and of different feeding frequencies on retained nutrition value of chill stored *Pavlova lutheri* concentrate diets. Growth and survival of Sydney rock oyster *Saccostrea glomerata* (=*commercialis*) larvae were used as direct indicators of retained nutritional value of the diets.

Materials and Methods

Experimental design:
Twelve experimental treatments in a 3 x 2 x 2 factorial design were employed to evaluate individual and interactive effects of harvest method (chemical flocculation; low speed centrifugation and high speed centrifugation), storage temperature (chilled storage at 2.0 ± 0.5°C or frozen storage at -15 ± 1°C) and frequency of feeding (once or six times per day) on the nutritional value of stored *P. lutheri* concentrates. An additional 6 treatments, an unfed control and five fresh (daily harvested) algae control diets, were also included. Four of the five fresh diets comprised a fresh algal culture and fresh algae concentrates diets harvested daily by each of the 3 alternative techniques (flocculation and high or low speed centrifugation) and fed once daily. The remaining fifth fresh diet comprised high speed centrifuged algae fed 6 times daily. Four replicates of each of the eighteen dietary treatments were randomly distributed throughout a common water bath.

Preparation of diets:
Cultures of *P. lutheri* were grown from axenic inocula in 500 L 80 µm clear polyethylene film bags. The cultures were propagated in oceanic water (34 - 35 g/L) settled for 6 to 8 days, filtered to 1 µm (nominal), and disinfected using sodium hypochlorite at an effective concentration of 10mg/L for approximately 12 h. Prior to inoculation, residual chlorine was deactivated using sodium thiosulphate. Cultures were enriched with f/2 beta growth medium (Guillard, 1983) held at 23 ± 0.5°C with a 16 h : 8 h light : dark cycle and illuminated with 58 watt 'cool white' fluorescent tube lights to an intensity of 4,000 lux at the bag surface. Cultures were continuously aerated with 1.5% carbon dioxide enriched air to maintain pH in the range of 8.0 to 8.4.

Three different methods of concentrating the algae were used. These comprised chemical flocculation, high speed centrifugation, and low speed centrifugation. Chemical flocculation (also see Section 5.3.4) was achieved by adding a solution of 0.5% Chitosan (Aldrich 41,941-9) dissolved in 0.5M Citric acid (BDH Prod No 10081), used at an optimum rate of 80 mg/L chitosan to cultures. A resultant sedimentary floc was subsequently harvested by simple separation from the supernatant. High speed centrifugation was performed using a 'Sharples Super Centrifuge at 13,000 g. Algal cultures in mid to late exponential phase were delivered to the centrifuge with a

diaphragm pump at a flow rate of approximately 25 L/minute. The algal concentrate was recovered from the super-centrifuge bowl using a plastic spatula. Low speed centrifugation involved the use of a Hitachi (Model 05P-21B) lab scale centrifuge operated at 1,330g for 5 minutes. The resultant slurry was then recovered by decanting the supernatant.

In order to combat culture variability, four separate 500 L batch cultures of *P. lutheri* were used for each method of harvesting. After harvesting, concentrates were thoroughly blended. Both flocculated and low speed centrifuge harvested product were in a slurry form while the super-centrifuged product was a characteristic firm paste. All treatments were held at 2 ± 0.5°C and stored in 120 mL clear polystyrene containers with a 1:2 ratio of slurry to air-space as either stiff pastes or diluted slurries at 10-20 g paste/L in accordance with optimum densities previously identified in Section 6.5. The containers were stored on a 30° incline and under light. All treatments stored at -15°C ± 1°C were gently blended with glycerol (SIGMA G-7893) at the rate of 15% (w/w). These were left at ambient room temperature for a minimum of half an h to allow penetration of the glycerol prior to immersion quick freezing (IQF) using an agitated 25% NaCl solution held at -15°C. All stored products were maintained in "Fisher and Paykel", New Zealand, chest freezers in which temperature was monitored and controlled at 2 ± 0.5°C with a "Thermo Eye-P", Tokyo, Japan, system. These had been held for a period of 33 days at the commencement of feeding.

Acquisition, husbandry and measurement of larvae:
One hundred and twenty broodstock Sydney Rock Oysters (*Saccostrea glomerata*) were road freighted to Port Stephens Research Centre from an oyster farmer at Tuross Head (approximately 36° S latitude, 150° E longitude). They were then held in a 1,500 L conditioning system for a period of 17 days. During this time they were subject to 20°C with 100% water change every 48 - 72 h and were fed with 100 - 120 L of batch cultured algae per day according to the standard conditioning regime developed by Frankish et al., 1991. The broodstock were induced to spawn using thermal and salinity shocks again as discussed by Frankish et al. 1991. A total of 10.66 million fertilized eggs were stocked into a 1000 L tank of one week aged and settled coastal seawater (salinity 34-36g/L) that was filtered to 1 µm through a wound cartridge depth filter, pre-warmed to 24°C ± 1°C and treated with sodium ethylenediaminetetra-acetic acid (EDTA) at 1 mg/L.

Each day, stored concentrated alga diets were resuspended in filtered seawater, with the flocculated treatments requiring re-acidification to a pH of approximately 3.0 to 3.5 using a 0.5N hydrochloric acid solution. Algal culture density of each treatment was measured using an 'Improved Neubauer' haemocytometer slide (*Superior Co.*, Berlin, Germany) at 200X using a 'Jenamed' compound light microscope. The feed rate for larvae in each treatment was adjusted daily in accordance with a prescribed optimum feeding regime described by Frankish et al., 1991. All treatments involving multiple feeds were fed at four hourly intervals, i.e. six times per day. Every 48 h larvae were wet sieved on 45 µm screens before being restocked into clean vessels containing freshly filtered replacement seawater (34 - 35 g/L) pre-treated as previously described.

After approximately 48 h, the D-veliger larvae were harvested onto a 45 µm screen, counted, measured, and found to have a mean (± s.d) shell length of 77.0 ± 0.4 µm. The larvae were stocked into 72 x 8 L cylindrical plastic aquaria at a density of 2 larvae/ml. Each aquarium was gently aerated and maintained at a near optimum temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998), within a common water bath housed in an air-conditioned room maintained at 23 ± 1°C. The 18 treatments each with four replicates were randomly distributed within the common water bath.

The experiment was terminated after 8 days when the larvae were collected and preserved using a 4% formaldehyde in seawater solution. Mean (± s.e.) growth of the larvae was evaluated by measuring a minimum of 50 individuals from each replicate at 200X using an Olympus (Model CH-2) compound light microscope fitted with a graticule and a 'Sedgewick Rafter' counting slide. Percentage survival was obtained by counting total and live larvae within a minimum sample of 100 larvae. Live larvae were distinguished from dead by lack of necrotic tissue and presence of food in the gut at the time of fixation.

Table 7.3.1. Description of experimental dietary treatments.

Treatment No.	Unfed and Fresh diet Treatments	Treatment No.	Stored Diet Treatments
1A (1)	Unfed	1B(6)	High speed centrifugation; stored 2°C; 1 daily feed
2A(2)	Fresh algal culture; 1 daily feed	2B(7)	Flocculated; stored 2°C; 1 daily feed
3A(3)	Fresh daily centrifuged; 1 daily feed	3B(8)	Low speed centrifugation; stored 2°C; 1 daily feed
4A(4)	Fresh daily flocculated ; 1 daily feed	4B(9)	High speed centrifugation. stored -15°C; 1 daily feed
5A(5)	Fresh daily gentle harvest; 1 daily feed	5B(10)	Flocculated ; stored -15°C; 1 daily feed
6A(18)	Fresh daily centrifuged ; multi feed	6B(11)	Low speed centrifugation; stored -15°C; 1 daily feed
		7B(12)	High speed centrifugation; stored 2°C; multi feed
		8B(13)	Flocculated; stored 2°C; multi feed
		9B(14)	Low speed centrifugation; stored 2°C; multi feed
		10B(15)	High speed centrifugation; stored -15°C; multi feed
		11B(16)	Flocculated; stored -15°C; multi feed
		12B(17)	Low speed centrifugation; stored -15°C; multi feed

Statistical analyses:
Homogeneity of variance for growth data and arcsine $X^{0.5}$ transformed survival data of *S. glomerata* larvae across the full array of 18 dietary treatments were assessed using Cochran's test. A subsequent one way analysis of variance was used to evaluate treatment differences. Growth data for the 18 diets were compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Growth data and arcsine $X^{0.5}$ transformed survival data for the 12 stored diets were grouped according to a hierarchy of the three main dietary treatment factors in a balanced orthogonal array. The latter comprised: feeding frequency (once daily or 6 times daily); storage temperature (+2 ± 0.5°C or -15±0.5°C) and method of harvesting. The data was then subjected to analysis of variance to separate out significant effects (or absence thereof) of the main dietary treatment factors and interactions thereof.

All three main dietary treatment factors being found to significantly effect both $X^{0.5}$ growth and survival, multiple range analyses was applied to growth data and to arcsine transformed survival data for the 12 stored diets. This entailed pooling of data with a common storage temperature and the resultant pooled means compared using Student-Newman-Keuls procedures (Winer et al., 1991). This procedure was repeated for the same set of data pooled according to feeding frequency and repeated once again for data pooled according to harvesting technique. In the latter case, three pair-wise comparisons of means of pooled data were required, namely high speed versus low speed

centrifugation; flocculation versus high speed centrifugation and flocculation versus low speed centrifugation.

Results

Overview of performance of the 18 diets – growth:
As an initial procedure, homogeneity of variance for growth data of *S. glomerata* larvae across the full array of 18 dietary treatments was confirmed using Cochran's test (P= 0.0821). A one way analysis of variance on these data revealed significant differences among dietary treatments ($P<0.0001$).

Growth data for the 18 diets compared using Student-Newman-Keuls procedures (Winer et al., 1991) are provided in Table 7.3.2. Highest mean growth increments in the approximate range 32 to 38 µm were exhibited by oyster larvae fed fresh high speed centrifuged *P. lutheri* concentrates and chill stored low speed centrifuged fed concentrates, whether fed as a single of multiple feeds. All these diets performed as well as or better than the fresh *P. lutheri* culture control diet and growth rates supported were within the range of 4 to 5 µm/day normally experienced with early stage *S. glomerata* larvae (Frankish et al., 1991).

High speed centrifuged *P. lutheri* stored concentrates whether held chilled or frozen and whether administered as single or multiple daily feeds, all exhibited moderate growth increments in the approximate range 20 to 26 µm as did the low speed centrifuged frozen stored *P. lutheri* concentrate diet when administered as multiple daily feeds.

Five of the six poorest performing test diets were those harvested by flocculation. The sixth was the low speed centrifuged, frozen stored, single daily feed diet. These six diets supported growth increments of only about 40% or less (3 to 13 µm) that of the fresh *P. lutheri* culture control diet and only about one third or less growth of the best performing (fresh, high speed centrifuged, multi feed) diet (38 µm). A measure of the growth performance of the three poorest diets, namely the chilled and frozen stored single feed, flocculated diets and the above cited low speed centrifuged, frozen stored, single daily feed diet, is that they were unable to support growth in excess of the unfed control (9.9 µm growth increment). This result was surprising in that the only source of nutrients for unfed (starved) larvae were endogenous reserves and micro particulate organic matter including micro-flagellates and bacteria mainly below 1 µm and dissolved organic nutrients within the aged filtered seawater used to rear them!

Table 7.3.2. Means for growth data of all 18 diets could then be compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell length increment (µm)	Homogeneous Groups*
5B(10)	Flocculated ; stored -15°C; 1 daily feed	3.52 ± 0.93	a
2B(7)	Flocculated; stored 2°C; 1 daily feed	8.61 ± 1.28	ab
6B(11)	Low speed centrifuged.; stored -15°C; 1 daily feed	9.53 ± 0.57	abc
1A(1)	Unfed CONTROL	9.92 ± 1.29	abc
8B(13)	Flocculated; stored 2°C; multi feed	11.53 ± 4.52	abcd
4A(4)	Fresh daily flocculated; 1 daily feed	13.55 ± 1.13	abcd
11B(16)	Flocculated; stored -15°C; multi feed	13.99 ± 6.45	abcd
4B(9)	High speed centrifuged ;stored -15°C; 1daily feed	19.63 ± 0.80	bcde
12B(17)	Low speed centrifuged.; stored -15°C; multi feed	19.77 ± 6.40	bcde
10B(15)	High speed centrifuged ;stored -15°C; multi feed	22.82 ± 2.08	bcdef
(6)1B	High speed centrifuged; stored 2°C; 1 daily feed	24.15 ± 1.53	cdef
7B(12)	High speed centrifuged; stored 2°C; multi feed	25.57 ± 1.90	defg
5A(5)	Fresh daily low speed centrifuged; 1 daily feed	31.42 ± 4.65	efg
2A(2)	Fresh algal culture; 1 daily feed CONTROL	32.49 ± 3.25	efg
3B(8)	Low speed centrifuged.; stored 2°C; 1 daily feed	32.76 ± 1.19	efg
9B(14)	Low speed centrifuged.; stored 2°C; multi feed	33.00 ± 2.27	efg
3A(3)	Fresh daily high speed centrifuged; 1 daily feed	36.25 ± 3.47	fg
6A(18)	Fresh daily high speed centrifuged; multi feed	38.64 ± 3.62	g

* Means sharing common letter are not significantly different at $P<0.05$

Overview of performance of the 18 diets – survival:
As an initial procedure, homogeneity of variance for arcsine $X^{0.5}$ transformed survival data of *S. glomerata* larvae across the full array of 18 dietary treatments was confirmed using Cochran's test (P= 0.0821). A one way analysis of variance (Table 7.3.4) on these data revealed that between group source of variation that was large and highly significant ($P<0.0001$).

High rates of mean survival in the range 86 to 95% were achieved by oyster larvae in 11 of the 18 test diets including both the control fresh *P. lutheri* culture diet and the unfed control. The nine well performing non-control diets all comprised high speed centrifuged *P. lutheri* concentrates, regardless of whether they were fresh or had been stored chilled or frozen.

By contrast rates of survival supported by the seven remaining diets were all significantly lower, falling within the universally low range 3 to 35%. These comprised all 5 diets produced by flocculation. The very worst performers amongst these were those that had been chilled rather than frozen and administered in single rather than multiple daily feeds. The remaining two poor performers were frozen low speed centrifuged diets administered as either single or multiple daily feeds. The very low survival rates of 7.5% in the case of the once daily fed diet and 15.1% for its

multiple fed companion, contrasted extremely with their chill stored counterparts that supported excellent survival rates of 94.5 and 95.1% respectively.

Table 7.3.3. Means for survival data of all 18 diets could then compared using Student-Newman-Keuls procedures on arcsine transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival	Homogeneous Groups*
2B(7)	Flocculated; stored 2°C; 1 daily feed	3.58 ± 0.35	a
12B(17)	Low speed centrifuged.; stored -15°C; multi feed	7.54 ±1.40	ab
5B(10)	Flocculated ; stored -15°C; 1 daily feed	7.55 ± 1.40	ab
8B(13)	Flocculated; stored 2°C; multi feed	8.99 ± 1.80	ab
4A(4)	Fresh daily flocculated; 1 daily feed	11.36 ± 2.10	b
6B(11)	Low speed centrifuged.; stored -15°C; 1 daily feed	15.07 ± 2.78	b
11B(16)	Flocculated; stored -15°C; multi feed	35.05 ± 4.80	c
5A(5)	Fresh daily low speed centrifuged; 1daily feed	86.74 ±4.54	d
2A(2)	Fresh algal culture; 1 daily feed CONTROL	90.33 ± 2.39	d
4B(9)	High speed centrifuged; stored -15°C; 1 daily feed	91.57 ± 1.00	d
1A(1)	Unfed CONTROL	92.18 ± 1.24	d
6A(18)	Fresh daily centrifuged; multi feed	92.61 ± 1.08	d
10B(15)	High speed centrifuged; stored -15°C; multi feed	93.05 ± 1.08	d
3A(3)	Fresh daily centrifuged; 1 daily feed	93.30 ± 1.71	d
7B(12)	High speed centrifuged; stored 2°C; multi feed	93.85 ± 0.98	d
3B(8)	Low speed centrifuged; stored 2°C; 1 daily feed	93.76 ± 1.81	d
9B(14)	Low speed centrifuged; stored 2°C; multi feed	94.50 ± 0.46	d
(6)1B	High speed centrifuged; stored 2°C; 1 daily feed	95.05 ± 0.76	d

* Means sharing common letter are not significantly different at $P<0.05$ level.

Further evaluation of main dietary treatment factors on the performance of the 12 stored P.lutheri concentrate diets – growth:

Three way Analyses of variance of growth data for the 12 stored diets (Table 7.3.4) confirmed that all three main dietary treatment factors (feeding frequency; storage temperature and method of harvesting) significantly affected growth ($P<$ 0.0001 to 0.02). The interaction of harvest method and cold storage temperature was also significant ($P<0.002$).

Results of S.N.K. for growth increment data for the stored diets are presented in Table 7.3.5. Mean (±s.e.) growth increment (pooled data) for chill stored *P. lutheri* concentrate diets (22.6±2.2 μm) were significantly greater than that of their frozen stored counterparts (14.9±2.0 μm). Similarly, the mean (± s.e.) growth increment for *P. lutheri* concentrate diets administered as multiple (6x) daily feeds (21.1±2.2 μm) significantly ($P<0.05$) exceeded that of their single daily feed counterparts (16.4±2.1 μm).

Mean (±s.e.) growth promoted by *P. lutheri* concentrate diets harvested by high and low speed centrifugation (Table 7.3.5) were very similar (23.8±3.0 and 23.0±1.0 µm, respectively) and significantly exceeded that of oyster larvae fed stored diets harvested by flocculation diets (9.4± 2.0 µm).

Table 7.3.4. Three way analysis of variance of growth data relating to stored *P. lutheri* diets.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F - ratio	Significance level
Main effects					
• A: Harvest Method	2091.7856	2	1045.8928	24.989	$P<0.0001$
• B: Storage Temperature	716.1848	1	716.1848	17.111	$P<0.0002$
• C: Feeding Frequency	270.0380	1	270.0380	6.452	$P<0.02$
Interactions					
• AB	672.3235	2	336.1617	8.032	$P<0.002$
• AC	39.8241	2	19.9121	0.476	n.s. $P>0.6$
• BC	124.3242	1	124.3242	2.970	n.s. $P>0.09$
• ABC	0.06950	2	0.03475	11.405	n.s. $P>0.6$
Residual	1506.7754	36	41.8549		
Total (Corrected)	5456.7524	47			

Table 7.3.5. Mean (± s.e.) growth rate and survival of for oyster larvae based on data pooled according to the main treatment factors for stored *P. lutheri* concentrate diets.

Treatment Factor	Mean (±s.e.) shell length increment (µm)	Mean (±s.e.) % survival
Storage Temperature		
• Frozen (-15°C ± 1°C)	14.9 ± 2.0^a	41.6 ± 7.6^a
• Chilled (+2 ± 0. 5°C)	22.6 ± 2.2^b	65.0 ± 8.7^b
Feeding Frequency		
• once per day	16.4 ± 2.1^a	51.1 ± 8.9^a
• 6 times daily	21.1 ± 2.2^b	55.5 ± 8.2^a
Harvesting Method		
• flocculation	9.4 ± 2.0^a	13.8 ± 3.4^a
• high speed centrifugation (super-centrifuge @ 12,000g)	23.0 ± 1.0^b	93.4 ± 1.0^b
• low speed centrifugation (bucket centrifuge @ 1,200g)	23.8 ± 3.0^c	52.7 ± 10.8^c

Further evaluation of main dietary treatment factors on the performance of the 12 stored P. lutheri concentrate diets – survival:

Analysis of variance of corresponding survival data for the 12 stored diets (Table 7.3.6) revealed that all three main dietary treatment factors, feeding frequency, storage temperature and method of harvesting, significantly effected survival ($P<$ 0.0001 to 0.0007). The two way interactions of harvest method with cold storage temperature and harvest method with feeding frequency both significantly effected survival ($P<0.0001$ in both cases) as did the three way interaction of harvest method, storage temperature and feeding frequency ($P<0.0001$).

Results of SNK for oyster larvae survival data for the 12 stored diets are also presented in Table 7.3.5. They were generally in line with equivalent results already described in relation to growth i.e. mean rate of survival supported by multiple daily feeds exceeded that of single feeds; chilled storage of *P. lutheri* concentrate diets promoted significantly higher survival than frozen storage and mean survival associated with the alternative harvesting techniques followed the sequence as growth, namely high speed centrifugation >>low speed centrifugation >> flocculation.

Table 7.3.6. Analysis of variance of arcsine $X^{0.5}$ transformed survival data relating to stored *P. lutheri* diets.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: Harvest Method	7.43925	2	3.71963	1220.871	< 0.0001
• B: Storage Temperature	0.91887	1	0.91887	301.597	< 0.0001
• C: Feeding Frequency	0.04204	1	0.04204	13.800	< 0.0007
Interactions					
• AB	3.22858	2	1.61429	529.850	< 0.0001
• AC	0.18586	2	0.09293	30.502	< 0.0001
• BC	0.00930	1	0.00930	3.051	n.s. P>0.08
• ABC	0.06950	2	0.03475	11.405	< 0.0001
Residual	0.10968	36	0.00305		
Total (Corrected)	12.00309	47			

All F-ratios are based on residual mean square error

Summary and Conclusions

While all three main treatment factors investigated significantly effected retained nutritional value of *P. lutheri* concentrates, their relative importance followed the sequence harvesting method > cold storage temperature > feeding frequency. Slow speed (bucket) centrifuged concentrates, when stored chilled at 2.0 ± 0. 5°C and administered either as single or multiple daily feeds, supported outstanding growth and survival of oyster larvae. However, the benefits of slow speed centrifugation were totally reversed by subsequent frozen storage of concentrates. Growth rates of larvae fed frozen low speed centrifuged concentrates ranged from less than one third (single daily feed) to two thirds (multiple feeds) those fed the fresh *P. lutheri* culture control diet. Detrimental effects on survival were equally dramatic with single and multiple daily feeding of frozen *P. lutheri* concentrate resulting in low survival rates of 7% and 15%, respectively.

P. lutheri cells were shown in a preliminary screening experiment (Section 5.3.2) to suffer considerably more physical damage when harvested by a high speed centrifugation (8%) than when harvested by low speed (1%) centrifugation. Nevertheless, super-centrifuged *P. lutheri* concentrates supported rates of survival above 90% survival and growth rates of 74 % to 79% those obtained on the fresh *P. lutheri* culture control diet. Moreover, high speed centrifuged diets (unlike their low speed centrifuged counterparts) still supported satisfactory growth rates 60 to 70% of those of the fresh *P. lutheri* culture control diet when stored frozen.

Chitosan flocculation was shown to have an extremely deleterious effect on retained nutritional value of *P. lutheri* concentrates. Growth of larvae on fresh flocculated concentrates was barely one third (13.6 µm) that of fresh *P. lutheri* culture control diet. Growth increments of 3.6 to 14 µm on chill or frozen stored chitosan flocculated diets fed as single or multiple daily feeds, were poor even relative to the unfed control diet (9.9 µm). The negative effect of chitosan flocculation on

larval survival was just as dramatic. Survival associated with 11 of the 18 diets, including the unfed control, fell within the high and narrow range of 87 to 95%. By contrast, survival rates on the five flocculated *P. lutheri* diets ranged from 3.6% when chill stored and fed once daily, to 35.0% when stored frozen and fed 6 times daily. These results are not readily explainable in so far that chitosan, a polymer of acetyl-glucosamine, is not documented as being toxic to invertebrate larvae. The negative effects of chitosan flocculated diets may have arisen through adverse ancillary factors such as the promotion of high bacterial loads or interference to normal ingestion or digestion of food by the oyster larvae.

7.4. Bio-Assay Experiment 4: Evaluation of Optimum Harvesting Techniques for Tahitian *Isochrysis* and *Thalassiosira pseudonana*

Introduction and Aims

This bio-assay experiment served as a companion to Bioassay Experiment 3 to clarify whether or not poor performance achieved to date with aged high speed centrifuged concentrates of two other species of micro-algae, namely T. *Iso* and *Thalassiosira pseudonana,* could be overcome. As with *P. lutheri,* both these species when fed as fresh cultures are of high nutritional value to bivalve larvae (Nell and O'Connor, 1991).

Evaluation was made of effects of various combinations of alternative harvesting technique, cold storage temperature, presence or absence of common food additives and density of stored concentrate (as pastes or slurries), on retained nutrition value of T. *Iso* and *Thalassiosira pseudonana* concentrates. As in the preceding Bioassay Experiment 3, growth and survival of Sydney rock oyster *Saccostrea glomerata* (=*commercialis*) larvae were used as direct indicators of retained nutritional value of diets.

Materials and Methods

Experimental design:
Twelve experimental treatments were employed for each of T. *Iso* and *T. pseudonana.* These included 8 treatments in a balanced 2 x 2 x 2 factorial design to evaluate individual and interactive effects the three main treatment factors namely, harvesting technique (high speed or low speed centrifugation); food additives (presence or absence thereof) and storage temperature (chilled storage at 2.0 ± 0.5°C or frozen storage at -15 ± 1°C) on the nutritional value of stored concentrates. Of the other 4 dietary treatments, two comprised a fresh (daily harvested) culture control and a common unfed control. The remaining two dietary treatments comprised high speed centrifuged concentrate reduced from a stiff paste to slurry of the same consistency produced by low speed centrifugation (density 10 to 20 g/L), one with food additives the other without and both chill stored at 2.0 ± 0.5°C. Four replicates for each of the dietary treatments were randomly distributed throughout a common water bath maintained at a favorable temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998), within a common water bath housed in an air conditioned room maintained at 23 ± 1°C.

Preparation of diets:
Cultures of T. *Iso* and *T. pseudonana* were grown from axenic inocula in 500 L 80 µm clear polyethylene film bags. The cultures were propagated in oceanic water (34 - 35 g/L) settled for 6 to 8 days, filtered to 1 µm (nominal), and disinfected using sodium hypo-chlorite at an effective concentration of 10mg/L for approximately 12 hours. Prior to inoculation, residual chlorine was deactivated using sodium thio-sulphate. Cultures were enriched with f/2 beta growth medium (Guillard, 1983) held at 23 ± 0.5°C with a 16 h : 8 h light : dark cycle and illuminated with 58 watt 'cool white' fluorescent tube lights to an intensity of 4,000 lux at the bag surface. Cultures were continuously aerated with 1.5% carbon dioxide enriched air to maintain pH in the range of 8.0 to 8.4.

The two alternative methods of concentrating the algae were high and low speed centrifugation. High speed centrifugation was performed using a 'Sharples' Super Centrifuge at 13,000 g. Algal cultures in mid to late exponential phase were delivered to the centrifuge with a diaphragm pump at a flow rate of approximately 25 L/minute. The micro-algae concentrate was recovered from the super-centrifuge bowl using a plastic spatula. Low speed centrifugation involved the use of a

Hitachi (Model 05P-21B) laboratory scale bucket centrifuge operated at 1,330g for 5 minutes. The resultant slurry was then recovered by decanting the supernatant.

In order to combat culture variability, four separate batch cultures each of T. *Iso* and *T. pseudonana* were used for each method of harvesting. After harvesting, concentrates of the same micro-algae were combined and thoroughly blended. Low speed centrifuge harvested concentrate was in a characteristic slurry form while the super-centrifuged product was a typical firm paste. The latter was diluted to a density of 10 to 20g/L to create two additional slurry treatments described below, aimed at differentiating the effects of harvesting technique and cell density during chilled storage.

Sub samples of concentrate used to assess the inclusion of additives were weighed and additives thoroughly blended in at the following rates:

- glycerol (SIGMA G-7893) at 10% (w/w)
- ascorbic acid (Vitamin C) (SIGMA A-7506) at 1% (w/w)
- citric acid (BD. Prod No. 10081) until pH was lowered to the range 4 - 4.5

All chill-stored dietary treatments were held at 2 ± 0.5°C in 120 mL clear polystyrene containers with a 1:2 ratio of slurry to air-space as either stiff pastes or diluted slurries at 10-20 g paste/L in accordance with optimum densities previously identified in Section 6.5. The containers were stored on a 30° incline and under light.

Prior to freezing all treatments stored at -15°C ± 1°C with additives were left at ambient room temperature for a minimum of half an hour to allow penetration of the glycerol prior to immersion quick freezing (IQF) using a 25% NaCl solution held at -15°C. All cold stored diets were maintained at prescribed chilled or frozen temperatures in Fisher and Paykel Pty Ltd., New Zealand, chest freezers in which temperature was monitored and controlled with a Thermo Eye-P system (Tokyo, Japan). These had been held for a period of 33 days at the commencement of feeding.

Acquisition, husbandry and measurement of larvae:
One hundred and twenty brood-stock Sydney Rock Oysters (*Saccostrea glomerata*) were road freighted to Port Stephens Research Centre from an oyster farmer at Tuross Head (approximately 36° S latitude, 150° E longitude). They were then held in a 1,500 litre conditioning system for a period of 40 days. During this time they were subject to 20°C with 100% water change every 48 - 72 hrs and were fed with 100 - 120 litres of batch cultured algae per day according to the standard conditioning regime developed by Frankish et al., 1992.

The broodstock were induced to spawn using thermal and salinity shocks again as discussed by Frankish et al. 1992. Several million fertilized eggs were stocked into a 1000 L tank of one week aged and settled coastal sea-water (salinity 34-36g/L) that was filtered to 1 µm through a wound cartridge depth filter, pre-warmed to 24°C ± 1°C and treated with sodium ethylenediaminetetra-acetic acid (EDTA) at 1 mg/L.

After approximately 48 hours, the D-veliger larvae were harvested onto a 45µm screen, counted, measured, and found to have a mean (± s.d) shell length of 77.0 ± 0.4µm. The larvae were stocked into 92 x 8 litre cylindrical plastic aquaria at a density of 2 larvae/ml. Each aquarium was gently aerated and maintained at a near optimum temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998), within a common water bath housed in an air conditioned room maintained at 23 ± 1°C. The 23 treatments (11 x T. *Iso* and 11 x *T. pseudonana* based diets plus a

common unfed control) with four replicates each were randomly distributed within the common water bath.

Each day, stored concentrated alga diets were re-suspended in filtered seawater. Alga culture density of each treatment was measured using an 'Improved Neubauer' haemocytometer slide (*Superior Co.*, Berlin, Germany) at 200X using and an Olympus (Model CH-2) compound light microscope. The feed rate for larvae in each treatment was adjusted daily in accordance with a prescribed optimum feeding regime described by Frankish et al., 1991. Every second day, larvae were wet sieved on 45 µm screens before being restocked into clean vessels containing freshly filtered replacement sea-water (34 - 35 g/L) pre-treated as previously described.

The experiment was terminated after 8 days when the larvae were collected and preserved using a 4% formaldehyde in sea-water solution. Mean (± s.e.) growth of the larvae was evaluated by measuring a minimum of 50 individuals from each replicate at 200X using an Olympus (Model CH-2) compound light microscope fitted with a graticule and a 'Sedgewick Rafter' counting slide. Percentage survival was obtained by counting total and live larvae within a minimum sample of 100 larvae. Live larvae were distinguished from dead by lack of necrotic tissue and presence of food in the gut at the time of fixation.

Table 7.4.1. Experimental Treatments Applied to each of T. *Iso* and *T. pseudonana.*

Treatment No.	Description of Treatment
1	Unfed Control (Common to both species)
2	Fresh Algal culture
3	Low speed centrifuged slurry.; stored 2°C
4	Low speed centrifuged slurry .; stored 2°C; +Additives
5	Low speed centrifuged slurry ; stored -15°C
6	Low speed centrifuged slurry ; stored -15°C; +Additives
7	High speed centrifuged paste; stored 2°C
8	High Speed centrifuged paste ; stored 2°C; +Additives
9	High speed centrifuged paste ; stored -15°C
10	High speed centrifuged paste ; stored -15°C; +Additives
11	High speed centrifuged slurry; stored 2°C
12	High Speed centrifuged slurry; stored 2°C; +Additives

Statistical analysis:
For trials conducted with each of the algal species, homogeneity of variance for *S. glomerata* larvae growth data and arcsine transformed survival data of across the full array of 12 dietary treatments were assessed using Cochran's test. A subsequent one way analysis of variance was used to evaluate within and between group source of variation. Growth data for the 12 diets were compared using Student-Newman-Keul's procedures (Winer et al., 1991).

For each species of micro-algae, growth data and arcsine transformed survival data for the 8 stored diets in the 2x2x2 factorial array (diets 3 to 10), were then grouped according to a hierarchy of the three main dietary treatment factors. The latter comprised method of harvesting (high speed or low speed centrifugation); storage temperature (chilled at +2 ±0.5°C or frozen at -15±0.5°C) and absence or presence of food additives. These data were checked for homogeneity of variance then subjected to 3 factor analysis of variance to separate out significant effects (or absence thereof) of the main dietary treatment factors and interactions thereof. Where any of the three main dietary treatment factors were found to significantly effect growth or survival, multiple range analyses were applied to relevant data for stored diets 3 to10. This entailed pooling of data with a common dietary treatment factor and the resultant pooled means were then compared using Student-

Newman-Keuls procedures (Winer et al., 1991). For example in the case of treatments 3 to 10 for T. *Iso* only storage temperature was found to significantly effect growth. Accordingly, data for treatments involving chilled storage at +2 ±0.5°C (treatments 3,4,7 and 8) were pooled and the resultant mean compared with equivalent data for treatments involving frozen storage at -15±0.5°C (treatments 5, 6, 9 and 10).

Finally, to distinguish the effects of storage density from centrifugation speed, an equivalent analysis was made of growth and arcsine transformed survival data of dietary treatments 7,8,11, &12. As indicated in Table 7.4.1, these four diets comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been either stored as pastes with or without additives (diets 7 & 8) and their direct counterparts that had been diluted to low density slurries (diets 11 & 12).

Results

T. Iso diets - larval growth:
Homogeneity of variance for growth data of *S. glomerata* larvae across the full array of twelve dietary treatments was confirmed using Cochran's test (P=0.5795). A one way analysis of variance (Table 7.4.2) on these data revealed that between group source of variation that was large and highly significant (P<0.0001).

Means for growth data of the 12 diets (Table 7.4.3) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). Oyster larvae fed the fresh T. *Iso* culture diet exhibited very acceptable mean ± s.e. shell height increase of 47.3± 0.4 µm over the 8 day experiment that compared favourably with a range of 4 to 6 µm/day normally experienced with early stage *S. glomerata* larvae when fed a standard fresh diet comprising equal amounts of T. *Iso, P.lutheri* and *C.calcitrans* (Frankish et al., 1991).

By contrast, growth rates of unfed larvae and of larvae fed the 10 stored T. *Iso* concentrate diets were all significantly lower and unsatisfactory. Indeed even the best performing stored diet, slow speed centrifuged *T. Iso* stored chilled without additives, supported a mean increment (23.6± 0.3 µm) less than half that of the fresh culture control diet. The two worst performing stored diets, slow speed centrifuged frozen concentrates with or without additives, supported mean increments of only 8.2± 6.7 and 8.6± 6.0 µm, respectively that were not significantly better than that of unfed larvae (6.8±7.1 µm).

Three factor analyses of variance of these growth data (Table 7.4.4) showed that of the three main dietary treatment factors, storage temperature, speed of centrifugation and use of additives, only storage temperature significantly influenced growth. Results of a multiple range analysis of data from treatments 3 to10 pooled according to storage temperature confirmed that overall mean growth achieved on chilled high speed centrifuged concentrates with or without additives (16.1 µm) was significantly higher than achieved on their frozen counterparts (11.8 µm).

Table 7.4.2. Means for growth data of all 12 T. *Iso* diets compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell length increment (μm)	Homogeneous Groups*
1	Unfed Control (Common to both species)	6.83±1.11	a
2	Fresh Algal culture	47.32±1.28	d
3	Low speed centrifuged slurry.; stored 2°C	23.62±1.79	c
4	Low speed centrifuged slurry .; stored 2°C; +Additives	15.63±1.14	b
5	Low speed centrifuged slurry ; stored -15°C	8.18±2.09	a
6	Low speed centrifuged slurry ; stored -15°C; +Additives	8.64±0.73	a
7	High speed centrifuged as paste; stored 2°C	11.06±0.78	ab
8	High Speed centrifuged as paste; stored 2°C; +Additives	14.10±2.35	ab
9	High speed centrifuged paste ; stored -15°C	15.26±1.50	ab
10	High speed centrifuged paste ; stored -15°C; +Additives	15.00±0.61	b
11	High speed centrifuged as slurry; stored 2°C	11.52±2.02	ab
12	High Speed centrifuged as slurry; stored 2°C; +Additives	20.79±1.02	bc

* Means sharing common letter are not significantly different at P<0.05

Table 7.4.3. Three way analysis of variance of growth data relating to stored T. Iso diets 3-10.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level P
Main effects					
• A: Storage Temperature	150.5100	1	150.5100	16.610	.0004
• B: Additives	11.2600	1	11.2600	1.243	.2760
• C: Centrifugation speed	.2080	1	.2080	.023	.8824
Interactions					
• AB	13.26	1	13.26	1.464	.2381
• AC	378.95	1	378.95	41.840	.0000
• BC	53.20	1	53.20	5.873	.0233
• ABC	69.03	1	69.03	7.621	.0109
Residual	217.39	24	9.058		
Total (Corrected)	893.81	31			

Results (Table 7.4.4) of a two way analysis of variance of growth data of dietary treatments 7,8,11, & 12 that comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been stored as pastes with or without additives (diets 7 & 8 respectively) or diluted to form slurry counterparts (diets 11 & 12) showed that that dilution to a slurry did not significantly influence growth but that additives did have a positive influence.

