# Investigation into development and preparation of 'artificial' food particles for tropical rock lobster, *Panulirus ornatus*, phyllosoma.

Thesis submitted by

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A late stage phyllosoma of the tropical rock lobster, *Panulirus ornatus*.

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## *Abstract*

This study investigated development of an 'artificial' inert food for tropical rock lobster, *Panulirus ornatus*, phyllosoma, of similar consistency to their natural diet of soft-bodied organisms. Aquaculture hatcheries worldwide rely either partially or totally on production of live foods to rear the larvae of target species. The cultivation of live foods still remains expensive, often unpredictable and, from a nutritional perspective, they maybe sub-optimal. Suitable foods for the culture of rock lobster phyllosoma include *Artemia* during early stages and, mussel gonad and other live zooplankton for later stages of phyllosoma. However, some problems associated with water quality and with tangling of the spiky pereipods of phyllosoma on large fleshy prey, have been reported. Developing an inert food particle could potentially enhance survival and nutrient delivery. Inert food particles have been trialed successfully for a variety of larval crustaceans including penaeid prawns, crabs and freshwater prawns.

Production of a moist microbound diet (MBD) particle using the atomisation method was successfully scaled-up during this study from hand-held glass atomiser to a spraying system capable of producing 1-1.3 kg of moist MBD within 5 minutes. The preferred size ranges of food particles for rock lobster phyllosoma stage 1-3 was 355 – 500 μm and for stages 3-5 were 500 – 850 μm. Size ranges of MBD produced during the study were between 100 and >850 μm, under the conditions trialed however, the size distribution of resulting MBD was broad.

Techniques for preparing multi-walled microcapsules (MC), to encapsulate a broad range of dietary ingredients, were assessed during this study based on similar methods developed by the biomedical and pharmaceutical industries. Multi-walled particles prepared during this study were between 0.5 - 4 mm in diameter. Multi-walled MC allow greater retention of valuable dietary material such as low molecular weight water-soluble nutrients, and allowed for more effective delivery of nutrients to the target species.

The technique for evaluating ingestion of MBD by early *P. ornatus* phyllosoma, stages was determined using a dietary radioisotope tracer ($^{14}C$). The quantity of ingested MBD significantly increased with progressive stages and ingestion period (up to 4 h). Consumption of MBD by stages 1 and 2 phyllosoma was very low however, consumption by stages 3 and 4 phyllosoma was significantly higher and they consumed 3.6 μg larvae$^{-1}$ and 2.75 μg larvae$^{-1}$ after 4 h ingestion duration, respectively. Stage 4 phyllosoma could potentially be the age at which inert food particles are accepted by phyllosoma however, further research is required to confirm this.

Low ingestion rates of inert food particles are characteristic of the larvae of many marine species, with the exception of penaeid prawns. Feeding strategies of other crustacean larvae, including phyllosoma, involve a series of processes detection (chemical, mechanical and/or visual), seeking and capture strategies to successfully capture prey. Phyllosoma are attracted to the swimming action of *Artemia,* and capture and extract nutrients from them. Ingestion and assimilation of $^{14}C$-labelled live *Artemia* metanauplii (1.5 day old) was determined for stage 1 phyllosoma, over a

4 h period. After 4 h, stage 1 phyllosoma consumed approximately one (1.5 day old) *Artemia* metanauplus (8.282 ± 0.501 μg larvae$^{-1}$), when supplied at a density 1 mL$^{-1}$. The highest assimilation of live *Artemia* occurred during the 2 h and 3 h feeding periods 0.62 ± 0.21 or 5.31 ± 1.84 μg larvae$^{-1}$ and 0.63 ± 0.343 or 5.35 ± 2.9 μg larvae$^{-1}$ (mean ± SE), respectively; however, these did not differ significantly ($P>0.05$). The second and third hour of feeding also had the highest assimilation efficiency of *Artemia* by stage 1 phyllosoma.

Substitution of live feeds with MBD was investigated for phyllosoma stages 1 and 3. Survival of early stage phyllosoma fed *Artemia* (1 metanauplus mL$^{-1}$) was shown to be greater than that of phyllosoma fed combinations of *Artemia* and MBD or fed MBD alone. However, both stage 1 and stage 3 phyllosoma fed entirely on MBD survived longer than unfed phyllosoma.

The results of this study allowed a suggested feeding protocol for *P. ornatus* phyllosoma to be proposed using both *Artemia* and inert food particles. Newly hatched *Artemia* should be fed to stage 1 and 2 phyllosoma, while larger metanauplii (500-3500 μm) are suitable in size for later phyllosoma stages. The possible weaning process or introduction of inert food particles could begin at the earliest with stage 4 phyllosoma. Apart from major cost savings associated with reduced live food use, delivery of an adequate formulated food would increase survival of phyllosoma through their long and relative complex larval life. As the nutritional composition of inert food particles can accurately be manipulated, the opportunity to determine the nutritional requirements of phyllosoma could then be achieved, at least for phyllosoma stages which consume such particles.

This study has provided valuable new information relating to the feeding biology and nutrition of *P. ornatus* phyllosoma. The results will allow further development towards production of appropriate hatchery foods for *P. ornatus* phyllosoma and facilitate progress towards more efficient hatchery techniques for this species.

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# Chapter 1

# A review of the development and preparation of artificial diets for larval stages, with particular emphasis on their potential for spiny lobster phyllosoma

## 1.1 Introduction

As worldwide demand for seafood continues to increase and current fisheries are either maximally exploited or over-exploited, aquaculture production is a favourable option to meet increasing demand. Lobsters are decapod crustaceans of the class Malacostraca and globally represent one of the most valuable seafood resources with well established fisheries (Jeffs and Hooker 2000). The commercial lobster market is comprised mainly of the Nephropoidea clawed lobsters, representing 62.6% of the total value. However, Palinurid or spiny lobsters are of significant importance, representing 34.9% of total harvest value (Fig. 1.1). The Australian spiny lobsters include *Panulirus cygnus, Panulirus* (tropical) spp. and *Jasus edwardsii* all of which have established domestic and international markets and interest. However, all of these fisheries are at maximum harvest capacity with demand exceeding supply. The only avenue to meet this demand is through aquaculture (Linton, 1998). Nevertheless, the major impediment for closed life-cycle lobster aquaculture is successful mass rearing of larvae which have a very long and complex planktonic phase (Kittaka, 1994; Kittaka and Booth, 2000). Despite decades of research overseas only marginal success has been obtained in larval rearing, with limited numbers of larvae, called phyllosoma, reared through to the juvenile stage. Unfortunately little is known about

the preferred culture conditions and in particular the nutritional requirements of phyllosoma.

**Fig. 1.1. The world fishery production of lobsters (Source: ABARE 1998-99).**


## 1.2 Live foods

The majority of aquaculture hatcheries rely either partially or totally on production of live foods to rear the larvae of the target species. The live foods often include at least one of the following; microalgae, brine shrimp (Artemia spp.) and rotifers (Langdon et al., 1985; Langdon, 2000; Southgate and Kolkovski, 2000). Although progress towards routine economical production of live foods is continual, the cultivation of live foods still remains expensive, often unpredictable and, from a nutritional perspective they maybe sub-optimal (Jones et al., 1993; Southgate and Kolkovski, 2000). Some of the general problems associated with live food production are outlined in Table 1.1.

Table 1.1. The problems associated with live food production for larval culture (Modified from Southgate, 2003).

| **Characteristic** | **Comments** |
|---|---|
| Nutritional deficiency | The majority of live foods have nutritional deficiency, particularly in fatty acid composition and require enrichment additives, which increases production cost. |
| Nutritional inconsistency | Live feed organisms may vary in their nutritional composition, according to source, i.e., the genetic strain of *Artemia* and nutritional status of the live feed varies. |
| Reliability of supply | Hatcheries world-wide rely heavily on supply of live food from over-seas, for example, *Artemia* cysts which vary from year-to-year. Live feed cultures are also susceptible to "disease crashes". |
| Health status | Live feeds may themselves be pathogen vectors resulting in health problems in the larvae. |
| Adequate size | The physical size of the live food must be optimised to larval characteristics, i.e., mouth gape, capture ability, etc. |

However, the main advantage of using live foods is that they are readily accepted by early stage larvae, which is important in achieving high survival.

Table 1.2. The various foods assessed for phyllosoma of spiny lobsters (*Jasus* spp. *Panulirus* spp. and *Palinurus* spp.) and slipper lobster (*Ibacus* spp.).

| Species | Food | Comments | Author(s) |
|---|---|---|---|
| *Jasus edwardsii* | *Artemia* nauplii | 1-3/mL (0.47mm) | Batham (1967) Tong *et al.* (1997) Kittaka *et al.* (1998) Moss *et al.* (1999) |
| | *Artemia* metanauplii | 1-3/mL | Kittaka *et al.* (1988) Tong *et al* (2000) Ritar *et al.* (2002) Smith *et al*. (2003) |
| | *Mytilus* spp. (mussel), *Katelesia scalarina* (Clam), *Crassostrea gigas* (Pacific oyster). | Suitable for later staged larvae (0.04-0.08 pieces/mL) | Kittaka *et al.* (1988) Kittaka (1997b) Ritar *et al.* (2002). |
| *J. sagmariasus* | *Artemia* nauplii | 1-3 /mL early stages | Kittaka *et al*. (1997) |
| | Mussel gonad | Suitable for older stages | Kittaka *et al*. (1997) |
| *J. lalandii* | *Artemia* nauplii | + Stage 1 larvae | Kittaka *et al*. (1988) |
| | Mussel gonad | + For stages 2+ | Kittaka *et al.* (1988) |
| *Panulirus japonicus* | *Artemia* nauplii | Suitable for stages 1-3. | Inoue (1978, 1981) Kittaka *et al.* (1988) |
| | Mussel gonad | Suitable for stages3 + | Kittaka and Kimura, (1989) Yamakawa *et al.* (1989) |
| | Fish larvae | + For stages 10 and above. | Saisho (1966a; 1966b) |
| *P. argus* | *Artemia* metanauplii, Angelfish (alive), Mangrove snapper (alive), Jelly fish (fresh strips), Conch (fresh grated), Coral banded shrimp, Dolphin flesh, Jewelfish (larvae) | *Artemia* were superior for early stage. While fish larvae for older phyllosoma | Moe (1991) |

| | | | |
|---|---|---|---|
| *P. interruptus* | *Artemia* nauplii, *Artemia* metanauplii, Ctenophores Fish larvae, Chaetognaths Sea urchin (gonad), Mytilus gonad, Tubifex | *Artemia* superior for early stages. Fish larvae and mussel for older stages | Dexter (1972) |
| *P. cygnus* | *Artemia* metanauplii | 3 *Artemia*/mL | Liddy *et al*. (2003) |
| *Palinurus elephas* | Mussel gonad | + for early instar | Kittaka and Ikegami (1988), Kittaka *et al*. (1988) |
| *Ibacus ciliatus* and *I. novemdentatus* | *Artemia* nauplii | Complete development | Takahashi and Saisho (1978) |
| | *Tapes philippinarum* (flesh) | Complete development | Takahashi and Saisho (1978) |

*Artemia* are the most extensively used larval food for rock lobster phyllosoma (Table 1.2). Phyllosoma are assumed to be attracted to the swimming action of *Artemia* and have the ability to catch, eat and extract nutrients (Ritar *et al.,* 2002, Cox and Johnston 2003, Nelson *et al*., 2004, Jeffs *et al*., 2004). The optimum densities of *Artemia* for feeding of the different stages of phyllosoma have been investigated for a variety of species (Kittaka, 1997b; Tong *et al.,* 1997; Ritar *et al.,* 2002). The number and particularly the size of *Artemia* generally increases with progressive phyllosoma stages (Table 1.2). Mussel gonad and other live zooplankton have also been shown to be suitable foods for the culture of later stages of phyllosoma (Kanazawa, 1994; Kittaka, 1994, 1999). However, some problems associated with water quality and with tangling of the spiky periepods of phyllosoma on the large fleshy prey, have been reported (Kittaka, 1997b). Feeding a combination of foods throughout their long larval phase may enhance survival of phyllosoma.