Table 7.4.4. Two way analysis of variance of growth data relating to stored *T. Iso* diets 7,8,11 & 12

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level P *=significant
Main effects					
• A: Storage density	51.09	1	51.09	4.049	.0672
• B: Additives	151.47	1	151.47	12.006	.0047*
Interactions					
• AB	38.72	1	38.72	3.069	.1053
Residual	151.39	12	12.62		
Total (Corrected)	392.67	15			

T. Iso diets - larval survival:
Homogeneity of variance for arcsine transformed survival data of *S. glomerata* larvae across the full array of 12 *T. Iso* dietary treatments was confirmed using Cochran's test (P=0.2833). A one way analysis of variance (Table 7.4.6) on these data revealed that between group source of variation that was large and highly significant (P<0.001).

High rates of survival (Table 7.4.5) in the range 90 to 97% were achieved by oyster larvae under 9 of the 12 T. *Iso* dietary treatments including the fresh algae culture control diet and 8 of the stored concentrate diets. The three remaining diets supported significantly lower rates in the range 64 to 83% were also those that supported the poorest growth rates namely, the unfed control and frozen low speed centrifuged slurries with and without additives.

Table 7.4.5. Mean survival data of all 12 T. *Iso* diets compared using Student-Newman-Keuls procedures on arcsine transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival	Homogeneous Groups*
1	Unfed Control (Common to both species)	64.51±7.08	a
2	Fresh Algal culture	93.45±3.38	bc
3	Low speed centrifuged slurry.; stored 2°C	96.51±0.27	c
4	Low speed centrifuged slurry .; stored 2°C; +Additives	90.1225±2.18	bc
5	Low speed centrifuged slurry ; stored -15°C	73.75±6.74	a
6	Low speed centrifuged slurry ; stored -15°C; +Additives	83.19±6.00	ab
7	High speed centrifuged paste; stored 2°C	91.763±1.79	bc
8	High Speed centrifuged paste ; stored 2°C; +Additives	93.59±3.29	bc
9	High speed centrifuged paste ; stored -15°C	92.315±2.77	bc
10	High speed centrifuged paste ; stored -15°C; +Additives	91.2825±4.00	bc
11	High speed centrifuged slurry; stored 2°C	92.15±4.08	bc
12	High Speed centrifuged slurry; stored 2°C; +Additives	96.66±1.02	c

* Means sharing common letter are not significantly different at P<0.05 level.

Three factor analyses of variance of these growth data (Table 7.4.8) showed that of the three main dietary treatment factors, storage temperature and centrifugation speed significantly influenced growth but inclusion of additives did not. Results of a multiple range analysis of data from

treatments 3 to10 pooled according to storage temperature confirmed that overall mean survival achieved on chilled high speed centrifuged concentrates (93%) was significantly higher than achieved on their frozen counterparts (85%). Results of a multiple range analysis of the same data pooled according to centrifugation speed confirmed that a significantly higher rate of survival is supported by high speed centrifuged pastes (92%) than by low speed centrifuged slurries (86%).

Table 7.4.6. Three way analysis of variance of arcsine transformed survival data for oyster larvae fed stored T. *Iso* diets.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: Storage Temperature	494.709	1	494.709	7.986	.0093*
• B: Additives	7.431	1	7.431	.120	.7358
• C: Centrifugation Speed	322.199	1	322.199	5.201	.0317*
Interactions					
• AB	84.175	1	84.175	1.359	.2552
• AC	390.462	1	390.462	6.303	.0192*
• BC	2.565	1	2.565	.041	.8426
• ABC	174.752	1	174.752	2.821	.1060
Residual	1486.78	24	61.949		
Total (Corrected)	893.8136	31			

Results (Table 7.4.9) of a two way analysis of variance of survival data of dietary treatments 7,8,11, &12 that comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been either stored as pastes (diets7 &8 respectively) and their diluted slurry counterparts (diets 11 &12) showed that that dilution to a slurry did not significantly influence survival.

Table 7.4.7. Two way analysis of variance of survival data relating to stored T. *Iso* diets 7,8,11 & 12.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level P *=significant
Main effects					
• A: Storage density	73.06	1	73.06	.4.671	.0516
• B: Additives	4.07	1	4.07	0.260	.6246
Interactions					
• AB	39.34	1	39.34	2.515	.1387
Residual	187.70	12	15.64		
Total (Corrected)	304.17	15			

T. pseudonana diets – growth:
Homogeneity of variance for growth data of *S. glomerata* larvae across the full array of 12 *T. pseudonana* related dietary treatments was confirmed using Cochran's test (P= 0.3855). A one way analysis of variance (Table 7.4.10) on these data revealed that between group source of variation was large and highly significant (P<0.001).

Means for growth data of the 12 diets (Table 7.4.11 were compared using Student-Newman-Keul's procedures (Winer et al., 1991). Oyster larvae fed the fresh *T.pseudonana* culture diet exhibited acceptable growth rate that fell at the lower end of a range of 4 to 6 μm/day normally exhibited by

early stage *S. glomerata* larvae when fed an optimised diet comprising equal amounts of fresh *T. Iso, P.lutheri* and *C. calcitrans* (Frankish et al., 1992).

Three factor analysis of variance (Table 7.4.12) for growth data of *S. glomerata* larvae across the 2x2x2x factorial array of stored *T. pseudonana* diets (3 to 10 in Table 7.4.1) showed that two of the three main dietary treatment factors, storage temperature and additives, significantly (P<0.002) influenced growth. This analysis also showed highly significant (P<0.0001) interaction between all combinations of the thee main treatment factors. Results of a subsequent multiple range analysis of the same data pooled according to storage temperature confirmed that growth was significantly greater on chilled (18.6 μm) than on frozen (13.5 μm) concentrates. Results of a multiple range analysis of these same data pooled according to use of additives confirmed that growth on raw concentrates was significantly higher (18.5 μm) than on diets with additives (13.6 μm).

Table 7.4.8. Means for growth data of all 12 *T. pseudonana* related diets compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell length increment (μm)	Homogeneous Groups*
1	Unfed Control (Common to both species)	6.83±1.11	a
2	Fresh Algal culture	30.36±1.71	c
3	Low speed centrifuged slurry.; stored 2°C	38.17±3.20	c
4	Low speed centrifuged slurry .; stored 2°C; +Additives	10.45±0.96	ab
5	Low speed centrifuged slurry ; stored -15°C	7.31±0.50	a
6	Low speed centrifuged slurry; stored -15°C; +Additives	8.76±1.03	a
7	High speed centrifuged paste; stored 2°C	11.85±2.02	ab
8	High Speed centrifuged paste ; stored 2°C; +Additives	13.98±0.54	b
9	High speed centrifuged paste ; stored -15°C	16.74±2.68	b
10	High speed centrifuged paste ; stored -15°C; +Additives	21.33±2.64	b
11	High speed centrifuged slurry; stored 2°C	19.26±2.77	b
12	High Speed centrifuged slurry; stored 2°C; +Additives	15.11±1.91	b

* Means sharing common letter are not significantly different at P<0.05

Table 7.4.9. Three way analysis of variance of growth data relating to stored T. pseudonana diets

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level P
Main effects					
• A: Storage Temperature	206.248	1	206.248	13.372	.0012
• B: Centrifugation speed	.320	1	.320	.021	.8882
• C: Additives	191.101	1	191.101	12.390	.0018
Interactions					
• AB	1003.07	1	1003.070	65.032	.0000
• AC	500.54	1	500.540	32.452	.0000
• BC	544.17	1	544.170	35.280	.0000
• ABC	356.71	1	356.710	23.127	.0001
Residual	370.18	24	15.424		
Total (Corrected)	3172.35	31			

Results (Table 7.4.10) of a two way analysis of variance of growth data of dietary treatments 7,8,11, & 12 that comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been either stored as pastes (diets 7 & 8 respectively) or as their diluted slurry counterparts (diets 11 & 12) showed that that dilution to a slurry did not significantly influence growth but that additives significantly enhanced growth.

Table 7.4.10. Two way analysis of variance of growth data relating to *T. pseudonana* stored diets 7,8,11 & 12.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level P *=significant
Main effects					
• A: Storage density	51.09	1	51.09	4.049	.0672
• B: Additives	151.47	1	151.47	12.006	.0047*
Interactions					
• AB	38.72	1	38.72	3.069	.1053
Residual	151.39	12	12.62		
Total (Corrected)	392.67	15			

T. pseudonana diets - larval survival:
Homogeneity of variance for arcsine transformed survival data of *S. glomerata* larvae across the full array of 12 *T. pseudonana* dietary treatments was confirmed using Cochran's test (P=0.0803). A one way analysis of variance (Table 7.4.14) on these data revealed that between group source of variation that was large and highly significant ($P<0.001$).

High rates of survival in the range 87 to 99% were achieved by oyster larvae when fed 9 of the 12 *T. pseudonana* test diets including the fresh algae culture control diet and 8 of the stored concentrate diets (Table 7.4.11). The three remaining dietary treatments, the unfed control and stored low speed centrifuged frozen concentrates with and without additives, supported lower survival rates in the range 60 to 72% and were the same three treatments previously cited as supporting the poorest growth rates.

Table 7.4.11. Means for survival data of *T. pseudonana* diets compared using Student-Newman-Keuls procedures on arcsine transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival	Homogeneous Groups*
1	Unfed Control (Common to both species)	64.51±7.08	a
2	Fresh Algal culture	89.00±5.60	ab
3	Low speed centrifuged slurry.; stored 2°C	83.00±3.24	ab
4	Low speed centrifuged slurry .; stored 2°C; +Additives	98.74±0.66	b
5	Low speed centrifuged slurry ; stored -15°C	60.41±6.13	a
6	Low speed centrifuged slurry ; stored -15°C; +Additives	71.89±6.08	ab
7	High speed centrifuged paste; stored 2°C	93.62±2.41	b
8	High Speed centrifuged paste ; stored 2°C; +Additives	89.97±1.84	b
9	High speed centrifuged paste ; stored -15°C	87.68±4.73	b
10	High speed centrifuged paste ; stored -15°C; +Additives	91.69±1.74	b
11	High speed centrifuged slurry; stored 2°C	86.88±9.94	ab
12	High Speed centrifuged slurry; stored 2°C; +Additives	94.66±0.57	b

* Means sharing common letter are not significantly different at P<0.05 level.

Three factor analyses of variance of these arcsine transformed survival data (Table 7.4.12) showed that the all three main dietary treatment factors, storage temperature, speed of centrifugation and use of additives, had highly significant effects (P = 0.0033; 0.0035 and 0.0127 respectively) on growth.

Results of a multiple range analysis of data from treatments 3 to 10 pooled according to storage temperature confirmed that mean survival promoted by chilled concentrates (90.3%) was significantly higher than equivalent frozen concentrates (78.5%). Results of a multiple range analysis of the same data pooled according to centrifugation speed confirmed that mean survival rate of 90.2% supported by high speed centrifugation was significantly greater than low speed centrifugation (78.5%). Finally, results of a multiple range analysis of the data pooled according to additives also confirmed that additives significantly reduced mean rate of survival from 89.2% in raw concentrates down to 79.5%.

Table 7.4.12. Three way analysis of variance of arcsine transformed survival data relating to stored *T. pseudonana* diets.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: : Storage Temperature	1116.40	1	1116.40	10.632	.0033
• B Speed of centrifugation	1098.52	1	1098.52	10.462	.0035
• C: Additives	761.57	1	761.57	7.253	.0127
Interactions					
• AB	1331.4100	1	1331.4100	12.680	.0016
• AC	.1313	1	.1313	.001	.9725
• BC	119.3100	1	119.3100	1.136	.2970
• ABC	32.3400	1	32.3400	.308	.5899
Residual	2520.1000	31			
Total (Corrected)	6976.7800				

All F-ratios are based on residual mean square error

Results of two way analyses of variance of growth and survival data of dietary treatments 7,8,11, &12 that comprised a 2x2 factorial array of high speed centrifuged chilled pastes that had been either stored as pastes (diets 7 & 8 respectively) or their diluted slurry counterparts (diets 11 & 12), showed that that dilution to a slurry did not significantly influence survival.

Summary and Conclusions

The performance of chilled raw slurry concentrates of both T. *Iso* and *T. pseudonana* produced by gentle slow speed (bucket) centrifugation were far superior to any other dietary treatment (Table 7.4.13). For both T. *Iso* and *T. pseudonana*, the great benefits of slow speed over high speed centrifugation were negated by freezing and to a lesser extent by inclusion of additives.

In the case of T. *Iso,* growth of oyster larvae fed the best performing stored concentrates was only half that of the corresponding fresh T. *Iso* control diet (Fig 7.4.1) and therefore unsatisfactory. By contrast, the best stored *T. pseudonana* concentrate diet (Fig 7.4.2) actually outperformed its fresh algal culture counterpart and can therefore be regarded as a good candidate for replacing fresh micro-algal culture diets used to rear bivalve larvae.

Table 7.4.13. Summary of results for T. *Iso* and *T. pseudonana* concentrate diets.

	Fresh algae control	**Centrifugation speed** (low/high)	**Use of additives** (Raw/ +additives)	**Storage Temp.** (Chilled/ Frozen)	**Concentrate density** (Paste/ slurry)	**Best performing concentrate** (mean growth/ survival)
T. *Iso*						
Mean growth µm	47.32	14.02 / 13.86	14.53 / 14.02	**16.10*** / 11.77	12.58 / **16.15**	Raw; chilled; low speed cent. slurry (23.62um / 96.5%)
Mean Survival %	93.45	85.89 / **91.24***	88.58 / 85.54	**93.00*** / 85.13	92.7 / 94.4	
T. pseudonana						
Mean growth µm	30.36	16.2 / 16.0	**18.5*** / 13.6	**18.6** */ 13.5	12.91/ **17.19***	Raw; chilled; low speed cent. slurry (38.12um / 89.0%)
Mean Survival %	93.8	78.5 / **90.2***	**89.2** * / 79.5	**90.3*** / 78.4	91.80 / 90.77	

Fig 7.4.1 Effects of harvest method, storage temperature and additives on the nutritional value of T.*Iso* concentrates when fed to oyster (*Saccostrea glomerata*) larvae

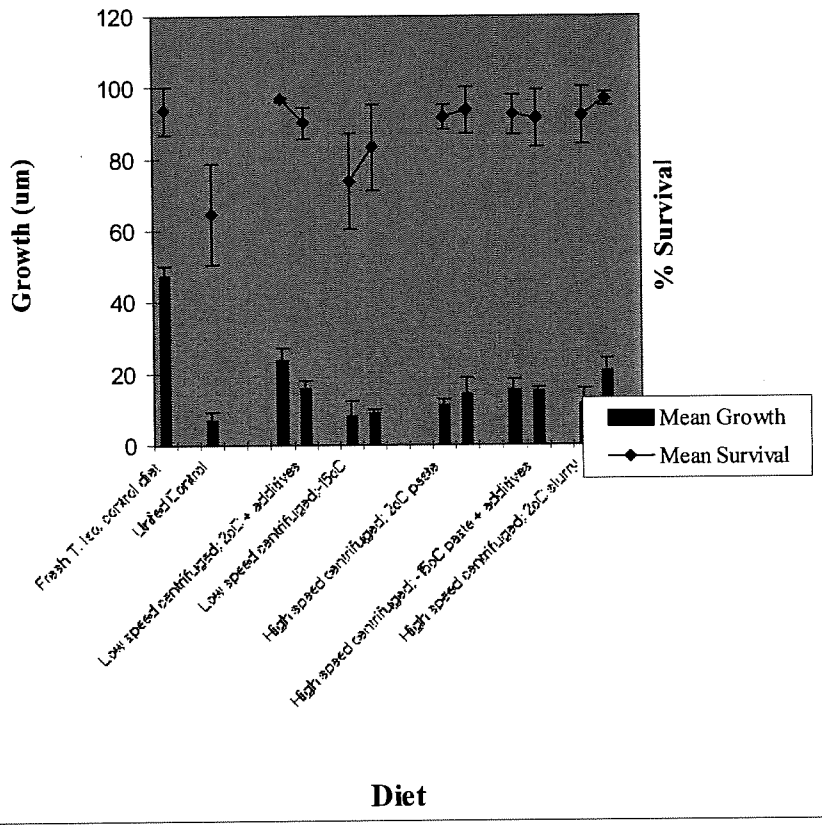
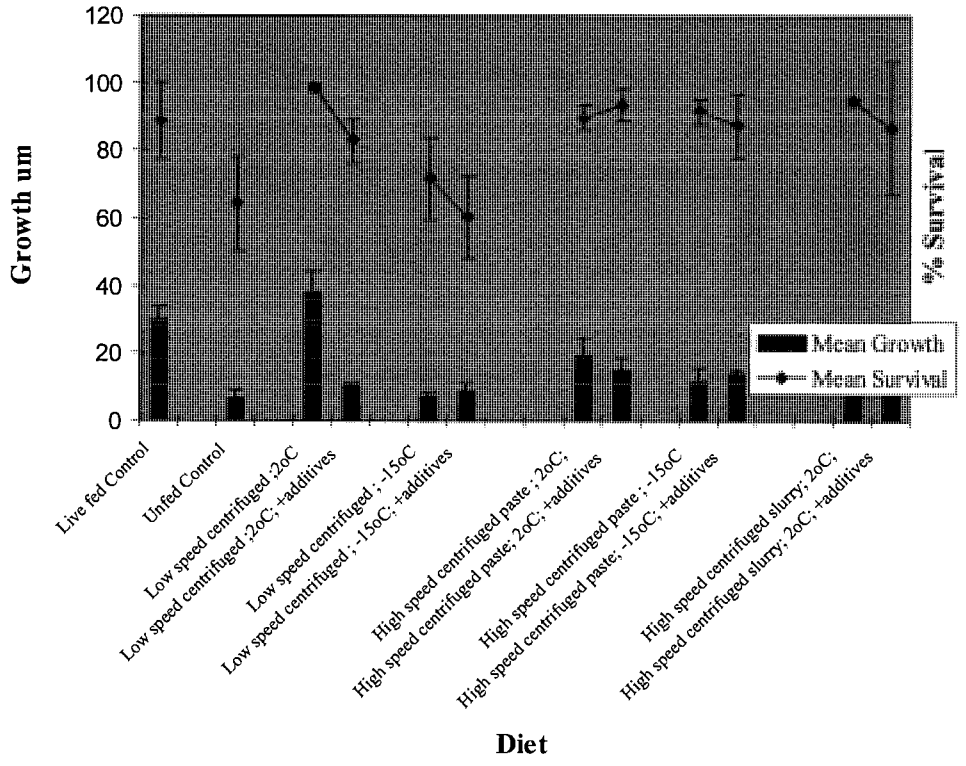


Fig 7.4.2 Effects of harvest method, storage temperature and additives on the nutritional value of *T. pseudonana* concentrates when fed to Sydney rock oyster (*Saccostrea glomerata)* larvae.



7.5. Bio-Assay Experiment 5: Evaluation of Optimum Harvesting Techniques for *Chaetoceros calcitrans* and *Skeletonema costatum*

Introduction and Aims

This bio-assay experiment served as a companion to Bio-assay Experiments 3 and 4 to clarify whether encouraging results with aged high speed centrifuged concentrates of two diatom species *C. calcitrans* and *S. costatum* based on indirect indicators of retained quality (Screening Trials 2, 4 & 6), would translate to good performance as hatchery diets. This experiment was also to determine if the nutritional value of stored concentrates of these diatoms could be enhanced by optimal methods of preservation and storage. As with *P. lutheri,* T. *Iso* and *T. pseudonana,* both *C. calcitrans* (Wilson, 1978; Waldock and Nascimento, 1979; Helm and Laing, 1987; Utting, 1986; Nell and O'Connor, 1991; Fildago and Herrero, 1993) and to a lesser extent *S. costatum* (Baylon, 1989), are of recognised nutritional value to some species of bivalve larvae when fed as fresh cultures.

The experiment was also to assess effects of various combinations of harvesting technique, cold storage temperature, presence or absence of common food additives and density of stored concentrate (as pastes or slurries) on retained nutrition value of *S. costatum* and *C. calcitrans.* As in the preceding Bio-assay Experiments 3 and 4, growth and survival of Sydney rock oyster *Saccostrea glomerata* (=*commercialis*) larvae were used as direct indicators of retained nutritional value of diets.

Materials and Methods

Experimental design:
The same twelve experimental treatments used in Bio-assay Experiment 4 (Table 7.5.1) were employed for each of *S. costatum* and *C. calcitrans*. These included eight treatments in a balanced 2 x 2 x 2 factorial design to evaluate individual and interactive effects the three main treatment factors namely, harvesting technique (high speed or low speed centrifugation); food additives (presence or absence thereof) and storage temperature (chilled storage at 2.0 ± 0.5°C or frozen storage at -15 ± 1°C) on the nutritional value of stored concentrates of *S. costatum* and *C. calcitrans*. Of the other four dietary treatments, two comprised an unfed and a fresh (daily harvested) culture control. The remaining two dietary treatments comprised high speed centrifuged concentrate reduced from a stiff paste to slurry of the same consistency produced by low speed centrifugation (density 10 to 20 g/L), one with food additives the other without and both chill stored at 2.0 ± 0.5°C.

Four replicates for each of the dietary treatments were randomly distributed throughout a common water bath maintained at a favourable temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998), within a common water bath housed in an air-conditioned room maintained at 23 ± 1°C.

Preparation of diets:
Cultures of *S. costatum* and *C. calcitrans* were grown from axenic inocula in 500 litre 80 µm clear polyethylene film bags. The cultures were propagated in oceanic water (34 - 35 g/L) settled for 6 to 8 days, filtered to 1 µm (nominal), and disinfected using sodium hypochlorite at an effective concentration of 10mg/L for approximately 12 h. Prior to inoculation, residual chlorine was deactivated using sodium thiosulphate. Cultures were enriched with f/2 beta growth medium (Guillard, 1983) held at 23 ± 0.5°C with a 16 h:8 h light : dark cycle and illuminated with 58 watt 'cool white' fluorescent tube lights to an intensity of 4,000 lux at the bag surface. Cultures were

continuously aerated with 1.5% carbon dioxide enriched air to maintain pH in the range of 8.0 to 8.4.

The two alternative methods of concentrating the algae were high and low speed centrifugation. High speed centrifugation was performed using the previously described "Sharples P/L" super-centrifuge at 13,000 g. Algal cultures in mid to late exponential phase were delivered to the centrifuge with a diaphragm pump at a flow rate of approximately 25 L/minute. The algal concentrate was recovered from the super-centrifuge bowl using a plastic spatula. Low speed centrifugation involved the use of the previously described "Hitachi " laboratory scale bucket centrifuge operated at 1,330g for 5 minutes. The resultant slurry was then recovered by decanting the supernatant.

In order to combat culture variability, four separate batch cultures each of *S. costatum* and *C. calcitrans* were used for each method of harvesting. After harvesting, concentrates of common algal species were combined and thoroughly blended. Low speed centrifuge harvested concentrate was in a characteristic slurry form while the super-centrifuged product was a typical firm paste. The latter was diluted to a density of 10 to 20g/L to create two additional slurry treatments described below, aimed at differentiating the effects of harvesting technique and cell density during chilled storage.

Sub-samples of concentrate used to assess the inclusion of additives were weighed and additives thoroughly blended in at the following rates:

- glycerol (SIGMA G-7893) at 10% (w/w)
- ascorbic acid (Vitamin C) (SIGMA A-7506) at 1% (w/w)
- citric acid (BD. Prod No. 10081) until pH was lowered to the range 4 - 4.5.

All chill-stored dietary treatments were held at 2 ± 0.5°C in 120 mL clear polystyrene containers with a 1:2 ratio of slurry to air-space as either stiff pastes or diluted slurries at 10-20 g paste/L in accordance with optimum densities previously identified in Section 6.5. The containers were stored on a 30° incline and under light.

Prior to freezing all treatments stored at -15°C ± 1°C with additives were left at ambient room temperature for a minimum of half an hour to allow penetration of the glycerol prior to immersion quick freezing (IQF) using a 25% NaCl solution held at -15°C. All cold stored diets were maintained at prescribed chilled or frozen temperatures in Fisher and Paykel Pty Ltd, New Zealand, chest freezers in which temperature was monitored and controlled with a Thermo Eye-P system (Tokyo, Japan). These had been held for a period of 33 days at the commencement of feeding.

Acquisition, husbandry and measurement of larvae:
One hundred and twenty broodstock Sydney Rock Oysters (*Saccostrea glomerata*) were road freighted to Port Stephens Research Centre from an oyster farmer at Tuross Head (approximately 36° S latitude, 150° E longitude). They were then held in a 1,500 litre conditioning system for a period of 3 weeks. During this time they were maintained at 25± 1°C with a 100% water change every 48 - 72 hours and were fed with 100 - 120 L of batch cultured algae per day according to the standard conditioning regime developed by Frankish et al., 1991.

The broodstock were induced to spawn using thermal and salinity shocks again as discussed by Frankish et al., 1991. Several million fertilised eggs were stocked into a 1000 L tank of one week aged and settled coastal seawater (salinity 34-36 g/L) that was filtered to 1µm through a wound cartridge depth filter, pre-warmed to 24°C ± 1°C and treated with sodium ethylenediaminetetra-acetic acid (EDTA) at 1 mg/L.

After approximately 48 hours, the D-veliger larvae were harvested onto a 45µm screen, counted, measured, and found to have a mean (± s.d) shell length of 73.7 ± 2.7 µm. The larvae were stocked into 92 x 8 litre cylindrical plastic aquaria at a density of 2 larvae/ml. Each aquarium was gently aerated and maintained at a near optimum temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998), within a common water bath housed in an air-conditioned room maintained at 23 ± 1°C. The 23 treatments (11 x *S. costatum* and 11 x *C. calcitrans*) based diets plus a common unfed control) with four replicates each were randomly distributed throughout the common water bath.

Each day, stored concentrated algae diets were re-suspended in filtered seawater. Algal culture density of each treatment was measured using an 'Improved Neubauer' haemocytometer slide (*Superior Co.*, Berlin, Germany) at 200X using and an Olympus (Model CH-2) compound light microscope. The feed rate for larvae in each treatment was adjusted daily in accordance with a prescribed optimum feeding regime described by Frankish et al., 1991. Every second day, larvae were wet sieved on 45µm screens before being restocked into clean vessels containing freshly filtered replacement seawater (34 - 35 g/L) pre-treated as previously described.

The experiment was terminated after 8 days when the larvae were collected and preserved using a 4% formaldehyde in seawater solution. Mean (± s.e.) growth of the larvae was evaluated by measuring a minimum of 50 individuals from each replicate at 200X using an Olympus (Model CH-2) compound light microscope fitted with a graticule and a 'Sedgewick Rafter' counting slide. Percentage survival was obtained by counting total and live larvae within a minimum sample of 100 larvae. Live larvae were distinguished from dead by lack of necrotic tissue and presence of food in the gut at the time of fixation.

Table 7.5.1. Experimental Treatments Applied to each of *S. costatum* and *C. calcitrans*.

Treatment No.	Description of Treatment
1	Unfed Control (Common to both species)
2	Fresh Algal culture
3	Low speed centrifuged slurry; stored 2°C
4	Low speed centrifuged slurry; stored 2°C; +Additives
5	Low speed centrifuged slurry; stored -15°C
6	Low speed centrifuged slurry; stored -15°C; +Additives
7	High speed centrifuged paste; stored 2°C
8	High speed centrifuged paste; stored 2°C; +Additives
9	High speed centrifuged paste; stored -15°C
10	High speed centrifuged paste; stored -15°C; +Additives
11	High speed centrifuged slurry; stored 2°C
12	High speed centrifuged slurry; stored 2°C; +Additives

Statistical analyses:
For trials conducted with each of the algal species, homogeneity of variance for *S. glomerata* larvae growth data and arcsine transformed survival data of across the full array of 12 dietary treatments were assessed using Cochran's test. A subsequent one way analysis of variance was used to evaluate within and between group source of variation. Growth data for the 12 diets were compared using Student-Newman-Keuls procedures (Winer et al., 1991).

For each species of micro-algae, growth data and arcsine transformed survival data for the eight stored diets in the 2x2x2 factorial array (diets 3 to 10), were then grouped according to a hierarchy of the three main dietary treatment factors. The latter comprised: method of harvesting (high speed or low speed centrifugation); storage temperature (chilled at +2 ±0.5°C or frozen at -15±0.5°C)

and absence or presence of food additives. These data were checked for homogeneity of variance then subjected to 3 factor analysis of variance to separate out significant effects (or absence thereof) of the main dietary treatment factors and interactions thereof. Where any of the three main dietary treatment factors were found to significantly effect growth or survival, multiple range analyses were applied to relevant data for stored diets 3 to10. This entailed pooling of data with a common dietary treatment factor and the resultant pooled means were then compared using Student-Newman-Keuls procedures (Winer et al., 1991). For example in the case of treatments 3 to 10 for T. *Iso* only storage temperature was found to significantly effect growth. Accordingly, data for treatments involving chilled storage at +2 ±0.5°C (treatments 3,4,7 and 8) were pooled and the resultant mean compared with equivalent data for treatments involving frozen storage at -15±0.5°C (treatments 5,6,9 and 10).

Finally, to distinguish the effects of storage density from centrifugation speed, an equivalent analysis was made of growth and arcsine transformed survival data of dietary treatments 7,8,11, &12. As indicated in Table 7.5.1, these four diets comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been either stored as pastes with or without additives (diets 7 & 8) and their direct counterparts that had been diluted to low density slurries (diets 11 & 12).

Results

C. calcitrans diets – growth:
Homogeneity of variance for growth data of *S. glomerata* larvae across the full array of 12 dietary treatments was confirmed using Cochran's test. A one way analysis of variance on these data revealed that between group source of variation that was large and highly significant ($P<0.0001$).

Mean growth data for the 12 diets (Table 7.5.2) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). Oyster larvae fed the fresh *C.calcitrans* culture diet exhibited a very acceptable mean ± s.e. shell height increase of 49.3± 1.88 µm over the 8 day experiment. This fell within the range of 4 to 6 µm/day normally experienced with early stage *S. glomerata* larvae when fed a standard fresh diet comprising equal amounts of T. *Iso, P.lutheri* and *C.calcitrans* (Frankish et al., 1991).

Poor growth rates less than 30% that of the fresh control diet were exhibited by unfed larvae and by larvae fed all four of the low speed cold stored *C.calcitrans* concentrate diets. Significantly higher growth rates (42 to 96% those fresh algae fed larvae) were promoted by high speed centrifuged diets. The best performing of the latter were those kept chilled at +2 ±0.5°C as either raw (without additives) paste or raw paste diluted to a slurry. The latter two diets supported mean increments of 47.5 ± 1.0 µm and 43.9 ± 1.0 µm representing 89.1 and 96.3% respectively those of larvae fed the fresh *C. calcitrans* culture control diet.

Homogeneity of variance for growth data of *S. glomerata* larvae across stored diets 3 to 10 in 2x2x2 factorial array was confirmed using Cochran's test. Three factor analyses of variance of growth data of *S. glomerata* larvae of these same (Table 7.5.2) showed that of the three main dietary treatment factors, additives and speed of centrifugation significantly influenced growth but that storage temperature did not. There was a significant interactive effect between speed on centrifugation and additives, between additives and storage temperature and also between all three factors. Results of a multiple range analysis of data from treatments 3 to10 pooled according to centrifugation speed confirmed that overall mean growth achieved on high speed centrifuged concentrates (34.10 µm) was significantly higher than achieved on low speed centrifuged diets (11.31 µm). Analysis of data from treatments 3 to10 pooled according to inclusion of additives confirmed that overall mean growth achieved with additives (20.0 µm) was marginally but significantly lower than without (25.4 µm) i.e. additives significantly depressed growth rate.

Table 7.5.2. Means for growth data of all 12 *C. calcitrans* diets compared using Student-Newman-Keul's procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell height gain (µm)	Homogeneous Groups*
1	Unfed Control (Common to both species)	14.79±1.20	a b
2	Fresh Algal culture	49.25±1.88	f
3	Low speed centrifuged slurry; stored 2°C	10.98±1.20	a
4	Low speed centrifuged slurry; stored 2°C; + Additives	15.05±0.49	b
5	Low speed centrifuged slurry; stored -15°C	10.85±1.78	a
6	Low speed centrifuged slurry; stored -15°C; + Additives	8.35±1.66	a
7	High speed centrifuged paste; stored 2°C	47.45±1.00	e f
8	High Speed centrifuged paste; stored 2°C; + Additives	20.93±1.78	c
9	High speed centrifuged paste; stored -15°C	32.35±1.70	d
10	High speed centrifuged paste; stored -15°C; + Additives	35.70±1.11	d
11	High speed centrifuged paste; stored as slurry; @ 2°C	43.87±1.45	e
12	High speed centrifuged paste; stored as slurry; @ 2°C; + Additives	37.50±1.23	d

* Means sharing common letter are not significantly different at P<0.05

Table 7.5.3. Three way analysis of variance of growth data relating to stored *C. calcitrans* diets.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: Centrifugation Speed	4155.98	1	4155.98	611.99	P=0.0000
• B: Additives	232.95	1	232.95	34.30	0.0000
• C: Storage Temperature	25.92	1	25.92	3.82	0.0625
Interactions					
• AB	305.54	1	305.54	44.99	0.0000
• AC	20.90	1	20.90	3.078	0.0922
• BC	271.68	1	271.68	40.01	0.0000
• ABC	664.48	1	664.48	97.85	0.0000
Residual	162.98	24	6.79		
Total (Corrected)	5840.44	31			

Homogeneity of variance for growth data of *S. glomerata* larvae across dietary treatments 7,8,11, & 12 that comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been stored as pastes with or without additives (diets 7 & 8, respectively) or diluted to form slurry counterparts (diets 11 & 12) was confirmed using Cochran's test (P= 0.7068).

Results (Table 7.5.4) of a two way analysis of variance of these same growth data, showed that both dilution to a slurry and additives did have a significant effect as did the interaction of the two factors. Results of a multiple range analysis of data from these treatments pooled according to density confirmed that overall mean growth achieved on the diluted slurries (40.6 µm) was

significantly (P<0.05) higher than achieved on low speed centrifuged diets (34.2). An equivalent analysis of the same data pooled according to inclusion of additives also confirmed that additives resulted in a very large and significant reduction of mean growth from 45.7 µm to 29.1 µm.

Table 7.5.4. Two way analysis of variance of growth data relating to stored *C. calcitrans* diets 7,8,11 &12.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level P *=significant
Main effects					
• A: Storage density	163.65	1	163.65	21.12	.0006*
• B: Additives	1094.78	1	1094.78	141.26	.0000*
Interactions					
• AB	397.90	1	397.90	51.34	.0000*
Residual	93.00	12	7.75		
Total (Corrected)	1749.34	15			

C. calcitrans diets – survival:
Homogeneity of variance for arcsine transformed survival data of *S. glomerata* larvae across the full array of 12 dietary treatments was confirmed using Cochran's test (P= 0.05). A one way analysis of variance on these data revealed that between group source of variation that was large and highly significant (P<0.0001).

Mean survival data for the 12 diets (Table 7.5.5) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). High rates of survival in the range of 90 to 98% were achieved by oyster larvae when fed 10 of the 12 test diets including the fresh *C. calcitrans* culture control diet and the unfed control. The two diets exhibiting reduced survival rates of 66 and 85% were those that also supported the poorest growth rates, namely, frozen low speed centrifuged concentrates with or without additives.

Table 7.5.5. Mean survival data of all 12 *C. calcitrans* related dietary treatments compared using Student-Newman-Keuls procedures on arcsine transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival	Homogeneous Groups*
1	Unfed Control (Common to both species)	96.45±0.57	b
2	Fresh Algal culture	93.45±3.38	b
3	Low speed centrifuged slurry; stored 2°C	89.63±6.77	a b
4	Low speed centrifuged slurry; stored 2°C; +Additives	94.92±0.42	b
5	Low speed centrifuged slurry; stored -15°C	66.31±7.0	a
6	Low speed centrifuged slurry; stored -15°C; +Additives	85.49±10.80	a b
7	High speed centrifuged paste; stored 2°C	98.47±0.37	b
8	High Speed centrifuged paste; stored 2°C; +Additives	97.48±0.72	b
9	High speed centrifuged paste; stored -15°C	97.72±0.37	b
10	High speed centrifuged paste; stored -15°C; +Additives	97.57±0.37	b
11	High speed centrifuged slurry; stored 2°C	97.59±0.98	b
12	High Speed centrifuged slurry; stored 2°C; +Additives	91.37±6.63	b

* Means sharing common letter are not significantly different at P<0.05 level.

Homogeneity of variance for arcsine transformed survival data of *S. glomerata* larvae across stored diets 3 to 10 in 2x2x2 factorial array was confirmed using Cochran's test. Three factor analyses of variance of growth data of *S. glomerata* larvae of these same (Table 7.5.6) showed that of the three main dietary treatment factors, speed of centrifugation and storage temperature significantly influenced growth but that additives did not. There were also highly significant interactive effects of all combinations of three factors. Results of a multiple range analysis of data from treatments 3 to10 pooled according to centrifugation speed confirmed that overall mean survival achieved on high speed centrifuged concentrates was significantly higher than achieved on low speed centrifuged diets. Analysis of these data pooled according to storage temperature confirmed that overall mean survival for larvae fed chilled *C. calcitrans* concentrates was significantly higher than those fed frozen concentrates.

Table 7.5.6. Analysis of Variance of arcsine $X^{0.5}$ transformed survival data relating to stored *C. calcitrans* diets 3 to 10.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: Centrifugation Speed	0.4177	1	0.4177	35.246	0.0000*
• B: Additives	0.0361	1	0.0361	3.044	0.0938
• C: Storage Temperature	0.1203	1	0.1203	10.155	0.0040*
Interactions					
• AB	0.0613	1	0.0613	5.173	0.0322*
• AC	0.0953	1	0.0953	8.045	0.0092*
• BC	0.0229	1	0.0229	1.929	0.0000*
• ABC	0.0118	1	0.0118	0.994	0.0000*
Residual	0.2843	24	0.0118		
Total (Corrected)	1.0494	31			

Variance for arcsine transformed survival data of *S. glomerata* larvae across dietary treatments 7,8,11, &12 that comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been stored as pastes with or without additives (diets 7 & 8 respectively) or diluted to form slurry counterparts (diets 11 & 12) was found to be highly heterogeneous using Cochran's test (P= 0.0007) and was not analysed using ANOVA.

S. costatum diets – growth:
Homogeneity of variance for growth data of *S. glomerata* larvae across the full array of 12 *S. costatum* related dietary treatments was confirmed using Cochran's test (P= 0.0001) A one way analysis of variance on these data revealed that between group source of variation was large and highly significant (P<0.0001).

Means for growth data of the 12 diets (Table 7.5.7) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). Oyster larvae fed the fresh *S. costatum* culture diet exhibited a very acceptable mean (±s.e) shell height increase of 50.4± 0.4µm over the 8 day experiment. This falls within the upper range of 4 to 6 µm/day normally experienced with early stage *S. glomerata* larvae when fed a standard fresh diet comprising equal amounts of T. *Iso, P.lutheri* and *C.calcitrans* (Frankish et al., 1991).

Poor growth rates, 22 to 32% of the fresh algae control diet, were exhibited by unfed larvae and by larvae fed frozen low speed centrifuged *S. costatum* concentrate diets stored with or without additives. Relatively poor growth rates, 45 to 59% those obtained on the fresh *S. costatum* control diet, were also exhibited by larvae fed 4 diets comprising chill stored low and high speed centrifuged pastes with or without additives.

Quite unexpectedly, the second best performing concentrate diets were frozen high speed centrifuged pastes with or without additives. These diets supported identical (shell height) increments of rates 34.4 µm being more than two thirds that of larvae fed the fresh *S. costatum* culture control diet.

However the best concentrate diet by far was high speed centrifuged paste that had been diluted to a slurry with additives and chill stored at +2.0 ± 0.5°C. This outstanding diet yielded a growth increment of 46. 4 µm i.e. 92% of the fresh *S. costatum* culture control diet. Its companion diet, lacking additives, supported a lower, albeit acceptable, mean growth increment of 41.4±6 um i.e. 82% of the fresh algae control.

Homogeneity of variance for growth data of *S. glomerata* larvae across stored diets 3 to 10 in a 2x2x2 factorial array was confirmed using Cochran's test (P= 0.6463). *S. costatum* diets 3 to 10 (Table 7.5.1) showed that of the three main dietary treatment factors only centrifugation speed significantly effected growth. The only interaction of main factors to significantly effect growth was that between centrifugation speed and storage temperature. Results of a multiple range analysis of data from treatments 3 to10 pooled according to centrifugation speed confirmed that overall mean growth achieved on high speed centrifuged pastes (31.03 µm) was far superior to that on low speed centrifuged slurries (19.04 µm).

In contrast to stored concentrates of *C. calcitrans* and indeed of all four micro-algae species investigated in preceding bioassay experiments 3 and 4, inclusion of food additives had no significant effect whatsoever on any of the *S. costatum* concentrate diets whether stored chilled or frozen or as pastes or slurries. These results again emphasise the lack of predictable effects when concentrates of different species of micro-algae are subjected to the same array at harvesting, preservation and storage techniques.

Table 7. 5.7. Means (± s.e.) for growth data of all 12 *S. costatum* related diets compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell height increment (µm)	Homogeneous Groups*
1	Unfed Control (Common to both species)	14.8±1.2	a
2	Fresh Algal culture	50.4±3.6	d
3	Low speed centrifuged slurry; stored 2°C	26.1±1.4	b
4	Low speed centrifuged slurry; stored 2°C; +Additives	22.8±.07	b
5	Low speed centrifuged slurry; stored -15°C	16.1±3.5	a b
6	Low speed centrifuged slurry; stored -15°C; +Additives	11.2±1.8	a
7	High speed centrifuged paste; stored 2°C	29.7±2.5	b
8	High Speed centrifuged paste; stored 2°C; +Additives	25.7±3.5	b
9	High speed centrifuged paste; stored -15°C	34.4±2.25	b c
10	High speed centrifuged paste; stored -15°C; +Additives	34.4±0.4	c
11	High speed centrifuged slurry; stored 2°C	41.4±1.6	c d
12	High Speed centrifuged slurry; stored 2°C; +Additives	46.4±1.3	d

* Means sharing common letter are not significantly different at P<0.05

Table 7.5.8. Three way analysis of variance of growth data relating to stored *S. costatum* diets 3 to 10 in 2x2x2 factorial array.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: Additives	8.69	1	8.69	0.388	0.5460
• B: Centrifugation Speed	1149.84	1	1149.84	51.26	0.0000*
• C: Storage Temperature	35.03	1	35.03	1.561	0.2235
Interactions					
• AB	76.08	1	76.08	3.391	0.0779
• AC	16.13	1	16.13	0.719	0.4138
• BC	613.03	1	613.03	27.33	0.0000*
• ABC	2.89	1	2.89	0.129	0.7265
Residual	538.40	24	22.43		
Total (Corrected)	2440.09	31			

Homogeneity of variance for growth data of *S. glomerata* larvae across dietary treatments 7,8,11, &12 that comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been stored as pastes with or without additives (diets 7 & 8 respectively) or diluted to form slurry counterparts (diets 11 & 12) was confirmed using Cochran's test (P= 0.4154).

Results (Table 7.5.9) of a two way analysis of variance of these same growth data, showed that only dilution to a slurry had a significant effect. Results of a multiple range analysis of data from these treatments pooled according to density confirmed that overall mean growth achieved on the diluted slurries (43.9 μm) significantly exceeded that achieved on the undiluted paste (27.7 μm) counterparts.

S. costatum diets – survival:
Homogeneity of variance for arcsine $X^{0.5}$ transformed survival data of *S. glomerata* larvae across the full array of 12 *S. costatum* dietary treatments was confirmed using Cochran's test. A one way analysis of variance on these data revealed that between group source of variation that was large and highly significant (P<0.0001).

Mean survival data for the 12 diets (Table 7.5.9) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). High rates of survival in the range of 88 to 97% were achieved by oyster larvae when fed 11 of the 12 test diets including the fresh for *S. costatum* culture control diet and the unfed control. The only diet exhibiting a markedly reduced mean survival rate of 67% was frozen low speed centrifuged concentrate without additives.

Table 7.5.9 Means for survival data of *S. costatum* diets compared using Student-Newman-Keul's procedures on arcsine transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival	Homogeneous Groups
1	Unfed Control (Common to both species)	96.0±1.2	b
2	Fresh Algal culture	88.5±7.5	a b
3	Low speed centrifuged slurry; stored 2°C	96.1±0.3	b
4	Low speed centrifuged slurry; stored 2°C; +Additives	96.6±1.0	b
5	Low speed centrifuged slurry; stored -15°C	67.2±6.8	a
6	Low speed centrifuged slurry ; stored -15°C; +Additives	87.8±5.7	a b
7	High speed centrifuged paste; stored 2°C	97.1±1.4	b
8	High Speed centrifuged paste; stored 2°C; +Additives	94.9±1.1	b
9	High speed centrifuged paste; stored -15°C	95.6±0.4	b
10	High speed centrifuged paste; stored -15°C; +Additives	95.8±0.6	b
11	High speed centrifuged slurry; stored 2°C	96.9±0.7	b
12	High Speed centrifuged slurry; stored 2°C; +Additives	96.5±0.7	b

* Means sharing common letter are not significantly different at P<0.05 level.

Homogeneity of variance for arcsine $X^{0.5}$ transformed survival data of *S. glomerata* larvae across stored *S. costatum* diets 3 to 10 in 2x2x2 factorial array was confirmed using Cochran's test. Three factor analysis of variance of growth data of *S. glomerata* larvae of these same data (Table 7.5.10) showed that all three main dietary treatment factors, additives, speed of centrifugation and storage temperature significantly influenced growth. There were also significant interactive effects of all combinations of three factors except additives and centrifugation speed.

Results of a multiple range analysis of data from treatments 3 to 10 pooled according to centrifugation speed confirmed that overall mean survival achieved on high speed centrifuged paste concentrates (95.8%) was significantly higher than that achieved on low speed centrifuged slurry concentrates (86.9%). Analysis of these data pooled according to use of additives showed that overall mean survival for larvae fed *S. costatum* concentrates with additives (94.3%) was significantly greater than those without additives (88.5%). Likewise analysis of these data pooled according to storage temperature showed that overall mean survival for larvae fed *S. costatum* chill stored concentrates (96.2%) was significantly greater than that of frozen concentrates (86.6%).

Table 7.5.10. Three way analysis of variance of arcsine transformed survival data relating to stored *S. costatum* diets 3 to 10 in 2x2x2 factorial array.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F -ratio	Significance level
Main effects					
• A: Additives	267.61	1	267.61	6.54	.0173
• B: Speed of Centrifugation	635.10	1	635.10	15.51	.0006
• C: Storage temperature	737.09	1	737.09	18.00	.0003
Interactions					
• AB	184.13	1	184.13	4.50	.0445
• AC	157.62	1	157.62	3.85	.0615
• BC	686.72	1	686.72	16.77	.0004
• ABC	253.58	1	253.58	6.19	.0202
Residual	982.83	24	40.95		
Total (Corrected)	3904.68	31			

N.B. All F-ratios are based on residual mean square error.

Homogeneity of variance for growth data of *S. glomerata* larvae across dietary treatments 7,8,11, &12 that comprised a 2x2 factorial array of high speed centrifuged chilled *S. costatum* concentrates that had been stored as pastes with or without additives (diets 7 & 8 respectively) or diluted to form slurry counterparts (diets 11 & 12) was confirmed using Cochran's test (P= 0.5626).

Results of a two way analysis of variance of these same survival data, showed that that neither concentrate density per se nor additives significantly effected survival.

Summary and Conclusions (Table 7.5.11 and Figs. 7.5.1 and 7.5.2)

In stark contrast to both T. *Iso* and *T.pseudonana* (see Bioassay Experiment 4) and also in stark contrast to apparent cell viability data, high speed (super) centrifuged concentrates of both *C. calcitrans* and *S. costatum* supported far superior rates of growth in oyster larvae than did their slow speed (bucket) centrifuged counterparts. The nutritional value of high speed centrifuged concentrates was markedly reduced by freezing.

In the case of *C. calcitrans,* there were two outstanding performers amongst the stored concentrate diets that supported growth rates of 96 and 92 % those of the fresh culture control diet. These comprised high speed centrifuged concentrates held chilled as a paste or slurry without additives. By contrast only one *S. costatum* stored concentrate diet supported a comparable growth rate to its corresponding fresh culture control diet. This was high speed centrifuged concentrate diluted to a slurry with additives.

Table 7.5.11. Summary of results for *C. calcitrans* and *S. costatum* concentrate diets.

	Fresh algae control	**Centrifugation speed** low/high	**Use of additives** Raw / +additives	**Storage Temp.** Chilled / Frozen	**Concentrate density** High density paste / low density slurry	**Best performing concentrate** (mean growth in µm /% survival)
C. calcitrans						
Mean growth µm	49.3	11.3 / **34.1***	**25.4*** / 20.0	**16.10*** / 11.77	23.6 / 21.8	Raw; chilled; high speed cent. paste (47.5µm / 98.5%)
Mean Survival %	93.5	84.1 / **97.8***	88.0 / 93.9	**95.1*** / 86.8	98.0 / 94.5	
S. costatum						
Mean growth µm	50.4	19.0 / **31.0***	26.5 / 23.5	26.1 / 24.0	27.7 / **43.9***	chilled; high speed cent. slurry with additives (46.4µm / 96.5%)
Mean Survival %	88.5	78.5 / **90.2***	/ 93.8	96.2 / 86.6	91.80 / 90.77	

Fig 7.5.1 Effect of centrifuge speed, additives, storage temperature and density of *C.calcitrans* concentrates on the growth and survival when fed to oyster (*S. glomerata*) larvae

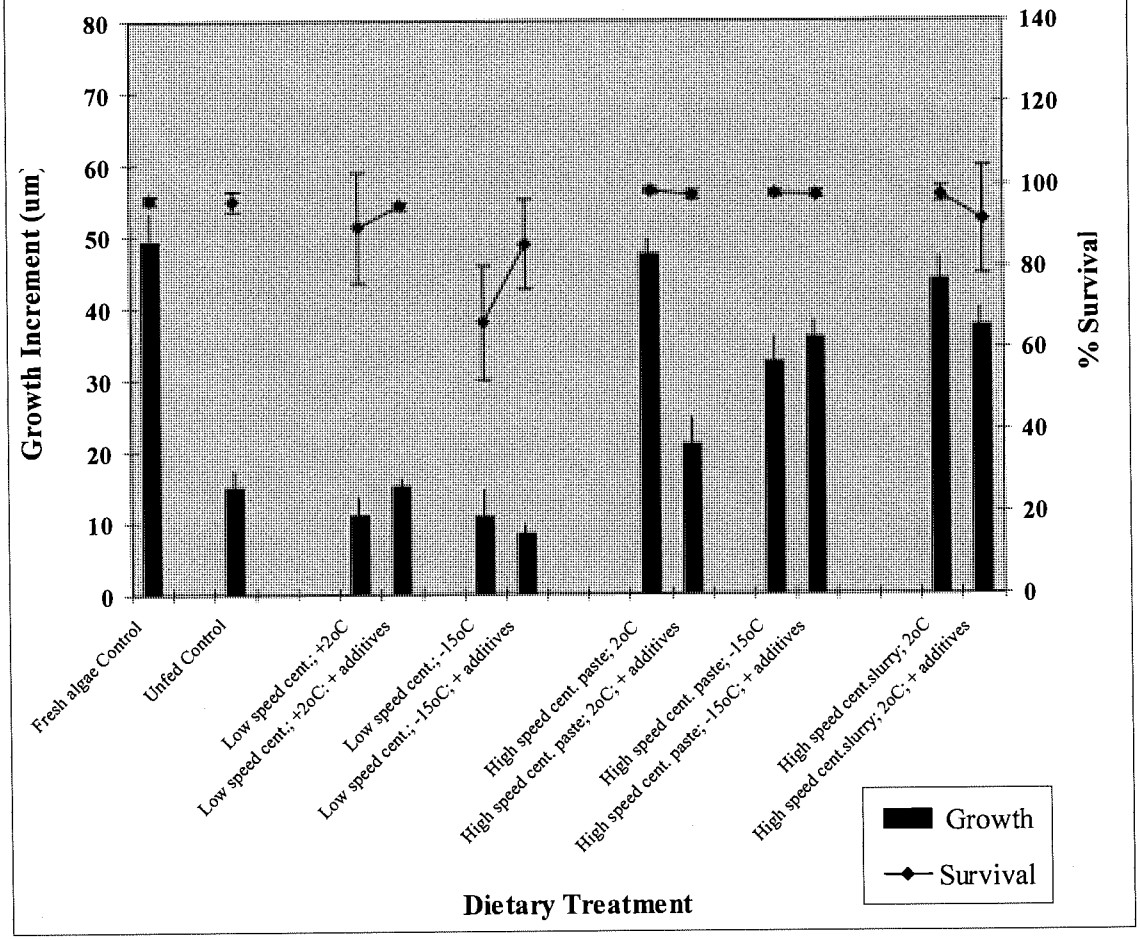
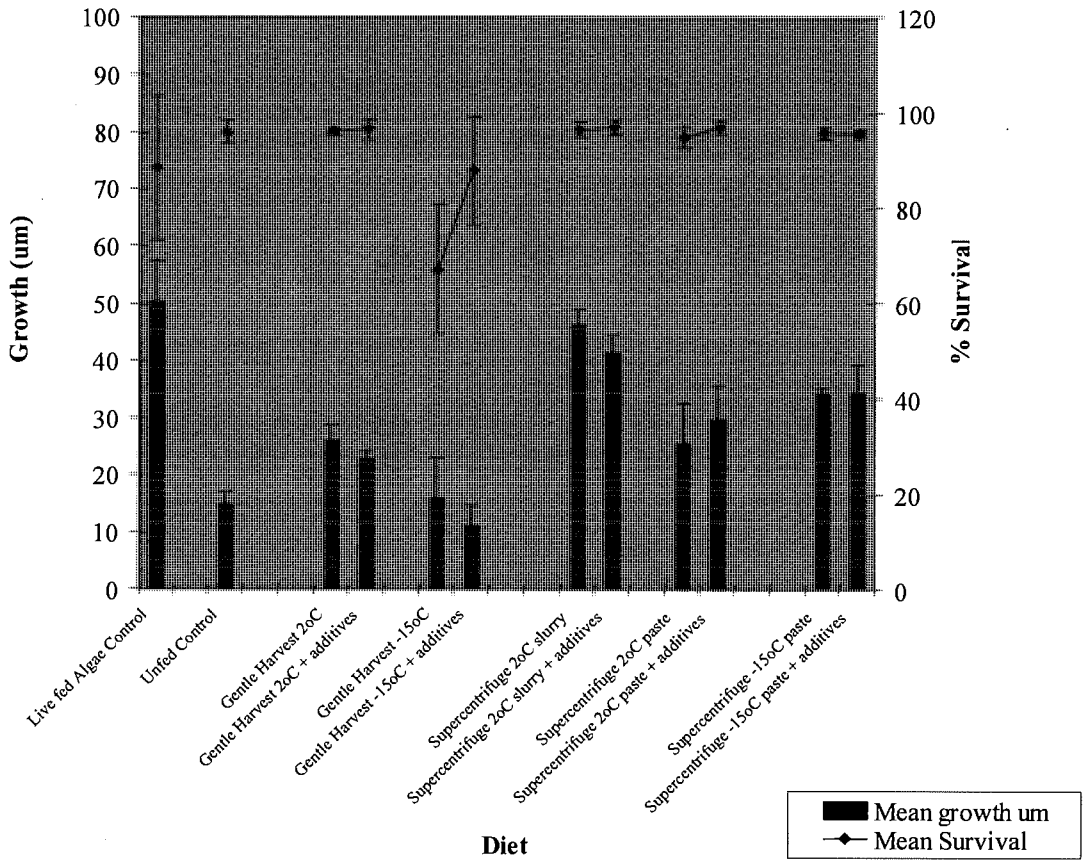


Fig 7.5.2 Effects of harvest method, storage temperature and additives on the nutritional value of *S. costatum* concentrates when fed to oyster (*Saccostrea glomerata*) larvae
NB error bars =s.d.



7.6. Bio-Assay Experiment 6: Evaluation of Optimum Harvesting Techniques for *Tetraselmis chui* and *Chaetoceros muelleri.*

Introduction and Aims

This bio-assay experiment, a companion to previous bio-assay experiments 3, 4 and 5 had two principal objectives. The first was to clarify whether rapid apparent loss of shelf life by both chilled (see Section 6.2) and frozen (see Section 6.6) super-centrifuged pastes of *Chaetoceros muelleri,* genuinely reflected loss of nutritional value. A basis of this uncertainty was that some of the same chill stored *C. muelleri* concentrates that exhibited rapid loss of cell viability otherwise remained in apparent good condition. For example, pH of stored concentrates was maintained within the targeted range of 4.0 to 4.5 by the addition of citric acid plus vitamin C with or without glycerol at all temperatures tested. Likewise, microbial contamination and decomposition as indicated by offensive odours, was totally prevented for up to 6 weeks by storing chilled at -2.0±0.5 ° C with or without additives and also at 0± 0.5 °C and at +2.0± 0.5 °C when all additives were used in combination.

The second objective, a corollary of the first, was to verify if apparent protracted retention of cell condition in chilled and frozen super-centrifuged pastes of *Tetraselmis chui,* as previously indicated by the results of Screening Experiments 2 and 6 (see Sections 6.2 and 6.6), genuinely reflected retention of the nutritional value of these pastes.

The experiment was also to assess effects of various combinations of alternative harvesting techniques, presence or absence of common food additives and density of stored concentrate (as pastes or slurries) on retained nutrition value of *T. chui* and *C. muelleri* concentrates. In contrast to preceding Bio-assay Experiments 3, 4 and 5, growth and survival of juvenile rather than larval doughboy scallops *Mimachlamys asperrima* were used as direct indicators of retained nutritional value of diets. This variation in protocol was adopted because mono-specific diets of both *C. muelleri* and *T.chui* had previously been shown to support satisfactory growth and survival of juvenile bivalves (O'Connor, et al., 1992) but not of their larvae (Nell and O'Connor, 1991).

Materials and Methods

Experimental design:
The same twelve experimental treatments (Table 7.6.1) used in Bio-assay Experiments 4 and 5 were employed for each of *T. chui* and *C. muelleri.* These included 8 treatments in a balanced 2 x 2 x 2 factorial design to evaluate individual and interactive effects the three main treatment factors namely, harvesting technique (high speed or low speed centrifugation); food additives (presence or absence thereof) and storage temperature (chilled storage at 2.0 ± 0.5°C or frozen storage at -15 ± 1°C) on the nutritional value of stored concentrates of *T. chui* and *C. muelleri.* Of the other 4 dietary treatments, two comprised an unfed and a fresh (daily harvested) fresh culture control. The remaining two dietary treatments comprised high speed centrifuged concentrate reduced from a stiff paste to slurry of the same consistency produced by low speed centrifugation (density 10 to 20 g/L), one with food additives the other without and both chill stored at 2.0 ± 0.5°C.

Four replicates for each of the dietary treatments were randomly distributed throughout a common water bath maintained at a favourable temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998), housed in an air-conditioned room maintained at 23 ± 1°C.

Table 7.6.1. Experimental dietary treatments applied to each of *C. muelleri* and *T. chui.*

Treatment No.	Description of Treatment
1	Unfed Control (Common to both species)
2	Fresh Algal culture
3	Low speed centrifuged slurry.; stored 2°C
4	Low speed centrifuged slurry .; stored 2°C; +Additives
5	Low speed centrifuged slurry ; stored -15°C
6	Low speed centrifuged slurry ; stored -15°C; +Additives
7	High speed centrifuged paste; stored 2°C
8	High Speed centrifuged paste ; stored 2°C; +Additives
9	High speed centrifuged paste ; stored -15°C
10	High speed centrifuged paste ; stored -15°C; +Additives
11	High speed centrifuged slurry; stored 2°C
12	High Speed centrifuged slurry; stored 2°C; +Additives

Preparation of diets:

Cultures of *T. chui* and *C. muelleri* were grown from axenic inocula in 500 litre 80 µm clear polyethylene film bags. The cultures were propagated in oceanic water (34 - 35 g/L) settled for 6 to 8 days, filtered to 1 µm (nominal), and disinfected using sodium hypochlorite at an effective concentration of 10 mg/L for approximately 12 hours. Prior to inoculation, residual chlorine was deactivated using sodium thiosulphate. Cultures were enriched with f/2 beta growth medium (Guillard, 1983) held at 23 ± 0.5°C with a 16 hr:8 hr light : dark cycle and illuminated with 58 watt 'cool white' fluorescent tube lights to an intensity of 4,000 lux at the bag surface. Cultures were continuously aerated with 1.5% carbon dioxide enriched air to maintain pH in the range of 8.0 to 8.4.

The two alternative methods of concentrating the algae were high and low speed centrifugation. High speed centrifugation was performed using the previously described "Sharples P/L" super-centrifuge at 13,000 g. Algal cultures in mid to late exponential phase were delivered to the centrifuge with a diaphragm pump at a flow rate of approximately 25 L/minute. The algal concentrate was recovered from the super-centrifuge bowl using a plastic spatula. Low speed centrifugation involved the use of the previously a "Hitachi " laboratory scale bucket centrifuge operated at 1,330 g for 5 minutes. The resultant slurry was then recovered by decanting the supernatant.

In order to combat culture variability, four separate batch cultures each of *T. chui* and *C. muelleri* were used for each method of harvesting. After harvesting, concentrates of common algal species were combined and thoroughly blended. Low speed centrifuge harvested concentrate was in a characteristic slurry form while the super-centrifuged product was a typical firm paste. The latter was diluted to a density of 10 to 20g/L to create two additional slurry treatments described below, aimed at differentiating the effects of harvesting technique and cell density during chilled storage.

Sub samples of concentrate used to assess the inclusion of additives were weighed and additives thoroughly blended in at the following rates:

- glycerol (SIGMA G-7893) at 10% (w/w)
- ascorbic acid (Vitamin C) (SIGMA A-7506) at 1% (w/w)
- citric acid (BD. Prod No. 10081) until pH was lowered to the range 4 - 4.5

All chill-stored dietary treatments were held at 2.0 ± 0.5°C in 120 mL clear polystyrene containers with a 1:2 ratio of slurry to air-space as either stiff pastes or diluted slurries at 10-20 g paste/L in accordance with optimum densities previously identified in Section 6.5. The containers were stored on a 30° incline and under light.

Prior to freezing all treatments stored at -15°C ± 1°C with additives were left at ambient room temperature for a minimum of half an hour to allow penetration of the glycerol prior to immersion quick freezing (IQF) using a 25% NaCl solution held at -15°C. All cold stored diets were maintained at prescribed chilled or frozen temperatures in Fisher and Paykel chest freezers in which temperature was monitored and controlled with a Thermo Eye-P system. These had been held for a period of 33 days at the commencement of feeding.

Acquisition, husbandry and measurement of spat:
At the commencement of the experiment, 92 miniature up-wellers to accommodate 4 replicates of 11 dietary treatments for each *T. chui* and *C. muelleri* plus a common unfed control, were individually housed within 8.5L plastic aquaria. The latter, held within a common temperature bath maintained at 25.0±1.0 °C, were stocked with 30 hatchery reared juvenile doughboy scallop *Mimachlamys asperrima* juveniles (spat) of an initial mean (± s.d.) shell height of 1823± 182µm (n=100). This and all subsequent measurement of shell height (distance from the centre of the hinge to centre point of anterior margin of the dorsal [right] valve) was made using an Olympus (model CH-2, Tokyo, Japan) stereo microscope fitted with a calibrated eyepiece micrometer.

The spat in all treatments were fed to allow satiation feeding by adding either fresh or resuspended chilled or frozen *T. chui* and *C. muelleri* concentrate cells at a dry weight equivalent of 7500 Tahitian *Isochrysis* cells/ml. The diets were administered as a morning feed at the rate of 2500/ml *T. Iso* equivalent cells/ml and an afternoon feed at the rate of 5000 T. *Iso* equivalent cells/ml in accordance with methods of O'Connor et. al., (1992).

Each day small samples of stored concentrate diets were resuspended, counted, and volume to feed/bucket calculated and feed administered. Complete water changes with fresh 1 µm filter seawater preheated to 25°C plus cleaning and disinfection of plastic aquaria was conducted three times a week.

The experiment was terminated after 20 days when the spat were collected and preserved using a 4% formaldehyde in seawater solution. Mean (± s.e.) growth of the spat was evaluated by measuring the shell height of all surviving spat from each replicate at 200X using an Olympus (Model CH-2) compound light microscope fitted with a graticule and a 'Sedgewick Rafter' counting slide. Percentage survival was obtained by counting all surviving spat in each replicate. Live spat were readily distinguishable from dead when counted prior to fixation.

Statistical analyses:
For trials conducted with each of the algal species, it was intended that homogeneity of variance be assessed using Cochran's test for *M. asperrima* spat growth data and arcsine $X^{0.5}$ transformed survival data of across the full array of 12 dietary treatments. However the total inexplicable mortality of *M. asperrima* spat fed low speed centrifuged diets of both *T. chui* and *C. muelleri* that that had been frozen narrowed the available data base to only 10 dietary treatments. One way analysis of variance was used to evaluate within and between group source of variation. Growth data for the 10 diets were compared using Student-Newman-Keuls procedures (Winer et al., 1991).

For each species of micro-algae, growth data and arcsine $X^{0.5}$ transformed survival data for the 4 high speed centrifuged paste diets (diets 7,8,9 and 10 in Table 7.6.1), in the 2x2 factorial array were then grouped according to a hierarchy of two main dietary treatment factors. The latter comprised storage temperature (chilled at +2.0 ±0.5°C or frozen at -15.0±0.5°C) and absence or

presence of food additives. These data were checked for homogeneity of variance then subjected to 2 factor analysis of variance to separate out significant effects of the main dietary treatment factors and interactions thereof. Where any of the main dietary treatment factors were found to significantly effect growth or survival, multiple range analysis was applied to relevant data. This entailed pooling of data with a common dietary treatment factor and the resultant pooled means were then compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Finally, to distinguish the effects of storage density from centrifugation speed, an equivalent analysis if warranted was made of growth and arcsine $X^{0.5}$ transformed survival data of dietary treatments 7,8,11, & 12. As indicated in Table 7.6.1, these four diets comprised a 2x2 factorial array of high speed centrifuged chilled concentrates that had been either stored as pastes with or without additives (diets 7 & 8) and their direct counterparts that had been diluted to low density slurries (diets 11 & 12).

Results

C. muelleri diets – growth:
All growth and survival data for scallops fed frozen stored low speed centrifuged *C. muelleri* with and without additives (treatments 5 and 6 in Table 7.6.1) were precluded when early in the experiment all juvenile scallops in all replicates inexplicably died from causes presumably related to these diets. Homogeneity of variance for growth data of *M. asperrima* spat across the remaining 10 dietary treatments was confirmed using Cochran's test (P= 0.8414). A one way analysis of variance of these data revealed a large sources of variation both within and between groups but that the latter was not significant (P=0.1048).

Mean *M. asperrima* spat growth data for the 10 *C. muelleri* diets (Table 7.6.3) were compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Table 7.6.2 Mean (± s.e.) growth data for *M. asperrima* spat for 10, *C. muelleri* related diets compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell height increment (µm)
1	Fresh Algal culture	292.11±72.4
2	Unfed Control (Common to both species)	64.58±41.1
3	Low speed centrifuged slurry.; stored 2°C	135.67±42.9
4	Low speed centrifuged slurry .; stored 2°C; +Additives	81.11±61.3
5	Low speed centrifuged slurry ; stored -5°C	-
6	Low speed centrifuged slurry ; stored -15°C; +Additives	-
7	High speed centrifuged paste; stored 2°C	120.68±25.1
8	High Speed centrifuged paste ; stored 2°C; +Additives	117.86±12.9
9	High speed centrifuged paste ; stored -15°C	184.6±58.9
10	High speed centrifuged paste ; stored -15°C; +Additives	167.05±6.5
11	High speed centrifuged slurry; stored 2°C	122.78±45.9
12	High Speed centrifuged slurry; stored 2°C; +Additives	209.54±35.0

M. asperrima spat fed the fresh C. muelleri culture diet exhibited a mean ± s.e. shell height increase of 292.11±72.4um over the 18 day experiment. This represented a modest rate compared with optimum rates for this size class of juveniles of 100 µm/day (O'Connor, 1998) achieved on mixed diatom diets.

Poor and highly variable mean growth increments were exhibited by larvae fed all eight cold-stored C. muelleri concentrate diets. Mean ± s.e. growth increments varied over a considerable range. The poorest increment of 81±61µm (28% that of fresh algae fed spat) occurred with low speed centrifuged chill stored concentrates with additives. The best increment, 209±35 µm (72% that of fresh algae fed spat) three fold range in mean growth increment, a very high degree of variability within 7 of the 9 treatments precluded statistically significant differences in mean growth rate between any two diets.

C. muelleri diets – survival:
Homogeneity of variance for survival data of *M. asperrima* spat across the array of 10 dietary treatments was confirmed using Cochran's test (P=0.3014). A one way analysis of variance on these data revealed that between group source of variation that was not significant ($P<0.3375$).

Universally high mean rates of survival in the range of 93 to 99% (Table 7.5.6) were achieved by *M. asperrima* spat in all treatments including the fresh *C. muelleri* culture and the unfed controls.

Table 7.6.6 Mean survival data of all 10 *C. muelleri* dietary treatments compared using Student-Newman-Keuls procedures on arcsine $X^{0.5}$ transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival
1	Unfed Control (Common to both species)	93.99±2.22a
2	Fresh Algal culture	96.66±1.93a
3	Low speed centrifuged slurry.; stored 2°C	99±1.00a
4	Low speed centrifuged slurry .; stored 2°C; +Additives	95.8±2.29a
5	Low speed centrifuged slurry ; stored -15°C	-
6	Low speed centrifuged slurry ; stored -15°C; +Additives	-
7	High speed centrifuged paste; stored 2°C	96.74±3.26a
8	High Speed centrifuged paste ; stored 2°C; +Additives	96.93±1.17a
9	High speed centrifuged paste ; stored -15°C	99.14±0.86a
10	High speed centrifuged paste ; stored -15°C; +Additives	92.62±2.16a
11	High speed centrifuged slurry; stored 2°C	97.89±1.31a
12	High Speed centrifuged slurry; stored 2°C; +Additives	94.52±2.06a

* Means sharing common letter are not significantly different at P<0.05 level.

Tetraselmis chui diets – growth:
As with *C. muelleri* diets, all growth and survival data for scallops fed frozen stored low speed centrifuged *T.chui* concentrates were omitted after all juvenile scallops in all replicates inexplicably died from causes presumably related to these diets, early in the experiment. Homogeneity of variance for growth data of *M. asperrima* spat across the remaining 10 *T. chui* related dietary treatments was confirmed using Cochran's test (P= 0.2273) A one way analysis of variance on these data revealed that between group source of variation was large and highly significant (P<0.0016).

Means for growth data of the 10 dietary treatments (Table 7.6.7) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). As with *C. muelleri*, scallop spat fed the fresh *T. chui* culture diet exhibited a mediocre mean shell height increase of only 326± 62µm over the 18 day experiment.

In stark contrast to *C. muelleri* concentrate diets that supported growth rates of *M. asperrima* spat well below (28 to72%) that of the fresh algae control diet, all *T. chui* concentrate diets supported mean growth rates that matched or exceeded (range 102 to 172 %) that of their fresh algae counterpart. The two top performing *T. chui* concentrate diets contrasted with one another comprising in one case, chilled low speed centrifuged slurry without additives and in the other, frozen high speed centrifuged paste with additives.

Table 7.6.7. Means for *C. asperrima* growth data of *T. chui* diets compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) shell height gain (µm)	Homogeneous Groups*
1	Fresh Algal culture	325.7±61.6	b
2	Unfed Control (Common to both species)	64.6±14.01	a
3	Low speed centrifuged slurry; stored 2°C	560.8±118.1	b
4	Low speed centrifuged slurry; stored 2°C; +Additives	455.4±60.3	b
5	Low speed centrifuged slurry; stored -15°C	**	
6	Low speed centrifuged slurry; stored -15°C; +Additives	**	
7	High speed centrifuged paste; stored 2°C	390.5±84.4	b
8	High speed centrifuged paste; stored 2°C;+ Additives	331.5±65.4	b
9	High speed centrifuged paste; stored -15°C	474.4±95.0	b
10	High speed centrifuged paste ; stored -15°C; +Additives	526.6±22.9	b
11	High speed centrifuged paste stored as slurry; @ 2°C	390.4±56.2	b
12	High speed centrifuged paste stored as slurry; @ 2°C; +Additives	447.4±18.2	b

Means sharing common letter are not significantly different at $P<0.05$
**All scallops died early in the experiment

T. chui diets – survival:
Homogeneity of variance for arcsine $X^{0.5}$ transformed survival data of *M. asperrima* spat across the array of 10 dietary treatments was confirmed using Cochran's test (P= 0.1241). A one way analysis of variance on these data revealed that between group source of variation that was large and significant ($P<0.0330$).

Table 7.6.8. Mean survival data of all 10 *T. chui* dietary treatments compared using Student-Newman-Keuls procedures on arcsine transformed data (Winer et al., 1991).