Live food reliance during hatchery culture of crustaceans can be minimised through partial or entire replacement with artificial alternatives (Jones *et al.,* 1993). The method of reducing (or completely replacing) the proportion of live foods fed to crustacean larvae is a process called 'weaning'. Weaning usually occurs towards the end of the larval period, when small quantities of 'artificial' food(s) are fed in combination with a live food source. Over time, the proportion of artificial diet is increased and live food reduced to achieve replacement without lowering survival. The weaning process is usually achieved within 30 days (Southgate, 2003).

According to Sorgeloos *et al*. (1986), Jones *et al*. (1993), Knauer and Southgate, (1999) and Southgate and Kolkovski, (2000) the major factors driving development of artificial diets to replace live foods are:

(1) expense;

(2) infrastructure and facility requirements;

(3) nutritional quality and deficiencies;

(4) availability;

(5) culture variation, source and age and;

(6) potential disease vectors.

The ability to substitute live foods with artificial diets would bring "off the shelf" convenience and also lower hatchery production costs.

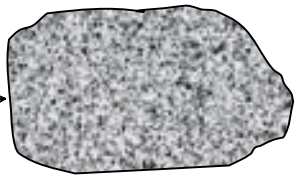
## 1.3 Artificial foods

"Artificial" food particles investigated as replacements for live foods in aquaculture hatcheries are broadly termed "microcapsules". Micro-capsules (MC) can be broadly classified as either micro-encapsulated (MED) or micro-bound (MBD) diets. MBDs can be further subdivided into pelleted, flaked, or cake feeds as well as micro-extruded marumised (MEM), particle assisted rotationally agglomerated (PARA) particles and sprayed beads (SB) (Langdon and Lellis, 2000). Whereas MEDs encapsulate nutrients, either solid or liquid, within a capsule wall, MBDs are particles held together with an internal binder with absorptive and adhesive properties. The final particle size may range from <10 μm to a few millimeters in size. The particles may be spherical, oblong or irregularly shaped, monolithic or aggregates, and may have single or multiple walls (Baken, 1973; Jackson and Lee, 1991). A third class of food particle is the complex microcapsules and can incorporate aspects of MBD and MED technology (Barrows, 2000). The majority of artificial diets developed for aquaculture, originated and were further modified from biomedical and pharmaceutical applications. Attempts to produce artificial diets for a variety of aquaculture species have resulted in several MC types, several of which are available commercially (Jones *et al*., 1984; Buchal and Langdon, 1998). Advancements in MC design are continually being made through research laboratories and commercial manufacturers.

**(1) Micro-bound diet particle (MBD)**

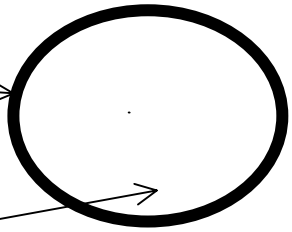
Matrix of alginate, carrageenan etc.

Nutrients trapped within matrix.



**(2) Micro-encapsulated diet particle (MED)**

Distinct wall or membrane.
e.g. cross-linked protein,
gelatin-acacia.



Nutrients such as protein, carbohydrate
and /or lipid, enclosed within a microcapsule wall.

**(3) Complex micro-encapsulated diet particles (CXM)**

Matrix of binder (alginate,
carrageenan etc **)**

A variety of other microcapsule types
trapped within the matrix.

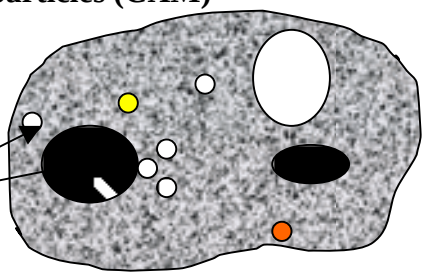


Fig. 1.2. The characteristics of the major types of MC. (1) Micro-bound diet particle; (2) Micro-encapsulated diet particle; and (3) Complex micro-encapsulated diet particles (CXM).

### 1.3.1 Artificial food production

The objective in preparing artificial foods is to encapsulate several ingredients in previously determined proportions to give a complete nutritionally adequate diet, which is protected from the external environment until ingested. Several processing

techniques have been assessed for their potential for providing diets to larval stages (Kondo and Wade Van Valkenburg, 1979; Jackson and Lee, 1991). These include:

(1) spray drying;

(2) spray chilling and spray cooling;

(3) extrusion;

(4) air suspension;

(5) multi-orifice extrusion;

(6) electrostatic bonding and

(7) co-crystillization.

Many of these methods are unsuitable for aquaculture largely due to the low digestibility of the resulting MC. Method selection largely depends on economics, the sensitivity of dietary material, the desired particle size, the physical/chemical properties of both diet and 'wall' material and water stability, which are designed for particular applications (Jones *et al*., 1984; Jackson and Lee, 1991). Assessment of these techniques has identified several MC types with potential as foods for larval culture (Jones *et al.,* 1984; Buchal and Langdon, 1998). MBDs may, for example, be separated into 3 groups according to production process (Barrows and Lellis, 2000; Langdon, 2000) including:

(1) crumbled;

(2) micro-bound complex particles; and

(3) on-sized.

Crumbled foods are produced via extrusion of diets and the particles are milled and crushed to an appropriate size (Barrows and Lellis, 2000). Microbound complex

particles are produced using two or more manufacturing techniques which incorporate aspects of each encapsulation process (Barrows and Lellis, 2000). On-size food particles are manufactured directly to the correct sized particle (Langdon *et al*., 1985; Barrows and Lellis, 2000) and can be separated into several groups (Barrows and Lellis, 2000) including:

(1) micro-extruded marumerized (MEM) particles;

(2) Particle-assisted rotational agglomerated (PARA) particles and,

(3) sprayed beadlets.

MEMs are manufactured from a wet mash of finely ground ingredients which is formed into thin noodles through a marumerizer. PARAs is a modification of the MEM process which elimates the extruder and just uses the marumerization process in the presence of inert particles (Barrows and Lellis, 2000). Sprayed beadlets are prepared using small hand-held glass atomizers. They have previously been used for the production of MC (Levine *et al*., 1983; Levine and Sulkin, 1984; Villamar and Langdon, 1993; Buchal and Langdon, 1998). This method is usually conducted using sodium alginate as the particle binder, which produces rigid gels when sprayed into a curing bath containing polyvalent metal ions (e.g. calcium chloride). The small-scale production of beadlets can be expensive and time consuming, but is useful for small scale experiments. Larger volumes can be produced by the industrial spraying apparatus described by Villamar and Langdon (1993).

### 1.3.2 Binder types

The binders used for MBD have a particular combination of physical and chemical properties (Appendix 1). The important criteria for MBD used as foods for larvae in aquaculture (Barrows, 2000) include:

(1) palatability;

(2) particle and nutrient stability; and

(3) nutritional composition.

Unfortunately, most binders have little or no nutritional value but, in general, they have the ability to bind a wide variety of ingredients (both dry and moist). Binders may be classified into various groups, which occur naturally and could potentially be used to form the matrix of MBD (Moller *et al.,* 1979) and include:

(1) pectins;

(2) starches;

(3) cellulose and chitin;

(4) hemicellulose;

(5) gums; and

(6) mono and oligosaccharides.

### 1.3.3 Micro-bound diet particles

Micro-bound diet (MBD) particles consists of a gelled matrix (Fig. 1.2) held together by an internal binder which maybe a complex carbohydrate or a protein having both absorptive and adhesive properties (Watanabe and Kiron, 1994). The properties of MBD include high palatability, nutrient stability, nutrient availability and particle

stability (Appendix 1) (Barrows and Lellis, 2000). Based on phyllosoma feeding behaviour observed in aquaria, they are believed to consume soft-bodied organisms and hence, the use of MBD with a consistent soft matrix would be logical option to replace live foods. MBDs can be designed to entrap high molecular weight, water-soluble nutrients such as proteins and carbohydrates but they have the disadvantage of having minimal ability to retain low-molecular weight water soluble nutrients, such as free amino acids, minerals and vitamins (Langdon, 1989; Langdon and DeBevoise, 1990), (Appendix 1). The use of a combination of binders in MBD can produce particles which have advantageous characteristics by increasing acceptability and digestibility, through changes in texture and binding properties (Partridge and Southgate, 1998). The majority of binder types used in MBDs commonly include polysaccharides from seaweed such as agar, carrageenan and alginate, and proteins such as zein and gelatin (Meyers *et al.,* 1972; Adron *et al.,* 1974; Chapman and Chapman, 1980; Heinen., 1981; Levine *et al.*, 1983; Teshima and Kanazawa, 1983; Person-Le Ruyet *et al*., 1993; Lee *et al.,* 1996). Many commercially produced MC are MBD based particles due to their ease of manufacture and low production cost.

### 1.3.4 Micro-encapsulated diet particles

Micro-encapsulated diet (MED) particles have a distinct membrane or capsule wall surrounding the capsule contents (dietary ingredients) (Langdon *et al*., 1985; Jones *et al*., 1993; Lopez-Alvarado *et al*., 1994) (Fig 1.2). One of the first MED particles used in biological applications was the nylon-protein MC (Chang *et al.,* 1966) used to produce artificial blood cells containing haemoglobin (Langdon *et al*., 1985). The technique was later modified by Jones *et al.* (1974) and further again by Levine *et al.*

(1983) for feeding crustacean larvae. These modified MC have been used successfully in feeding experiments with fish larvae, crustaceans and bivalves (Langdon, 2000b). MED also have a good ability to encapsulate high molecular weight, water soluble nutrients such as proteins and carbohydrates, but a generally low ability to retain low-molecular weight water soluble nutrients (Langdon, 1989; Langdon and DeBevoise, 1990) (Appendix 1). Examples of commonly used MED include, gelatin-acacia MC, nylon-protein MC, lipid-walled MC and protein-walled MC. All have their advantages and disadvantages. For example, lipid-walled MC have greater ability to retain low molecular weight water soluble nutrients compared with other MCs, but larvae must either have appropriate lipases to digest the capsule wall or peristaltic action of the gut must be sufficient to burst the capsules to release the nutrients (Jopez-Alvarado *et al.,* 1994; Langdon and Buchal, 1998).

### 1.3.5 Complex micro-encapsulated particles

Complex micro-encapsulated particles (CXM) are characterised by one or more MC smaller particle types embedded within a larger particle composed of another binder (Fig. 1.2). CXM incorporate the advantages and overcome the disadvantages of individual production methods (Burrows and Lellis, 2000) through a double encapsulation process. CXM have the ability to retain both high and low molecular weight water-soluble nutrients (Langdon, 2000; Barrows and Lellis, 2000). CXM have been used to deliver micro and macro-nutrients to shrimp larvae using lipid-walled MC embedded within alginate-gelatin (Villamar and Langdon, 1993). Ozkizkzlcik and Chu (1996) prepared protein-walled with lipid-walled MC inside which a high proportion of the amino acid lysine was retained. Although multi-step

processing is involved, the CXM have favourable characteristics for delivery of nutrients to the target species.

### 1.3.6 General characteristics of micro-capsules

In general, the materials used as binders or to form MC walls have little or no nutritional value, with the exception of beef plasma extract which has high protein (70%) content (Appendix 1) and gelatin. Most binders have the ability to encapsulate a wide variety of ingredients from fresh to synthetic powdered nutrients, although gelatin-acacia MC can only be used to encapsulate lipid material (Langdon *et al.*, 1985) or water insoluble particles. Many MBD and MED have high losses of low molecular weight water soluble nutrients (e.g amino acids, vitamins and minerals) (Lopez-Alvarado *et al.,* 1994). Means of avoiding these losses include coating MC with an impermeable membrane (Goldbatt *et al*., 1980; Hall, 1980 Patent No 4,182,778; Blenford, 1986) and production of CXM that utilise two or more encapsulating processes (Ozkizilcik and Chu, 1996). The general trend in artificial diet development is towards particles which are highly stable yet are digestible; a shift towards MBD type feeds because of ease of production, high digestibility, palatability and moderate nutrient stability.

## 1.4 Desired characteristics of formulated artificial food particles

Artificial diet particles must be attractive, acceptable, water-stable and digestible and have high nutritional value and long storage ability (Table 1.3) (Barrows, 2000; Langdon, 2000; Southgate and Kolkovski, 2000). The above criteria for food particle

preparation is influenced by the requirements of the individual species, but the physical characteristics must remain balanced for successful use in culture.

Table 1.3. The desired characteristics of artificial food particles for larval culture (modified from Southgate, 2003).

| **Characteristic** | **Comments** |
|---|---|
| Acceptability | Artificial food particles must be attractive and readily ingested. Diet particles must be the correct size, texture, flavour and must stimulate a feeding response. |
| Stability | Artificial food particles must maintain integrity in aqueous suspension and must retain encapsulated ingredients. Some nutrient leaching maybe beneficial in enhancing diet attractability. |
| Nutrient composition | Artificial food particles must have appropriate and adequate nutritional composition. Materials added to the diet as binders or components of MC walls must have nutritional value |
| Digestibility | Artificial food particles must be digestible and easily assimilated. |
| Storage | Artificial food particles must be suitable for long term (6-12 months) storage with nutrient composition and particle integrity remaining stable. |
| Buoyancy | Artificial food particles must remain in the water column to be available to target species. |

### 1.4.1 Acceptability

The acceptability of artificial food particles is initially the most important characteristic. Larval acceptance and consumption of food has been shown to be affected by the presence of attractants, visual stimuli including size, colour, movement and shape, taste, smell, density, buoyancy in the water column and physical stimuli such as texture and hardness (Kurmaly *et al*., 1990; Barrows and Lellis, 1996; Kolkovski, *et al*., 1997 a,b). These factors, which influence ingestion rate, vary in relevance between the larvae of different marine invertebrates, depending on their mode of feeding and specialised nutritional requirements (Langdon *et al*., 1985). The acceptability of inert particles can be enhanced by the addition of chemical attractants, which has been shown by Kurmaly *et al*. (1990) with lobster *Homarus gammarus,* larvae. Furthermore, artificial food particles fed in combination with live prey have been shown to stimulate feeding responses in larval fish (Kolkovski *et al.,* 1997 a, b).

### 1.4.2 Water stability

Water stability refers to the physical effects on artificial food particles when immersed and involves physical deterioration associated with the absorbance and/or loss of water, loss of valuable nutrients and particle clumping. A major objective of micro-encapsulating dietary ingredients for aquatic animals is to reduce nutrient leaching (Langdon, 2000c). Artificial food particles commonly used by researchers are the MBD and MED types which both retain nutrients of high molecular weight, such as protein, with high efficiencies (Langdon, 1989; Langdon and DeBevoise,

1990). But the retention of low molecular weight nutrients (e.g. free amino acids) is low, with up to 80% of free amino acids lost within the first few minutes of immersion (Lopez-Alvarado and Kanazawa, 1993; Lopez-Alvarado *et al.,* 1994). Factors influencing the level of leaching include binder type and strength, water solubility of the particles contents (e.g. nutrients) and the surface area to volume ratio (Lopez-Alvarado *et al*., 1994; Barrows, 2000). There are several problems associated with nutrient leaching. The most important aspect is the loss of both nutritionally valuable and expensive water-soluble components (e.g. amino acids, vitamins, minerals etc.) into the surrounding water, which can result in elevated bacterial concentrations (Langdon and Bolton, 1984; Muir and Sutton, 1994). The resulting decrease in water quality (Muir and Sutton, 1994) may result in out-breaks of disease (Langdon, 2000c).

In contrast, some nutrient leaching is beneficial (where leached nutrients act as a feeding stimulant) in enhancing the acceptability of MC (Southgate and Partridge, 1998; Southgate and Kolkovski, 2000). Many attempts have been made to reduce losses of dietary ingredients from MC. The use of MC coating materials, for example, has been proven to reduce leaching rates (Heinen, 1981; Jackson and Lee, 1991). Such coating materials include lipid (Langdon *et al.,* 1985; Lopez-Alvarado *et al.,* 1994; Buchal and Langdon, 1998).

### 1.4.3 Nutrient composition

The binders used to make food particles generally have the ability to encapsulate a wide range of nutritional components, which can be precisely manipulated. Nutrient

sources that can be incorporated into artificial food particles include crystalline nutrients (e.g. amino acids, supplements of vitamins and minerals) and fresh materials. Dietary ingredients in their free form are more easily assimilated compared with more complex molecules (i.e. amino acids vs dietary protein) and minimal energy is wasted during digestive processes (Jones *et al.,* 1997). Where as formulated diets must contain adequate nutritional composition, the nutritional requirements of marine crustacean larvae vary and, for all but penaeid prawns, are poorly known. Rearing through the larval phase using artificial foods alone is possible for penaeid prawns (Jones *et al*, 1997). However, little is know about the nutritional requirements of lobster phyllosoma.

### 1.4.4 Digestibility

Digestibility refers to the availability of nutrients from artificial food particles for absorption. Digestibility is influenced by the degree of gut development at a particular ontogenetic stage and, most importantly, the digestibility of the chosen binder (MBD or MED). Digestibility is also influenced by dietary ingredients and to a lesser degree by environmental factors on their performance (Sugiura, 2000). Larvae must have the ability to physically and/or chemically break down the artificial diet binder in order to access the nutrient within. Methods used to increase digestibility of artificial food particles include the use of live foods fed in combination with MC which helps stimulate feeding and enzyme responses (Jones *et al*., 1993; Kolkovski, 2000) or inclusion of digestive enzymes within artificial food particles which are released during their digestion (Kolkovski *et al.,* 1991; Kolkovski *et al*., 1993; 1997c; Jones *et al.,* 1997; Kolkovski *et al*., 2000; Kolkovski, 2001).

### 1.4.5 Buoyancy

Buoyancy of artificial food particles has shown to be a problem especially in systems with poor hydrodynamics where there is insufficient aeration and turbulence to maintain food particles in suspension. MC are usually maintained in suspension using mechanical means such as aeration or water movement within the tank. Alternative options to improve the buoyancy of artificial food particles include the addition of buoyant dietary ingredients (e.g. lipids) or potentially incorporating bubbles within the MC. The encapsulation of lipids would be unsuitable due to the resulting high fat content of the diet. Bubble encapsulation could change the specific gravity and possibly the digestibility of the particle through increasing the surface area for absorption. Incorporation of nitrogen gas would not degrade or oxidise valuable dietary components. The method would be useful in particles used to feed the larvae of carnivorous crustaceans, rather than whole ingestion feeders as the bubbles may interfere with their normal digestion processes.

### 1.4.6 Storage

Artificial food particles should have the ability to be stored over long periods of time (>6 months). The storage of MC within dry, cool and dark environments is necessary for maximum shelf life (Pillay, 1990).

## 1.5 Studies on the feeding of MC to crustacean larvae

As mentioned above, the use of live foods is expensive and labour intensive and their partial or complete replacement would be beneficial to the aquaculture industry. Use of artificial food particles for crustacean larval culture has been studied broadly using a variety of artificial binder types (Guillaume, 2001).

Successful use of MC for feeding crustacean larvae has been reported for the mud crab, *Scylla serrata,* where the level of ingestion of $^{14}$C-labelled MBDs progressively increased with advancing larval stages (Genopeda *et al.,* 2004). Similar results were observed for stage 5 of the freshwater prawn, *Macrobranchium rosenbergii,* larvae fed exclusively a high moisture content formulated MBD where larval survival was almost equivalent to that of larvae fed live *Artemia* nauplii (Kovalenko *et al.,* 2000). The use of nylon-protein MC in feeding trials with brachyuran crabs (*Eurypanupeus depressus*) were investigated by Levine *et al*. (1983) and Levine and Sulkin (1984). When fed MBDs alone, larvae achieved better survival than those in unfed treatments, but did not reach the megalopa stage. While larvae fed live *Artemia* reached megalopa stage at relatively high rate of survival. However, when *E. depressus* larvae were fed alginate-bound *Artemia* in combination with live rotifers they achieved 50-53% survival to megalopa stage (Levine and Sulkin, 1984), while larvae fed live rotifers and 'empty' MC had only 20% survival (Levine and Sulkin, 1984). Kurmaly *et al*. (1990) investigated the acceptability of various foods by larvae of the lobster, *Homarus gammarus*. All diets induced search responses from the larvae but frozen foods were superior to MC, possibly due to higher leaching of nutrients than in encapsulated forms. Survival of the larvae was significantly higher when fed MBD compared to MED, but growth was not different between the two particle types.

Continual developments of techniques for producing more appropriate methods for encapsulating nutrients are a result of changing criteria for MC applications. Phyllosoma require diets, which have high acceptability and digestibility, adequate size and nutritional composition, water and nutrient stability and are buoyant with long storage ability. Artificial food particles are currently unable to completely replace live foods used for phyllosoma culture. This is partly due to their low acceptability (particularly during early stages) and limitations in current culture methods for phyllosoma. Also, knowledge of their nutritional requirements and the ability to provide adequate diet for the duration of phyllosoma culture is limited. Development of a successful artificial food for phyllosoma would not only reduce reliance on live foods and simplify culture methods, but would also provide a tool, which could be used to identify specific nutritional requirements of phyllosoma.

The major objective of this study was development towards an appropriate 'artificial' food for phyllosoma larvae. The specific aims of the study were:

(1) Preparation of moist MBD particles, which contain adequate nutritional composition and are characteristic of phyllosoma predicted natural diet of gelatinous zoo-plankton (see section 2.1.3) (Chapter 3, 4);
(2) Determine the point at which the phyllosoma will physically accept and ingest MBDs (Chapter 5);
(3) Determine the ingestion and assimilation of *Artemia*, which is the current live food used to culture phyllosoma (Chapter 6); and
(4) Investigate the survival of phyllosoma fed *Artemia* substituted with artificial food particles (Chapter 7).

# Chapter 2

# Rock lobster biology and general methods

## 2.1 Biological aspects of rock lobster culture

### 2.1.1 Holding rock lobster broodstock

The breeding period for the tropical rock lobster, *Panulirus ornatus*, occurs during the summer season between December and March (Bell *et al.,* 1987; Junio-Menez and Estrella, 1994), and is stimulated by increasing water temperature and photoperiod as in other rock lobster species (Kittaka and MacDiarmid, 1994). The age of *P. ornatus* at sexual maturity is approximately 30-36 months (123 mm carapace length) on the Queensland coast (Phillips *et al.,* 1983).

Under culture conditions, broodstock density and sex ratios of greater than 4 females to 2 males have resulted in successful reproduction in a number of rock lobster species (Kittaka and Kimura, 1989; Kittaka and MacDiarmid, 1994). Sexually mature male lobsters are able to inseminate several females and females may produce 2-4 consecutive broods each season (Junio-Menez and Estrella, 1994; Pitcher *et al.,* 1995). The eggs are extruded in captive rock lobsters approximately 2 weeks after mating (Phillips *et al.,* 1983) and the eggs are incubated for approximately 3 weeks (pers. obs., 2002). Each batch contains approximately 20,000 to 500,000 eggs (Phillips *et al.,* 1983; pers. obs., 2003). Once the phyllosomes have utilised their yolk reserves and are fully developed (Kittaka and Kimura, 1989) they hatch at night

(Philips and Sastry, 1980; Kittaka, 1994). Immediately after hatch, they moult from a naupliosoma to the phyllosma stage and begin life in the plankton (Phillips and Sastry, 1980). Once the phyllosoma hatch they rise to the surface with offshore dispersal occurring due to wind driven currents. They undergo vertical migration patterns between the surface at night and deeper waters during the day throughout their planktonic period (Booth and Phillips, 1994).

### 2.1.2 Phyllosoma characteristics

Rock lobster phyllosoma are broad, thin, leaf-like schizopod larvae, highly planktonic, transparent in appearance and, without an obvious yolk sack upon hatching (Duggan and McKinnon, 2003). Phyllosoma grow through a series of 8-11 larval stages consisting of morphological moults but may also undergo several instar moults without any apparently morphological changes until they metamorphose into puerulus, the final non-feeding planktonic stage, with the appearance of a juvenile lobster, before benthic settlement (Fig 2.1). The length of the complex larval cycle ranges from 3-24 months, and is significantly shorter in tropical species (Phillips and Sastry, 1980; Kittaka and Ikegami, 1988; Kittaka, 1994, 1997b). The tropical rock lobster, *P. ornatus,* has been identified as an aquaculture candidate due its relatively short larval duration and high growth rate compared to temperate species (Linton, 1998).