Treatment No.	Description	Mean (±s.e.) % Survival	Homogeneous Groups*
1	Unfed Control (Common to both species)	96.66±1.93	ab
2	Fresh Algal culture	93.98±2.76	ab
3	Low speed centrifuged slurry.; stored 2°C	96.51±2.62	ab
4	Low speed centrifuged slurry .; stored 2°C; +Additives	93.59±4.42	ab
5	Low speed centrifuged slurry ; stored -15°C	**	
6	Low speed centrifuged slurry ; stored -15°C; +Additives	**	
7	High speed centrifuged paste; stored 2°C	98.52±0.87	b
8	High Speed centrifuged paste ; stored 2°C; +Additives	98.11±1.10	b
9	High speed centrifuged paste ; stored -15°C	93.96±0.96	ab
10	High speed centrifuged paste ; stored -15°C; +Additives	98.34±0.96	b
11	High speed centrifuged slurry; stored 2°C	96.63±0.13	ab
12	High Speed centrifuged slurry; stored 2°C; +Additives	85.30±1.92	a

* Means sharing common letter are not significantly different at P<0.05 level **All scallops died early in the experiment

Mean survival data for the 10 *T. chui* diets (Table 7.6.8) were compared using Student-Newman-Keuls procedures (Winer et al., 1991). High mean rates of survival in the range of 94 to 99% were achieved by *M. asperrima* spat in all treatments except the chilled high speed centrifuged paste diluted to a slurry with additives that supported a significantly lower survival rate of 85%.

Summary and Conclusions (Figs. 7.6.1 and 7.6.2)

Fresh algae control diets of both *C. muelleri* and *T. chui* supported poor growth but excellent survival of scallop spat relative to mixed diets of diatoms and prymnesiophytes routinely used to nursery rear juvenile bivalves. Growth rates supported by 8 types of variously harvested and stored concentrates of *T.chui* equalled or exceeded (range 102 to172%) that of a fresh culture of *T. chui.* By contrast, growth rates supported by the same array of centrifuged concentrates of *C. muelleri* were all considerably inferior (range 28 to 72%) to that of a fresh culture of *C. muelleri.* Best performing *C. muelleri* concentrate diet (72% the growth rate of the fresh control) was the chilled high speed centrifuged paste diluted to a slurry with additives.

Results of previous nutritional research with *T chui* on juvenile bivalves have been variable depending on the species involved. For example *T. Chui* was shown by O'Connor et al., 1992 to support good rates of growth in Sydney rock oyster spat but very poor reproductive conditioning in of the scallop *Pecten fumatus* (Reeve), by Heasman et al., 1996. The improved performance of *T. chui* concentrates over their fresh counterparts may be ascribed to loss of motility and increased digestibility. The two best performing *T. chui* diets were in fact greatly contrasting. One comprised chilled, slow speed centrifuged raw slurry the other, high speed centrifuged paste with additives. However the ease and efficiency of harvesting and storing frozen paste sets it well ahead of the slurry as a practical diet.

Fig 7.6.1 Effects of harvest method, storage tempertature and additives on the nutritional value of *Chaetoceros muelleri* concentrate diets on mean(+/-s.e.) growth and survival of doughboy scallop *(C asperrima)* spat

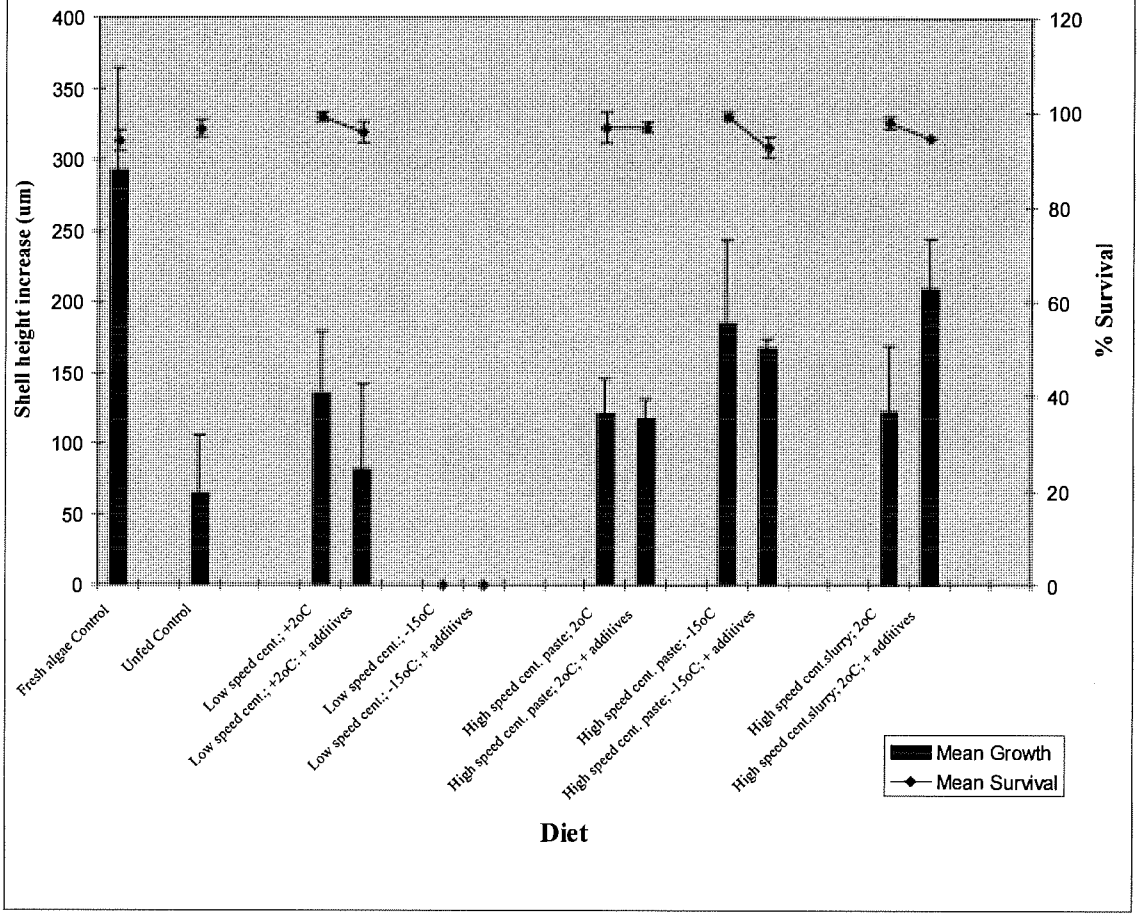
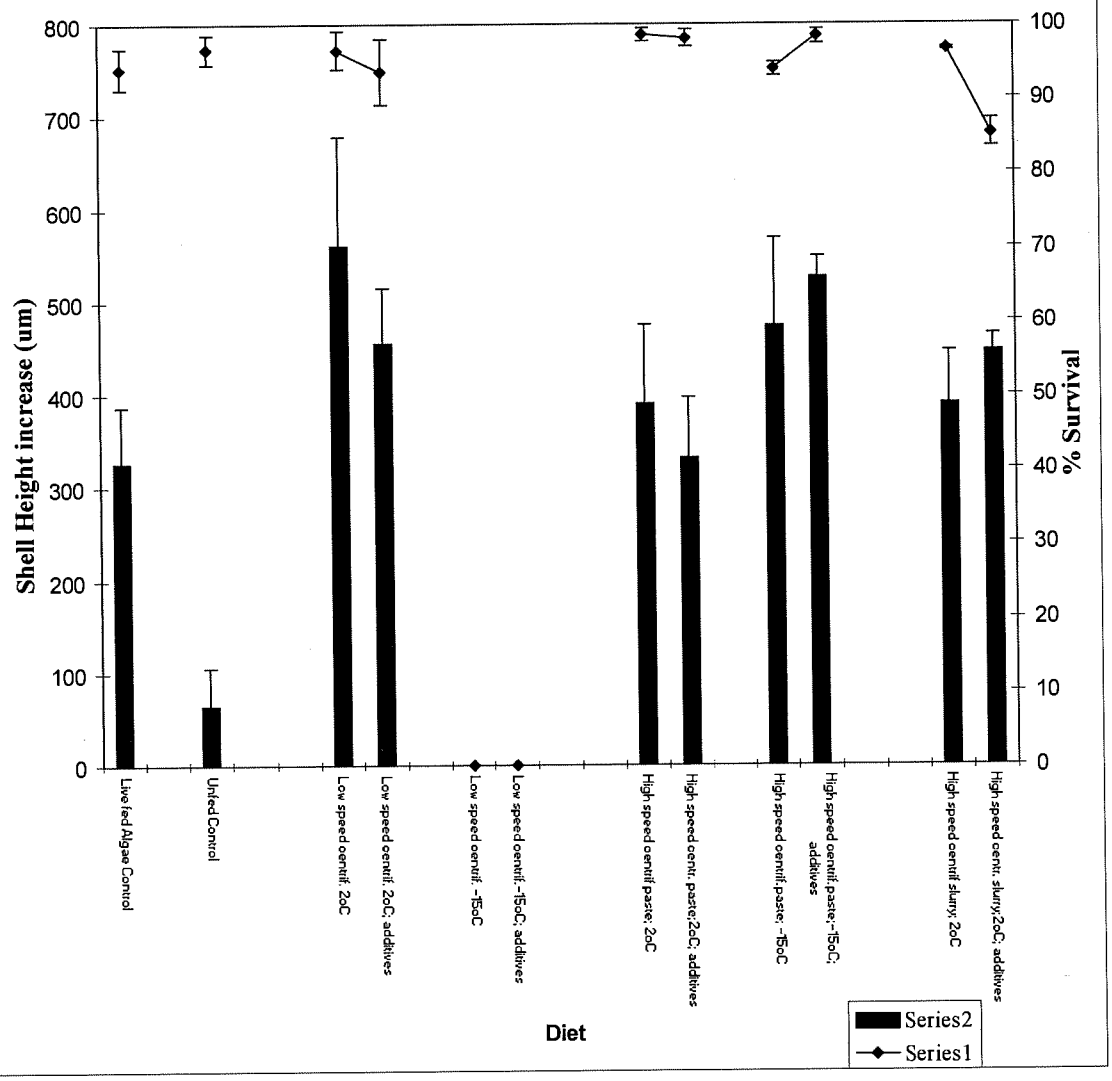


Fig. 7.6.2 Effects of harvest method, storeage temperature and additives on nutritional value of *Tetraselmis chui* concentrate diets as measured by mean (+/-s.e.) growth and survival of juvenile scallops *(Mimachlamys asperrima)*



7.7. Bio-Assay Experiment 7: Evaluation of Optimum Stored Mono-Specific and Binary Diets for Sydney Rock Oyster Larvae.

Introduction and Aims

The seventh and penultimate bio-assay experiment of this project was to evaluate the nutritional value of the 7 to 8 week old concentrates of seven mono-specific micro-algae (*S. glomerata*) diets and all 21 binary combinations thereof for rearing Sydney rock oyster larvae. Concentrates of each micro-algae were produced using species specific harvesting, preservation and storage protocols previously identified as maximising their respective shelf lives and nutritional values.

Materials and Methods

Algae production:
Bulk cultures of all species of micro-algae were grown from axenic inocula. *P. lutheri* and T. *Iso* were produced in 500 L 80 µm clear polyethylene film bags while those of the other five micro-algal species were bulk produced in 1000L cylindrical polyethylene vats. All species of micro-algae were propagated in oceanic water (34 - 35 g/L) settled for 6 to 8 days, filtered to 1 µm (nominal), and disinfected using sodium hypo-chlorite at an effective concentration of 10mg/L for approximately 12 hours. Prior to inoculation, residual chlorine was deactivated using sodium thio-sulphate. Cultures were enriched with f/2 beta growth medium (Guillard, 1983) held at 23 ± 0.5°C with a 16 h : 8 h light : dark cycle. Bag cultures were illuminated with 58 watt 'cool white' fluorescent tube lights to an intensity of 4,000 lux at the bag surface while open top vat cultures were illuminated with metal halide lights to an intensity of 4,000 lux at the surface. Cultures were continuously aerated with 1.5% carbon dioxide enriched air to maintain pH in the range of 8.0 to 8.4.

Preparation of diets:
Concentrates for each of the 7 species of micro-algae were prepared and stored in accordance with criteria provided in Table 7.7.1 to maximize their nutritional quality. The two alternative methods used to concentrate the algae were high and low speed centrifugation. High speed centrifugation was performed as in previous experiments a "Sharples P/L" super-centrifuge at 13,000 g. Algal cultures in mid to late exponential phase were delivered to the centrifuge with a diaphragm pump at a flow rate of approximately 25 L/minute. The algal concentrate was recovered from the super-centrifuge bowl using a plastic spatula. Low speed centrifugation as previously performed involved the use of a "Hitachi " laboratory scale bucket centrifuge operated at 1,330g for 5 minutes. The resultant slurries were recovered by decanting the supernatant. In order to combat culture variability, four separate batch cultures were used to prepare concentrates of each of the 7 species of micro-algae. In the case of *C. muelleri* and *S. costatum* pastes were subsequently diluted to an approximate density of 20g/L to create low density slurries prior to storage. High speed centrifuged pastes of *S. costatum* and *T. chui* that included additives were weighed and additives thoroughly blended in at the following rates:

- glycerol (SIGMA G-7893) at 10% (w/w)
- ascorbic acid (Vitamin C) (SIGMA A-7506) at 1% (w/w)
- citric acid (BD. Prod No. 10081) until pH was lowered to the range 4 - 4.5

All chill-stored concentrates were held at 2.0 ± 0.5°C in 300 mL clear polystyrene containers with a 1:2 ratio of concentrate to air-space and stored under light.

Prior to freezing, *T. chui* paste was blended with additives, left at ambient room temperature for a minimum of half an hour to allow penetration of the glycerol and finally subjected to immersion quick freezing (IQF) by submersing in a stirred 25% NaCl solution held at -15°C. All concentrate diets were maintained at prescribed chilled or frozen temperatures in Fisher and Paykel chest freezers in which temperature was monitored and controlled with a Thermo Eye-P system. These had been held for a period of 42 days at the commencement of feeding and of 50 days at the completion of the experiment.

Table 7.7.1. Optimal methods used to prepare and store concentrates of the 7 species of micro-algae used in this experiment.

Species	Method of Harvesting	Storage density	Additives	Storage Temperature	Light or dark	Source
P. lutheri	Low speed centrifugation	low density (20g/L) slurry	No additives	Chilled +2 .0±0.5° C	Under light	Section 7.2 & 7.3
T. Iso	Low speed centrifugation	low density (20g/L) slurry	No additives	Chilled +2 .0±0.5° C	Under light	Section 7.4
T pseudonana	Low speed centrifugation	low density (20g/L) slurry	No additives	Chilled +2 .0±0.5° C	Under light	Section 7.4
C. muelleri	High speed centrifugation	low density (20g/L) slurry	No additives	Chilled +2 .0±0.5° C	Under light	Section 7.6
S. costatum	High speed centrifugation	low density (20g/L) slurry	All 3 Additives	Chilled +2 .0±0.5° C	Under light	Section 7.5 & 6.4
C. calcitrans	High speed centrifugation	high density paste	No additives	Chilled +2 .0±0.5° C	Dark	Section 7.5
T. chui	High speed centrifugation	high density paste	All 3 Additives	Frozen -15.0±0.5° C	Dark	Section 7.6

Experimental treatments comprised seven optimally prepared and stored mono-specific micro-algae concentrate diets (Table 7.7.1) and all 21 binary combinations thereof for rearing Sydney rock oyster larvae. Two additional reference treatments were included bringing total experimental treatments to 30. The first reference treatment comprised an unfed control and the second an equal mix of *C. calcitrans, P. lutheri,* and T. *Iso* previously shown as optimal for the rearing of oyster (Frankish et al. 1991) larvae and scallop larvae (O'Connor, 1998).

Day one *S. glomerata* D veliger larvae, mean (± s.d.) shell height of 74.21± 3.08 µm (n=50) were used as bio-indicators of diet quality. The larvae were stocked at 2/mL into 90 x 8.5 L plastic aquaria accommodating 3 replicates of 28 concentrate diet treatments plus an unfed control and optimum fresh reference diet comprising equal amounts (on a dry weight basis) of *C. calcitrans, P. lutheri* and T. *Iso*. The three replicates for each of the dietary treatments were randomly distributed throughout a common water bath maintained at a favourable temperature of 24.5 ± 0.5°C (Heasman et al., unpublished data, 1998). The water bath was housed in an air-conditioned room maintained at 23 ± 1°C. All measurements of shell height were made using an Olympus (model CH-2, Tokyo, Japan) stereo microscope fitted with a calibrated eyepiece micrometer.

The larvae in all treatments were fed on an equal dry weight basis rate in accordance with an optimised feeding regimen developed by Frankish et al., 1991 for *S. glomerata* larvae and ratified by O'Connor, 1998 in relation to scallop (*Mimachlamys asperrima)* larvae. Each day small samples of stored concentrates were resuspended, cell densities determined and required volumes for each treatment calculated. Complete water changes using fresh 1µm filter seawater preheated to 24°C was undertaken every second day as was cleaning and disinfection of the plastic aquaria.

The experiment was terminated after 8 days when the larvae were collected and preserved using a 4% formaldehyde in seawater solution. Mean increase in shell height of the larvae was determined by measuring 50 surviving larvae from each replicate at 200X using an Olympus (Model CH-2) compound light microscope fitted with a graticule and a 'Sedgewick Rafter' counting slide. Percentage survival was obtained by counting surviving and dead larvae in each replicate. Live larvae were distinguished from dead by a full compliment of soft tissue free of apparent necrosis and in all cases except the unfed control, by the presence of some food in the gut.

Statistical analyses:
Homogeneity of variance for growth data and arcsine $X^{0.5}$ transformed survival data of *S. glomerata* larvae across the full array of 30 dietary treatments were assessed using Cochran's test. A subsequent one way analysis of variance was used to evaluate within and between group source of variation. Growth data for the 30 diets were compared using Student-Newman-Keuls procedures (Winer et al., 1991).

Results

Homogeneity of variance for growth data of *S. glomerata* larvae across the full array of 30 dietary treatments was confirmed using Cochran's test (P= 0.0674). A one way analysis of variance on these data revealed that between group source of variation that was large and highly significant (P<0.0001).

Growth data for the 30 diets compared using Student-Newman-Keuls procedures (Winer et al., 1991) are provided in Table 7.7.2. As indicated in Table 7.7.2 and Figure 7.7.1 the two best performing mono-specific concentrate diets *P. lutheri, C. calcitrans* yielded growth rates little more than half those of the optimal fresh ternary reference diet while the third best, *S. costatum,* yielded a growth rate only marginally more than one third of optimal. At the opposite end of the performance spectrum was *T. chui* concentrate that yielded a growth rate lower even than the unfed control.

Fortunately, the best performing binary concentrate diets of *P. lutheri* in combination with either *C.calcitrans* or *S. costatum*, (i.e. the best single species diet in combination with either the second or third best) both supported excellent growth rates 85% and 91% respectively those of the optimal fresh reference diet.

Homogeneity of variance for arcsine $X^{0.5}$ transformed survival data of *S. glomerata* larvae across the full array of 30 dietary treatments was confirmed using Cochran's test (P= 0.7654). A one way analysis of variance on these data revealed that between group source of variation that was statistically significant (P<0.048).

Survival data for the 30 diets compared using Student-Newman-Keuls procedures (Winer et al., 1991) are provided in Table 7.7.2 and Figure 7.7.1. The best performing binary concentrate diet of *P. lutheri* in combination with either *C. calcitrans* supported an excellent mean survival rate of 95.9%. This was identical to that of larvae fed the optimum ternary reference diet and substantially higher than the survival rate of 79.6% exhibited by larvae fed the second best binary diet of *P. lutheri* and *S. costatum* concentrates. Poorest rates of survival supported by single species concentrate diets *T. chui* (41%) and T. *Iso* (51%) and by the binary concentrate diets of T. *Iso* in combination with *P.lutheri, T.pseudonana, S. costatum* and *C.calcitrans,* were all lower than that of the unfed control (61%).

Table 7.7.2. Mean growth and survival data of *S. glomerata* larvae.

Dietary Treatment	Mean ±s.e. increase in shell height (µm)		Mean ±s.e. % Survival	
Unfed Control	5.78±1.09	a b c	62.04±8.57	a b
Live Fed Control	30.98±1.45	h	95.72±1.23	b
Mono specific diets				
Pav	17.49±5.33	c d e	70.80±12.67	a b
C.calc	16.99±1.09	b c d e	79.72±7.51	a b
Skel	11.48±2.54	a b c d e	72.99±8.14	a b
T.Iso	8.27±2.05	a b c d	50.57±14.21	a b
C.muell	7.04±1.35	a b c	84.93±3.06	a b
Thal	6.66±2.25	a b	63.56±20.62	a b
T.chui	3.90±2.58	a	41.07±18.20	a
Binary Diets				
Pav & C.calc	28.11±2.76	g h	95.85±2.31	b
Pav & Skel	26.62±0.41	f g h	79.57±0.10	a b
Pav & C.muelleri	19.42±3.94	d e f	67.77±9.46	a b
Pav & Thal	15.64±1.29	a b c d e	68.55±4.93	a b
Pav & T.chui	13.20±1.76	a b c d e	65.61±9.05	a b
Pav & T.Iso	12.58±2.61	a b c d e	58.06±17.79	a b
C.calc & C.muell	16.69±1.01	b c d e	79.18±6.70	a b
C.calc & Skel	12.29±2.59	a b c d e	76.78±10.94	a b
C.calc & Thal	11.90±2.20	a b c d e	55.55±9.04	a b
C.calc & T.Iso	11.16±2.34	a b c d e	64.87±13.04	a b
C.calc & T.chui	10.26±1.99	a b c d e	70.03±12.69	a b
Skel & C.muell	21.23±1.17	e f g	85.71±4.39	a b
Skel & Thal	13.24±1.89	a b c d e	71.88±8.43	a b
Skel & T.Iso	11.92±1.76	a b c d e	53.36±3.86	a b
Skel & T.chui	11.71±3.24	a b c d e	84.70±4.80	a b
T.Iso & C.muell	11.89±1.39	a b c d e	81.18±1.93	a b
T.Iso & T.Chui	9.45±1.20	a b c d	80.43±7.75	a b
T.Iso & Thal	9.43±1.89	a b c d	59.18±18.59	a b
C.muell & T.chui	11.35±1.49	a b c d e	84.68±4.11	a b
C.muell & Thal	10.53±2.26	a b c d e	77.21±7.26	a b
Thal & T.chui	5.28±1.70	a b	65.92±16.61	a b
* Means sharing common letter are not significantly different at P<0.05				

Conclusions and Discussion

Monospecific concentrate diets all failed to support satisfactory growth (shell height increases of 3.9 to 17.5 µm) relative to the optimum fresh ternary control diet (31.5 µm). By contrast binary diets combining the best mono-specific concentrate *P. lutheri* with either the second or third best namely *C. calcitrans* and *S. costatum* supported growth rates of 91 and 86% respectively that of the fresh optimal ternary reference diet.
A scattergram plot of corresponding growth and survival data across all 30 dietary treatments (Fig 7.7.2) and associated regression analysis (Table 7.7.3) illustrate an apparent strong link (correlation) between the two variables. Thus the better the growth rate up to a peak of about 40 µm over the first 8 days of larval life (in this case), the closer survival approached 100 percent.

Table 7.7.3. Regression analysis (linear model: Y=a+bX) growth and survival data of larval oyster (*S. glomerata*) fed optimised binary diets.

Parameter	**Estimate**	**Standard error**	**T Value**	**Probability Level**
Intercept	50.92380	3.659180	13.91670	0.00000
Slope	1.53985	0.240538	6.40171	0.00000

Analysis of Variance					
Source	Sum of Squares	Df	Mean Square	F-Ratio	Probability Level
Model	10975.38200	1	10.975382	40.98	0.00000
Residual	23567.33900	88	267.811000		
Lack-of-fit	22842.45200	84	271.934000	1.50	0.38293
Pure error	724.88730	4	181.221830		
Total: (Corr.)	34542.72100	89			

Correlation Coefficient = 0.563679
R-squared = 31.77%
Standard error of est. = 16.3649

Fig 7.7.1 Effect of optimized mono-specific and binary concentrate diets on the mean +/- s.e. growth and suvival of oyster (*S. crassostrea*) larvae

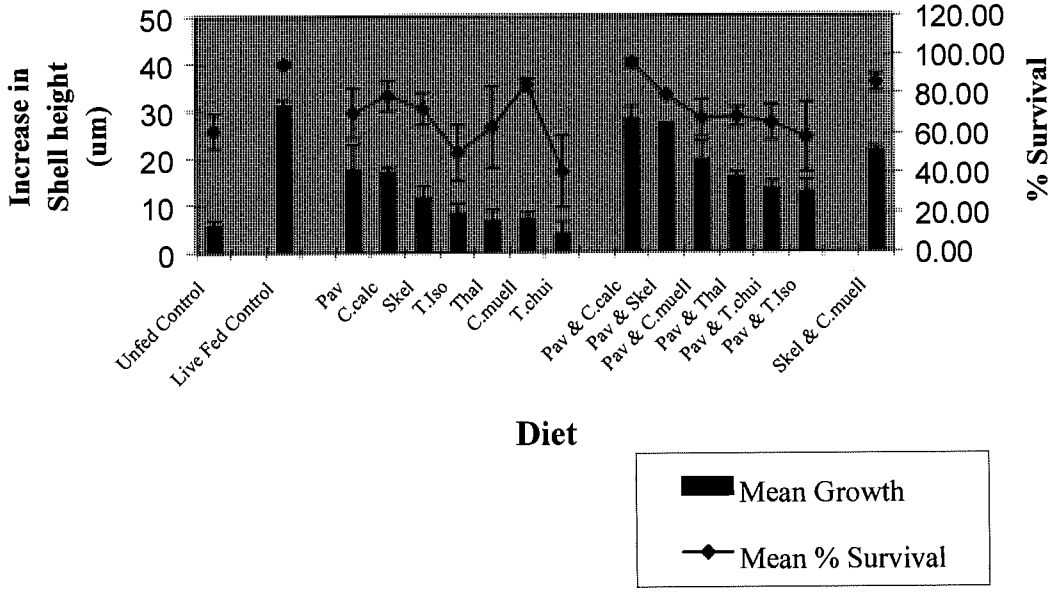
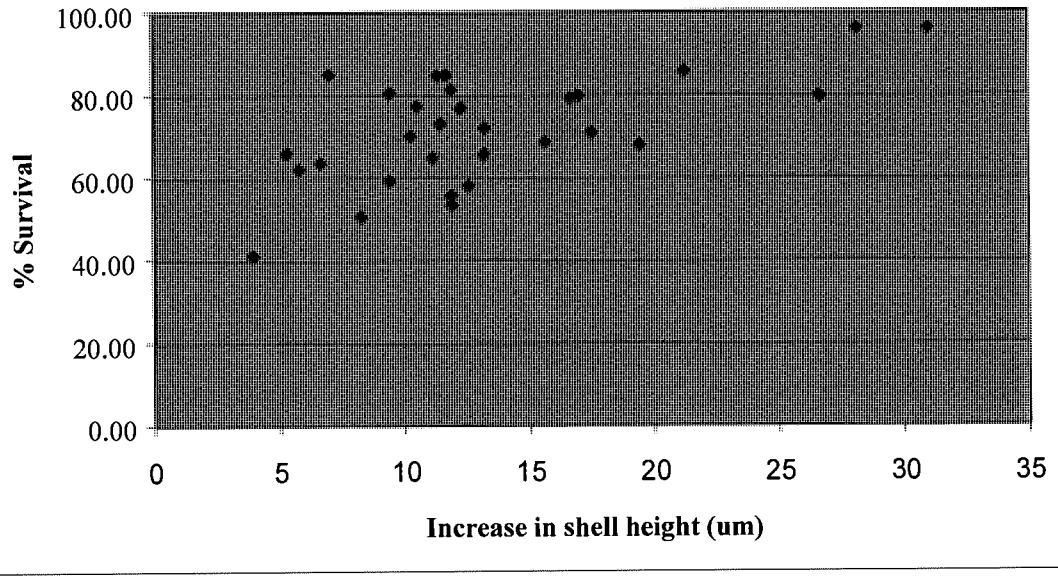


Fig 7.7.2 Scattergram plot of growth and survival data of SRO larvae fed single and binary diets



7.8. Bio-Assay Experiment 8: Evaluation of Optimally Harvested and Stored Binary Diets using Juvenile Sydney Rock Oyster (*Saccostrea glomerata*).

Introduction and Aims

This final bio-assay experiment of the project, a companion to Bioassay Experiment 7, was to evaluate the nutritional value of the 7 to 9 week old concentrates of 7 mono-specific micro-algae concentrate diets and all 21 binary combinations thereof for rearing Sydney rock oyster (*S. glomerata*) spat. Concentrates of each micro-algae were produced using species specific harvesting, preservation and storage protocols previously identified as maximising their respective shelf lives and nutritional values.

Materials and Methods

Algae production and preparation of experimental diets:
Techniques of algal production and preparation of diets were identical to those described for the preceding companion experiment Bio-assay Experiment 7. The 30 experimental treatments (Table 7.7.2) comprised 7 optimally prepared and stored mono-specific micro-algae concentrate diets (Table 7.7.1), all 21 binary combinations of the mono-specific diets and two additional reference treatments. The latter comprised an unfed control and a ternary reference diet made up of an equal mix (on a dry cell weight basis) of *C. calcitrans, P. lutheri,* and T. *Iso* previously shown as optimal for the rearing of oyster larvae (Frankish et al. 1991) and scallop larvae (O'Connor and Heasman, 1997).

Acquisition, husbandry and measurement of spat:
At the commencement of the experiment, 90 miniature up-wellers to accommodate 3 replicates of the 30 dietary treatments were individually accommodated within 8.5L plastic aquaria. The latter, housed within a common temperature bath maintained at 25.0±1.0 °C, were stocked with 30 hatchery reared juvenile Sydney rock oysters of an initial mean (± s.d.) ash-free dry weight of 4.03± 0.33mg (n=90). The experiment was run for 16 days by which live weight of spat fed the reference fresh ternary control diet had increased to 15.57±1.98 mg, i.e. by a factor of almost four. Ash free dry weight was determined by difference between dry weight (after oven drying to constant weight at 100°C) and ash weight (after ashing at 475 °C in a muffle furnace for 5 hours).

The spat in all treatments were fed to allow satiation feeding by adding either fresh or resuspended chilled or frozen concentrate cells at an initial dry weight equivalent of 30 000 Tahitian *Isochryis* cells/ml/day. This rate was gradually increased up to 80,000 cells/ml/day in line with daily monitoring of residual cell concentrations in the fresh algae control diet immediately prior to daily additions of new feed. Residual (=minimum) cell concentrations were maintained at >15 000 cells/ml. Each day small samples of stored concentrate diets were resuspended, counted, and volume to feed/bucket calculated and feed administered. Complete water changes with fresh 1 µm filter seawater preheated plus cleaning of plastic aquaria was conducted three times a week.

The experiment was terminated after 16 days. Total collective live-weight of the spat, measured to ±0.1mg with a Mettler model PB303 (Switzerland), electronic balance. This procedure entailed blot and evaporative air drying in an air-conditioned room at 25°C separating out and counting dead spat and repeat weighing the remaining live spat until constant weight was achieved. Once dry, the gaping valves of dead spat readily distinguished them from their live counterparts. Percentage survival was obtained by counting all surviving and dead spat in each replicate.

Statistical analyses:
Homogeneity of variance for data of live-weight and ash free dry weight increments and of survival across the full array of 30 dietary treatments were assessed using Cochran's test. Once homogeneity of variance had been demonstrated (requiring log transformation in the case of live-weight data and arcsine $X^{0.5}$ transformation in the case of survival data), one way analysis of variance was used to evaluate within and between group source of variation for each of these three sets of data. Finally, multi range analysis using Student-Newman-Keuls procedures (Winer et al., 1991) were used to compare mean live weight and ash free dry weight gains and to compare mean survival rates achieved with each of the 30 dietary treatments.

Results

Growth:
Homogeneity of variance for log transformed live-weight increment data and for ash free dry weight increment data of *S. glomerata* spat across the full array of 30 dietary treatments was confirmed using Cochran's test (P= 0.2277 and 0.2061 respectively). One way analysis of variance (Table 's 7.8.2a & b) on these same sets of data revealed that between group source of variation that was large and highly significant (P<0.001for live-weight and P<0.00001 for ash free dry-weight).

Mean live-weight increments and ash free dry weight increments of *S. glomerata* spat across the full array of 30 dietary treatments were compared using Student-Newman-Keuls procedures (Winer et al., 1991). Results are summarized in Table 7.8.3 and Figure 7.8.1.

Although the relative performance of the diets as measured by gains in both live weight and in ash-free dry weight (organic matter) were very similar, ash free dry weight, that measures increased organic matter, is clearly the more appropriate measure of growth and hence the nutrition value of food responsible for that growth. Growth performance on the mono-specific concentrate diets, as measured by increased ash free dry weight followed the sequence *T. chui.* > *T.pseudonana* > *S. costatum*, *P. lutheri, T. Iso* and *C.calcitrans* and *C. muelleri.*

T. chui was the outstanding performer as a mono-specific diet and also as a binary diet but only when combined with second and third best mono-specific diet species namely *T. pseudonana* and *S. costatum*. Dry weight increase achieved on a mono-specific diet of *T. chui* was 88% that of the fresh ternary control diet while a binary diet of *T. chui* and *T pseudonana* yielded a growth rate equal (103%) to that achieved on the fresh ternary control diet. Combining *T. chui* with the other four concentrates (*T. Iso, P. lutheri, C. calcitrans* and *C. meulleri* either had nil or a negative effect on growth. This was also true of all other binary diets except for *C. muelleri* (the worst performing mono-specific concentrate diet) which exhibited greatly improved performance when combined with either *P. lutheri* or *T. Iso* (both of which also performed poorly as mono-specific diets).

Table 7.8.1. Mean growth and survival data of *S. glomerata* spat.

Treatment	Mean ± s.e. live-weight gain (mg)	Mean ± s.e ash -free dry weight gain (mg)	Mean ± s.e % survival
Unfed Control	5.03±0.24 a	0.33±0.11a b	93.33±3.33 a
Live Fed Control	15.57±1.90 i j	0.74±0.09d e	96.67±1.92 a
Mono-specific Concentrate Diets			
T. chui	13.18±0.56 f g h i	0.65±0.11 b c d e	100.00±0.00 a
Thal	10.77±1.20d e f g	0.49±0.08 a b c d e	97.78±1.11 a
Skel	9.81±0.19c d e f	0.41±0.01 a b c d	92.22±2.94 a
Pav	9.69±0.82c d e	0.42±0.00 a b c d	96.67±1.92 a
T.Iso	7.84±0.24b c	0.43±0.05 a b c d	97.78±1.11 a
C.calc	7.29±0.52b	0.42±0.09 a b c d	94.44±4.01 a
C.muell	7.16±0.32b	0.36±0.02 a b	96.67±3.33 a
Binary Concentrate Diets			
T.chui & Thal	17.05±0.38 j	0.75±0.02 e	91.11±2.94 a
T.chui & Skel	16.50±0.99 i j	0.70±0.09 c d e	94.44±1.11 a
T.Chui & T.Iso	14.33±0.28 h i j	0.65±0.10 b c d e	97.78±2.22 a
T.chui & Pav	13.21±0.51 f g h i j	0.62±0.10 a b c d e	93.33±1.92 a
T.chui & C.calc	12.61±1.07 e f g h i	0.62± 0.10 a b c d e	96.67±1.92 a
T.chui & C.muell	13.38±0.12 g h i j	0.59±0.01 a b c d e	95.56±4.44 a
Thal & Pav	11.36±1.02 d e f g h	0.47±0.05 a b c d e	93.33±1.92 a
Thal & C.muell	11.06±0.99 d e f g h	0.41±0.05 a b c d	97.78±1.11 a
Thal & T.Iso	10.76±0.51 d e f g	0.40±0.03 a b c d	93.33±1.92 a
Thal & Skel	9.83±0.24 c d e f	0.40±0.02 a b c d	96.67±1.92 a
Thal & C.calc	10.05±0.40 c d e f	0.38±0.03 a b c	97.78±2.22 a
Skel & C.muell	11.24±0.72 d e f g h	0.56±0.04 a b c d e	97.78±2.22 a
Skel. & T.Iso	9.15±0.70 b c d	0.35±0.01 a b	93.33±5.09 a
Skel & C.calc	8.70±0.49 b c d	0.33±0.02 a b	96.67±1.92 a
Skel & Pav	10.03±0.96 c d e f	0.30±0.10 a	95.56±1.11 a
Pav & C.muell	10.70±0.23 d e f g	0.65±0.16 a b c d e	93.33±1.92 a
Pav & T.Iso	9.70±0.23 c d e	0.43±0.01 a b c d	85.56±2.94 a
Pav & C.calc	8.47±0.44 b c d	0.40±0.05 a b c	93.33±1.92 a
T.Iso & C.muell	9.97±0.29 c d e f	0.57±0.07 a b c	95.56±2.22 a
T.Iso & C.calc	7.40±0.15 b	0.35±0.01 a b	92.22±1.11 a
C.calc & C.muell	13.37±0.12 b e d	0.39±0.00 a b c	95.56±4.43 a
* Means sharing common letter are not significantly different at P<0.05			

Survival:
Homogeneity of variance for arcsine $X^{0.5}$ transformed percent survival data of *S. glomerata* spat across the full array of 30 dietary treatments was confirmed using Cochran's test (P= 0.2823). One way analysis of variance (Table 7.8.3) of these data however revealed that between group source of variation was small and not significant (P=0.340).