Culture of rock lobster phyllosoma has been carried out for more than 50 years in Japan (Nonaka *et al.,* 1958; Nishimura, 1983; Nishimura and Kawai, 1984; Nishimura and Kamiya, 1986; Kamiya *et al*., 1986; Kittaka, 1994, 1997a, b, 2000). To date, extensive culture attempts have been relatively unsuccessful predominantly because

of the length of their larval phase, the fragility of larvae, and lack of understanding of biological, technical, cultural and nutritional requirements. Nevertheless, small numbers of cultured pueruli have been produced in culture (Silberbauer, 1971; Bardach *et al.,* 1972; Dexter, 1972; Van Olst *et al*., 1980; Kittaka, 1988, 1994b; Kittaka *et al*., 1988; Kittaka and Ikegami, 1988; Kittaka and Kimura, 1989; Yamakawa *et al*., 1989; Booth and Kittaka, 1994; Illingworth *et al*., 1997; Tong *et al*., 1997).

### 2.1.3 Phyllosoma feeding

Knowledge of the natural diet and dietary requirements of phyllosoma is still limited (Phillips and Sastry, 1980; Kittaka, 1994b; Tong *et al*., 1997). The gut contents of wild caught Palinurid and Scyllarid phyllosoma have proven difficult to identify, however the mouth morphology suggest that they eat a high proportion of soft-bodied organisms (Phillips and Sastry, 1980; Mikami *et al*., 1994), such as ctenophores, medusae, chaetognaths, fish larvae, salps and other soft organisms (Heron *et al*., 1988; Kittaka, 1997b; Cox and Johnston, 2003). To predict the feeding habits of phyllosoma, a variety of factors must be considered, including their morphological ability to track, capture and digest prey and the plankton most available to phyllosoma during their larval stage.

### 2.1.4 Prey detection

The mechanisms used by phyllosoma to locate their prey probably involve several chemical and mechanical processes (Laverack, 1988; Nishida and Kittaka, 1992) and

potentially visual sensory apparatus. Cox and Johnston (2003) described a combination of integument organs in the temperate species, *Jasus edwardsii,* were plumose setae, possibly to detect near-field water movement, simple putative mechano-reception setae, aesthetasc hairs (chemo-reception), porous setae, dorsal cuticular organs (chemoreception and baroreception), simple pores and pinnate setae. The large well-developed eyes of phyllosoma on long stalks (Ritz, 1972; Rimmer and Phillips, 1979; Duggan and McKinnon, 2003) may also influence their ability to track and capture prey.

### 2.1.5 Limb morphology

Phyllosoma primarily use sharp dactyls, spines along the length and long terminal spikes on their pereiopods, to capture their prey, which are then passed onto the maxillipeds for processing (Kittaka, 1994a, b; Abrunhosa and Kittaka 1997; Cox and Johnston, 2003). During phyllosoma development, periepods increase in length and during mid-stages they develop an additional appendage indicating that their ability to capture and handle prey advances with age (Kittaka, 1990; Kittaka, 1994b; Cox and Johnston, 2003).

### 2.1.6 Mouthparts

Once the prey has been captured the next processing function is mechanical breakdown. The mouthpart morphology of phyllosoma from an early age through to advanced stages may indicate the type, texture and possibly size of their natural diets (Mikami and Takashima, 1993; Mikami *et al.,* 1994; Johnston and Ritar, 2001; Cox

and Johnston, 2003). Johnston and Ritar (2001) described the mouthpart morphology of *J. edwardsii,* which has well developed teeth, incisor and molar processes on the mandibles, sharp spinose projections on the first maxillae and spinose setae on the second maxillipeds. These suggest that their mouthparts are well adapted from an early age for manipulating, cutting and masticating soft foods such as gelatinous–bodied zooplankton and flesh. Although few changes occur during larval development, their mouthparts become more effective at handling larger prey items (Johnston and Ritar, 2001; Cox and Johnston, 2003). These findings are consisted with other Palinurid and Scyllarid lobsters (Wolfe and Felgenhauer, 1991; Lemmens and Knott, 1994; Johnston and Alexander, 1999).

### 2.1.7 Digestive capacity

Knowledge of the chemical and physical digestive capability throughout their larval cycle provides useful information in relation to their natural diet, digestive capacity and the importance of particular dietary components (Cox and Johnston, 2003). Palinurid phyllosoma have a single foregut chamber with a number of well-developed grooves, ridges and setae, but they lack a gastric mill. Throughout phyllosoma development, significant changes in complexity and function occur. In particular their filter press develops in mid-stage larvae, while the foregut develops from early, mid and later staged phyllosoma, enabling them to consume and digest larger and fleshier prey (Wolfe and Felgenuer, 1991; Johnston and Ritar, 2001). The digestive enzyme profiles of *Jasus edwardsii* phyllosoma indicate that they have the ability to digest protein, lipid carbohydrate and chitin during all stages of development (Johnston *et al*., 2004).

### 2.1.8 Nutritional requirements

Development and survival of the larvae of a given species is largely influenced by adequate delivery of nutrition (Guillaume, 2001). However, as discussed above, the knowledge of the nutritional requirements of phyllosoma is still relatively limited. The fatty acid profile of 'wild' phyllosoma has been reported for *J. edwardsii* and *J. sagmariasus* and was found to be similar to a range of Antarctic zooplankton, although content varied between phyllosoma at different locations (Phleger, 2001; Nichols *et al.,* 2001, Jeffs *et al*., 2004). The fatty acid composition of *J. edwardsii* revealed relatively high levels of DHA and EPA (Nichols *et al.,* 2001), indicating that these fatty acids maybe important dietary components. Further research into the nutritional requirements of phyllosoma is required to assist the development of formulated diet.

## 2.2 Study site

### 2.2.1 Australian Institute of Marine Science aquaculture facility

The Australian Institute of Marine Science (AIMS) aquaculture facility is located 40 km south of Townsville, north Queensland, Australia. The research facility focuses primarily on penaied prawn research and, more recently, tropical rock lobster, *Panulirus ornatus*, propagation. The facility operates on a high quality flow-through seawater system. Water is pumped from the ocean into a series of settlement systems including holding towers, a small dam and into another holding tank until required.

All water is UV sterilized and filtered down to 5 μm, and further to 1 μm for larval rearing applications.

### 2.2.2. MARFU aquaculture complex

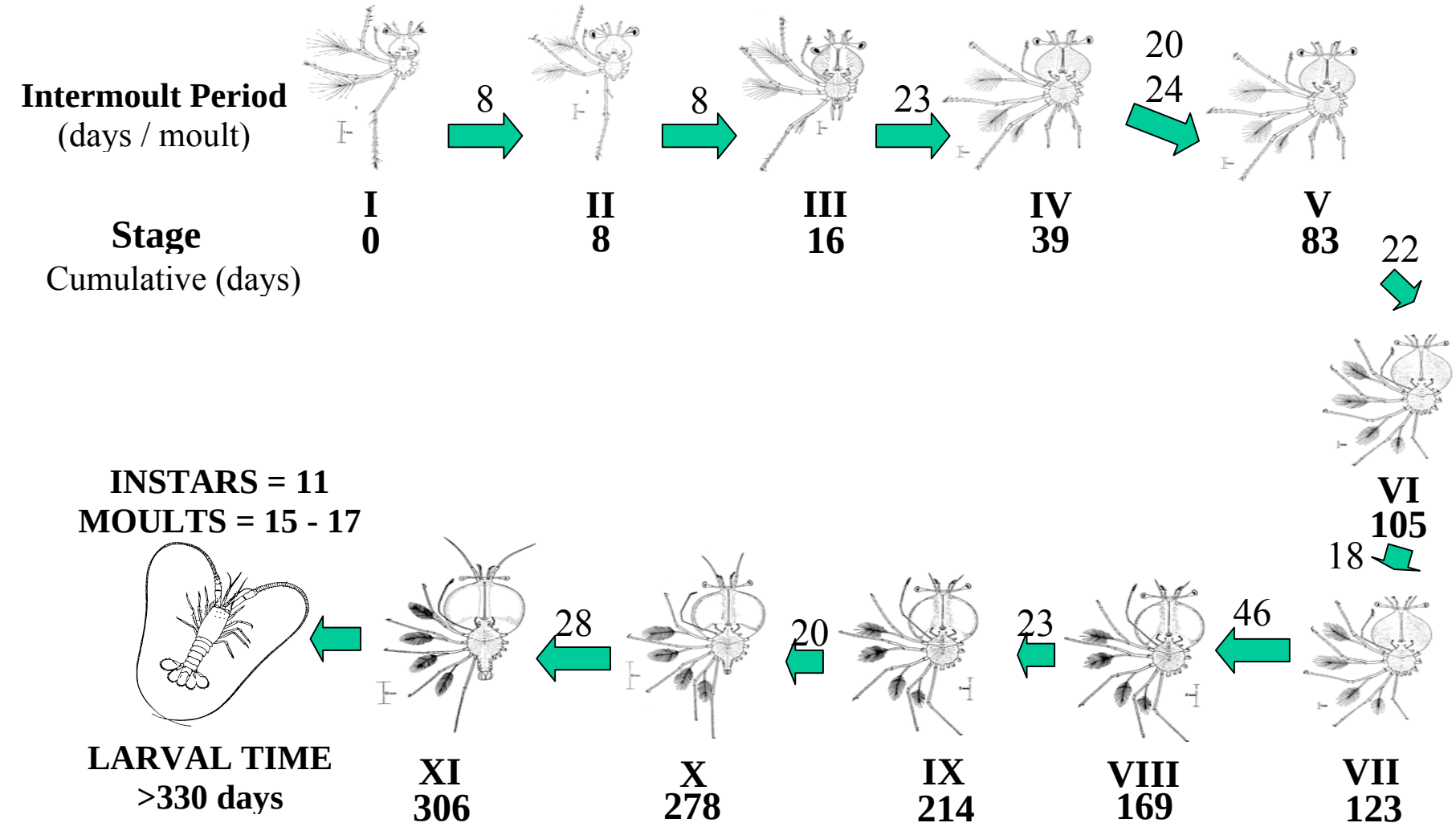
The James Cook University (JCU) aquaculture Facility (MARFU-Marine and Aquaculture Research Facility Unit) at the Douglas campus of JCU is located in Townsville, north Queensland, Australia. The facility uses a high quality recirculating water system, and the seawater is filtered by a series of sand filters, biological filtration and "algal scrubbers" to maintain quality. The seawater is trucked in to the facility and chlorinated in large storage tanks before use. The research conducted at this facility is largely on bivalves, mud crabs and finfish propagation. The facility is composed of several rooms that are temperature and photoperiod controlled for individual research projects.

## 2.3 General materials and methods

### 2.3.1 Larval production

Phyllosoma were obtained from captive broodstock held at the AIMS aquaculture facility under natural and artificial climatic conditions. Once berried females had completed incubating their eggs, they were treated in a bath of 40 ppm formalin for 1 h and then placed into a sterilised hatching tank containing clean 1 μm filtered seawater. The day of hatch can be predicted by the development of eyes and the consumption of their yolk reserves, which can easily be observed. The phyllosoma hatched in the holding tank at night and were transferred to larval rearing tanks the

Fig. 2.1. An estimated larval development cycle for temperate rock lobster, phyllosoma.



following day. Several tank sizes and designs were used, although the large-scale holding of phyllosoma was conducted in 5000 L parabolic tanks. All larvae required for the current study were reared through a semi-intensive green-water system. The approx. density of the microalga was between 5.0 x $10^4$cells/mL and 2.0 x $10^5$cells/mL. The general larval rearing protocol is shown in Table 2.1.

Table 2.1. The daily culture protocol and conditions within the 5000 L larval rearing tank used for larval rearing of *P. ornatus*.

| Parameter | Range |
|---|---|
| Water exchange: | 25-35% 1µm UV filtered |
| Temperature | 27-28 $^oC$ |
| Salinity | 30-35 ppt |
| Micro-algae spp. | 50 L *T-isochrysis* |
| *Artemia* | 1-2 metanauplii $ml^{-1}$ |

The phyllosoma were reared under conditions detailed in Table 2.1 until the desired larvae stage was reached. The larval stages used in experiments in this study were 1, 2, 3 and 4 (Fig. 2.1). The experimental tanks used during this study are described in the relevant sections.

## 2.4 Diet preparation

The general methods used for the preparation of artificial food particles in this study are described below. However, a significant part of the thesis focused on the

development of specific types of food particles. This research is described in detailed in Chapters 3 and 4. The method of preparing the spraying dietary mixture is similar to that described by Levine *et al*. (1983). The binder mixture, was individually prepared. For sodium alginate (GMB), three litres of distilled water was heated to 40 $^{o}$C and pH was adjusted to 12 with 1 M NaOH, positioned on the magnetic/heater stirrer. Gelatin (Sigma: Bloom 180) and carrageenan (Sigma: Kappa) binders were prepared in a similar way without the addition of NaOH. The binders (w/v) were added gradually to the heated water and stirred using a glass rod. Once the binder was completely dissolved, the mixture was stored at 4 $^{o}$C in a sealed container under high purity nitrogen, until required.

### 2.4.1 Preparation of dietary mixtures

The dietary components of the experimental diets used in this study consisted of fresh, frozen and dried ingredients as well as supplements. All fresh or frozen components were homogenised (Ultra-turrax T25 homogeniser) individually until a thin consistency was achieved. The paste was then pushed through a 100 μm sieve to remove lumps, which may otherwise have blocked spray nozzles of the encapsulation devices. The dietary mixture, binder mixture and dried and supplemented ingredients were added together step-by-step with the mixture being homogenised between each step to ensure the final mixture has a thin consistency (Fig. 2.2). The resulting mixture was then stored at 4 $^{o}$C in a sealed container and under high purity nitrogen until use. The nitrogen gas was applied every time the container was opened and the mixture was stored for a maximum of two weeks.

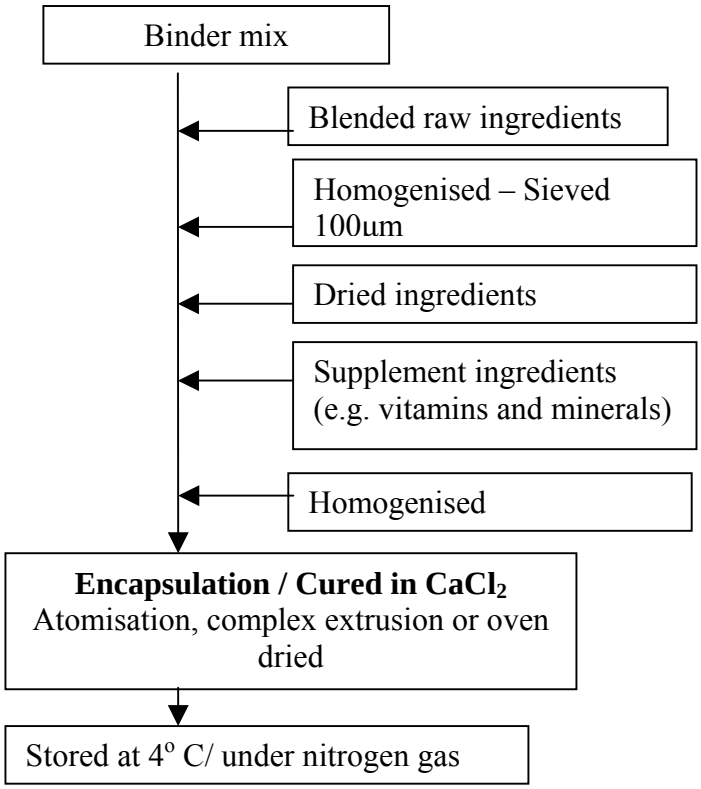



Fig 2.2 the generalised procedure for the preparation of artificial diets

2.4.2 Method for $^{14}$C-labelling of *Artemia*

Labelling *Artemia* with $^{14}$C was conducted using the feeding apparatus shown in Fig. 2.3.

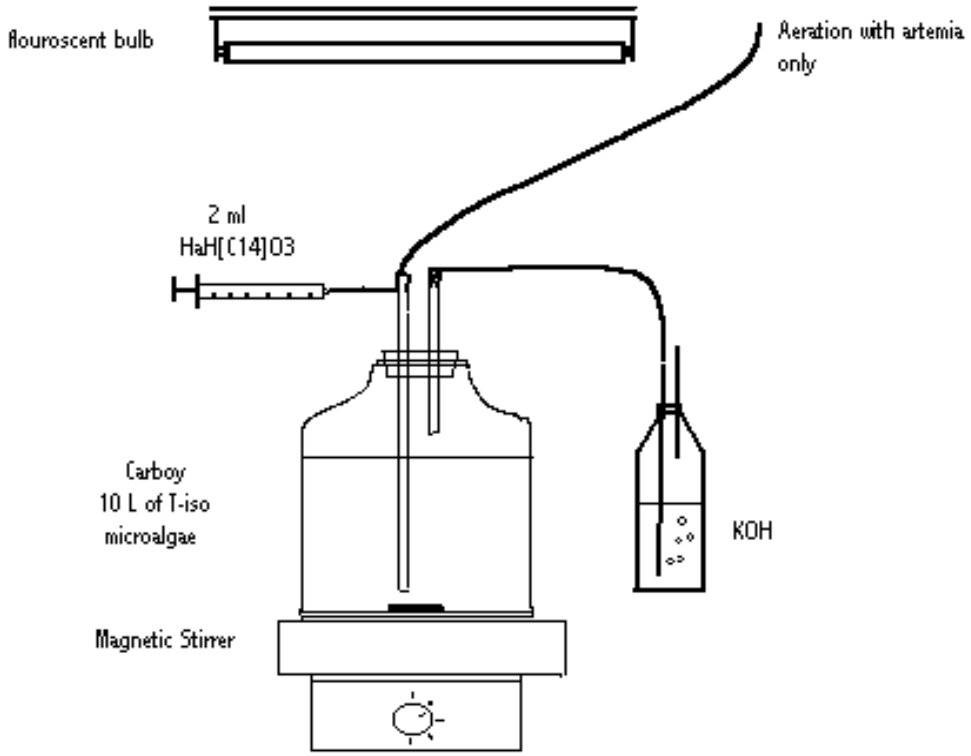



Fig. 2.3 The apparatus used to label T-*Isochrysis* sp. (T-iso) microalgae and *Artemia*. The apparatus was under constant illumination.

The method of $^{14}$C-labelling the MBDs was based on the technique described by Kolkovski *et al.* (1993). Briefly, microalgae (*T- Isochrysis* spp.) was incubated in seawater containing [$^{14}$C]-sodium bicarbonate (Amersham Biosciences, 925 MBq) and *Artemia* were allowed to graze on the labelled algae. Two mL of aqueous NaH [$^{14}$C]$O_3$ (2 mCimL$^{-1}$) was injected into a sealed 20 L carboy, containing 10 L of T-*Isochrysis* microalgae culture (Fig. 2.3). The culture was illuminated with two lights and stirred using a magnetic stirrer. The algal culture containing the labelled medium was incubated for 24 h. Aeration was then supplied and the exhaust was passed through a potassium hydroxide trap, to remove remaining [$^{14}$C]$O_2$.

*Artemia* metanauplii were added to the carboy at a density of 2.97 x $10^5$ $L^{-1}$. The *Artemia* were allowed to graze on the microalgae for 22 h. The labelled *Artemia* were collected on a 150 μm sieve, washed and oven-dried at 45 $^oC$ for 24 h they were then ground with motar and pestle. The dried $^{14}C$-labelled *Artemia* were required for the experiment described in Chapter 5. Live $^{14}C$-labelled *Artemia* were required for the experiment described in Chapter 6 and they were fed T-*Isochrysis* (as a cold-chase) for 3 h to flush $^{14}C$-labelled microalgae from their gut and held until required.

### 2.4.3 Preparation of samples for counting

The $^{14}C$-labelled *Artemia*, prepared diets containing $^{14}C$ and phyllosoma fed $^{14}C$-labelled diets were used or produced in a number of experiments during this study and had to be measured for $^{14}C$ content (specific activity). In separate scintillation vials 30 mg of $^{14}C$-labelled diet, 50 mg of $^{14}C$-labelled *Artemia*, 10 live *Artemia* and 20 phyllosoma from each experimental replicate were dissolved with 1 mL tissue solubilizer (Soluene-350, Packard chemicals) at 45 $^oC$ for 24 h, until completely dissolved. Then 5 mL of the scintillation cocktail (Hionic-Fluor, Parkard Biosciences) was added. Samples were counted in a beta scintillation counter (Beckaman) and corrected for background radiation.

# Chapter 3

# Production of moist micro-capsules using atomisation

## 3.1 Introduction

The major impediment for successful closed life-cycle aquaculture of rock lobsters is the completion of their long and complex planktonic larval phase. Despite decades of research into larval rearing of phyllosoma, all attempts have been plagued by high rates of attrition such that, by completion of the long planktonic larval phase, too few phyllosoma survive to make it commercially viable (Kittaka, 199). One cause of high mortality is assumed to be inadequate foods during larval rearing. The problem is further exacerbated by the paucity of knowledge of the natural prey of phyllosoma. It is generally believed that phyllosoma consume soft-bodied organisms, such as gelatinous zooplankton, in the wild (Phillips and Sastry, 1980). However there have been no reports on the routine production of such live foods. Several sources of fresh food have been trialed in artificial propagation of phyllosoma including mussel (*Mytilus edulis*) gonad and fish larvae (Kittaka, 1997).

Due to the relatively large size of the phyllosoma, most substantial live foods are inadequate, with the exception of *Artemia* and cropped mussel gonad. *Artemia* has been shown to be most effective as a food source for phyllosoma due to ease of culture (Ritar *et al.*, 2002). However, due to the large change in body size of phyllosoma during their planktonic phase (from 2 mm to 10-30 mm body length), live foods such as *Artemia*, are inadequate for the entire larval cycle. Although *Artemia* is a suitable live food source for early staged phyllosoma, a shift to other foods, and

especially fresh rather than live foods, with more adequate nutritional profiles is required (Ritar *et al.,* 2002).

As phyllosoma are assumed to prey on soft-bodied organisms, the use of artificial food particles with a consistent soft matrix could be the most logical option. Mass production methods may be possible for preparation of a soft 'artificial' food particle by using microencapsulation techniques that were originally developed for the food, medical and pharmaceutical industries. Several microencapsulation types have been used successfully to produce artificial food particles suitable for bivalves, fish and crustacean larvae (Kanazawa *et al*., 1982; Levine *et al*., 1983; Langdon and Siegfried, 1984; Jones *et al*., 1987; Knauer *et al*., 1993; Nell, 1993; Southgate and Partridge, 1998; Southgate and Kolkovski, 2000).

Sprayed beadlets are a type of microcapsule made using atomisation. They have been extensively used as food particles for marine larvae (Levine *et al.,* 1983; Levine and Sulkin, 1984; Buchal and Langdon, 1998). Diet ingredients and binder are blended together in a low viscosity liquid state. This method usually relies on sodium alginate as the binder. Its advantage is that it may be sprayed in a liquid form to produce a soft, but rigid gel. Alginate gels on contact with polyvalent metal ions and beadlets are normally produced by spraying a diet mixture contacting sodium alginate into a curing bath of calcium chloride ($CaCl_2$). Production methods for spray beadlets vary from small-scale laboratory to industrial scale (Villamar and Langdon 1993). Small-scale methods include the use of hand-held glass atomisers while scale-up methods use large reservoirs of diet-binder solution before atomisation. Production relies on extruding a liquid solubilized diet including sodium alginate binder using gas pressure

through a small orifice; atomisation of the diet-alginate mixture at the spray head results in a fine spray. The spray may be directed into a curing bath of $CaCl_2$ resulting in gelling and production of sprayed beadlets. The use of nitrogen as the pressuring and atomising gas eliminates oxidation of critical dietary ingredients such as highly unsaturated fatty acids.

One of the major problems associated with sprayed beadlets is their negative buoyancy. They sink through the water column too rapidly and may decompose on the bottom of larval rearing tanks leading to deteriorating water quality. Beadlet suspension in the water column may be manipulated through more appropriate tank design and hydrodynamics and modification of the beadlet encapsulation process. As phyllosoma are delicate, and high turbulence or vigorous aeration is inappropriate during larval rearing, one possible option for manipulating buoyancy of beadlets is to encapsulate bubbles within the diet particle at the time of manufacture.

The main aims of these experiments were to:

(1) Design and manufacture suitable 'artificial' food particles for *Panulirus ornatus*, phyllosoma.

(2) Examine scale-up production of the particles by modifying industrial spraying equipment; and

(3) Assess potential for manipulating buoyancy of the food particles to increase the time that they remain in the water column and remain available for phyllosma in a up-welling tank.