Mean arcsine $X^{0.5}$ transformed percent survival data *S. glomerata* spat across the full array of 30 dietary treatments were compared using Student-Newman-Keuls procedures (Winer et al., 1991) with the anticipated result that there were no significant differences in survival between any two diets. Results are summarized in Table 7.8.1. and Figure 7.8.1. Uniformly high rates of survival in the range 92 to 100 % occurred across 29 of dietary treatments including the unfed control. The only notable departure from this pattern was a lower survival rate of 86% recorded for spat fed the *P. lutheri* and *T. Iso* binary diet but as already stated, even this did not differ significantly (at P<0.05) from that of any other diet.

Conclusions and Discussion

Results of this experiment are encouraging in that best growth and survival was achieved with *T. chui*, a very vigorous and relatively easy to propagate species shown in a companion research project (Borowitzka et al., 1998) to be well suited to efficient intensive production in outdoor tubular bio-reactors operating under ambient conditions in southern Australia (Perth). An additional advantage of *T.chui* as a stored concentrate diet, is that it performs best after being harvested to a stiff paste by high speed centrifugation and stored in the simplest and most practical method assessed, namely held frozen at a common domestic freezer temperature of -15°C.

Results of the present study are compared in Table 7.8.4 with equivalent results for *S. glomerata* spat fed the same array of micro-algae diets but as fresh cultures rather than as stored concentrates by O'Connor et al. (1992). The degree to which the nutritional value of micro-algae is altered by the combined effects of centrifugation and cold storage is highly species specific. While cold stored concentrates of 6 of the 7 mono-specific diets compared in table 7.8.4 suffered loss of nutritional value, *T. chui* exhibited an opposite response of enhanced nutritional value both after short term (1 to 2 weeks) and protracted (7 to 9 weeks) storage.

Fresh binary micro-algae diets were also generally superior to their cold stored concentrate counterparts. A very important exception was the binary diet that combined high speed centrifuged *T. chui* stored as frozen paste with high speed centrifuged *T. psuedonana* stored as a chilled slurry. The performance of this diet not only matched the reference fresh ternary diet but also rivalled the three fresh diatom binary diets identified by O'Connor et al. (1992) as those best for culturing *S. glomerata* spat.

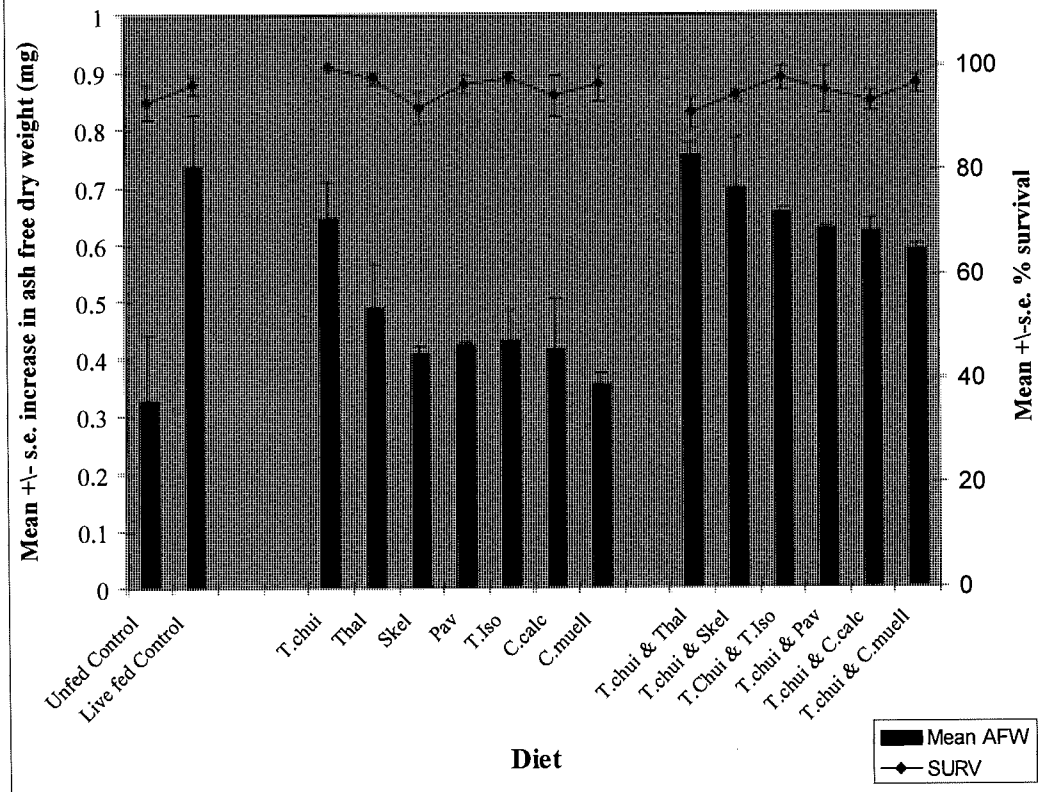
Brown et al. (1998) demonstrated that flocculated concentrates of several species of diatom could be cost effectively used to enhance the growth of oyster (*Crassostrea gigas*) spat reared at high density in field nursery upwellers supplied with estuarine phyto-plankton as their only source of food. Results of this experiment suggest an equivalent future role for frozen *T.chui* paste as a cheap dietary supplement.

Table 7.8.2 Comparative growth data for Sydney rock oyster spat reared on equivalent fresh and cold stored concentrate mono-specific and binary diet.

Algal Species	**Algae diets fed as centrifuged concentrates cold stored for 7 to 9 weeks (This Study)**	**Algae diets fed as fresh cultures Fresh cultures (O'Connor et al.1992)**
Mono-specific diets	*Initial spat weight: 4.0mg, Duration: 16 days Temperature 24±1℃*	*Initial spat weight: 9.5mg Duration: 21 days Temperature 25±1℃*
Tetraselmis chui	(1) 17.2mg ≡ 9.5% per day	(6) 42.7mg ≡7.4% per day
Thalassiosira pseudonana	(2) 14.8mg ≡ 8.4% per day	(4) 49.6mg ≡ 8.2% per day
Pavlova lutheri	(3) 13.7mg ≡ 8.0% per day	(7) 39.8mg ≡ 7.0% per day
Skeletonema costatum	(4) 13.8mg ≡ 8.0% per day	(1) 61.1mg ≡ 9.3% per day
Tahitian *Isochrysis*	(5) 11.8mg ≡ 7.0% per day	(5) 46.8mg ≡ 7.9% per day
Chaetoceros calcitrans	(6) 11.3mg ≡ 6.7% per day	(2) 57.9mg ≡ 9.0% per day
Chaetoceros muelleri(=gracilis)	(7) 11.2mg ≡ 6.7% per day	(3) 51.1mg ≡ 8.3% per day
Binary Diets	*Initial spat weight: 4.0mg Duration: 16 days*	*Initial spat weight: 1.9mg Duration: 19 days*
S costatum + T. chui	(1)20.5mg ≡ 10.7%per day	(2)16.2mg ≡ 12.0% per day
S costatum + C. muelleri(=gracilis)	(2)15.3mg ≡ 8.8% per day	(1)19.8mg ≡ 13.1% per day
S costatum + P. lutheri	(3)14.3mg ≡ 8.1% per day	(4)13.1mg ≡ 10.7 % per day
S costatum + T. pseudonana	(4)13.8mg ≡ 8.0% per day	(5)12.6mg ≡ 10.5% per day
S costatum + T *Iso*	(5)13.2mg ≡ 7.8% per day	(3)15.3mg ≡ 11.6% per day
S costatum + C. calcitrans	(6)12.7mg ≡ 7.5 % per day	(6)11.9mg ≡ 10.1% per day
T. chui + T.pseudonana (best diet)	21.4mg ≡ 11.0 % per day	NA

N.B. Results for each diet are expressed as rank (in parentheses); final weight and equivalent instantaneous growth rate.

Fig 7.8.1 Effects of optimum mono-specific and binary concentrate diets on the growth and survival of Sydney rock oyster (*S. glomerata*) spat



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9. BENEFITS

Results of this project are being currently being commercialized by NSW fisheries through the regular supply of alga concentrates for oyster and finfish. In 1998/99 concentrates extracted from 43 000L of algae culture were supplied to private hatcheries and a further 20,000 L used for successful quarantine hatchery production of selected breeding lines of Sydney rock oysters at Port Hacking in summer 1998 and again in 1999.

Results of recent collaborative research led by Dr Frances D'Souza at the CSIRO Marine Research Laboratories in Cleveland, Queensland suggest that use cold stored concentrates in emergencies or for partial substitution of live algae by could be adopted in the near future. Like-wise the successful demonstration by McCausland et al, (1999) of the use of algae concentrate supplements to enhance the output at one of Australia's largest commercial oyster spat nurseries and the very encouraging results achieved in this study, particularly with simple low cost concentrates *Tetraselmis chui*, point towards rapid commercial adoption of this technology once centralized large scale production is established.

One unforeseen benefit of this project has been the supply of algae pastes for small scale production and maintenance of a range of marine invertebrates required for unrelated research being conducted by a number of universities and government research organizations elsewhere in NSW, Tasmania the Northern Territory.

Surveys of the use of micro-algae in Australian aquaculture have been conducted by Brown et al., (1989) and more recently by DOSAQUA on behalf of Western Biotechnology Ltd. (WBL) in 1993 revealed that on average, 30 to 40% (max. 70%) of hatchery costs can be attributed to micro-algae culture. Successful commercialization of the technology developed in this project over the next few years will help reduce risks and high costs associated with small scale often remote hatcheries and will thereby contribute to the continuing rapid growth and diversification of marine aquaculture in this country.

Technology developed in this project meet all original targets, namely methods of producing algae concentrates that:

- can be reconstituted into low density suspensions of single, neutrally or very slightly negatively buoyant cells in sea-water under low turbulence culture conditions used to rear delicate marine larvae;
- have cells of a nutritional quality equal or close to that of the original fresh cultures;
- yield cells that can be to be efficiently filtered from suspension, ingested, digested and assimilated by the animals being cultured;
- have a minimum practical shelf life 4 to 6 weeks under storage temperature regimens that fall within the normal operating capability of domestic or industrial freezers and chillers (refrigerators) readily available throughout Australasia;
- appropriate packaging and shipping methods that ensure that end users reliably receive a cost-effective and nutritionally adequate product.

10. FURTHER DEVELOPMENT

The next step to ensure that the above benefits of this project are realised is to continue the expanding demand for centrally produced quality assured concentrates by private sector hatcheries and nurseries. This in turn will depend on an expanded production capacity to match increased demand and provision of adequate advisory and consultation service to ensure trouble free application. NSW Fisheries will assume interim responsibility for this role as part of a wider program of enhanced oyster industry technology transfer. The latter is being implemented to ensure the flow on of benefits of a long standing oyster genetics program to arm the ailing Sydney rock oyster farming industry with faster growing more disease resistant stock.

Other relevant and important issues to further development of this technology are discussed in Section 12.1 on Intellectual Property.

11. CONCLUSIONS

Results of bioassay experiments with cold stored concentrates of seven species of micro-algae showed that the influence of three common food additives, the anti-oxidant ascorbic acid, a food acid (citric acid) and the cryo-protectant glycerol,are highly species specific. In general the nutritional value of micro-algae concentrates suffering significant cell damage during centrifugation such as *P. lutheri,* T. *Iso* and *T. psuedonana* are further degraded rather than enhanced by these additives. By contrast those species of micro-algae suffering little or no observable cell damage during centrifugation such as *T. chui* and *S. costatum*, benefit from the use of additives used singly or in combination.

Harvesting technique, temperature and density of storage, mode of feeding as well as the use of additives, all have a major and significant effect on the nutritional value of micro-algae concentrates used to feed larval and juvenile bivalves. The relative importance of the three former factors follows the sequence harvesting technique > method of storage > feeding frequency.

Slow speed (bucket) centrifuged concentrates of the fragile celled algae *P. lutheri*, when stored chilled at 2.0 ± 0. 5°C for up to 6 weeks and administered either as single or multiple daily feeds, support good rates growth and survival of oyster larvae relative to fresh *P. lutheri* cultures. However, the benefits of slow speed centrifugation are negated by subsequent frozen storage of concentrates with or without the use of cryo-protectants.

As with *P. lutheri*, low speed centrifugation was found far superior to high speed centrifugation for harvesting concentrates of other fragile celled species T. *Iso* and *T. pseudonana* used to feed bivalve larvae. The best stored *T. pseudonana* concentrate diet developed in this study actually outperformed its fresh algae culture counterpart and therefore appeared to be a good candidate for replacing fresh micro-algae culture diets used to rear bivalve larvae. In the case of T. *Iso* however growth of oyster larvae fed the best performing of 10 variously harvested and stored concentrates, was only half that of the corresponding fresh T. *Iso* control diet and therefore unsatisfactory.

Chitosan flocculation, an alternative less traumatic technique to centrifugation for harvesting of fragile celled micro-algae species such as *P. lutheri,* T. *Iso* and *T. pseudonana*, was shown to have a catastrophic effect on the growth and survival of bivalve larvae. As chitosan is not documented as being toxic to invertebrate larvae, the negative effects of chitosan flocculation may have arisen through adverse ancillary factors such as the promotion of high bacterial loads or interference to normal ingestion or digestion of food by the oyster larvae.

In stark contrast to *P. lutheri*, T. *Iso,* and *T. pseudonana*, concentrates of the tougher celled species *C. calcitrans*, *S. costatum* and *T chui* harvested by high speed centrifugation support significantly better rates of growth and survival of oyster larvae than their low speed centrifuged counterparts. This occurs regardless of whether the concentrates are stored chilled or frozen and regardless of the inclusion of additives. Great benefits of high speed over slow speed centrifugation are however negated by freezing and to a lesser extent by inclusion of additives.
Growth rates of juvenile bivalves supported by eight variously harvested and stored forms of *T.chui* concentrate, equaled or exceeded (range 102 to 172%) that of fresh *C.chui* cultures. The improved performance of *T. chui* concentrates over their fresh counterpart may be ascribed to loss of motility and increased digestibility.
By contrast, growth rates of juvenile bivalves supported by the same array of centrifuged concentrates of *C. muelleri* were all considerably inferior (range 28 to 72%) to that of a fresh culture of *C. muelleri.* The best performing *C. muelleri* concentrate diet (72% the growth rate of

the fresh control diet) was the high speed centrifuged paste diluted to a slurry with additives and chill stored.

None of seven mono-specific concentrate diets for which optimal methods of harvesting and storage were developed during this study were able to support satisfactory rates of growth or survival of bivalve larvae after 6 to 7 weeks of storage. Fortunately however, 2 of 21 binary diets combining the best of these 7 optimally harvested and stored mono-specific concentrate diets (*P. lutheri)* with either the second or third best (*C. calcitrans* or *S. costatum,* supported satisfactory growth rates 91 and 86% respectively that of an optimal ternary reference diet.

A mono-specific diet of *T.chui* paste harvested by high speed centrifugation and stored frozen with additives at-15°C in a domestic freezer for 7 to 9 weeks supported good rates of growth and survival of juvenile oysters. A particular advantage of *T. chui* is its vigor and relative ease of propagation. As shown in a companion research project (Borowitzka et al., 1998), *C. chui* is well suited to efficient intensive production in outdoor tubular bio-reactors operating under ambient conditions in southern Australia (Perth). This result suggests an important future role for frozen *T.chui* paste as a cheap highly practical dietary supplement to enhance the growth of bivalve spat reared at high density in field up-weller nurseries.

A binary concentrate diet combining an equal mix of high speed centrifuged *T. chui* stored as frozen paste with high speed centrifuged *T psuedonana* stored as a chilled slurry is able to support growth and survival of juvenile bivalves rivaling that of three fresh diatom binary diets previously identified as optimum for this application.

Of the seven species of micro-algae investigated, satisfactory methods of harvesting and preserving alga concentrates with a minimum useful shelf life of 6 weeks as food for larval or juvenile bivalves were not achieved with either T. *Iso* or *C. muelleri.*

In contrast to the disappointing results achieved with *T. Iso* and *C. muelleri* in this study, results attained with samples of the same optimally harvested and preserved concentrates of *C. muelleri,* and T. *Iso* sent to the CSIRO for evaluation as diets for larval penaeid prawns (*Penaeus monodon)* by (D'Sousa et al., In prep), were very encouraging. Further testing of these and additional binary concentrate diets as partial or complete substitutes for fresh algae used to rear larval prawns are therefore warranted. Preliminary laboratory trials conducted by the CSIRO (D'Souza et al., in prep.) have also shown that these same centrifuged concentrates may be as effective as fresh algae when fed in conjunction with artificial micro-particulate diets.

12. APPENDICES

12.1. Intellectual Property

None of the technology for extended shelf life concentrates of micro-algae developed during this study is considered patentable. The technology is nevertheless hard won in terms of time and cost of development and would convey considerable competitive advantage if it were to remain exclusive to a particular producer of such products.

Mid to long-term indirect benefits of freely disseminating this technology are wider-spread cost and risk reduction and enhanced output volume and diversity from marine hatcheries in Australasia. On the other hand some immediate cost recovery for this project could be gained by retaining and selling the rights of the technology to a particular client(s), for example "Western Biotechnology P/L" or an alternative marine aquaculture enterprise with established marine micro-algae expertise.

Another related issue is that of the need and scope for ongoing refinement of this technology. As discussed in the conclusions (Section 11) encouraging results were attained with samples of optimally harvested and preserved concentrates of *C. muelleri,* and T. *Iso* sent to a collaborating team led by Dr Frances D'Souza, at the CSIRO Marine Laboratories, Cleveland, Queensland for feeding larval penaeid prawns.

Preliminary laboratory trials conducted by the same CSIRO team have also shown that these same centrifuged concentrates may be as effective as fresh algae when fed in conjunction with artificial micro-particulate diets. Further testing of algae concentrate diets as partial or complete substitutes for fresh algae and artificial diets for larval prawns are therefore warranted.

There also appears good scope for amalgamating successful micro-algae flocculation techniques recently developed by CSIRO (FRDC Project 94/083) with shelf life extending technology developed in the current project.

These important issues can only be resolved by negotiation and consultation between the three stake holders in this project namely, FRDC, Western Biotechnology Pty Ltd and NSW Fisheries.

12.2. Staff

The following members of staff were employed on the project:

Dr Michael Heasman	Principal Investigator
Dr Wayne O'Connor	Scientific Officer
Mr John Diemar	Technical Officer
Ms Lynne Foulkes	Technical Officer
Ms Tanya Sushames	Technical Officer
Mr David Keith	Technical Officer
Mr Brett Fitzhenry	Technical Officer (Temporary)

12.3. Publications

O'Connor, W.A., Heasman, M.P., O'Connor, S.J., 2000. Algal diets for broodstock maintenance of the doughboy scallop *Mimachlamys asperrima* (Lamarck). Aquaculture Research 31, 627-635.

Heasman, M., Diemar, J., O'Connor, W., Sushames, T., Foulkes, L., 2000. Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs – a summary. Aquaculture Research 31, 637-659.

D'Souza, F.M.L., Lecossois, D., Heasman, M.P., Diemar, J.A., Jackson, C.J., Pendrey, R.C., 2000. Evaluation of centrifuged microalgae concentrates as diets for *Penaeus monodon* Fabricius larvae. Aquaculture Research 31, 661-670.

[Copies of papers following]

Aquaculture Research, 2000, 31, 627–635

Algal diets for broodstock maintenance of the doughboy scallop *Mimachlamys asperrima* (Lamarck)

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Abstract

The effect of monospecific algal diets on filtration rate (algal cells h^{-1}) and fecundity of *Mimachlamys asperrima* was investigated. Filtration rates of seven algal species were monitored to indicate species preferences and to estimate maximum daily filtration rates. Cells of *Chaetoceros calcitrans* and *Pavlova lutheri* were filtered most rapidly; however, on a cell weight (mg h^{-1}) and cell volume (μL h^{-1}) basis, scallops filtered more *Rhodomonas salina* and *Tetraselmis chui* from the water column. Filtration rates when fed the diatom *Chaetoceros muelleri* were the lowest of the tested species, with a relatively low weight and volume of the algae filtered. Maximum filtration rates of the tested species were estimated to vary between 2.25×10^9 and 7.68×10^9 cells scallop^{-1} day^{-1}. Filtration of algal species by *M. asperrima* varied in accordance with both scallop size and water temperature. Five of the seven algal species previously tested were then selected for use as monoalgal diets for female *M. asperrima* and fed for 4 weeks. Fecundity of scallops after this treatment did not necessarily reflect filtration rates, being greatest for scallops fed *C. muelleri*, which was significantly greater than that of the scallops fed *C. calcitrans*. Percentage development of eggs to D-veliger larvae did not differ in accordance with the maternal diet. A combined diet of *C. muelleri*, *P. lutheri* and Tahitian *Isochrysis* aff. *galbana* averaging $\approx 2.5 \times 10^9$ cells scallop^{-1} day^{-1} was found to be suitable for the maintenance and conditioning of *M. asperrima* broodstock in recirculating systems.

Introduction

Scallop reproductive condition is a genetic response to the environment (Sastry 1979) in which exogenous factors, such as food, temperature and light, interact with endogenous factors, such as neuronal and hormonal actions (Barber & Blake 1991). Through the manipulation of exogenous factors, protocols have been established that can either initiate oogenesis or enhance broodstock reproductive condition (Bourne, Hodgson & Whyte 1989; Devauchelle & Mignant 1991; Heasman, O'Connor & Frazer 1996). Many protocols include dietary regimens that recommend types and quantities of algae that should be used. Such regimens, however, are rarely supported by relevant research concerning the value of different diets in broodstock conditioning.

The relationship between recently ingested food, stored energy reserves and gonadal development is poorly understood (Barber & Blake 1991; Devauchelle & Mignant 1991) and can vary within scallop species (Sastry 1970). The composition of the gonad can be greatly influenced by diet (Soudant, Marty, Moal, Robert, Quéré, Le Coz & Samain 1996), but the proximate composition of gonads is not altered, and neither are the relative compositions of certain fatty acids assumed to be metabolically important (Soudant *et al.* 1996). As a result of these and other palatability and digestibility factors, it is not yet possible to select a diet on the basis of chemical composition.

An alternative basis used in some hatcheries for selecting broodstock diets is algal preferences. This

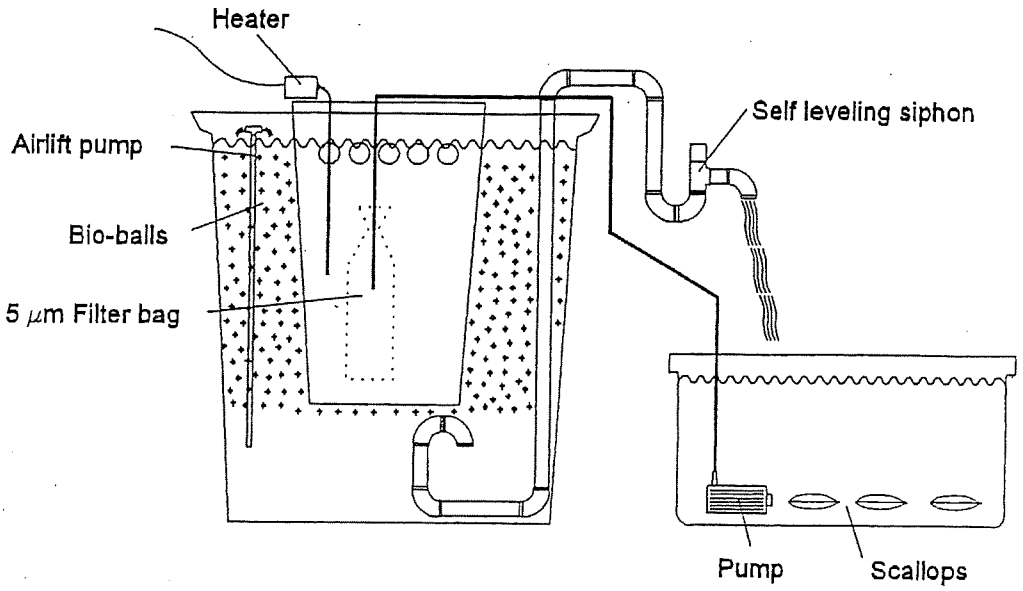

Figure 1 Scallop conditioning system with biofiltration reservoir (after Heasman, O'Connor & Frazer 1996).

assumes that particle selection can occur on a physiological basis, that of food quality (Jørgensen 1996), with those algal species rapidly filtered from the water being good diets. Scallops do regulate the amount of material ingested and discriminate among algal species with cells of similar size (Shumway, Cucci, Gainey & Yentsch 1985; Shumway, Cucci, Lesser, Bourne & Bunting 1991), although filtration is influenced by a number of other factors. Food quantity and physical and chemical factors in the environment (Ward & Cassell 1991), algal metabolites (Epifanio 1983; Ward & Cassell 1991), algal cellular structure (Epifanio 1979; Peirson 1983; Le Pennec & Rangel-Davalos 1985) and water temperature (Kirby-Smith 1970; McLusky 1973; Heasman *et al.* 1996) influence particle uptake; however, particle selection by bivalves on the basis of food quality has not been clearly established (Jørgensen 1996).

In the case of the doughboy scallop *Mimachlamys asperrima* Lamarck, the use of embryos in ecotoxicological studies (Krassoi, Everett & Anderson 1996) and in aquaculture research requires broodstock to be readily available and has encouraged the development of protocols for broodstock maintenance. Developing such protocols has been aided by the construction of conditioning systems for *Pecten fumatus* Reeve broodstock (see Heasman *et al.* 1996). These systems (Fig. 1), held at 14–15 °C (to suit *P. fumatus*), appear to be suitable for maintaining *M. asperrima* reproductive condition, although feed rates used for the larger *P. fumatus* broodstock are excessive (W O'Connor, pers. obs.). This study was conducted to investigate the relationship between filtration rate and fecundity, and to determine whether algal species preferences were indicative of the value of different algal species as broodstock diets. This information was used to develop dietary regimens for use in conjunction with recirculating systems.

Materials and methods

All *M. asperrima* used in these trials were collected from wild populations at Murrays Beach, Jervis Bay, NSW, Australia. Scallops were placed in an aerated 500-L fish transporter and transferred by road to the laboratory (≈ 6 h) at Port Stephens Research Centre (PSRC). Spawnings were conducted in 1-L beakers and were induced using a total of 0.05 mL of a 10^{-3}M solution of serotonin (creatinine sulphate complex; Merck, Darmstadt, Germany) injected into the gonad (O'Connor & Heasman 1995). Each serotonin dose was divided among three areas of the gonad (proximal, middle and distal) to avoid localized egg release at the injection site. When individual scallops spawned, they were moved to successive containers of sea water at

intervals of ≈15 min and the eggs retained for counting. A scallop was considered to have failed to spawn if gametes had not been released within 2 h of the time of injection. Spawning was deemed to have ceased if no new releases of gametes had occurred over a period of 30 min. Egg numbers were determined from four replicate 1-mL samples of egg suspension, each counted using a Sedgwick–Rafter slide at 40 × magnification.

Temperature and algal species effects on filtration

Batches of eight randomly sampled *M. asperrima* (shell height 45–70 mm) were maintained at one of four temperatures, 14, 16, 18 or 20 °C (± 0.5 °C) for 1 week before experimentation and fed to satiation. On the day before the experiment, each scallop was placed in individual aerated white, plastic 8-L aquaria. Aeration was used to both simulate the conditions experienced in the broodstock conditioning systems and ensure that algal cells remained in suspension during experimentation. White aquaria had been adopted in earlier trials, so that any significant algal settlement was apparent.

Approximately 1 h before each trial, the sea water in each aquaria was exchanged with temperature-equilibrated, 1-μm-filtered sea water (35 p.p.t. salinity). One hour later, *Isochrysis* sp. clone T-ISO was added to achieve a density of 100 000 cells mL^{-1}. Water samples were collected from individual aquaria immediately after food addition and then half-hourly for 4 h. Algal cell density within each sample was determined by microscopic examination at 100 × magnification using an improved Neubauer haemocytometer ($n = 4$). If the algal cell density dropped to 50 000 cells mL^{-1}, an additional 50 000 cells mL^{-1} T-ISO was added. At the end of the experiment, the scallops were removed from the aquaria, and final cell densities were determined as above ($n = 8$). Filtration of T-ISO at 14 °C by an additional 15 scallops was determined as above to provide additional data for the estimation of change in filtration with scallop size.

Using the procedure described above, consumption of *Pavlova lutheri* (Droop) Green, *Chaetoceros calcitrans* (Paulsen) Takano, *Chaetoceros muelleri* Lemmermann, *Skeletonema costatum* (Greville) Cleve, *Rhodomonas salina* (Wislouch) Hill and Wetherbe and *Tetraselmis chui* Butcher by *M. asperrima* ($n = 8$) was compared with that of T-ISO at 14 °C (± 0.5 °C). As a result of significant increases in T-ISO consumption with increasing shell height, the size range of scallops used in this comparison was limited to the range 50–60 mm. Approximately 4 h after each experiment, faecal samples from scallops were collected and checked for the presence of algal cells using a binocular microscope (100 × magnification).

With the exception of *S. costatum*, the dry weight of algal cells of each species and relative cell dimensions were taken from Nell & O'Connor (1991) and O'Connor, Nell & Diemar (1992). Recent changes in the morphology of the *S. costatum* clone held at the PSRC necessitated the recalculation of cell weights and dimensions. To estimate cell volume, T-ISO, *P. lutheri*, *T. chui* and *R. salina* were assumed to be oblate ellipsoids, *C. muelleri* and *C. calcitrans* were assumed to be spheres and *S. costatum* a rectangle (after Jeffrey & Garland 1988).

Unialgal diets and fecundity

Seventy female scallops with shell heights in the range 55–65 mm were collected, and 20 were chosen at random to estimate initial mean fecundity of the group. Spawnings were conducted using serotonin injections as described previously. The remaining scallops were randomly allocated to one of five groups of 12 scallops. Each group of scallops was then divided into three aerated 90-L cylindroconical tanks (4 scallops $tank^{-1}$) held at a temperature of 14 °C (± 0.5 °C). Initially, each group was fed 2×10^9 cells $scallop^{-1}\,day^{-1}$ of T-ISO or the equivalent dry weight of one of the other species listed. After 3 days, the feed rates were reduced to 1×10^9 cells of T-ISO or its equivalent, as scallops did not clear the water of the higher algal ration. However, scallops fed *P. lutheri* and *C. calcitrans* did clear the reduced ration and so, 3 days later, the rate was raised to 1.5×10^9 cells for all treatments This rate was maintained for a further 3 weeks. Thrice weekly, the water in each tank was drained and replaced with temperature-equilibrated, 1-μm-filtered sea water.

After 2 weeks, each scallop was visually assessed for changes in reproductive condition using the scale of O'Connor & Heasman (1996) and, after a further 2 weeks, scallops were spawned and fecundities recorded. A sample of eggs (10 000) from each scallop that spawned was fertilized and placed into an individual 1-L plastic beaker held in a water bath

at 18 °C (± 0.5 °C). After 48 h, larvae were resuspended in the water column using a perforated plunger, and the number of D-veligers in each beaker was determined from four replicate 5-mL samples. Larvae in each sample were killed with several drops of formalin solution (10%) and counted using a binocular microscope. The number of D-veligers was expressed as a percentage of the initial egg stocking density.

Conditioning in recirculating systems

Seventy *M. asperrima* (45 females) of shell height 55–65 mm were placed in a recirculating conditioning system (Fig. 1) in a temperature-controlled room held at 14 °C. An additional 50 similar-sized scallops were placed in a second system and used to maintain the stocking density in the first system when scallops were removed for spawnings. All scallops were fed a combination of *P. lutheri*, T-ISO and *C. muelleri* to satiation ($\approx 2–3 \times 10^9$ cells scallop^{-1} day^{-1}). Upon arrival and then at weekly intervals, five male and 10 female scallops were selected at random from the first system and were measured and visually assessed for gonad condition. Macroscopic condition was ranked from one to five according to a previously developed arbitrary scale (O'Connor & Heasman 1996). These scallops were then spawned, the time elapsed before spawning commenced in both sexes, and the fecundity for females was recorded. In the interim, 15 tagged scallops from the second system were placed in the experimental system to maintain scallop stocking densities.

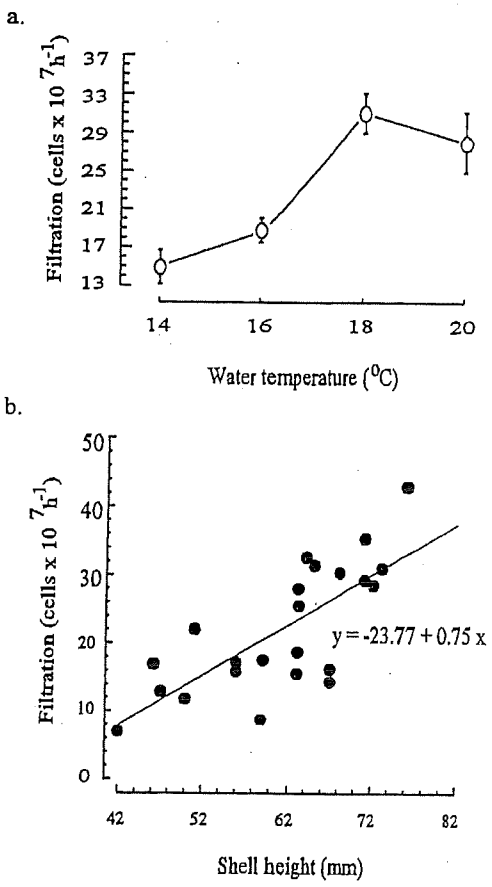

Figure 2 The effect of (a) water temperature and (b) scallop size (at 14 °C) on mean filtration of Tahitian *Isochrysis* aff. *galbana* by *Mimachlamys asperrima*.

Results

Temperature and algal species effects on filtration

The rate at which T-ISO cells were removed from the water column varied greatly, ranging from 70×10^6 to 402×10^6 cells h^{-1}. ANOVA, with temperature as a factor and scallop size as a covariate, identified significant variation in the consumption of T-ISO with both temperature and scallop size ($F = 14.33$ and 6.19, respectively, $P < 0.05$; Fig. 2). Scallops held at 14 °C and 16 °C filtered less algae from the water column than those held at 18 °C and 20 °C, while the larger scallops in each treatment filtered greater amounts of algae. There was no significant interaction between scallop size and water temperature on algae filtered from the water column ($F = 0.732$, d.f. 9/42, $P > 0.05$).

An ANOVA was used to confirm that shell height did not differ significantly between groups of scallops fed different algal species ($F = 2.225$, d.f. 6/49, $P > 0.05$). In terms of cell numbers, the two diatoms, *C. calcitrans* and *S. costatum*, and the prymnesiophyte, *P. lutheri*, were most rapidly filtered, followed by T-ISO, *R. salina*, *C. muelleri* and *T. chui* in that order (Fig. 3). Based on hourly cell depletion rates, maximal rates for 24 h fell within the range 2.25×10^9–7.68×10^9 cells.

No significant correlations were found between the rate at which cells were removed from the water column and either the volume (μL) or the dry weight (mg) of the cells of the various algal species tested ($r = -0.67$ and $r = -0.66$ respectively). In

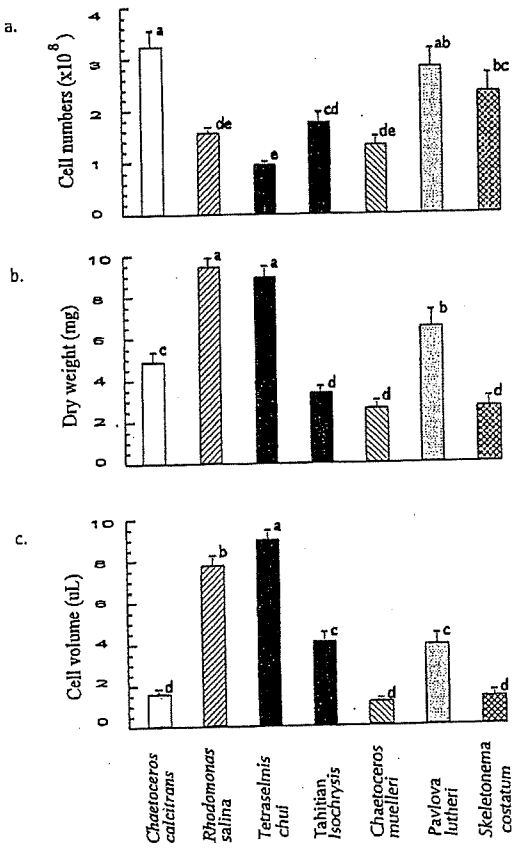

Figure 3 Mean hourly filtration of seven algal species used as food for *Mimachlamys asperrima* on a (a) cell number, (b) dry weight and (c) cell volume basis. Data from scallops (50–60 mm shell height, $n=8$) held at 14 °C. Values are means ± SE. Values within graphs with a common superscript do not differ significantly ($P>0.05$).

terms of the dry weight of cells filtered, larger weights of the heavier species tested, *T. chui* and *R. salina*, were removed from the water column (Fig. 3), whereas a relatively low weight of the two species most rapidly removed from the water column, *C. calcitrans* and *S. costatum*, were filtered. Total dry weights of algae filtered ranged from 2.25 mg h^{-1} (62 mg day^{-1}) for *C. muelleri* to 9.35 mg h^{-1} (225 mg day^{-1}) for *R. salina*. The pattern for total volume of cells removed from the water column was similar to that of dry weights, with greater volumes of the larger cells (*T. chui* and *R. salina*) removed than the smaller cells of *C. calcitrans* and *S. costatum* (Fig. 3).

Cells of all species were found undigested within the faeces of *M. asperrima*. In the case of the prymnesiophytes, intact cells were difficult to find;

Table 1 Correlation matrices for filtration rate of five algal species by *Mimachlamys asperrima*, measured in terms of cell numbers, cell volume and dry weight of cells filtered, and mean fecundity of scallops fed one of the five species for 4 weeks

Filtration rate	Fecundity	Cell numbers filtered	Cell volume filtered
Cell numbers filtered	$r=0.25$ ($P=0.67$)		
Cell volume filtered	$r=-0.59$ ($P=0.29$)	$r=-0.85$ ($P=0.06$)	
Dry weight filtered	$r=-0.61$ ($P=0.27$)	$r=-0.79$ ($P=0.11$)	$r=0.99$ ($P=0.001$)

however, they were more evident in the case of *R. salina* and markedly more so for *T. chui*.

Unialgal diets and fecundity

Initial mean fecundity of scallops collected for unialgal diet evaluations was $0.81 \pm 0.21 \times 10^6$ eggs. After 2 weeks, macroscopic examination of gonads failed to detect any change in condition from the time of stocking or to detect differences between treatments. However, after 4 weeks, gonads of scallops fed *C. muelleri* and *P. lutheri* were more turgid and less granular in appearance.

ANOVA was used to confirm that shell height did not differ significantly between treatments fed one of five algal diets ($F=2.389$, d.f. 4/55, $P>0.05$). Variation in fecundity between treatments was initially analysed using a one-factor model with data nested for tanks. As the nested factor was not significant ($F=1.066$; $P=0.351$), a single-factor model was used (Underwood 1981). Fecundity was then found to vary significantly according to diet ($F=2.628$, d.f. 4/55, $P<0.05$), with greatest fecundities recorded from scallops fed *C. muelleri* and the lowest in treatments fed *T. chui* and *C. calcitrans*. Mean fecundity for each treatment did not correlate with filtration rates in terms of cell numbers, cell volume or dry weight of cells consumed (Table 1). Percentage development of eggs to D-veligers was arcsin $x^{0.5}$ transformed (Winer 1971), and single-factor ANOVA indicated no significant differences between scallops fed differing diets ($F=0.279$, d.f. 4/55, $P>0.05$). Fecundity and percentage development of eggs to D-veligers was not significantly correlated ($r=-0.12$, $P=0.36$, $n=60$).

Conditioning in recirculating systems

No significant difference was detected among the mean shell heights of females used in weekly spawnings ($F = 0.26$, d.f. 4/45, $P > 0.05$), and no significant correlation ($P < 0.05$) was found between fecundity and the size of female scallops in any of the five spawnings conducted. Of the scallops injected with serotonin in this experiment, 43 out of 50 females and 24 out of 25 males released gametes commencing 40.9 ± 8.3 min and 17.2 ± 4.7 min (mean $\pm$ SD), respectively, after injection. Mean time elapsed before spawning commenced did not differ significantly for either sex over the 4 weeks of this experiment (females $F = 1.85$, d.f. 4/39, $P > 0.05$; males $F = 1.64$, d.f. 4/20, $P > 0.05$).

Mean fecundity and mean macroscopic ranking of female scallops were significantly correlated ($P < 0.001$), with both showing a tendency to increase by around 20% over the 4 weeks in the conditioning system (Fig. 4). Linear regression of fecundity over time showed a significant increase in egg numbers released over the duration of the experiment (slope significantly greater than zero, $P < 0.05$). Fecundity varied greatly among scallops, ranging from 0.21×10^6 to 4.20×10^6 eggs from a single female. There was also a trend for mean male ranking to increase with time, although male rankings were lower on most occasions than those of females (Fig. 4).

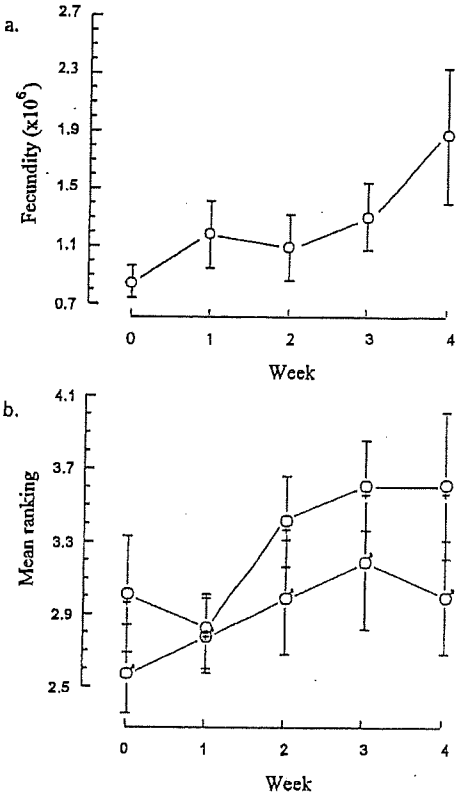

Figure 4 Changes in (a) fecundity and (b) mean ranking of *Mimachlamys asperrima* held for 4 weeks in recirculating conditioning systems. Values are means $\pm$ SE, $n = 10$ and $n = 5$.

Discussion

Variation in the filtration rates of the seven algal species tested illustrates the complexity of the feeding process in pectinids. Particle filtration is affected by size, with the effective lower limit for pectinids about 5–7 μm, below which retention efficiency varies with scallop species (Bricelj & Shumway 1991). In this study however, reductions in filtration of algal species could not be solely ascribed to small particle size. Rather, the inverse was apparent. The two smallest algae, *C. calcitrans* and *S. costatum*, were among the most rapidly depleted from the water column, despite both being less than 5 μm in size (4.0×3.1 and 4.2×3.1 μm respectively). If *C. muelleri* is excluded, filtration of the other algae was significantly negatively correlated with both cell size and cell weight ($r = -0.87$ and $r = -0.81$ respectively). One possible explanation is that *M. asperrima* reduced particle filtration as they approached satiation. Scallops can regulate the amount of material ingested by varying the time spent filtering, altering particle retention rates and/or increasing pseudofaeces production (Foster-Smith 1975). While pseudofaeces production (which did occur) may still have resulted in reduced cell numbers in the water column, reductions in filtering time and algal cell retention could explain the observed reductions in algal cell filtration.

Despite its small size, *C. muelleri* was not filtered from the water column as rapidly as other species. While this supports the results of Heasman *et al.* (1996) with *P. fumatus*, studies with *Placopecten magellanicus* (Gmelin) (Ward & Cassell 1991; MacDonald & Ward 1994) and *Mercenaria mercenaria* (Linne) (Marinucci in Epifanio 1983) have indicated that the metabolites of *C. muelleri* stimulated filtration. Semura (1995) found that, while filtering rates of *Pecten albicans* Schröter were

Table 2 Fecundity and percentage development of eggs to D-veligers of *Mimachlamys asperrima* fed one of five algal diets for 4 weeks

Algal diet	Fecundity ($\times 10^6$)	Percentage D-veliger development
Chaetoceros muelleri Lemmermann	1.30 ± 0.38^{a}	42.5 ± 5.2^{a}
Pavlova lutheri (Droop) Green	1.12 ± 0.33^{a}	48.9 ± 6.1^{a}
Tahitian *Isochrysis* aff. *galbana* Parke	1.02 ± 0.62^{ab}	41.6 ± 7.9^{a}
Tetraselmis chui Butcher	0.84 ± 0.29^{ab}	44.6 ± 4.3^{a}
Chaetoceros calcitrans (Paulsen) Takano	0.79 ± 0.51^{b}	47.1 ± 6.4^{a}

Values are means ± SD ($n = 12$). Values within columns with a common superscript do not differ significantly ($P > 0.05$, LSD).
Initial mean fecundity $0.81 \pm 0.21 \times 10^6$ and development percentage 47.4 ± 4.9 ($n = 20$).

highest in the presence of *C. muelleri* (higher than for *P. lutheri*), ingestion of the species was the lowest of the four species tested. If this was the case with *M. asperrima*, filtration may have been rapid, but the scallops failed to ingest *C. muelleri* and returned it to the water column, either directly or loosely bound in pseudofaeces. This would have led to an incorrect estimate of filtration rate.

The case of *C. muelleri* illustrates the need for care in the use of filtration rates in selecting diets for conditioning scallops. Filtration rates are not direct indicators of nutritional value. Clearly, filtration does not imply ingestion, nor does ingestion imply digestion. While only small quantities of *C. muelleri* were ingested, examination of the faeces indicated that near-complete digestion had taken place. *T. chui* was ingested in relatively large quantities but was poorly digested, with numerous intact cells in the faeces. When compared with those fed other monospecific diets, scallops fed *C. muelleri* were in macroscopically better condition and released the greatest mean number of eggs (Table 2). Along with scallops fed *P. lutheri*, those fed *C. muelleri* also released significantly more eggs than scallops fed the most rapidly consumed species, *C. calcitrans*. The relatively poor performance of *C. calcitrans* in failing to raise fecundity above the initial (preconditioning) level is consistent with the findings of Soudant *et al.* (1996), who found the species inferior to T-ISO as a diet for broodstock *P. maximus*. However, this need not preclude the use of *C. calcitrans* in broodstock diets. This species is among the most productive (yield volume^{-1} day^{-1}) at the PSRC and, in the case of *P. maximus*, completely spawned broodstock were capable of resuming gametogenesis and spawning when fed solely *C. calcitrans*. If broodstock are in suitable condition and only require a maintenance diet, *C. calcitrans* may make economic sense. Alternatively, if scallops are to be conditioned, diets involving several species, including *C. muelleri*, are recommended.

Food consumption rates estimated (2.25×10^9–7.68×10^9 cells scallop^{-1} day^{-1}) and used in monodiets (1×10^9–2×10^9 cells scallop^{-1} day^{-1}) in this study are generally lower than other values reported in the literature for scallops but, given the small size of *M. asperrima* broodstock used (55–65 mm), were not considered to be inconsistent on the basis of scallop biomass. Cochard & Devauchelle (1993) fed *P. maximus* at a 'high' rate of 8×10^9 cells scallop^{-1} day^{-1}, Aviles-Quevedo & Muncino-Diaz (1988) fed *Argopecten circularis* (Sowerby) 4–5×10^9 cells scallop^{-1} day^{-1} and Heasman *et al.* (1996) fed 55- to 75-mm *P. fumatus* 6×10^9 cells scallop^{-1} day^{-1} in the same conditioning systems used in this study. The move from 90-L tanks to the recirculating systems required an increase in the feed rate used to satiate *M. asperrima* (1.5×10^9–3×10^9 cells scallop^{-1} day^{-1}). This is likely to have been a product of the cessation of discontinuous feeding regimens in which high initial algal concentrations can decrease clearance rates (Bricelj & Shumway 1991), although the continuous feeding regimen was used in conjunction with a recirculating system in which algal cells may have been trapped in the biofilter. Regardless, these types of estimates are used to set species-specific feeding maxima, beyond which expensive food is wasted and biofilters can become clogged or fouled. However, it should not be assumed that these amounts of food are necessary for scallop conditioning. While reductions in feed rate reduced spawning percentages in *P. maximus* (Devauchelle & Mignant 1991) and reproductive condition in *P. fumatus* (Heasman *et al.* 1996), the possibility of overfeeding during hatchery conditioning has been raised. Scallops have seasonal cycles in energy utilization associated with reproduction, in which the adductor muscle is considered to be a source of energy used during gonadogenesis. Therefore, observations that

hatchery-conditioned *P. magellanicus* do not selectively increase only gonad weight (Grant & Cranford 1989) and have adductor weights twice that of wild stocks at the same stage of reproductive development (Paon & Kenchington 1995) suggest that hatcheries may provide more food than is necessary for conditioning. In the latter study, negative correlations between temporal changes in adductor and gonadal RNA/DNA ratios were suggestive of neurosecretory involvement. An explanation put forward was that of hormonal suppression of protein synthesis in the muscle, while protein synthesis occurred within the gonad. At such times, digestion products would be used preferentially by the gonad. Understanding these mechanisms could lead to lower feed rates through encouragement of better utilization of endogenous reserves (Paon & Kenchington 1995) and enhance out-of-season conditioning.

The extended temporal availability of spawnable *M. asperrima* in Jervis Bay (O'Connor & Heasman 1996) is not mirrored in some southern populations (Zacharin 1994) and, thus, the regimen used in this study may only satisfy the needs of potential scallop farmers in NSW. In particular, size-dependent changes in filtration by *M. asperrima* warrant attention. Sexually mature *M. asperrima* in Jervis Bay range in size from ≈30 to 80 mm and have demonstrated more than a fourfold increase in algal cell filtration over this size range (Fig. 3b). In Tasmanian waters, *M. asperrima* grow to in excess of 100 mm and could reasonably be expected to have much greater satiation rates. The increase could, however, be ameliorated to a small extent by temperature, if, as is the case with the commercial scallop *Pecten fumatus*, *M. asperrima* from Tasmania can be conditioned at lower temperatures.

In summary, care should be taken in the selection of broodstock diets. While a suitable diet must be actively filtered from the water column, the rate of filtration was not correlated with reproductive output. While none of the monoalgal diets resulted in significant reductions in fecundity, a diet of between 2×10^9 and 3×10^9 cells scallop^{-1} day^{-1} of *C. muelleri*, *P. lutheri* and T-ISO steadily improved the condition of scallops in the size range 55–65 mm held in a recirculating conditioning system.

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Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs – a summary

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Abstract

On the basis of initial harvesting efficiency trials and screening trials to evaluate apparent cell damage and viability, high-speed centrifugation was selected as the most appropriate microalgae harvesting method for developing extended shelf-life concentrates that would collectively meet the requirements of marine hatcheries and nurseries. Bioassay evaluation of stored microalgae concentrates revealed major discrepancies between closely related species of microalgae with regard to the impact of harvesting method on both short-term nutritional quality and shelf-life of stored concentrates. At one extreme, very good retention of nutritional quality was exhibited by high-speed-centrifuged concentrates of *Tetraselmis* spp. and *Chaetoceros calcitrans* beyond 8 weeks storage. In contrast, the naked flagellates *Pavlova lutheri* and Tahitian *Isochrysis* and the diatom *Chaetoceros muelleri* exhibited rapid and profound losses in nutritional quality as a consequence of supercentrifugation. Likewise, the impact of storage conditions and the effects of preservatives and other common food additives on the quality and extended shelf-life of stored concentrates was found to be unpredictable and highly species specific. Accordingly, optimum combinations of harvesting and storage, including optimum cell densities, presence or absence of food additives, temperature and, in some cases, gaseous atmosphere and light, had to be specifically tailored to individual species of microalgae in order to maximize the effective shelf-life of their concentrates. Data are presented demonstrating that the best binary concentrate diets developed during the course of this study could sustain growth and survival of larval and juvenile bivalves at rates similar to fresh microalgae culture even after storage periods of 6–8 weeks.

Introduction

Microalgae are essential for the commercial rearing of many aquatic animals, especially the larvae and juveniles of bivalve molluscs, penaeid prawns and zooplankton, such as rotifers *Brachionus* spp., used in turn as live feeds to rear larval marine finfish and crustaceans. Live microalgae were first used as food in bivalve hatcheries in 1940 (Bruce, Knight & Parke 1940) and for commercial shrimp hatcheries in the 1960s (Hudinaga & Kittaka 1967). However, the culture of microalgae to feed marine invertebrate larvae and juveniles has also been recognized as a 'bottleneck' to some forms of marine hatchery and nursery production, especially that of bivalve seed (for reviews, see Coutteau & Sorgeloos 1992; Knauer & Southgate 1999). Consequently, there is a great need to replace or supplement live microalgae as food in commercial and research hatcheries (Knauer & Southgate 1999).

The main constraint to microalgae production is cost. Surveys of the use of microalgae in Australian aquaculture have been conducted by Brown, Jeffrey & Garland (1989) and, more recently, by Borowitzka (1999). The latter showed that, on average, 30–40% of hatchery costs can be attributed to algal production. Comparable information gathered from the USA by Fulks & Main (1991)

gave a cost exceeding \$US 50 kg^{-1} dry algal biomass for a large specialized oyster hatchery. However, for smaller hatcheries, costs are likely to be much higher. For example, Coutteau & Sorgeloos (1992) and Borowitzka (1999) in worldwide surveys reported upper costs of microalgae by small hatcheries in the range \$US 300–600 kg^{-1} dry algal biomass.

Problems of high costs may be compounded by risks to hatchery output posed by difficulties in continuously matching output of good-quality microalgae with high short-term fluctuations in demand. Algal production failures, particularly at times of high demand, cause considerable problems even under tightly controlled indoor conditions typical of most bivalve hatcheries. Commonly encountered difficulties for cheaper outdoor bulk production reported to the authors by penaeid prawn hatcheries in Australasia are adverse environmental climatic factors.

Adverse climatic factors include excessive or suboptimal ambient temperatures and rainfall, light intensities, variable chemistry of coastal sea water used as media to culture the algae and contamination of cultures. Contaminants include aerosol-borne bacteria, other non-target algae, protozoans and metazoans, especially *Artemia* when produced as live food for larval finfish or decapod crustaceans at the same facility. Such problems particularly afflict shrimp *Penaeus monodon* hatcheries and, to a lesser extent, pearl oyster *Pinctada maxima* and *P. margaritifera* and marine finfish hatcheries in northern Australia and elsewhere in the tropics.

Shrimp hatcheries need to produce large amounts of microalgae, mainly *Chaetoceros* spp. (especially *C. muelleri*), *Tetraselmis* spp. and *Skeletonema costatum* over brief but critical periods of 5–7 days that encompass protozoeae and early mysis larval stages, within a total hatchery production cycle spanning 25–35 days (Griffith, Murphy-Kenslow & Ross 1973; Chu 1989; Liao 1992; Smith, Beidenbach & Lawrence 1992). In Australia, the annual hatchery production period can stretch to 9 or 10 months, but is mainly confined to a very distinct seasonal peak demand for post-larvae from August to December (C Robinson, Queensland Department of Primary Industries and Fisheries, pers. commun., June 1995).

High costs and risks posed by on-site production of live microalgae has prompted a search for alternatives, such as preserved microalgal concentrates, dried microalgae grown heterotrophically and microalgae substitutes. The latter comprise formulated microparticulate, microbound and encapsulated diets plus alternative unicellular organisms, namely yeasts and bacteria (see reviews by Couteau & Sorgeloos 1992; Knauer & Southgate 1999). However, commercial hatcheries throughout the world, especially bivalve hatcheries (Coutteau & Sorgeloos 1992), have tried and in the main discarded substitutes for live microalgae. This is in spite of some very encouraging experimental results achieved by researchers and developers of such products.

A notable exception has been the partial substitution of live algae by formulated microparticulate diets by penaeid shrimp hatcheries in Australia (M Zipf, pers. commun., 1999) and elsewhere (Yashiro, Bautista, Daza & Kanazawa 1985; Amjad & Jones 1992; Liao & Sheen 1993). Another exception has been the use of concentrates of the diatoms *Thalassiosira pseudonana* and *Skeletonema costatum* as a short-term interim food source for remote setting of hatchery-produced Pacific oyster *Crassostrea gigas* pediveligers by farmers mainly on the coast of North America (Jones & Jones 1988; Roland & Broadley 1990). Indeed, the major seed supply for the 25 000 tonne Pacific oyster industry in that part of the world is dependent upon this method of remote settlement (Donaldson 1989, 1991).

Different techniques used for concentrating and preserving microalgae cells as food for bivalve molluscs have included spray- and freeze-drying, refrigerating and freezing (see review by Knauer & Southgate 1999). Spray- or freeze-dried microalgae have been used with some success as complete diets for larval clams *Mercenaria mercenaria* by Hidu & Ukeles (1962) and as a partial diet for oysters *Saccostrea glomerata = commercialis* by Numaguchi & Nell (1991). Comparative trials with juvenile mussels *Mytilus galloprovincialis* by Cordero Esquivel & Voltolina Lobina (1996) have, however, demonstrated superior performance of those fed frozen microalgae over those fed an equivalent freeze-dried microalgae. These results reflected a lower rate of biochemical degradation of the microalgae caused by cold storage than by air- or freeze-drying reported by Cordero Esquivel, Voltolina Lobina & Correa Sandoval (1993).

Heightened interest in algal concentrates has been generated by findings that the chemical and microbial loads associated with direct feeding of

microalgal cultures have been found to be deleterious to some mollusc larvae (Nalewajko, Dunstal & Shear 1976, 1984; Watson 1986; Lewis, Garland, O'Brien, Fraser, Tong, Ward, Dix & McMeekin 1988) and crustacean larvae (Zein-Eldin in Griffith *et al.* 1973). Elimination of growth media via the use of microalgal concentrates may therefore explain reports of enhanced larval growth and survival of some species when fed such concentrates (Ukeles in Watson 1986; Nell & O'Connor 1991; O'Connor & Nell 1992).

A major constraining factor to wider commercial adoption of microalgae concentrates has been the inability to create pastes or slurries that retain high nutritional value through protracted storage. To encourage use by commercial marine hatcheries, microalgae concentrate diets must be able to be easily reconstituted into low-density suspensions of unclumped cells. The latter need to remain neutrally or very slightly negatively buoyant in sea water under low-turbulence culture conditions used to rear delicate marine larvae. The retained nutritional quality of resuspended cells needs to rival that of the original live cells. They must also retain palatability and be able to be efficiently filtered from suspension, ingested, digested and assimilated by the animals being cultured. Ideally, the shelf-life of stored microalgae concentrates should at least span the hatchery/nursery-rearing cycles of important species of farmed marine bivalves, crustaceans and finfish. In practice, this equates to 4–6 weeks. Finally, storage requirements for these concentrates need to fall within normal operating capabilities of the smallest and most remote of commercial hatcheries.

The aims of this research project were to extend the initial work of Nell & O'Connor (1991) and O'Connor, Nell & Diemar (1992) in developing harvesting, preservation and storage techniques for a representative array of microalgae species that provide a minimum practical shelf-life of 6 weeks.

The overall plan of research was to evaluate alternative harvesting, preservation, storage and feeding techniques (and combinations thereof) in extending the shelf-life of a representative array of microalgae species using both direct (bioassay) and indirect indicators of retained nutritional quality. The following is a brief overview of the scope and outcomes of research reported in full by the authors in their final report to the Fisheries Research and Development Corporation of Australia.

Materials and methods

Selection of microalgae species

In terms of both frequency of use and volume of production data presented by Coutteau & Sorgeloos (1992), the 10 microalgae species used in this research appear collectively to account for more than 90% of global usage in marine aquaculture. They comprised two prymnesiophyte species, *Pavlova lutheri* and Tahitian *Isochrysis* (T-Iso); five diatoms (*Chaetoceros muelleri, C. calcitrans, Skeletonema costatum, Thalassiosira pseudonana* and *Phaeodactylum tricornutum*); one green flagellate (*Tetraselmis chui*); one yellow green (*Nannochloropsis oculata*) and one cryptomonad (*Rhodomonas salina*).

Harvesting techniques

Preliminary evaluation was made of a number of harvesting techniques, including sedimentation, filtration, chemical flocculation, electroflocculation and three types of centrifugation. Of these, only centrifugation and chemical flocculation using chitosan proved suitable for further evaluation in terms of speed and efficiency of harvesting and of the density and quality of cells in resultant concentrates. Limits of acceptable efficiency and speed of harvesting were arbitrarily defined as recovery of 80% or more of cells in original microalgae cultures and within a period of 24 h respectively. Acceptable quality concentrates were defined as those that can be easily resuspended as unclumped cells and those suffering detectable damage to less than 10% of cells, as evident by micoscopic examination at 200 × or by returning a positive response to the stain Evan's Blue. Evan's Blue has been used as a dead-cell stain for various species of marine microalgae (Reynolds, Mackiernan & Van-Valkenberg 1978; Walsh 1983; Gallagher 1984; Molina Grima, Sanchez Perez, Garcia Camacho, Acien Fernandez, Lopez Alonso & Segura del Castillo 1994; Amsler & Raymond 1995). Its diagnostic action is that it stains the organic matter within dead cells a deep blue. In contrast, it is repelled by live cells with a functional cell membrane (Molina Grima *et al.* 1994)

Centrifugation

Three alternative types of centrifuge were evaluated. These included two forms of high-speed centrifuge, namely a conventional cream separator

(Elecrem, 220V; Farm Services, Rossmore, NSW, Australia) and an industrial supercentrifuge (Sharples P/L 'super-centrifuge' Type MV 35 32Y 22 KY32; Alfa Laval, Sydney, Australia) with applied acceleration factors of 6000 *g* and 13 000 *g* respectively. The third type of centrifuge was a slower speed laboratory-style bucket centrifuge (Hitachi, model 05P-21B; Meeco Holdings, Sydney, Australia) with an applied acceleration factor of 1300 *g*.

All three types of centrifuge were evaluated over a suite of nine of the previously cited 10 species of microalgae, the only omission being *R. salina*. Different batch cultures of each species of microalgae were used in each of three replicated harvesting trials to offset interbatch variability. The densities of the cultures were determined before concentration by microscopic examination at 200× using a haemocytometer (Improved Neubauer, Superior Co., Berlin, Germany). During the centrifugation process, samples of the supernatant (effluent) were collected to evaluate harvest efficiency. Each culture was also examined for retention of apparent cell viability, as indicated by a negative response to the Evan's Blue stain. At the completion of centrifugation, subsamples of the resultant concentrate were resuspended in 1 µm nominal-filtered sea water by vigorous shaking, stained with Evan's Blue and examined microscopically at 200× to evaluate apparent cell damage.

Harvest efficiency and apparent cell viability data were arcsine $x^{0.5}$ transformed before analysis by two-way ANOVA. For viability data, homogeneity was confirmed using Cochran's test; however, transformation of harvest efficiency data failed to remove heterogeneity of variances. Means were compared using Student–Newman–Keul's procedures (Winer, Brown & Michels 1991).

Chemical flocculation using chitosan

Chemicals such as lime and ferric-alum have been widely used for treating wastewater and effluents (McGarry 1970; Dodd 1979; Benemann, Koopman, Weissman, Eisenberg & Geobel 1980; Moraine, Shelef, Sandbank, Mar-Moshe & Shvartzburd 1980; Koopman & Lincoln 1983; Lincoln 1985), although from an aquaculture perspective, these chemicals pose the potential risk of toxic residues if used as a food source. An edible non-toxic alternative is chitosan, a flocculant used to concentrate various species of microalgae (Nigam, Ramanathan & Venkataraman 1980; Lavoie & de la Noüe 1983; Morales, de la Noüe & Picard 1985; Lubian 1989).

The lack of reported toxicity, ease of manufacture and low dosage rates for chitosan (Richmond & Becker 1986) encouraged an assessment of its abilities for concentrating algae, despite its reduced efficiency in salt water. In addition, the resuspension of microalgae flocs was assessed using a variety of dilute acid solutions.

Samples of each of seven species of microalgae, namely *C. calcitrans*, *S. costatum*, *T. pseudonana*, *P. lutheri*, T-Iso and *T. chui*, were poured into 1-L glass beakers and placed on a magnetic stirrer. The pH of each culture was determined using a Metrohm (model 605) pH meter fitted with an intermediate junction Ionode pH electrode. A stock solution of 0.5% (w/v) chitosan (Aldrich Chemicals) was made up in a 1% acetic acid solution (Lubian 1989) and was progressively added to each microalgae suspension. Chitosan concentrations tested ranged from 10 mg L^{-1} to 150 mg L^{-1}.

At each of the tested concentrations of chitosan, the culture and flocculating agent were mixed for 1 min before adjusting the pH to 8.0 with the addition of 1N sodium hydroxide solution. The culture was then removed from the magnetic stirrer and allowed to stand for several minutes.

The dose rate of chitosan was raised progressively by increments of 10 mg L^{-1} until a sedimentary floc formed on the bottom of the beaker. The clear aqueous supernatant was siphoned off, and the remaining floc was dewatered on a 75-µm pore size polyester mesh screen. The flocculated product was then transferred to a sterile 70-mL plastic screw-cap container (Disposable Products) and stored at 2 ± 0.5 °C for several days before undertaking resuspension trials. For each trial, pH changes and the effective dose of chitosan were recorded. Owing to the aggregation of the harvested cells within flocs, the effectiveness of chitosan was calculated by deducting density counts of cells within the siphoned fluid from those of the original cultures. These counts were determined by microscopic examination.

Resuspension of flocs

Using flocs of *Chaetoceros calcitrans*, several methods of dissociating aggregated cells were tested. Initially, 10 mL of flocculated material was added to either 100 mL of filtered sea water, distilled water or to a

hypersaline solution of either 70 or 140 mg kg^{-1}. Each suspension was then poured into a beaker, placed on a magnetic stirrer and agitated for several minutes. Each suspension was then acidified to pH 5.0 using 0.1 N hydrochloric acid.

Hayes, Davies & Munroe (1978) noted that the choice of solvent for chitosan influenced its solubility and, hence, six 'non-toxic' organic acid solvents were evaluated at the following concentrations:

Tartaric acid (Sigma T-0375)	0.5 N
Lactic acid (Sigma L-1893)	0.5 N
Dichloroacetic acid (Sigma D-6399)	0.2 N
Citric acid (BDH product 10081; Australian Scientific, Newcastle, Australia)	0.5 N
Glycolic acid (Sigma G-1884)	0.5 N
Acetic acid (Ajax UN no. 2789, Ajax Chemicals, Sydney, Australia)	1.0% w/v.

Six samples each of *P. lutheri*, *C. calcitrans* and *S. costatum* were collected, and the pH of each was adjusted to 6.5 using 0.5 N HCl. Chitosan solutions were then made using each of the six listed acids. In each case, sufficient acid was added to permit enough chitosan to be dissolved to form a 0.5% w/v solution. Each chitosan solution was used at a rate of 80 mg chitosan L^{-1} to flocculate cells within samples of each of the three microalgae species. The microalgae samples were allowed to stand until a floc formed and settled on the bottom of the vessels. Microalgae flocs were harvested by siphoning off the supernatant, and the resultant slurry was stored at 2 ± 0.5 °C in sealed 70-mL plastic screw-cap specimen jars for several days.

Two subsamples of each of the 18 flocculated slurries were then added to 100 mL of 1-μm-filtered sea water before one of the samples was reacidified to a pH of 5.0 using the same acid used as the chitosan solvent for that particular sample. The remaining sample from each pair was reacidified using 0.1 N HCl. The number of individual cells successfully resuspended was then assessed by microscopic examination at 200 ×, and the results were recorded as a percentage of the original number of flocculated cells.

Preservation and storage techniques

Extension to the shelf-life of microalgae concentrates was investigated using one or a combination of three techniques used by the food industry. The first was the use of non-toxic preservatives (food additives) that can be classified by their mode of action into the following categories:

1. Antioxidants – prolong the shelf-life of food by preventing oxidation, which causes rancidity and colour changes. These are particularly useful in retaining the organic chemical integrity of essential lipids, especially highly unsaturated fatty acids (HUFAs) and vitamins (especially C, B1 and E).
2. Food acids – help to induce and maintain constant low pH (< 5) to inhibit autolysis and microbial decomposition.
3. Other preservatives – additional additives that help to protect against spoilage, especially that caused by microorganisms. Common examples are high osmolarity levels of salt and sugar inhibitory to autolysis and microbial decomposition.
4. Cryoprotectant – agents that prevent damage to cell membranes caused by the formation of intracellular ice crystals, thereby enhancing the benefits of subzero storage temperatures.
5. Vitamins – make up for losses in processing and storage and are added to certain foods to supplement dietary intake. Vitamins C and E also double as antioxidants.

Three additives initially selected and evaluated for enhancing the shelf-life of microalgae concentrates were glycerol (category 4), ascorbic acid (categories 1, 2 and 5) and citric acid (category 2). Glycerol has been widely used (Aujero & Millamena 1979; Fenwick & Day 1992; Day & Fenwick 1993; Molina Grima *et al.* 1994) and was selected ahead of dimethyl sulphoxide (DMSO) because of the potential toxicity of the latter to both microalgae (Canavate & Lubian 1994) and invertebrate larvae, including those of molluscs (Chao, Chiang, Hsu, Tsai & Lui 1994) and crustaceans (Fisher, Courtney, Glas & Rayburn 1996).

Ascorbic acid (vitamin C) was the first choice antioxidant because of its widespread availability and low cost, low toxicity, dual action as a food acid and potential benefit as a dietary vitamin supplement. Citric acid is a non-toxic naturally occurring food acid widely used in the food industry that was included in some treatments to maintain a constant low pH in stored microalgal concentrates.

The second and simplest preservation technique evaluated was low-temperature storage. Reduced temperature slows both metabolic processes while cells remain alive (viable) and post-mortem changes, including oxidative denaturation of essential vitamins and HUFAs, autolysis and microbial degradation.

Percentage survival was also obtained by counting live and total numbers of larvae in replicate 1.0-mL samples.

Daily samples of stored paste were drawn with a 1-mL syringe from each treatment while working within a laminar flow cabinet. The sample of microalgae concentrate was resuspended into 1-μm-filtered sea water at the rate of approximately 0.1 mL of paste per 100 mL of sea water using a hand-held food processor. Cell density within resuspended pastes was calculated using microscopic examination at 200×. Feed rates were calculated according to methods described by Frankish *et al.* (1991).

Rearing techniques – juveniles (spat)

For oyster spat, initial mean (± SD) live weight was determined by taking eight representative subsamples each containing 30 individuals. The corresponding initial mean (± SD) dry weight (DW) was then determined by oven drying to a constant weight at 80 °C. Finally, mean (± SD) initial ash-free DW was determined after grinding and combusting the same eight subsamples for 5 h in a muffle furnace at 475 °C, thereby avoiding thermal decomposition of carbonates, especially calcium carbonate within the shell (Walne & Millican 1978). The same procedures were used to determine final mean (± SD) live weight, DW and ash-free DW for all four replicates in each of the dietary treatments at the conclusion of the experiments.

For scallop spat, initial mean shell height was determined from a randomly selected sample of 100 juveniles. Mean final shell height was determined by measuring the final shell height of all survivors in each replicate.

Statistical methods

Homogeneity of variance for growth data and survival data (arcsine $x^{0.5}$ transformed if necessary) across the full array of dietary treatments in each experiment was assessed using Cochran's test. A subsequent one-way analysis of variance was used to evaluate treatment differences. Growth data were compared using Student–Newman–Keul's procedures (Winer *et al.* 1991).

In more complex experiments, growth data and survival data for tested diets were grouped according to a hierarchy of the main dietary treatment factors that, in most cases, were in balanced orthogonal array. The latter variously comprised: feeding frequency (once daily or six times daily); storage temperature (+2 ± 0.5 °C or −15 ± 0.5 °C); harvest method (high- or low-speed centrifugation of chitosan-induced flocculation); additives (vitamin C and/or citric acid and/or glycerol); storage density of concentrates (pastes or slurries). These data were then subjected to analysis of variance to separate out significant effects (or the absence thereof) of the main dietary treatment factors and the interactions thereof.

When any or all of the main dietary treatment factors being investigated were found to affect growth or survival significantly, multiple-range analyses were applied to growth data and survival data for the stored diet. This entailed pooling data with a common form of harvesting, preservation or storage temperature, and the resultant pooled means were compared using Student–Newman–Keul's procedures (Winer *et al.* 1991). Pairwise comparisons of means of pooled data were undertaken where appropriate.

Table 2 Optimum chitosan dosage and associated flocculation data

Species	Optimum chitosan dosage (mg L^{-1})	Culture pH	pH after chitosan addition	Cell recovery (%)
Chaetoceros muelleri	150	8.06	5.03	95
Chaetoceros calcitrans	80	7.29	5.27	80
Skeletonema costatum	80	8.66	5.42	70
Thalassiosira pseudonana	40	8.29	6.31	90
Tetraselmis chui	40	7.69	6.03	80
Pavlova lutheri	80	7.28	5.30	80
Tahitian *Isochrysis*	40	7.43	6.26	90

Results

Harvesting techniques – chitosan flocculation

Harvest efficiency was enhanced with the manipulation of pH. The best results obtained for a range of microalgae species are summarized in Table 2. Optimal chitosan dosages ranged from 40 mg L^{-1} for *Tetraselmis chui*, T. *Isochrysis* sp. and *T. pseudonana* to as high as 150 mg L^{-1} for *C. muelleri*, with no apparent consistency within algal taxonomic groups. For example, within the diatoms, optimum chitosan concentration ranged from 40 mg L^{-1} for *T. pseudonana* up to 150 mg L^{-1} for *C. muelleri*.