## 3.2 Materials and methods

The micro-capsules (MC) used in this study were of the sprayed beadlet type which were made by a method based on that described by Villamar and Langdon (1993). The following series of experiments and modifications to manufacturing techniques were carried out to produce adequate quantities of spray beadlets of the specific sizes suitable for the various stages of phyllosoma.

### 3.2.1 Progressive steps in preparation of MC

Experiments were conducted to examine methods for small-scale production of spray beadlets. The methods and apparatus used in these trials are outlined below.

### 3.2.2 Laboratory small-scale production: Glass atomisers and $CaCl_2$ rotating chamber

The principal production apparatus consisted of a hand-held glass chromatography atomiser (Alltech, Fig. 3.1) and a rotating chamber containing a curing solution of $CaCl_2$ (20 % w/v)(Fig. 3.2). This apparatus has been used for production of MC at small laboratory scale (Levine *et al.,* 1983; Langdon and Buchal, 1998). The atomiser produces an aerosol of diet-alginate droplets that gel when sprayed into a rotating collecting chamber containing a curing solution of $CaCl_2$. The maximum volume of the atomisers was 250 mL. High purity nitrogen was used a propellent to avoid oxidation of dietary ingredients as the gas drives the diet through an internal tube to produce an atomisation action. The rotating curing chamber was built according to a

design supplied by Dr C.J. Langdon, Oregon State University (November, 2000) and was constructed by the AIMS workshop.

**A**


**B.**


Fig. 3.1 (A and B). The Alltech Inc. hand held glass atomisers. A, Apparatus used for production of fine particles and B, Coarse particles. The atomisers each held 250 mL. The diet mixture can be preheated within a water bath prior to spraying.

**A.**

**B.**

Fig. 3.2 (A and B). The rotating curing chamber/cylinder used to cure sprayed beadlets, containing the curing $CaCl_2$ solution. A, Top/ front view of rotating chamber; B, Side view and opening to chamber bath (Design based on C.J. Langdon and M.A. Buchal, 1998). The chamber volume for the curing bath is 2 L. The rotation speed of the chamber is determined by the user.

### 3.2.3 Laboratory scale-up production: Industrial spraying atomiser apparatus and rotating collecting chamber

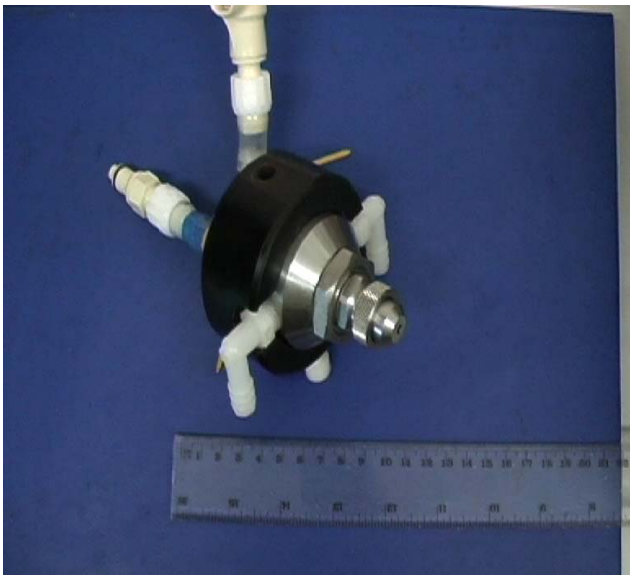
The basic scale-up concept was based on, and modified from, that described by Langdon (1989). The apparatus comprised a water jacket 1.35 L reservoir cylinder, containing the diet-alginate solution, a pressurised gas-driven piston and stainless steel atomisation spray head (Fig. 3.3). The heated water jacket could be maintained at a desired temperature via a water bath reservoir to maintain the diet-alginate solution at a low viscosity. A spray head (Model: Steam jacket 1/4 JBCJ Source: Spraying Systems Co.) comprised of an internal bore head orifice, through which the diet-alginate solution was extruded into a narrow chamber which was pressurised using atomisation gas. The resulting diet-alginate droplets were then passed through a nozzle cap, into a bath of $CaCl_2$ to form sprayed beadlets.

By varying the diet-alginate extrusion rate and the volume of atomisation gas, beadlets of varying rheology could be produced. In addition, beadlet morphology could be manipulated by the type of bore head orifice and nozzle shape. To further extend the range of beadlet morphology, bore heads and nozzle caps (Spraying Systems Co.) were modified and constructed at AIMS. The spraying head was also water-jacket heated to prevent nozzle blockages. The cylinder was mounted vertically and filled with diet-alginate solution and capped with a free-floating piston head. A plastic cap was screwed onto the top of the cylinder which had an intake for the pressurised gas into the air space above the piston. The water jacket kept cylinder and spray head at a constant temperature ($35^{o}C$) that ensured consistent production conditions between batches. The diet piston was forced down through the cylinder by

pressurised nitrogen gas. The speed of travel and hence flow rate of the extruded of diet-alginate solution through the bore cap orifice, could be controlled by the flow and pressure of the gas into the header space. The degree of atomisation was controlled by varying the flow and pressure of feeder gas into the space between the bore cap and nozzle unit. The atomised aerosol of diet-alginate was directed into a curing bath containing $CaCl_2$ solution, resulting in the formation of sprayed beadlets.

In the first instance, the apparatus was used with the cylinder lying horizontally and the atomised spray directed into a rotating cylinder of curing solution. Particle diameter could partially manipulated by controlling the speed of piston travel, and hence flow rate, as well as varying the pressure, and hence flow of the atomising gas into spray head. The resultant beadlets could be further sieved into appropriate size ranges.

**A**



**B**

Fig. 3.3 (A and B). The sprayer device and dietary cylinder used to prepare sprayed beadlets. A, the atomisation apparatus with heated water jacket from Spraying Systems Co. and the dietary cylinder B, which is $N_2$ piston driven and heated to desired temperature of 35$^{o}$C by surrounding water jacket. (Sourced and further modified from D.F. Villamar and C.J. Langdon, 1993; C.J. Langdon pers. comm, 2001).

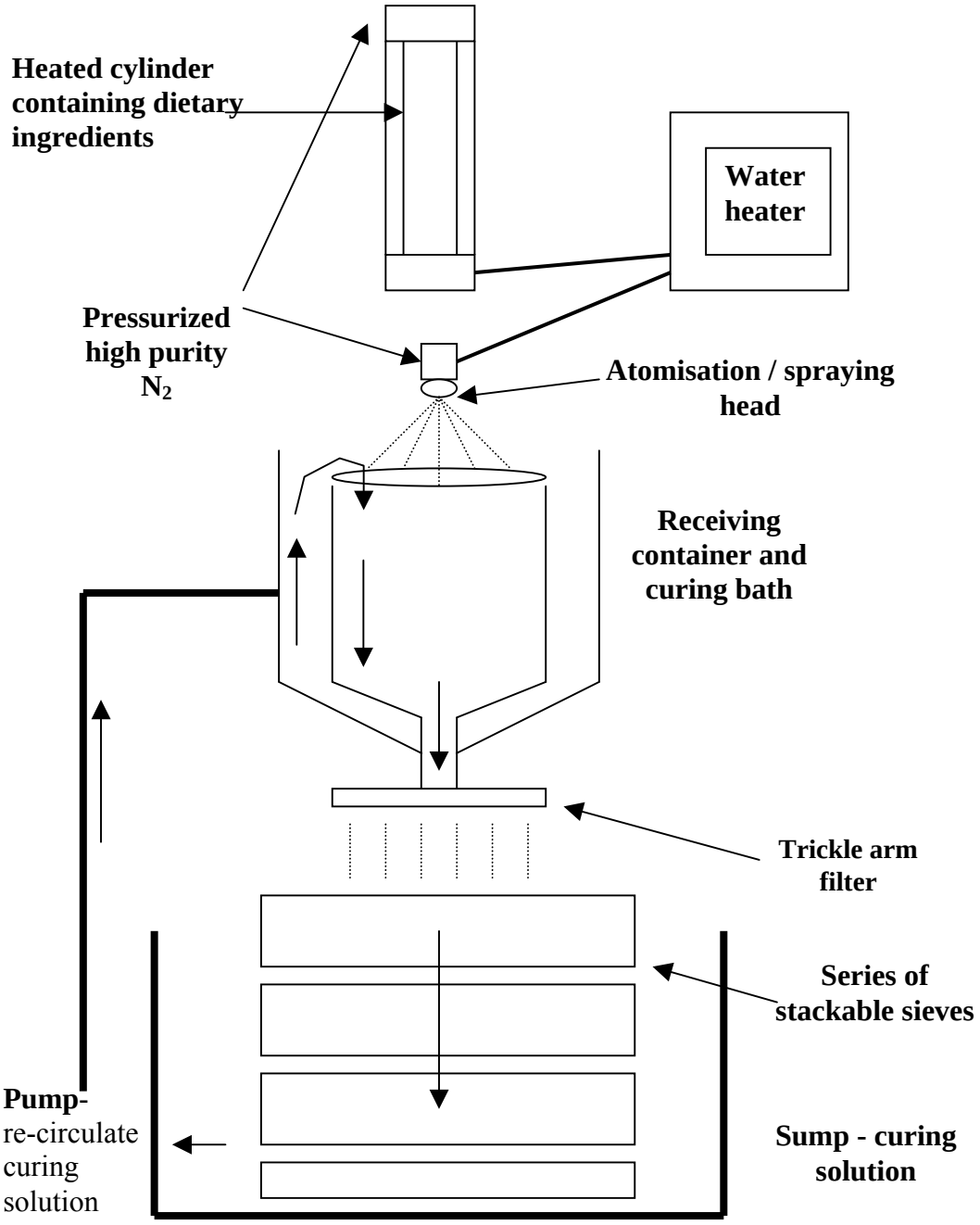



Fig. 3.4. The spray tower apparatus used to prepare atomised microcapsules. Including the dietary cylinder, the atomisation head, the curing bath and collection sieves (>850, 500, 355, 210 and 100 μm).

### 3.2.4 Scaled-up production: Development of the "Spraying tower"

Based on previous methods described for preparing sprayed beadlets, a large-scale apparatus was developed to produce larger batches of sprayed beadlets. The production device was referred to as the "Spraying tower" (Fig. 3.4, Fig. 3.5 and Fig. 3.6). The device included an industrial sized sprayer, the use of industrial grade $CaCl_2$ and a semi-automated sieving system to collect size-selected sprayed beadlets.

The spraying tower unit was mounted vertically above a receiving container and curing bath. Upon atomisation the extruded aerosol was directed into a "circular waterfall" of $CaCl_2$ curing solution. The receiving container was a double-walled tank containing $CaCl_2$ solution which upwells in the outer compartment and overflows into the inner chamber (Fig. 3.4). Diet-alginate aerosol droplets solidified upon contact with the "circular waterfall" and passed into the sequential sieving tower. The $CaCl_2$ solution drained into a sump and was then pumped back into the outer compartment of the receiving container.

The solution containing the cured beadlets flowed through a sequential series of sieves and beadlets were separated into 5 size classes: >850, 500-850, 355-500, 210-355, 100-210 μm. The collected beadlets were kept separate by continuous gentle distribution of the curing solution onto the sieves using a rotating trickle arm. The total volume of the industrial grade solution of $CaCl_2$ was 70 L (20% w/v) and it was recirculated using an Onga 414 pump at a regulated flow rate of 0.5-0.7 L/sec. During the production of sprayed beadlets the curing solution was replaced after every 15

batches and between exchanges the solution was maintained at a specific gravity of 200 ppt and was 1 μm filtered using a cartridge filter.

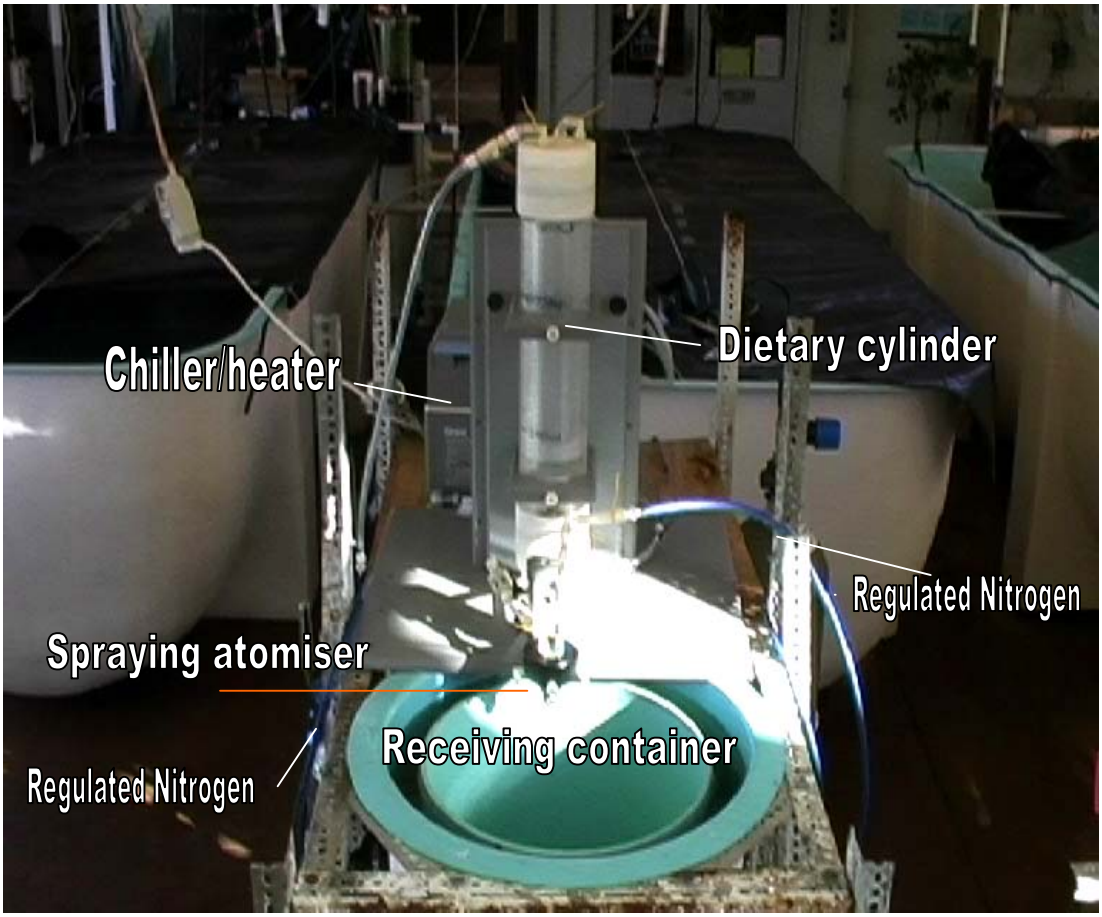



Fig. 3.5. The spraying tower used to produce sprayed beadlets (top view). The heated (35 $^{o}$C) dietary cylinder supplies the diet mixture to spraying nozzle and the mixture is atomised using high purity $N_2$. Note the central position of the spraying atomiser and the "water fall" effect where the sprayed beadlets are formed as curing solution overflows the outer chamber into the inner chamber of the receiving container.

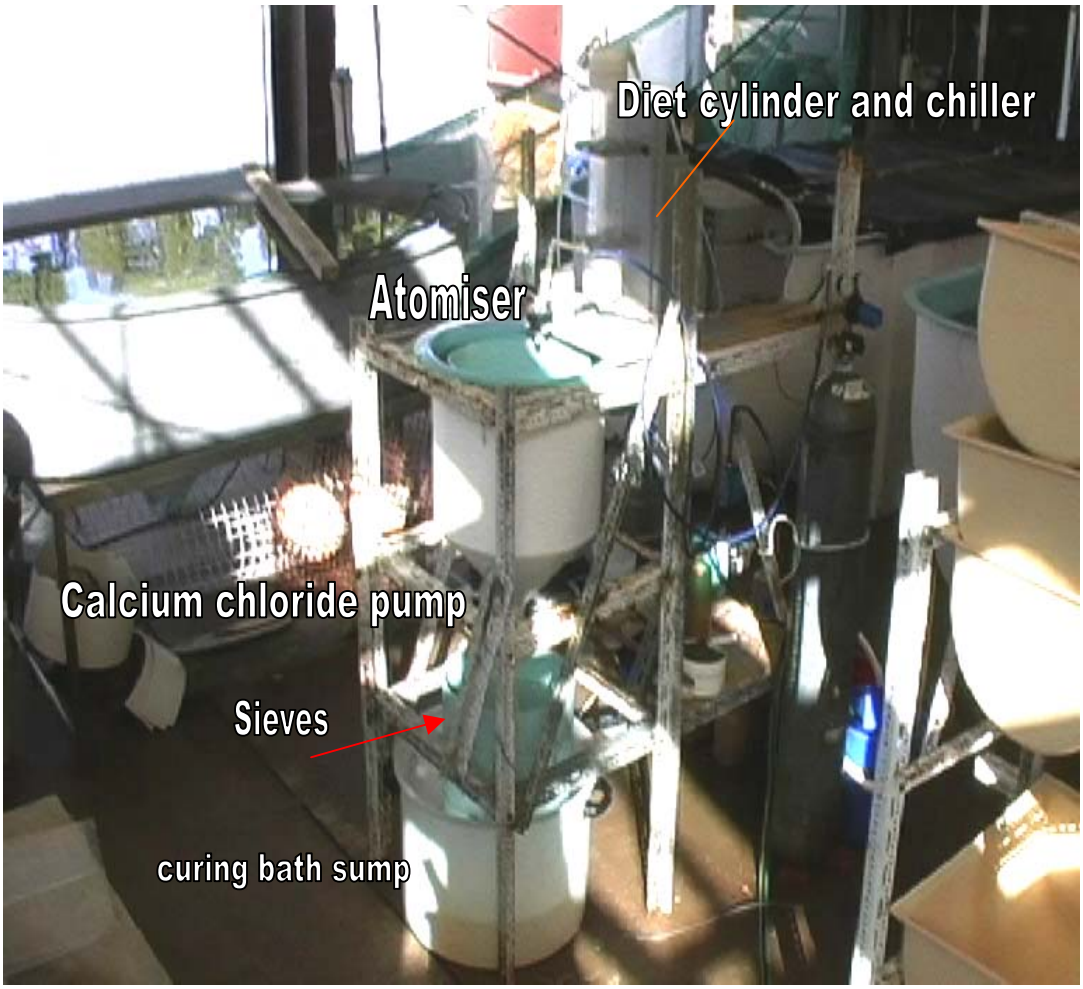



Fig. 3.6. The spraying tower used to produce sprayed beadlets (side view). (Note the recirculating $CaCl_2$ curing bath (75 L) with sump containing desired sized stackable sieves >850, 500, 355, 210 and 100 µm).

### 3.2.5 Experimental production of sprayed beadlet microcapsules

This chapter describes experiments conducted, using the apparatus described above, to determine the effects of various system parameters on the characteristics of the resulting sprayed beadlets. The experiments examined the effects of different

combinations of spraying heads and nozzle cap and flow rates and pressures of diet mixture and atomisation gas.

### 3.2.6 Preparation of the diet-binder mixture

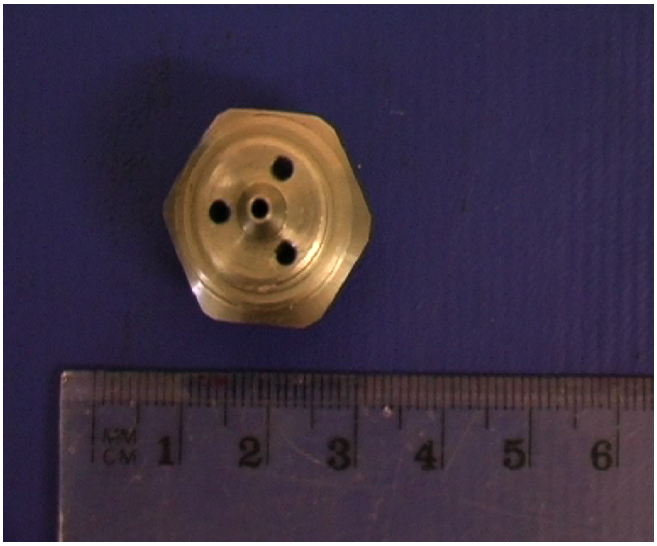
The method used for preparing the spraying mixture was similar to that of Levine et al. (1983). The method for preparing the binder mixture was described in Chapter 2 (section 2.4). The MC produced during the following experiment contained no diet. The sodium alginate solution was added to the dietary cylinder (511 g, 500 mL) for each trial, the diet injection tube and fittings were filled prior to each series of production sessions.

Sprayed beadlets were prepared using the apparatus described in section 3.2.3 and the system parameters used to produce the sprayed beadlets are outlined in Table 3.1.

Table 3.1 Combinations of bore head and fluid cap and nozzle cap examined

| **Apparatus combination number** | **Head bore orifice** | **Nozzle cap** | **Figure** |
|---|---|---|---|
| 1 | 2 mm (1/4 JN-SS) | 3.5 mm (SU11-SS) | Fig. 3.7 A,B |
| 2 | 2 mm (1/4 JN-SS) | 3.5 mm/2.75 mm step (modified SU11-SS) | Fig. 3.8 A,B |
| 3 | 2 mm (1/4 JN-SS) | 3.5 mm/0.95 mm split (SU13A-SS) | Fig. 3.9 A,B |

**A**




B

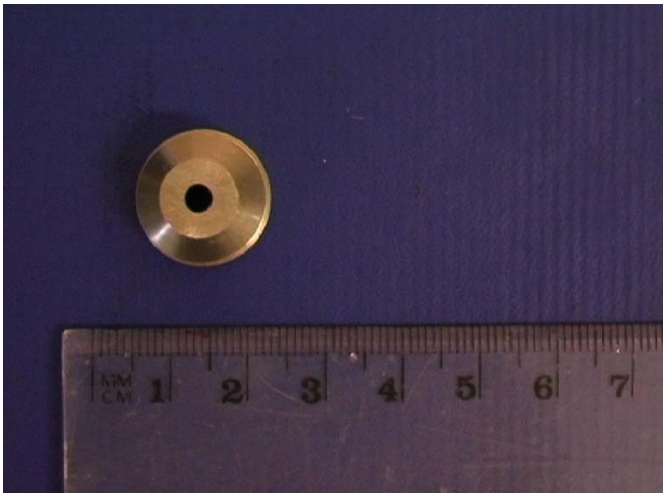
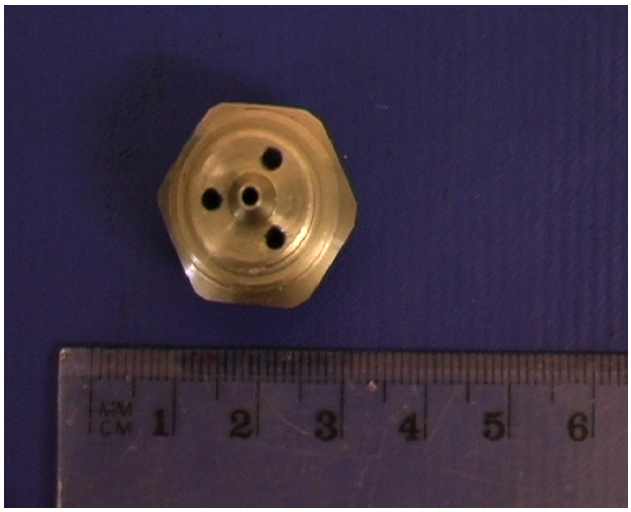



Fig. 3.7 A and B. Spraying combination: Head bore orifice 2 mm bore (A) and nozzle cap 3.5 mm bore (B).