Attempts to resuspend flocs by agitation alone were unsuccessful. Six alternative organic acid solvents used to prepare chitosan solutions successfully flocculated all species of microalgae tested, with no observed differences between solvents (Table 3). However, when the same acid solvents used to prepare chitosan solutions were used to reacidify resultant flocculated cells, the percentage of cells dissociating from flocs as single cells varied greatly. Among the tested acids, citric and hydrochloric acids appeared to be most suitable for resuspending flocs, irrespective of algal species, yielding from 50% to 90% of cells as singles.

Harvesting techniques – centrifugation

The mean apparent cell viability (as indicated by a negative staining response to Evan's Blue) of all nine species of microalgae harvested using the three centrifuges (Table 4) ranged from 88% to 100% and varied significantly with respect to species, method of centrifugation and the interaction of the two factors (Table 5). *C. muelleri*, the most vulnerable species, suffered damage to 12% of cells. The prymnesiophytes *P. lutheri* and T. *Isochrysis* also sustained moderate levels of apparent physical damage during supercentrifugation, but ostensibly low cell damage rates of 1% and 2%, respectively, when harvested using a cream separator. The remaining six microalgae species tested exhibited very low apparent cell damage (0–3%) regardless of the method of centrifugation, including supercentrifugation at 13 000 ***g***.

Within each centrifugation method, there was no significant correlation ($P > 0.05$) between the harvest efficiency and apparent cell viability across the various species tested ($r = 0.22$, 0.18 and 0.23 for supercentrifugation, cream separation and bucket centrifugation respectively). Harvesting efficiency (Table 5) also varied significantly with species and type of centrifuge, and interactions thereof.

On the basis of these results, the supercentrifuge was initially adopted as the standard bulk-harvesting method. Ultimately, this decision had to be reversed for those microalgae species that sustained $\geqslant 2\%$ loss of cell viability. In all such cases, the results of subsequent bioassay experiments discussed below showed that an acceptable shelf-life of 6–8 weeks could not be achieved. For example, worthwhile nutritional value to bivalve larvae of supercentrifuged pastes of delicate species, such as T-Iso and *C. muelleri*, could not be retained for more than a few days after harvesting, regardless of storage or preservation technique.

Table 3 The percentage of single resuspended cells for three microalgae species flocculated and resuspended using various combinations of chitosan solvents and reacidification solutions

Chitosan solvent/reacidifiction solutions	Chaetoceros calcitrans	Pavlova lutheri	Skeletonema costatum
Citric acid/citric acid	69.7	65.4	89.0
Citric acid/HCl	50.5	68.6	81.1
Acetic acid/acetic acid	67.5	45.0	72.0
Acetic acid/HCl	50.6	49.1	85.4
Tartaric acid/tartaric acid	59.7	51.9	92.2
Tartaric acid/HCl	53.9	64.5	86.4
Glycolic acid/glycolic acid	47.3	62.3	78.1
Glycolic acid/HCl	46.0	64.2	80.7
Lactic acid/lactic acid	42.8	60.7	35.1
Lactic acid/HCl	47.1	59.7	73.0
Dichloroacetic acid/dichloroacetic acid	40.8	65.2	76.8
Dichloroacetic acid/HCl	38.6	67.6	72.3

Table 4 Comparison of three types of centrifugation across an array of species of microalgae on the basis of harvest efficiency and apparent viability after resuspension

	Apparent cell viability (%)			Harvest efficiency(%)		
	Supercentrifuge	Cream separator	Bucket centrifuge	Supercentrifuge	Cream separator	Bucket centrifuge
P. lutheri	92 ± 2^{bc}	99 ± 1^{ab}	100 ± 1^{ab}	100 ± 0^{a}	79 ± 3^{abc}	66 ± 2^{bcd}
Isochrysis sp.	88 ± 1^{c}	98 ± 0^{ab}	100 ± 3^{a}	100 ± 0^{a}	65 ± 4^{bcd}	54 ± 6^{de}
C. calcitrans	98 ± 1^{ab}	100 ± 0^{ab}	100 ± 0^{ab}	97 ± 0^{ab}	52 ± 3^{de}	48 ± 8^{de}
S. costatum	100 ± 0^{ab}	98 ± 1^{ab}	100 ± 0^{ab}	98 ± 0^{a}	47 ± 8^{de}	39 ± 11^{e}
T. pseudonana	97 ± 0^{ab}	99 ± 0^{ab}	100 ± 0^{ab}	99 ± 0^{a}	76 ± 5^{bcd}	57 ± 8^{cde}
P tricornutum	100 ± 0^{ab}	99 ± 1^{ab}	100 ± 0^{ab}	94 ± 3^{ab}	65 ± 7^{bcd}	56 ± 7^{cde}
C. muelleri	88 ± 3^{c}	88 ± 8^{c}	95 ± 4^{abc}	96 ± 1^{ab}	46 ± 7^{de}	15 ± 3^{f}
T. chui	100 ± 0^{ab}	100 ± 0^{ab}	100 ± 0^{ab}	100 ± 0^{a}	96 ± 1^{ab}	5 ± 3^{f}
N. oculata	100 ± 0^{ab}	99 ± 1^{ab}	99 ± 1^{ab}	95 ± 1^{ab}	53 ± 2^{de}	65 ± 7^{bcd}

Mean percentage ± SE apparent cell viability and harvest efficiency data. Means within columns with a common superscript do not differ significantly (Student–Newman–Keul's, $P < 0.05$).
NB. Data for harvest efficiency should be interpreted with care as variances are heterogeneous.

Table 5 ANOVA of (A) cell viability and (B) harvest efficiency for nine species of microalgae concentrated using a supercentrifuge, a cream separator or a bucket centrifuge

Factor	SS	d.f.	*F*	P
(A) Cell viability				
Species	659.5	8	6.2	<0.001
Method	225.6	2	8.5	<0.001
Interaction	453.1	16	2.1	0.0193
Residual	713.8	54		
(B) Harvest efficiency				
Species	5524.4	8	9.8	<0.0001
Method	38288.0	2	271.1	<0.001
Interaction	12739.3	16	11.3	<0.001
Residual	3812.8	54		

Screening trials and bioassay experiments

Evaluation of chitosan-flocculated concentrates

Chitosan flocculation was shown in bioassay expt 3 (Table 6) to have an extremely deleterious effect on the retained nutritional value of microalgae (*P. lutheri*) concentrates when fed to oyster larvae. Growth of oyster larvae on fresh flocculated concentrates was one-third of that on a fresh culture control diet, while those fed chilled or frozen stored chitosan-flocculated diets performed as poorly as unfed larvae. The negative effect of chitosan flocculation on larval survival was just as dramatic. Survival of unfed larvae and those fed centrifuged diets was within the high and narrow range of 87–95%. In contrast, larval survival rates on five flocculated *P. lutheri* diets ranged from 3.6% to 35.0%. These results are not readily explainable insofar as chitosan, a polymer of acetylglucosamine, is not documented as being toxic to invertebrate larvae. The negative effects of chitosan-flocculated diets may have arisen through adverse ancillary factors associated with cell clumping, such as the promotion of high bacterial loads or interference with normal ingestion or digestion of food by the oyster larvae.

Optimization of harvesting, preservation and storage techniques

Key indicative findings of the six screening trials were tested in bioassay expts 1–6 inclusive, the results of which are summarized in Table 7.

The seventh bioassay experiment of this project compared the nutritional value of concentrates of

Table 6. Summary of Bio-assay Experiments 1 to 6 to evaluate harvesting, preservation and storage techniques

<table>
<tr><th>Experiment title, aims and outline</th><th colspan="3">Key Findings</th></tr>
<tr><td>1. Foundation Bio-assay Experiment to define the effect of chilled storage period on retained nutritional quality of a standard bivalve mollusc larval diet comprising equal amounts (on a dry weight basis) of P. lutheri, T. Iso and C. calcitrans concentrated by super-centrifugation.</td><td colspan="3">Storage of super-centrifuged paste beyond one week resulted in progressively poorer growth rates. The progressive decline in growth rate was most protracted and pronounced from ash-free dry-weight data with rates declining to about 10% those supported by live algae and fresh (0-1 week old) pastes after 6 to 8 weeks of storage. This progressive and profound decline in the nutritional value of stored super-centrifuged paste of a standard hatchery diet collectively comprising two thirds by dry matter content of P. lutheri and T. Iso observed in the current experiment was consistent with indicative results of centrifugation trials and of Screening Experiments 2 and 5 (Table 7) based primarily on staining response to Evan's blue as an indicator of cell viability.</td></tr>
<tr><td>2. Effects of combinations of three types of common food additives on the shelf life of P. lutheri as indicated by direct bio-assay using bivalve larvae.

The aim of this experiment was to use growth of larval scallops (P. fumatus) to verify (or refute) the disappointing results obtained during Screening Experiment 2 in relation to effects of additives on the keeping qualities of centrifuged concentrates of P. lutheri, T. Iso and C. muelleri. P. lutheri was selected as the test species ahead of T. Iso or C. muelleri because of the former's demonstrated superior performance as a mono-specific diet for rearing bivalve larvae (Nell & O'Connor 1991).</td><td colspan="3">As shown by the growth and survival data below and consistent with indicative results of Screening Trials 2 and 5 (Table 7), inclusion of three additives alone or in combination in all seven aged super-centrifuged paste diets failed to enhance growth and survival of P. fumatus larvae. The statistically significant ($P<0.05$) margin of about 10% in growth rate exhibited by larvae fed fresh super-centrifuged concentrate control diet over that of larvae fed the live culture of P. lutheri, was consistent with results achieved elsewhere using Sydney rock oyster (S. glomerata) larvae, e.g. see Bioassay experiment 3.
<table>
<tr><th>Diet Treatment</th><th>Mean ± s.d Growth (um)</th><th>Mean % Survival ± s.d</th></tr>
<tr><td>Fresh Pavlova culture</td><td>44.71±2.08^{b}</td><td>72.61±4.74^{ab}</td></tr>
<tr><td>Fresh Paste</td><td>50.76±3.95^{a}</td><td>66.50±3.07^{abcd}</td></tr>
<tr><td>No Additives @ 2°C</td><td>39.12±2.57^{bc}</td><td>78.16±11.50^{a}</td></tr>
<tr><td>Glycerol @ 2°C</td><td>36.07±1.55^{cd}</td><td>55.11±5.68^{de}</td></tr>
<tr><td>Low pH @ 2°C</td><td>36.12±1.83^{abcd}</td><td>60.44±2.93^{bcde}</td></tr>
<tr><td>Vit C @ 2°C</td><td>34.66±4.01^{cde}</td><td>65.48±10.40^{bcde}</td></tr>
<tr><td>Low pH + Vit C @ 2°C</td><td>30.60±1.58^{def}</td><td>70.98±13.97^{abc}</td></tr>
<tr><td>All additives @ 2°C</td><td>28.92±1.38^{ef}</td><td>60.09±7.25^{cde}</td></tr>
<tr><td>All additives @ -2°C</td><td>27.97±1.08^{f}</td><td>53.90±4.62^{e}</td></tr>
<tr><td colspan="3">NB Means within columns with a common superscript do not differ significantly (SNK, P<0.05)</td></tr>
</table>
</td></tr>
</table>

Table 6. continued

Title, aims and outline	Key Findings		
3. Evaluation of optimal harvesting technique for *P. lutheri* (super centrifuge vs bucket centrifuge vs flocculation), stored as paste or slurry, chilled or frozen. This experiment was devised to address whether or not poor nutritional value of aged *P. lutheri* pastes is due in part to a very rapid deterioration of cells once removed from cold storage and added to hatchery tanks. Towards this end an evaluation was made of combinations of alternative harvesting and cold storage techniques and of different feeding frequencies on retained nutrition value of chill stored *P. lutheri* concentrate diets. Growth and survival of Sydney rock oyster (*S. glomerata*) larvae were used as direct indicators of retained nutritional value of the diets.	**Treatment Description**	**Mean (±s.e.) % Survival**	**Mean (±s.e.) shell length growth (µm)**
	Flocculated ; stored -15°C; 1 daily feed	7.55 ± 1.40 ab	3.52 ± 0.93 a
	Flocculated; stored 2°C; 1 daily feed	3.58 ± 0.35 a	8.61 ± 1.28 ab
	Low speed centrifuged.; stored -15°C; 1 daily feed	15.07 ± 2.78 b	9.53 ± 0.57 abc
	Unfed CONTROL	92.18 ± 1.24 d	9.92 ± 1.29 abc
	Flocculated; stored 2°C; multi feed	8.99 ± 1.80 ab	11.53 ± 4.52 abcd
	Fresh daily flocculated; 1 daily feed	11.36 ± 2.10 b	13.55 ± 1.13 abcd
	Flocculated; stored -15°C; multi feed	35.05 ± 4.80 c	13.99 ± 6.45 abcd
	High speed centrifuged ;stored -15°C; 1daily feed	91.57 ± 1.00 d	19.63 ± 0.80 bcde
	Low speed centrifuged.; stored -15°C; multi feed	7.54 ±1.40 ab	19.77 ± 6.40 bcde
	High speed centrifuged ;stored -15°C; multi feed	93.05 ± 1.08 d	22.82 ± 2.08 bcdef
	High speed centrifuged; stored 2°C; 1 daily feed	95.05 ± 0.76 d	24.15 ± 1.53 cdef
	High speed centrifuged; stored 2°C; multi feed	93.85 ± 0.98 d	25.57 ± 1.90 defg
	Fresh daily low speed centrifuged; 1 daily feed	86.74 ±4.54 d	31.42 ± 4.65 efg
	Fresh algal culture; 1 daily feed CONTROL	90.33 ± 2.39 d	32.49 ± 3.25 efg
	Low speed centrifuged.; stored 2°C; 1 daily feed	93.76 ± 1.81 d	32.76 ± 1.19 efg
	Low speed centrifuged.; stored 2°C; multi feed	94.50 ± 0.46 d	33.00 ± 2.27 efg
	Fresh daily high speed centrifuged; 1 daily feed	93.30 ± 1.71 d	36.25 ± 3.47 fg
	Fresh daily high speed centrifuged; multi feed	92.61 ± 1.08 d	38.64 ± 3.62 g
	* Means sharing common letter are not significantly different at $P<0.05$ level SNK		
	The main treatment factors significantly effected retained nutritional value of *P. lutheri* concentrates. Their relative importance followed the sequence: harvesting method > cold storage temperature > feeding frequency. As indicated by the data above, low speed (bucket) centrifuged concentrates, when stored as a chilled slurry at 2.0 ± 0. 5°C and administered either as single or multiple daily feeds, supported outstanding growth and survival of oyster larvae. However, the benefits of slow speed centrifugation were totally reversed by subsequent glycerol protected frozen storage of concentrates. Growth rates of larvae fed frozen low speed centrifuged concentrates ranged from less than one third (single daily feed) to two thirds (multiple feeds) those fed the fresh *P. lutheri* culture control diet. Detrimental effects on survival were equally dramatic with single and multiple daily feeding of frozen *P. lutheri* concentrate resulting in low survival rates of 7% and 15% , respectively. Chitosan flocculation was shown to have an extremely deleterious effect on retained nutritional value of *P. lutheri* concentrates. Growth of larvae on fresh flocculated concentrates was barely one third (13.6 µm) that of fresh *P. lutheri* culture control diet. Growth increments of 3.6 to 14 µm on chilled or frozen stored chitosan flocculated diets fed as single or multiple daily feeds, were poor even relative to the unfed control diet (9.9 µm). The negative effect of chitosan flocculation on larval survival was just as dramatic. Survival associated with 11 of the 18 diets, including the unfed control, fell within the high and narrow range of 87 to 95%. By contrast, survival rates on the five flocculated *P. lutheri* diets ranged from 3.6% when chill stored and fed once daily, to 35.0% when stored frozen and fed six times daily.		

Table 6. continued

Title, aims and outline	Key Findings					
	Treatment Factor					
	Fresh algae control	Centrifugation speed (low/ high)	Use of additives (Raw/ +additives)	Storage Temp. (Chilled/ Frozen)	Concentrate density (Paste/ slurry)	Best performing concentrate (mean growth/survival)
4. Evaluation of optimum harvesting techniques for T.*Iso* and *T. pseudonana*. This companion to Bio-assay Experiment 3 was to clarify whether poor performance achieved to date with aged high speed centrifuged concentrates of two other species of micro-algae, namely T. *Iso* and *T. pseudonana*, could be overcome. As with *P. lutheri*, both these species when fed as fresh cultures are of high nutritional value to bivalve larvae (Nell & O'Connor 1991).	***T. Iso***					
	Mean growth (µm)	47.32	14.02 / 13.86	14.53 / 14.02	**16.10*** / 11.77	12.58 / **16.15**	Raw; chilled; low speed cent. slurry (23.62 um / 96.5%)
	Mean Survival %	93.45	85.89 / **91.24***	88.58 / 85.54	**93.00*** / 85.13	92.7 / 94.4	
	T. pseudonana					
	Mean growth (µm)	30.36	16.2 /16.0	**18.5*** / 13.6	**18.6** */ 13.5	12.91/ **17.19***	Raw; chilled; low speed cent. slurry (38.12 um / 89.0%)
	Mean Survival %	93.8	78.5 /**90.2***	**89.2** * / 79.5	**90.3*** / 78.4	91.80 / 90.77	
	N.B. * Bold type denotes statisically significant difference ($P<0.05$) using SNK						
Evaluation was made of various combinations of alternative harvesting technique, cold storage temperature, presence or absence of common food additives and density of stored concentrate (as pastes or slurries) on retained nutrition value of T. *Iso* and *T. pseudonana* concentrates. As in Bio-assay Experiment 3, growth and survival of Sydney rock oyster larvae were used as direct indicators of retained nutritional value of diets.	The performance of chilled raw slurry concentrates of both T. *Iso* and *T. pseudonana* produced by gentle slow speed (bucket) centrifugation were superior to any other dietary treatment. For both T. *Iso* and *T. pseudonana*, the great benefits of slow speed over high speed centrifugation were negated by glycerol protected freezing and to a lesser extent by inclusion of additives. In the case of T. *Iso*, growth of oyster larvae fed the best performing stored concentrates was only half that of the corresponding fresh T. *Iso* control diet and therefore unsatisfactory. By contrast, the best stored *T. pseudonana* concentrate diet actually outperformed its fresh algal culture counterpart and can therefore be regarded as a good candidate for replacing fresh micro-algal culture diets used to rear bivalve larvae.						

Table 6. continued

Title, aims and outline	Key Findings						
		Fresh algae control	**Centrifugation speed** low/ high	**Use of additives** Raw / +additives	**Storage Temp.** Chilled / Frozen	**Concentrate density** High density paste/ low density slurry	**Best performing concentrate** (mean growth in µm / % survival)
5. Evaluation of optimum harvesting techniques for *C. calcitrans* and *S. costatum* This companion to Bio-assay Experiments 3 and 4 was to clarify whether encouraging results with aged high speed centrifuged concentrates of two diatom species *C. calcitrans* and *S. costatum* based on indirect indicators of retained quality (Screening Trials 2, 4 & 6), would translate to good performance as hatchery diets. It also aimed to determine if the nutritional value of stored concentrates of these diatoms could be enhanced by optimal methods of preservation and storage. The experiment was also to assess effects of various combinations of harvesting technique, cold storage temperature, presence or absence of common food additives and density of stored concentrate (as pastes or slurries) on retained nutrition value of *S. costatum* and *C. calcitrans*. As in the preceding Bio-assay Experiments 3 and 4, growth and survival of Sydney rock oyster (*S. glomerata*) larvae were used as direct indicators of retained nutritional value of diets.	***C. calcitrans***						
	Mean growth µm	49.3	11.3 / **34.1***	**25.4*** / 20.0	**16.10*** / 11.77	23.6 / 21.8	Raw; chilled; high speed cent. paste (47.5 µm / 98.5%)
	Mean Survival %	93.5	84.1 / **97.8***	88.0 / 93.9	**95.1*** / 86.8	98.0 / 94.5	
	S. costatum						
	Mean growth (µm)	50.4	19.0 / **31.0***	26.5 / 23.5	26.1 / 24.0	**27.7 / 43.9***	Chilled; high speed cent. slurry with additives (46.4 µm / 96.5%)
	Mean Survival %	88.5	78.5 / **90.2***	89.2/ 93.8	96.2 / 86.6	91.8 / 90.8	
	In stark contrast to *P. lutheri*, T. *Iso* and *T. pseudonana* (see Bio-assay Experiments 3 & 4), high speed (super) centrifuged concentrates of both *C. calcitrans* and *S. costatum* supported far superior rates of growth in oyster larvae than did their slow speed (bucket) centrifuged counterparts. The nutritional value of high speed centrifuged concentrates was markedly reduced by freezing. In the case of *C. calcitrans*, there were two outstanding performers amongst the stored concentrate diets that supported growth rates of 96 and 92% those of the fresh culture control diet. These comprised high speed centrifuged concentrates held chilled as a paste or slurry without additives. By contrast only one *S. costatum* stored concentrate diet supported a comparable growth rate to its corresponding fresh culture control diet. This was high speed centrifuged concentrate diluted to a slurry with additives.						

Table 6. continued

Title aims and outline	Key Findings			
	Dietary Treatment	*T. chui*		*C. muelleri*	
		Mean (±s.e.) shell height gain(μm)	Mean (±s.e.) % Survival	Mean (±s.e.) shell height increment (μm)	Mean (±s.e.) % Survival
6. Evaluation of optimum harvesting techniques for *T. chui* and *C. muelleri*. The first aim was to clarify whether rapid apparent loss of shelf-life by both chilled (see Screening trial 2) and frozen (see Screening trial 6) super-centrifuged pastes of *C. muelleri*, genuinely reflected loss of nutritional value. A basis of this uncertainty was that some of the same chill stored *C. muelleri* concentrates that exhibited rapid loss of cell viability otherwise remained in apparent good condition. The second objective, a corollary of the first, was to verify if apparent protracted retention of cell condition in chilled and frozen super-centrifuged pastes of *T. chui*, as previously indicated by the results of Screening Experiments 2 and 6 genuinely reflected retention of the nutritional value of these pastes. The experiment was also to assess effects of various combinations of alternative harvesting techniques, presence or absence of common food additives and density of stored concentrate (as pastes or slurries) on retained nutrition value of *T. chui* and *C. muelleri* concentrates. In contrast to preceding Bio-assay Experiments 3, 4 and 5, growth and survival of juvenile rather than larval doughboy scallops (*Mimachlamys asperrima*) were used as direct indicators of retained nutritional value of diets. This variation in protocol was adopted because mono-specific diets of both *C. muelleri* and *T. chui* had previously been shown to support satisfactory growth and survival of juvenile bivalves (O'Connor et al. 1992) but not of their larvae (Nell & O'Connor 1991).	Fresh Algal culture	325.7±61.6 b	96.66±1.93 ab	292.11±72.4a	93.99±2.22a
	Common Unfed Control	64.6±14.01 a	93.98±2.76 ab	64.58±41.1a	96.66±1.93a
	Low speed cent. slurry; 2°C	560.8±118.1 b	96.51±2.62 ab	135.67±42.9a	99±1.00a
	Low speed centrifuged slurry; stored 2°C; +Additives	455.4±60.3 b	93.59±4.42 ab	81.11±61.3a	95.8±2.29a
	Low speed cent. slurry; -15°C	**	**	**	**
	Low speed cent. slurry; -15°C; +Additives	**	**	**	**
	High speed cent. paste; 2°C	390.5±84.4 b	98.52±0.87 b	120.68±25.1a	96.74±3.26a
	High speed centrifuged paste; stored 2°C;+ Additives	331.5±65.4 b	98.11±1.10 b	117.86±12.9a	96.93±1.17a
	High speed cent. paste; -15°C	474.4±95.0 b	93.96±0.96 ab	184.6±58.9a	99.14±0.86a
	High speed centrifuged paste ; stored -15°C; +Additives	526.6±22.9 b	98.34±0.96 b	167.05±6.5a	92.62±2.16a
	High speed centrifuged paste stored as slurry; @ 2°C	390.4±56.2 b	96.63±0.13 ab	122.78±45.9a	97.89±1.31a
	High speed centrifuged paste stored as slurry; @ 2°C; Add's	447.4±18.2 b	85.30±1.92 a	209.54±35.0a	94.52±2.06a

Means sharing common letter are not significantly different at P<0.05 **All scallops died early in the experiment

Fresh algae control diets of both *C. muelleri* and *T. chui* supported poor growth but excellent survival of scallop spat relative to mixed diets of diatoms and prymnesiophytes routinely used to nursery rear juvenile bivalves. Growth rates supported by 8 types of variously harvested and stored concentrates of *T.chui* equalled or exceeded (range 102 to172%) that of a fresh culture of *T. chui*. By contrast, growth rates supported by the same array of centrifuged concentrates of *C. muelleri* were all considerably inferior (range 28 to 72%) to that of a fresh culture of *C. muelleri*. Best performing *C. muelleri* concentrate diet (72% the growth rate of the fresh control) was the chilled high speed centrifuged paste diluted to a slurry with additives.

Results of previous nutritional research with *T. chui* on juvenile bivalves have been variable depending on the species involved. For example *T. chui* was shown by O'Connor et al. (1992) to support good rates of growth in Sydney rock oyster spat but very poor reproductive conditioning in of the scallop *Pecten fumatus* (Reeve), by Heasman et al. (1996). The improved performance of *T. chui* concentrates over their fresh counterparts may be ascribed to loss of motility and increased digestibility. The two best performing *T. chui* diets were in fact greatly contrasting. One comprised chilled, slow speed centrifuged raw slurry, the other, frozen high speed centrifuged paste with additives. However, the ease and efficiency of harvesting and storing frozen paste sets it well ahead of the slurry as a practical diet.

Table 7. Summary of six screening trials using indirect criteria to evaluate harvesting, preservation and storage techniques

Trial	Title, aims and outlines	Key Findings
1	**Preliminary evaluation of three additives.** This trial aimed to evaluate the anti-oxidant ascorbic acid (Vitamin C), citric acid induced low pH and the cryo-protectant glycerol for enhancing the shelf-life of super-centrifuged microalgal pastes chill stored at 2 ± 0.5°C. The diatom *Skeletonema costatum* was used because of its widespread use in bivalve molluscs and penaeid shrimp hatcheries and high resistance to high speed centrifugation	The best result was obtained with *S. costatum* paste protected by the combination of all 3 additives ie. Vitamin C, citric acid induced low pH and glycerol. It retained >90% cell viability and remained essentially free of microbial contamination and associated offensive odours for up to 10 weeks. The pH of raw paste rose above 10 within a fortnight of storage and remained so for the full 10 week duration of the trial. Only the combination of Vitamin C and citric acid (with or without glycerol) was successful in maintaining pH within the targeted range of 4 to 4.5.
2	**Evaluation of alternative low temperature storage regimens in combination with additives.** This large screening trial was prompted by encouraging results of Trial 1. It incorporated 3 additional micro-algae species, *C. muelleri;* T. *Iso* and *P. lutheri* to evaluate the same suite of 3 food additives over 3 additional chilled storage temperature regimes namely 4 ± 6°C (provided by a refrigerator subject to routine daily use), 0.0 ± 0.5°C and 2.0 ± 0.5°C.	**Species of algae** / **Influence of Temperature** / **Best combination of additives** / **Best cell viability retention** / **Comments** *S.costatum* — +2°C ≥ -2°C >0°C >+4°C — All additives — 85% after 15 weeks — *P.lutheri* — +2°C ≥ -2°C >0°C >+4C° — No additives — — High % damaged cells after high speed centrifugation but all other indirect quality criteria high for up to 5 weeks T.*Iso* — -2° C ≥ +2°C >0°C >+4C° — All additives — 40% after 6-8weeks — *C.muelleri* — -2° C ≥ +2°C >0°C >+4C° — No additives — 14% after 2 weeks — High % damaged cells following high speed centrifugation but all other quality criteria remained high for 6 weeks

Table 7. continued

3	**Evaluation of additional additives** This screening trial was designed to compliment Screening Trials 1 and 2 in which all three common food additives (Vitamin C; citric acid and glycerol) initially selected for evaluation, were found to be ineffectual in extending the very poor shelf-life of chilled super-centrifuged pastes of more fragile species of micro-algae. The latter had been shown to include the diatom *C. muelleri* and two prymnesiophytes, *P. lutheri* and T. *Iso*, all of which are of major importance to marine aquaculture. Alternative food additives assessed included three preservatives, common salt (sodium chloride, BDH Prod. code 10241) at 5, 10, 15, and 20% (w/w); propionic acid (SIGMA, P-1386) at 0.5 mg/g of paste and potassium sorbate (SIGMA, S-1751) at 0.3 mg/g of paste. Two alternative food grade anti-oxidants were also assessed. These were ethoxyquin (SIGMA, E-8260) at 0.125 mg/g of paste and butylated hydroxytoluene (B.H.T.) (SIGMA, B-1378) at 0.1 mg/g of paste. The aim of this trial was to evaluate whether an alternative array of common food additives to Vitamin C, citric acid and glycerol, alone or in combination, could be used to prolong the shelf-life of super-centrifuged pastes of both *P. lutheri* and T. *Iso*.	T. *Iso:* As in Screening Trial 2, a rapid fall in cell viability was exhibited by raw super-centrifuged paste of T. *Iso*. Cell viability fell from a post-harvest mean ± s.e value of 88±1% to 40±2% after a week of chilled storage and thence to 13±4% after 4 weeks. Effects of additives were rather inconclusive. None of the new additive treatments trialed were sufficiently effective to maintain a satisfactorily high degree of cell viability. Propionc acid, various combinations of propionic acid, potassium sorbate, BHT and ethoxyquin did nevertheless improve retained cell viability values significantly above those of untreated, raw paste and paste blended with citric acid and Vitamin C. Amongst the new additives trialed, the two preservatives, especially propionic acid, appeared to offer best prospects of further improving retained cell viability above that of raw super-centrifuged T. *Iso* paste. The addition of to 5 to 20 % of NaCl did not however improve retention of cell viability over that of the raw paste control and neither of the alternative anti oxidants appeared to be superior to Vitamin C. *P. lutheri:* Results indicated that raw super-centrifuged paste of *P. lutheri* exhibited a very rapid initial decline in retained cell viability falling from a post harvest value of 92±2 0% to 26.5±9.1% after only one week of chilled storage. Mean cell viability continued to decline thereafter falling to less than 10% after 2 weeks and thence to less than 5% after 4 weeks storage at 2.0±0.5°C. All of the additive treatments tested on chill stored super-centrifuged pastes of *P. lutheri* reduced cell viability to values below that of the raw paste control after one week of storage at 2.0±0.5°C. In this regard effects of additive treatments with *P. lutheri* were opposite to those achieved with pastes of T. *Iso* already discussed above.

Table 7. continued

4	**Evaluation of additives across an expanded array of micro-algae.** Effects of Vitamin C, citric acid and glycerol, alone or in combination in extending the shelf life of centrifuged pastes of yet more species of micro-algae were assessed. Additional algae included C. *muelleri* and *S. costatum*, already demonstrated in Trials 1 and 2 as poor and good shelf life reference species respectively, plus three other commercially important diatoms, *P. tricornutum*, *T. pseudonana* and *C. calcitrans*. The remaining two species selected were the green flagellates *T. chui* and *N. oculata*.	Apparent shelf-lives of raw pastes of *T. chui* and *N. oculata* exceeded the 12 weeks duration of the trial. By contrast, apparent shelf life of the five diatom species were generally brief and followed the sequence *S. costatum* and *C. calcitrans* (8weeks)>>*P. tricornutum* (5weeks) >*T. pseudonana* (4 weeks) >> *C. muelleri* (less than 2 weeks). Effects of additives were highly species specific in their effect on retained cell viability. At one extreme the shelf-life of *S. costatum* was substantially extended. The shelf life of *T. pseudonana* was also improved but only marginally. No discernible effects occurred with *C. calcitrans* or *T. chui*. Additives had a pronounced negative influence on *P. tricornutum* and a slight negative effect on *C. muelleri*.
5	**Evaluation of modified storage conditions in extending the shelf life of concentrates of *P. lutheri* and T. *Iso* initially produced as pastes but diluted to low density slurries of either 10,20,40 or 80 g/L prior to protracted chilled storage** Modified storage conditions evaluated included • illuminated as opposed to dark storage • alternative types of gaseous atmosphere • the inclusion of two additives Vitamin C and glycerol. Both of these additives had already been shown during Screening Trials 1, 2, 3 & 4 to enhance retention of cell viability and other quality criteria in chilled super-centrifuged pastes of 7 out of 9 species tested.	**Effects of treatment factors on retained cell quality**

Treatment factors	*P. lutheri*	T. *Iso*
Density of stored concentrate	10 g/L> 20 g/L>40 g/L>80 g/L	Original undiluted paste far out-performed all slurries that did not vary over the density range of 10 to 80 g/L
Additives	None or Glycerol or Vitamin C alone	No effect on diluted slurries but paste greatly enhanced by inclusion of both additives
Atmosphere	Air with cotton plug or sealed	No discernible effect of different atmospheres
Light/Dark	Light	No discernible effect
Combined factors giving best result (in parenthesis)	10 g/L; + Vit C; Air with cotton wool plug (60% cell viability after 7 weeks of storage).	Undiluted paste with both additives stored under air with cotton plug (45% cell viability after 2 weeks of storage).

Table 7. continued		
6	**Evaluation of frozen storage of micro-algal concentrates.**	Suitability for frozen storage of super-centrifuged pastes of the 9 species of micro-algae followed the sequence *T. chui* >*P. tricornutum*, *S. costatum* and *C. calcitrans* >>*T. pseudonana*, T. *Iso*, *P. lutheri* and *R. salina*. Only the first four species retained an acceptable degree of apparent quality. Both cryo-protectants provided protection against damage to frozen cells within super-centrifuged pastes. Best overall results across the full array of nine species tested occurred at the highest inclusion level of 15% for both DMSO and glycerol. However, as DMSO is toxic to animals especially aquatic larvae, glycerol at 10 to 15% w/w is clearly the cryo-protectant of choice.
	Relative advantages and disadvantages of storing super-centrifuged pastes of nine different micro-algal species at -15°C were evaluated as an alternative to chilled storage. The 9 species utilized included 5 diatoms; *S. Costatum*, *P. tricornutum*, *T. pseudonana*, *C. calcitrans*, *C. muelleri*, 2 prymnesiophytes *P. lutheri* and T. *Iso*; the cryptophyte *R. salina*; and the prasinophyte *T. chui*. The 2 cryo-protectants glycerol and DMSO (Dimethyl sulphoxide, BDH Prod. 10323) used, were tested at three inclusion levels, 0.15, 1.5, and 15% (w/w).	

seven species of microalgae and all binary combinations thereof stored for 7–8 weeks before being fed to oyster larvae. Concentrates of *C. muelleri*, *C. calcitrans*, *T. pseudonana*, *S. costatum*, *P. lutheri*, T-Iso and *T. chui* were produced using species-specific optimal harvesting, preservation and storage protocols identified in earlier screening trials and bioassay experiments. As indicated in Fig. 1, the best performing monospecific concentrate diets were *C. calcitrans*, *S. costatum* and *P. lutheri*. The best performing binary diets were *P. lutheri* in combination with either *C. calcitrans* or *S. costatum*. Both these diets supported excellent larval growth rates of 85% and 90%, respectively, of those achieved on an optimal fresh reference diet. The latter comprised equal amounts on a DW basis of three species, *C. calcitrans*, *P. lutheri* and T-Iso (O'Connor & Heasman 1998).

The final eighth bioassay experiment was essentially identical to expt 7 except that Sydney rock oyster spat were used instead of larvae, and the experiment was run over 2 weeks rather than 8 days. By that time, spat in the best treatments had exhibited three- to fourfold increases in mean live weight, DW and ash-free DW. As indicated by the results in Fig. 2, the best performing monospecific concentrate diets were those of *T. chui*, *T. pseudonana* and *S. costatum*. The best performing binary concentrate diets were *T. chui* in combination with either *T. pseudonana* or *S. costatum*. Both of these supported excellent growth rates, at least the equal of the fresh algae reference diet.

Conclusions and discussion

This study demonstrated that microalgae concentrate diets, when appropriately harvested, preserved and stored, have the potential of replacing fresh microalgae culture diets used to rear larval and juvenile bivalves. However, the results also showed that particular type(s) of microalgae concentrates that provide the best results with one life stage of a reared species, in this case larval bivalves, will not necessarily apply to another life stage, such as early juvenile development.

The results of this and a companion study (D'Souza, Lecossois, Heasman, Diemar, Jackson & Pendrey 2000), in which the same array of optimally harvested and preserved microalgae concentrates were evaluated for rearing penaeid shrimp larvae, revealed profound differences in the relative

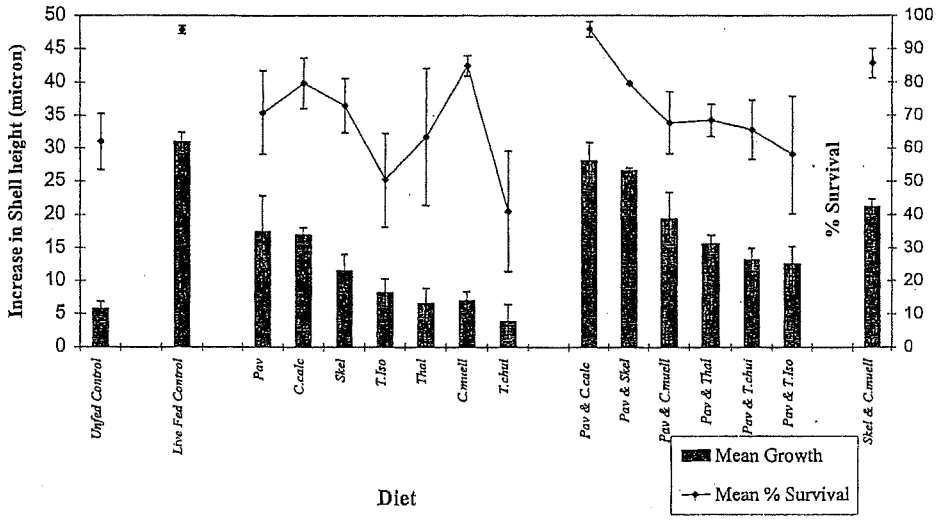

Figure 1 Effect of optimum monospecific and binary concentrate diets (stored for 7–8 weeks) on the mean ± SE growth and survival of Sydney rock oyster *S. glomerata* larvae.

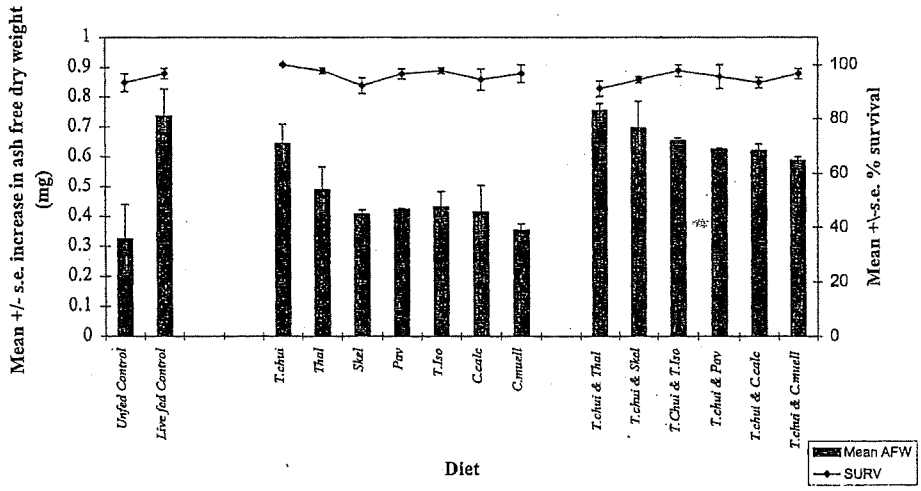

Figure 2 Effects of optimum monospecific and binary concentrate diets (stored for 7–8 weeks) on the growth and survival of Sydney rock oyster *S. glomerata* spat.

performance and shelf-life limitations of particular microalgal concentrates when applied to a different class of invertebrates.

The present study also indicated considerable scope for further development of improved microalgae harvesting techniques. This was particularly so for the more fragile species susceptible to damage by high-speed centrifugation, such as T-Iso, *P. lutheri* and *C. muelleri*. A technique with promise is a new method of bulk filtration being developed by Dr Alan Bunch, Research School of BioSciences, University of Kent, Canterbury, UK (pers. commun., 1999). An equally promising flocculation technique, based on pH manipulation, is being developed by CSIRO in Tasmania. Algal concentrates harvested by this technique are being used to supplement natural microalgae for rearing Pacific oyster *C. gigas* spat housed in field nursery upweller systems (McCausland, Brown, Barrett, Diemar & Heasman 1999)

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Evaluation of centrifuged microalgae concentrates as diets for *Penaeus monodon* Fabricius larvae

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Abstract

Concentrates of nine microalgae widely used in marine hatcheries were prepared by centrifugation, stored chilled for either 9 or 14 weeks and then compared with their fresh counterpart as feeds for *Penaeus monodon* larvae. The nine species – *Chaetoceros muelleri*, *C. calcitrans*, *Isochrysis* sp. 'Tahitian' (T-ISO), *Nannochloropsis oculata*, *Pavlova lutheri*, *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Tetraselmis chuii* and *Thalassiosira pseudonana* – in fresh and centrifuged forms were fed at high and low cell densities. The Larvatron, a computer-controlled apparatus for rearing planktonic animals, was used for the experiments. Fresh *C. muelleri* and *T. pseudonana* promoted the highest survival and fastest development of *P. monodon* to mysis 1. Although the survival rate of larvae fed concentrated *C. muelleri*, *P. lutheri* and T-ISO was comparable with those fed the fresh *C. muelleri* diet, the larvae fed these concentrates generally developed more slowly. The higher cell density, whether of fresh or concentrated algae, usually produced both higher survival and faster development of larvae. *Nannochloropsis oculata* and *P. tricornutum* were poor diets, regardless of their form or feeding density. Concentrating *C. calcitrans* and *S. costatum* markedly reduced their efficacy as diets. The centrifuged concentrates were unsuitable as complete replacements for fresh algae in larval culture when stored for either 9 or 14 weeks. However, the concentrates may be more successful in combination with other concentrates, fresh algae and/or artificial diets, or when stored for a shorter time.

Introduction

Fresh microalgae, in conjunction with artificial diets, are fed routinely to prawns being raised in hatcheries in Australia. To produce fresh microalgae, special facilities, daily maintenance and skilled labour are required. Changes in temperature and light and the presence of contaminating organisms can cause a crop failure or change the algal culture's nutritional quality. In addition, the demand for algae is variable depending on the availability of spawners and the stage of growth of the prawn larvae. Consequently, algal biomass is often produced in excess, which is expensive: algal production can represent 30–50% of hatchery operating costs (Jeffrey & Garland 1987).

One method of making the culture of fresh algae more efficient is to concentrate excess algae, especially when growing conditions are ideal, and to stockpile the concentrate until needed. Concentrates produced by drying, centrifugation or flocculation contain different amounts of moisture and can be stored at various temperatures. In this way, hatchery procedures are simplified and costs reduced.

However, substituting concentrates for all or part of prawn larvae diets has met with varying degrees of success. For example, Biedenbach, Smith & Lawrence (1990) fed spray-dried *Tetraselmis suecica* to *Penaeus vannamei* larvae as a partial replacement for fresh algae, and recorded similar growth, survival and development to larvae fed 100% fresh algae. However, Millamena, Aujero & Borlongan (1990) had more success feeding sun-dried *T. chuii* and *Chaetoceros calcitrans* to *P. monodon* larvae as

complete replacements for fresh algae. The survival rate was not compromised, but the development rate was not reported in this study. The development rate of larvae is often a more sensitive measure of the adequacy of the diet (D'Souza & Kelly, 2000).

Centrifuged concentrates of *T. chuii* and *Skeletonema costatum*, stored frozen and then fed to *P. setiferus* larvae as a complete replacement for fresh algae, resulted in a survival rate of ≈90% (Mock, Fontaine & Revera 1980). Again, however, the development rate was not reported. Similar concentrates, stored in the refrigerator, have been fed successfully to bivalve larvae and juveniles (Nell & O'Connor 1991; McCausland, Brown, Barrett, Diemar & Heasman 1999), but there are no published reports of feeding them to prawn larvae.

The aim of the present study was to test whether stored centrifuged concentrates could be used as full replacements for fresh algae fed to *P. monodon* larvae without affecting their survival and development rates.

The term 'fresh algae' has been used in this study in preference to 'live algae', as some algal concentrates contain live cells (Molina Grima, Sánchez Pérez, García Camacho, Acién Fernández, López Alonso & Segura del Castillo 1994; Montaini, Chini Zittelli, Tredici, Molina Grima, Fernández Sevilla & Sánchez Pérez 1995).

Materials and methods

Fresh algae cultures

Nine microalgae were tested as diets for prawn larvae: *Chaetoceros muelleri* Lemmermann CS176 (strain number–CSIRO Collection of Living Microalgae); *Chaetoceros calcitrans* (Paulsen) Takano CS178; *Isochrysis* sp. 'Tahitian' (T-ISO) CS177; *Nannochloropsis oculata* (Droop) Green CS179; *Pavlova lutheri* (Droop) Green CS182; *Phaeodactylum tricornutum* Bohlin CS29; *Skeletonema costatum* (Greville) Cleve CS181; *Tetraselmis chuii* Butcher CS26; *Thalassiosira pseudonana* (Hust.) Hasle and Heimdal CS173. The use of these nine microalgae in penaeid prawn culture has been reviewed by D'Souza (1998). To our knowledge, however, there have been no reports of feeding *N. oculata* and *P. tricornutum* to penaeid prawn larvae.

Stock cultures (100 mL) of the nine species were provided by New South Wales Fisheries, Port Stephens Research Centre, Taylors Beach, NSW, Australia, and maintained in f_2 medium (Gu Ryther 1962) without the vitamin biotin bi sodium metasilicate for diatom cultures. microalgae were originally sourced from the Collection of Living Microalgae, CSIRO Research, Hobart, Tasmania. Natural sea ($\approx 35\,g\,L^{-1}$ salinity) for the f_2 medium was through a sand filter (100 µm nominal por followed by a 1-µm cartridge filter.

The sea water was treated with $0.01\,g\,L^{-1}$ E chelate metal ions and stored for at least 2 before use. It was chlorinated with $10\,mg\,L^{-1}$ chlorine for 24 h and then dechlorinated sodium thiosulphate for inoculation with th cultures. Cultures were incubated at 23 ± 2 ' illuminated from one side with Osram Fluora giving 45 µmol photon $m^{-2}\,s^{-1}$ (measured Licor 4π PAR spherical sensor) under a 12 light:dark cycle. The stock cultures were subcu once a month to maintain actively growing Working cultures were prepared by inoculati of f_2 medium with 90 mL of stock culture in Erlenmeyer flasks. Working cultures were with continuous bubbling of 0.2-µm-filtered a

Concentrated algae

Centrifugation was chosen as an efficient (: recovery) way to concentrate micro Concentrates of the nine species of microalgae produced by centrifuging 500-L bag or 1000-L vat cultures at NSW Fisheries, Port Ste Research Centre. The cultures were grown i same medium as the fresh cultures described but in natural sea water that was aged and s for a minimum of 1 week before use. The sea was then passed through a cartridge filter nominal pore size), and chlorinated for at leas before dechlorination as above. All cultures grown at 23 ± 2 °C under 16 h:8 h light:dark and moderately agitated and buffered to main pH in the range 8.0–8.5 by continuous bub with 1% CO_2-enriched air.

Open-top 1000-L cylindrical polyethylene illuminated by a single 400 W metal halide that provided 300–350 µmol photon $m^{-2}\,s^{-1}$ a culture surface, were used to culture all microalgae except for the prymnesiophytes *P. l* and T-ISO. The latter were produced in 500-L polypropylene film bags with four 58 W cool fluorescent tube lamps that provided illuminati 70–100 µmol photon $m^{-2}\,s^{-1}$ at the culture su

Algal cells were harvested during mid- to late-logarithmic growth phase, and a minimum of three cultures was used for each species to reduce the effect of culture variability.

Different methods of harvesting, storage and preservation were used for different species of algae to ensure a minimum shelf life of 6–8 weeks. Details can be found in Heasman, Diemar, Sushames, O'Connor & Foulkes (2000). Concentrates of *P. lutheri*, T-ISO and *T. pseudonana* were harvested with a bucket centrifuge (Hitachi 05P-21B, Japan) at 2000 r.p.m. (800 ***g***) for 5 min (Table 1). All other concentrates were harvested by pumping the cultures (2000 L h^{-1}; Mono CP800 pump fitted with a variable speed drive) to a supercentrifuge (Sharples MV3532Y22KY32, Surrey, UK) operating at 15 000 r.p.m. (13 000 ***g***). The supercentrifuged concentrates, depending on species, were stored either as emergent stiff pastes or as more diluted slurries; they are both referred to here as concentrates. Except for the *T. chuii* concentrate that was stored at −18 ± 1 °C, concentrates of all other microalgae were stored at 2–4 °C for 9–14 weeks (Table 1). These storage periods were longer than recommended by Heasman *et al.* (2000) based on cell viability or direct bioassays with larval and juvenile bivalves. However, the difficulty of co-ordinating the production of prawn larvae with a particular age of concentrate meant that our storage periods were prolonged.

Each day, freshly harvested algal cultures and small aliquots of the algal concentrates were fed to the larvae in the Larvatron. The algal concentrates were resuspended and diluted in stock bottles by gentle shaking in filtered sea water. The bottles of fresh and concentrated algae were placed in a shaking water bath at 4 °C for feeding to the larvae. The density of cells in the bottles was monitored with a Neubauer haemocytometer.

Larvatron

These experiments would, under normal circumstances, be extremely labour intensive and require large numbers of rearing vessels and the space to house them. The Larvatron, a larval and zooplankton culturing apparatus, was used for the feeding trials because it is fully automated and capable of maintaining up to 200 culture vessels simultaneously in a small area (Jackson, Pendrey & Rothlisberg 1992).

In the current study, the Larvatron consisted of 100 individual culture vessels (housing 20 treatments × five replicates) made from 1.5-L plastic, round-bottomed, soft-drink bottles. They were moved continuously around a closed-circuit spiral track in a temperature- (28 ± 1 °C) and light-controlled room. The densities of algal cells in the fresh and concentrate stock bottles were entered into the Larvatron computer. Water was exchanged in each culture vessel at the rate of 10% approximately every 2 h. The culture water removed from each vessel was pumped through a fluorometer to determine the density of algae in that vessel. The fluorometric signal was sent to the computer, and the volume of algae from the stock bottles that was added to each vessel was adjusted automatically to maintain the specified algal density.

The water make-up and feeding system of the original Larvatron (Jackson *et al.* 1992) were redesigned for the present series of experiments. The new design (Fig. 1) caters for up to 10 food sources and can be easily and cheaply expanded to accommodate many more.

In the new system, a single peristaltic pump (Alitea, model S1, Stockholm, Sweden) supplies food via the delivery probe to the culture vessels (Fig. 1). The food is selected by opening the appropriate food pinch valve (Neptune Research, model 225P, NJ, USA); only one of these opens at any one time. Pinch valves were chosen for this purpose because there is little likelihood of contamination (the algal medium contacts only silicon and PTFE tubing), and because they are easy to clean and sterilize. The duration of pumping determines the quantity of food delivered. After the pumping time has elapsed, the food pinch valve closes and the air pinch valve opens; pumping then continues until all the suspended food is delivered to the probe. Finally, the air pinch valve closes and the main solenoid valve opens, delivering the sea water and flushing any remaining food into the culture vessel. The Larvatron computer controls the operation of all pumps and valves.

Cross-contamination between successive deliveries of different food sources was controlled using hydrophobic PTFE tubing wherever possible and by minimizing tubing lengths. Microscopic examination of the water from the culture vessels confirmed that contamination levels were at acceptable levels ($< 1 \times 10^3$ cells mL^{-1}).

Table 1 Cell densities, consistency of concentrate, centrifugation and storage conditions of microalgae fed to *Penaeus monodon* larvae

Alga	Feeding density (cells mL^{-1})		Consistency of concentrate	Centrifugation speed (r.p.m.)/	Storage time (weeks)		Recommended storage time (weeks)*
	High	Low		(°C)cold storage	Expt 1	Expt 2	
Chaetoceros calcitrans	1×10^5	5×10^4	Paste	15 000 2–4	9		⩽8
Chaetoceros muelleri	1×10^5	5×10^4	Slurry	15 000 2–4	9	14	<1
Pavlova lutheri	1×10^5	5×10^4	Slurry	2000 2–4	9		6–7
Isochrysis sp. (Tahitian)	1×10^5	5×10^4	Slurry	2000 2–4	9		1
Tetraselmis chuii	3×10^4	8×10^3	Paste	15 000 –18	9		>14
Nannochloropsis oculata	2×10^5	5×10^4	Paste	15 000 2–4		14	>14
Phaeodactylum tricornutum	1×10^5	5×10^4	Paste	15 000 2–4		14	2
Skeletonema costatum	1×10^5	5×10^4	Slurry	15 000 2–4		14	⩽ 8
Thalassiosira pseudonana	1×10^5	5×10^4	Slurry	2000 2–4		14	⩽3

*Recommended storage time based on bioassay results with larval and juvenile Sydney rock oysters *Saccostrea glomerata = commercialis* and other indirect criteria for retained quality (M P Heasman *et al.* 2000).

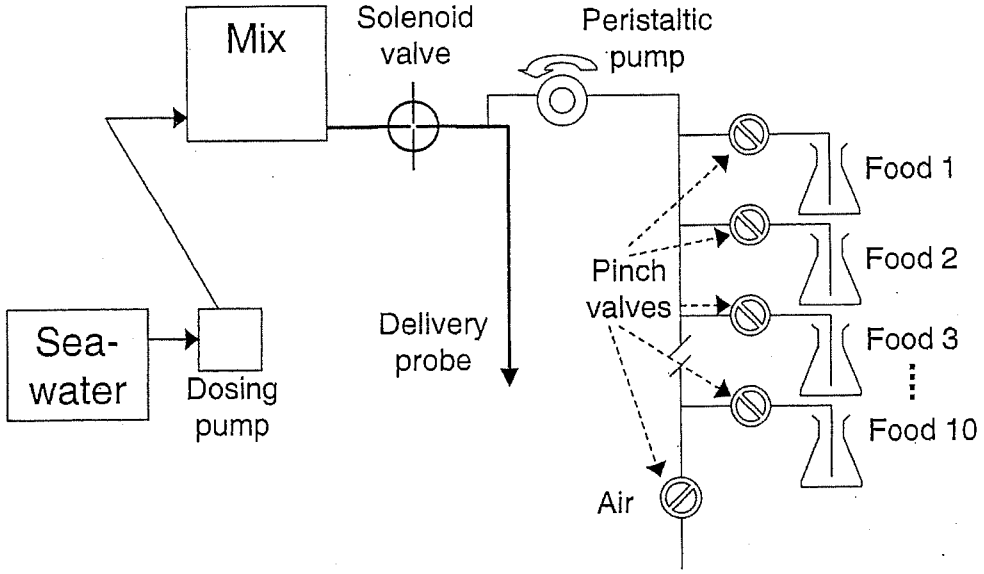

Figure 1 Modified Larvatron water make-up and feeding system. Under computer control, a peristaltic pump delivers metered amounts of selected food via the delivery probe to the culture vessels. The food is selected by opening the appropriate pinch valve. Air is used to clear the food lines, and the required amount of sea water is then delivered with a dosing pump, flushing the delivery probe of remaining food. See text for further details of operation.

Larvae

Penaeus monodon Fabricius nauplii were obtained from local hatcheries at about 24 h after hatching. The larvae in expt 1 were from a mixed spawning, while those in expt 2 were from a single spawning. The nauplii were counted with a custom-built electronic counter based on the light interruption principle and were stocked in the culture vessels at 100 larvae L^{-1} of filtered sea water.

The larvae were fed to excess with algae at either a high or a low density (Table 1). High densities were chosen as follows: *T. chuii* – 3×10^4 cells mL^{-1} [determined from density of *T. suecica* tested in a preliminary trial by D'Souza (1998)]; *C. muelleri* – 1×10^5 cells mL^{-1} [used by Villegas & Kanazawa (1979) and by Australian prawn hatcheries]. The remaining species were fed at high densities chosen by comparing their cell volumes (Smayda 1978) with those of *C. muelleri* and *T. suecica*. Low densities were set at about half the high cell densities. The cell densities in the culture vessels were monitored and adjusted, if necessary, every 2 h, although in most feeding experiments of this kind, cell densities are adjusted only once a day (e.g. Biedenbach *et al.* 1990; Nell & O'Connor 1991).

At the end of the experiments, all the larvae were preserved in a neutral-buffered (boric acid-saturated) solution of 10% formaldehyde in sea water before counting and development staging according to Motoh (1979).

A development index was calculated by applying the formula of Villegas & Kanazawa (1979): development index = A/total number of larvae staged, where A = Σ (stage value × number of larvae at that stage). The stage values increase as the larvae moult through the protozoeal substages and metamorphose to mysis 1 with values for protozoea 1 (PZ1) = 1, PZ2 = 2, PZ3 = 3 and mysis 1 = 4. Therefore, the greater the proportion of later stage larvae in a treatment, the higher the development index.

Experimental design

Two experiments were designed to test the effect of different algal species at different densities and in different forms on the growth and survival of prawn larvae. In expt 1, the larvae were fed with *C. muelleri*, *C. calcitrans*, *P. lutheri*, *T. chuii* or T-ISO at both densities (high and low) and in both forms [fresh and concentrated (9 weeks old)] (Table 1).

There were five replicates per treatment. In expt 2, the larvae were fed with *C. muelleri*, *N. oculata*, *P. tricornutum*, *S. costatum* or *T. pseudonana* at both densities and in the fresh and concentrated (14 weeks old) form. A control treatment of fresh *C. muelleri*, fed at high density, was included in each experiment, as this diatom is widely used in Australian hatcheries. The experiments were stopped when ≈ 50% of the larvae in the control had metamorphosed to mysis 1, which occurred within about 5 days. The fresh T-ISO treatments in expt 1 were stopped at day 4, because there was insufficient algal culture to continue feeding. Artificial diets, which are commonly used in hatcheries along with fresh algae, were not used in these experiments.

Statistical analysis

The data from these experiments were analysed with a one-way analysis of variance (ANOVA) to test for differences in survival and development of larvae fed different algal species at high density in fresh or concentrated form (SAS Institute Inc. 1989). Tukey's test was used to compare the mean values. Before statistical analysis, the survival data were arcsine square-root transformed. A *t*-test was used to compare the effect of algal density on larval survival and development. The fresh T-ISO treatments were not included in the ANOVA because these treatments were stopped before the others. When there were less than 10 larvae in any replicate, this replicate was not included in the development index analysis. Thus, the number of replicates for the development index was not always five for each treatment.

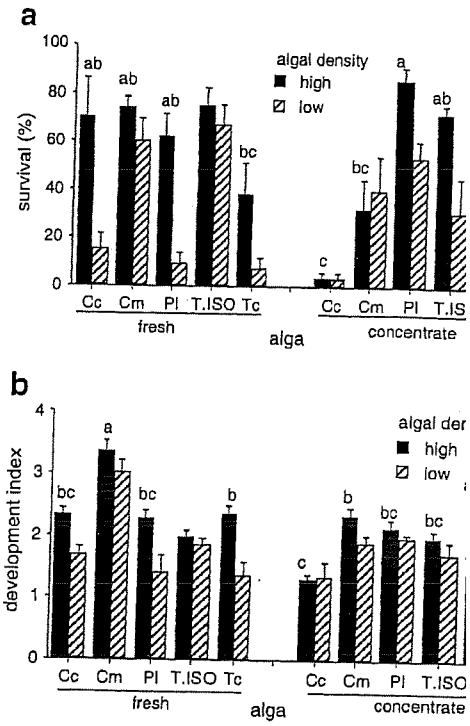

Figure 2 (a) Mean survival (± SE) and (b) m development index (± SE) of *Penaeus monodon* larvae different species of algae in a fresh and concentra form at a high and low density of algal cells. *Chaetoceros calcitrans*; Cm, *Chaetoceros muelleri*; Pl, *Pavl lutheri*; T-ISO, *Isochrysis* sp. (Tahitian); Tc, *Tetrasel chuii*. $n = 5$ for each mean. Means with the sa superscript are not significantly different ($P > 0.05$); o high density-fed larvae compared. Fresh T-ISO treatme were stopped early and therefore are not included statistical analyses.

Results

Experiment 1

Survival of prawn larvae

Larval survival was high (≥ 65%) with three of the fresh (*C. calcitrans*, *C. muelleri* and *P. lutheri*) and two of the concentrated (*P. lutheri* and T-ISO) algal diets fed at the higher density (Fig. 2a). There were no significant differences in survival of larvae fed these diets ($P > 0.05$). The survival of larvae fed the fresh T-ISO at either density was also high (≥ 70%) but, as this treatment was stopped earlier than the other treatments, care should be taken in interpreting the results.

The survival of larvae fed a particular algal diet either high or low density was compared. A lo feeding density of fresh *C. muelleri* or T-ISO concentrated *C. calcitrans*, *C. muelleri* or *T. chuii* d not affect larval survival ($P > 0.05$, Fig. 2a However, larvae fed fresh *C. calcitrans*, *P. lutheri* *T. chuii* ($P = 0.0591$) or concentrated *P. lutheri* or ISO ($P = 0.0565$) at high density had a significantl ($P < 0.05$) better survival rate than when fed the at low density.

Development of prawn larvae

Of the 10 diets tested in expt 1, the fresh *C. muelle* fed at the higher density promoted the faste development of larvae ($P < 0.05$, Fig. 2b). Th

development index (DI) was greater than 3.3, indicating that some of the larvae had moulted to the mysis 1 stage within 5 days. The larvae fed fresh or concentrated *T. chuii* were apparently next best, with DI values of about 2.5. However, statistically, these DI values did not differ ($P > 0.05$) from several other diets at the higher density (fresh *C. calcitrans* and *P. lutheri* and concentrated *C. muelleri*, *P. lutheri* and T-ISO).

The development of larvae fed a particular algal diet at the two densities was compared. A low feeding density of fresh *C. muelleri* or concentrated *C. calcitrans*, *P. lutheri*, T-ISO or *T. chuii* did not affect larval development ($P > 0.05$, Fig. 2b). However, larvae fed fresh *C. calcitrans*, *P. lutheri* ($P = 0.0510$) or *T. chuii* ($P = 0.0505$) or concentrated *C. muelleri* at high density developed significantly ($P < 0.05$) faster than when fed them at low density.

Survival and development rates combined

When the algal diets were compared on the basis of both larval survival and larval development (Fig. 3), fresh *C. muelleri* fed at the higher density performed the best, producing larvae with high survival and the fastest development of all the diets. It was closely followed by fresh *C. muelleri* fed at the lower density. Although the *P. lutheri* concentrate fed at the higher density supported higher survival than all the other diets, the corresponding rate of development was slower than that obtained with fresh *C. muelleri* fed at the higher density. In fact, the development rate of larvae fed this *P. lutheri* diet was similar to the development rates (DI ≈ 2) obtained with a majority of the other diets (Fig. 3).

The poorest diets in terms of low larval survival (< 5%) and rates of development less than half (DI < 1.3) that of the best diet were the concentrated *C. calcitrans* fed at either density. Fresh *P. lutheri*, *T. chuii* and *C. calcitrans* fed at the lower density also performed poorly (Fig. 3). Survival was below 20% in these treatments, with most larvae remaining at the PZ1 stage of development (DI < 1.5).

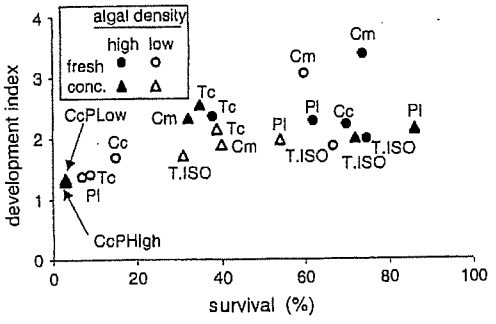

Figure 3 Combined plot of mean survival and development index of *Penaeus monodon* larvae fed different species of algae in a fresh or concentrated (conc.) form at a high or low density of algal cells. Cc, *Chaetoceros calcitrans*; Cm, *Chaetoceros muelleri*; Pl, *Pavlova lutheri*; T-ISO, *Isochrysis* sp. (Tahitian); Tc, *Tetraselmis chuii*. When symbols overlap, P = concentrate, Low = low density-fed, High = high density-fed.

Experiment 2

Survival of prawn larvae

Again, larval survival was high (≥ 70%) with the fresh *C. muelleri* diet fed at the higher density (Fig. 4a). However, there were no significant differences ($P > 0.05$) in survival between larvae fed this

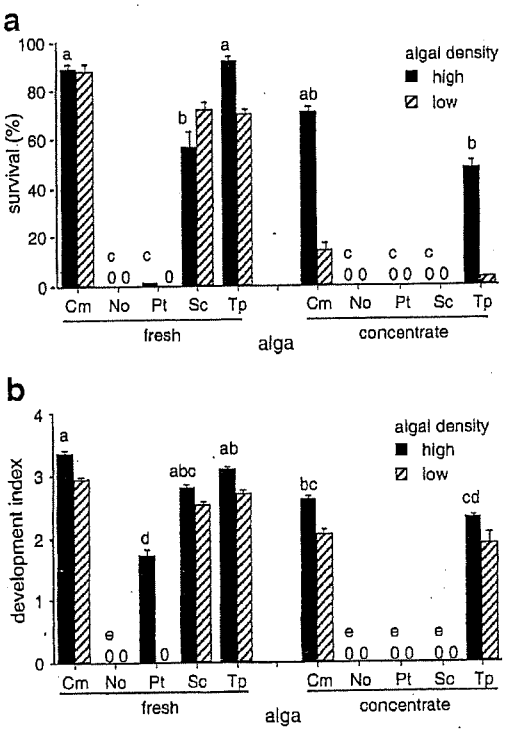

Figure 4 (a) Mean survival (± SE) and (b) mean development index (± SE) of *Penaeus monodon* larvae fed different species of algae in a fresh or concentrated form at a high or low density of algal cells. Cm, *Chaetoceros muelleri*; No, *Nannochloropsis oculata*; Pt, *Phaeodactylum tricornutum*; Sc, *Skeletonema costatum*; Tp, *Thalassiosira pseudonana*. $n = 5$ for each mean. Means with the same superscript are not significantly different ($P > 0.05$); only high density-fed larvae compared.

diet and larvae fed fresh *T. pseudonana* or concentrated *C. muelleri*.

The survival of larvae fed a particular algal diet at the two densities was compared. As in expt 1, the lower feeding density of fresh *C. muelleri* did not increase the mortality of larvae ($P > 0.05$, Fig. 4a). This was also the case with the lower density of fresh *S. costatum*. However, larvae fed fresh *T. pseudonana* or concentrated *C. muelleri* or *T. pseudonana* at high density had a significantly ($P < 0.05$) better survival rate than when fed them at low density.

Nannochloropsis oculata and *P. tricornutum*, whether fresh or concentrated, did not support, or barely supported, survival of larvae for the duration of the experiment (Fig. 4a). On the other hand, *S. costatum* supported reasonable survival of larvae when fed fresh, but did not support survival of larvae when fed as an aged concentrate (Fig. 4a).

Development of prawn larvae

The fastest rates of development (DI > 2.8) were obtained with fresh *C. muelleri*, *S. costatum* and *T. pseudonana* fed at the higher density ($P < 0.05$, Fig. 4b). Relatively high rates of development (DI > 2.5) were also obtained with the concentrates of *C. muelleri* and *T. pseudonana* fed at the higher density.

The development of larvae fed a particular algal diet at the two densities was compared. The lower density of fresh *S. costatum* and concentrated *T. pseudonana* did not affect larval development ($P > 0.05$, Fig. 4b). However, larvae developed significantly ($P < 0.05$) faster when fed fresh *C. muelleri*, *P. tricornutum* or *T. pseudonana* or concentrated *C. muelleri* at high density than when fed them at low density.

Survival and development rates combined

A simultaneous comparison of larval survival and development confirmed that fresh *C. muelleri* and *T. pseudonana* fed at the higher density were the best algal diets, again followed closely by the fresh *C. muelleri* fed at the lower density (Fig. 5). In contrast, the worst algal diets – *N. oculata* and *P. tricornutum* fed either fresh or concentrated, and *S. costatum* fed concentrated – all performed poorly regardless of cell density. Indeed, in all these treatments, less than 5% of the larvae survived, and those that did survive were mostly at the PZ1 stage.

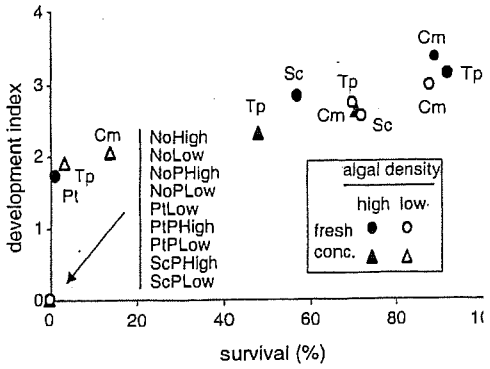

Figure 5 Combined plot of mean survival a development index of *Penaeus monodon* larvae different species of algae in a fresh or concentrat (conc.) form at a high or low density of algal cells. C *Chaetoceros muelleri*; No, *Nannochloropsis oculata*; *Phaeodactylum tricornutum*; Sc, *Skeletonema costatum*; *Thalassiosira pseudonana*. $n = 5$ for each mean. Wh symbols overlap, P = concentrated algae, Low = l density-fed, High = high density-fed.

Discussion

The results of this study show that the feedi density of fresh *C. muelleri* could be halved Australian penaeid prawn hatcheries witho greatly compromising survival or development larvae. This would reduce the reliance on expensi and problematic continuous production of fre algae.

Our study also showed that fresh *T. pseudona* was as good as fresh *C. muelleri*. However, *pseudonana* is more difficult to mass culture tropical Australia and so may not be an appropri substitute for the more commonly used *C. muelle*

The same *C. muelleri* concentrate was used both experiments, differing only in period of stora (either 9 or 14 weeks). There was no apprecial decrease in overall performance of larvae fed t 14 week-old concentrate compared with those the 9 week-old concentrate. In fact, the survival r of the larvae fed the older concentrate at the high density of cells was better than that of larvae fed t younger concentrate at the same density, althou this difference could be attributable to differences the quality of larvae from different spawnir (D'Souza & Kelly, 2000). Feeding younger conc trates to larvae may, as a general rule, improve t

larval development rate over feeding older concentrates (see below).

The results also exposed some stark inconsistencies in the effect of concentrated algal diets on different groups of invertebrates or even different life stages of the same species. Complementary research by Heasman *et al.* (2000) found that concentrates of *C. muelleri*, *T. pseudonana* and T-ISO, as used in the present study, appeared to have no nutritional value for larval and juvenile oysters *Saccostrea glomerata* within 1–3 weeks of storage. In contrast, the present study had very encouraging results feeding the same concentrates to larval *P. monodon*, even though the concentrates had been stored for 9–14 weeks. While excellent results were obtained with 6- to 7-week-old concentrated *C. calcitrans* fed to oyster larvae, extremely poor results were obtained with the 9-week-old diet fed to prawn larvae. In the same vein, encouraging results obtained with aged concentrates of *T. chuii* as diets for both juvenile oysters and larval prawns did not extend to larval oysters (Heasman *et al.* 2000).

Combinations of fresh algal diets have been shown to benefit prawn larvae (D'Souza & Loneragan 1999). Combinations of centrifuged concentrates of two species of algae (binary diets) yielded growth and survival rates for larval and juvenile oysters equal to the best known combination of fresh algae (Heasman *et al.* 2000). It would therefore be useful to identify similar binary concentrate diets for prawn larvae.

Loss of nutrients during storage of concentrates may affect their efficacy as diets for aquacultured animals (Brown 1995). Brown (1995) found that centrifuged *C. calcitrans* lost ascorbic acid with storage age. Molina Grima *et al.* (1994) and Montaini *et al.* (1995) found, however, that polyunsaturated fatty acids remained constant in centrifuged *Isochrysis galbana* and *Tetraselmis suecica*. Other losses of nutrients, such as total protein, carbohydrate and lipid, could have affected the development rate of larvae. In future experiments, it would be useful to measure the effect of storage age on a broader range of nutritional components, including vitamins, in the algal concentrates.

In addition, the storage conditions of the concentrates (light, temperature, oxygen, preservatives) and their feeding density need to be optimized to ensure a more stable and thus nutritious product for aquaculture.

Viable bacterial numbers and potential bacterial pathogens were not assessed in the algal concentrates or larval cultures. However, O'Connor & Nell (1992) found that centrifuging algae before feeding reduced the bacterial numbers introduced to larval oyster cultures.

The process of concentrating algal cells may affect the efficacy of the resultant concentrate. Cells can be damaged or leak their contents during centrifugation, and autolysis and microbial degradation can occur during long storage. To reduce the effects of centrifugation, a more gentle method of harvesting cells by flocculation was recently developed by Knuckey (1998). This flocculation process, along with shorter storage times, warrants evaluation as an alternative method of producing algal concentrate diets for prawn larvae.

Conclusion

The results of this study indicate that, although survival of larvae fed some concentrates is quite high and similar to that of their fresh-fed counterparts, development is often retarded. Thus, centrifuged concentrates of a single species of microalgae cold stored for 9–14 weeks are not recommended as stand-alone diets for prawn larvae. However, concentrates of some species, in particular *C. muelleri*, *P. lutheri* and T-ISO, may be a suitable supplement to artificial diets in the same way that fresh algae are currently used, or as a partial replacement for fresh algae. Preliminary laboratory trials have shown that these centrifuged concentrates may be as effective as fresh algae when fed in conjunction with an artificial diet. However, further testing of these aged concentrates as supplements to artificial diets will be needed to confirm their efficacy compared with fresh algal supplements.

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