A




B

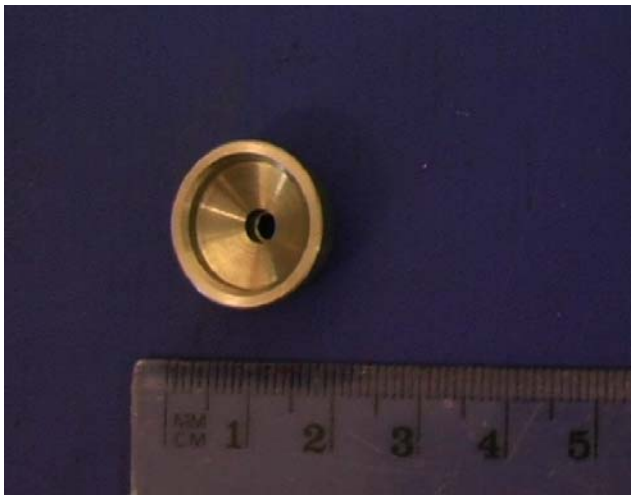
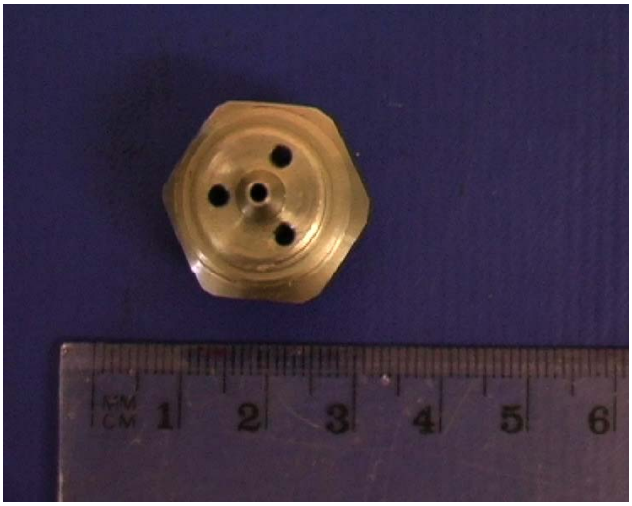



Fig. 3.8 A and B. Spraying combination: Head bore orifice 2 mm bore (A) and nozzle cap 3.5 mm bore/ 2.75 mm bore step nozzle cap (B).

A




B

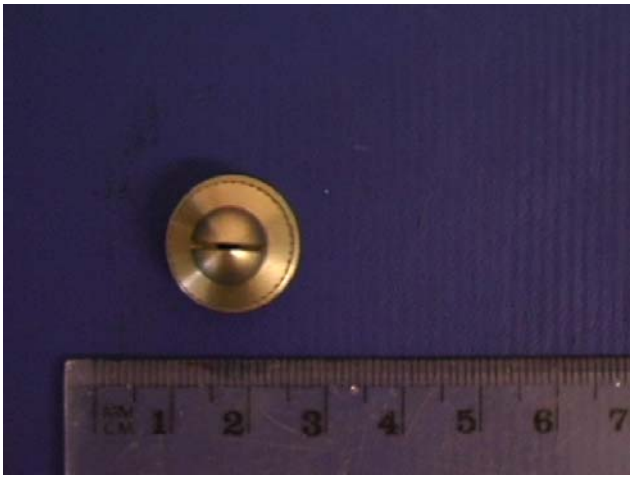



Fig. 3.9 A and B. Spraying combination: Head bore orifice 2 mm bore (A) and nozzle cap 3.5 mm bore/ 0.95 mm gap split nozzle cap (B).

### 3.2.7 The effect of flow rate and pressures on sprayed beadlet yield.

A series of experiments were conducted to determine whether the flow rate and pressures of the atomisation gas has an effect on resulting sprayed beadlet diameter and shape.

### 3.2.8 The effect of flow rate on sprayed beadlet yield

Two flow conditions were examined; each used a 0.5 L/min gas flow rate to the piston head space with one at 10 L/min atomisation gas to the spray head and the other at 15 L/min. Pressure remained constant at 10 psi at the piston and 20 psi in the piston chamber and atomiser chamber in the spraying head, respectively.

### 3.2.9 The effect of operating pressures on sprayed beadlet yield

The effect of varying nitrogen gas pressure to the piston and spray head were also examined. Two pressure conditions were examined, where the pressure at the piston head and atomisation gas was manipulated. Low pressure conditions were composed of 10 psi piston head and 20 psi atomisation gas to the spray head. High-pressure conditions involved 15 psi piston head and 30 psi atomisation gas to the spraying head. In each case, the flow rate of the extruded solution remained constant at 0.5 L/min and 10 L/min of atomisation gas to the spray head.

In all experiments described in sections 3.2.8 and 3.2.9, the speed of travel of the piston and feeder gas was maintained through flow meters fitted to the respective

nitrogen bottles. The resulting sprayed beadlets remained in the curing bath for 10 min and were then washed gently with freshwater for a further 5 min. Sprayed beadlets were collected from each sieve, were padded with paper towel underneath the sieve until interstitial water was removed and weighed. Yield of sprayed beadlets was recorded as proportions present on each sieve relative to the total weight on all sieves (>850, 500, 355, 210 and 100 μm).

### 3.2.10 Curing of sprayed beadlets.

The proportion of sprayed beadlets produced in each of the size classes was calculated on the basis of the recovered weight of sprayed beadlets after the curing reaction. This is because during the curing, some of the diet-alginate mixture is lost. The curing reaction occurs when the sodium alginate sprayed droplets undergo a substitution reaction where sodium is exchanged for calcium producing water. The weight of pre-sprayed alginate mixture was measured prior to and after gelling of the sprayed beadlets produced using two devices: (1) hand held glass atomiser; and (2) a laboratory syringe dropper. The $CaCl_2$ curing solution (20% w/v) containing the cured sprayed beadlets was collected onto sieves (>850 and 100 μm), rinsed with freshwater and the residue water was removed through a Whatman filter (GF/C) by vacuum. The filter was allowed to semi-dry and weighed, estimating the loss of weight in alginate mixture during the curing process.

### 3.2.11 Sedimentation rates of MC

#### *3.2.11.1 Preparation of sprayed beadlets containing diet and nitrogen gas bubbles*

The basic method of preparing the diet-alginate mixture is described in Chapter 2 (section 2.4). Sodium alginate at 1.5% w/v and 0.5% w/v gelatin (Sigma, type A porcine skin, 300 Bloom) were dissolved in distilled water at 40 $^{o}$C and gently stirred with a glass rod. To this was added a dietary mixture at 20% w/w consisting of 50% fish eggs and 50% green-lip mussel tissue blended to a paste and sieved to 355 μm to remove large cellular particles. The mixture was stirred thoroughly and heat reduced at room temperature (22$^{o}$C). The dietary mixture was divided into two and each was used to prepare sprayed beadlets, which were analysed for sedimentation properties. One batch of sprayed beadlets were made with the addition of bubbles and other as normal (section 3.2.4).

The diet-alginate solution used for bubble encapsulation was placed into the pre-heated (35.5$^{o}$C) cylinder for 10 min (Fig. 3.4) and the piston driven at a rate of 10 L/minute through a 10 mm hose attached to a small venturi (Brand: Mazzi) fed from a high purity nitrogen cylinder at a flow rate of 2 L/minute. The diet-alginate mixture was then sprayed using the 2 mm bore head with 3.5 mm/2.75 mm step nozzle cap, at a flow rate of 0.5 L/min under 10 psi with a atomisation gas of 15 L/min at a pressure of 20 psi. The resulting sprayed beadlets were left for 5-10 min in the curing bath and then washed gently for 5 min with freshwater, removed and sieved into appropriate size ranges (355-500 μm and 500-850 μm).

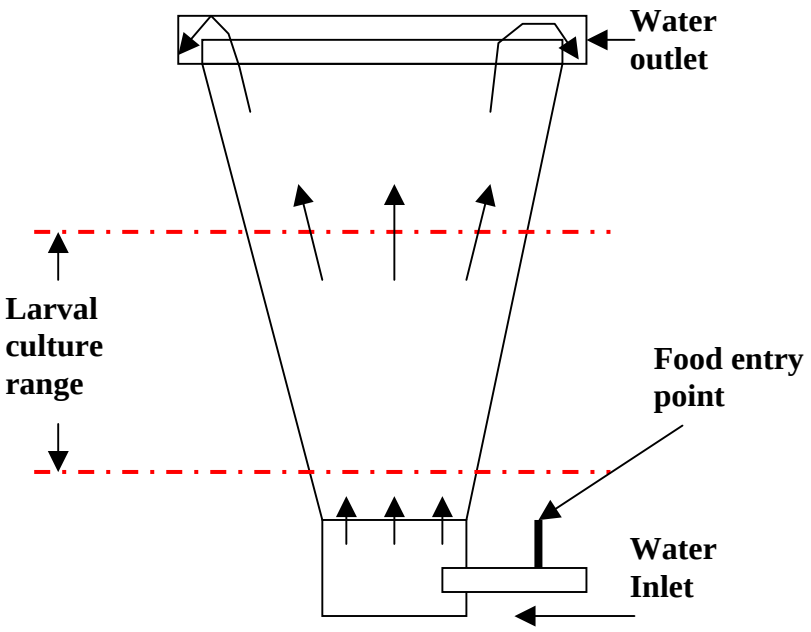



Fig. 3.10. The up-welling larval culture tank used for *P. ornatus* phyllosoma. The preferred larval culture depth range for phyllosoma is indicated. The filtered seawater travels through the adjustable water inlet then through a porous plate forming vertically laminar flow.

### 3.2.11.2 *Assessing the sedimentation rates of MC*

The sedimentation rates of sodium alginate sprayed beadlets was assessed to determine the required parameters for use in an up-welling larval rearing tank used for *P. ornatus* phyllosoma culture at AIMS (Fig. 3.10). Sprayed beadlets of 355-500 µm and 500-850 µm were assessed. Sedimentation rates of normal and "bubble" sprayed beadlets were determined immediately after curing and after 1 hour of hydration in aerated seawater. The sedimentation velocity was determined in a 1 L graduated cylinder filled with 1 µm filtered seawater held in a heated water bath 28-29$^{o}$C. Particles were released below the water surface. Time was recorded using a digital stopwatch, and commenced once the particle reached the 800 mL graduation and was stopped when sprayed beadlets reach the 200 mL mark, a vertical distance of 20 cm.

Assumptions made during these analyses were that the effect of frictional forces on the surfaces of the particles and between water pressure relative to depth is minimal. Given that the data generated was used for estimating particle behaviour in up-welling phyllosoma larval culture tank, it was also assumed the up-welling tanks flow is laminar and the rate of settlement is constant throughout the vertical cylinder (no acceleration).

### 3.2.12 Development of sedimentation model

A sedimentation model was calculated to determine the up-welling flow rate within larval rearing tank (Fig. 3.10) required, maintaining particles of a particular size range within the water column. As phyllosoma develop their body size and morphology changes which, necessitates changes in up-welling flow rates to keep them within the water column. The larval rearing tank design shown in Fig. 3.10 allows changes to be made to the flow rate of incoming water to produce a variation in up-welling flow at various heights in the tank. Tanks of this design are used at AIMS for *P. ornatus* phyllosoma culture. Phyllosomas congregate in specific areas of these tanks to minimized their energy cost for swimming to maintain their position in the water column. The desired up-welling flow rate for early-stage *P. ornatus* phyllosoma was determined in several trials. It was found that a flow rate of 2-5 L/min. (0.033-0.0833 L/s) positioned them within the bottom half of the tank. Clearly, to maximise ingestion of food particle, it is desirable that food particles occupy the same strata.

Positioning of food particles within the middle of the phyllosoma range can be achieved once the sedimentation rates of given size ranges of particles is known and the required flow rate to the tank can be calculated.

Preliminary experiments showed that in the tanks described in Fig. 3.10, an incoming water flow rate of between 0.033 L/sec and 0.0833 L/sec was required to maintain the phyllosoma a desirable height within the tank (between 100 mm and 860 mm above the tank bottom). On the basis of these desired water flow rates into larval culture tanks, the velocity of water at various heights within the tank could be calculated;

$$\text{Velocity}_{\text{(Position within tank)}} = Q_{\text{(incoming flow rate)}} / \text{Cross-sectional area}_{\text{(position within the tank)}}$$

Knowledge of the sedimentation rate of sprayed beadlets within both the 500-850 μm and 355-500 μm sizes ranges allowed a model to be developed whereby their position within the culture tanks could be predicted according to the water flow rate entering the larval rearing tank.

### 3.2.13 The analysis for sedimentation rates

Viewing Q-Q plots and a Levine's statistic checked the suitability of the data for analysis of variances. The confidence limit was allocated at P=0.05. Size frequency distribution of sprayed beadlets was analysed using a ANOVA, after results from apparatus combination number 2 was square root transformed. Effects of hydration times in seawater, size and the encapsulation of nitrogen bubbles on the sedimentation velocities of the sprayed beadlets were analysed using MANOVA, data was normally distributed without transformation.

## 3.3 Results

### 3.3.1 Production techniques for sprayed beadlets

The production of alginate sprayed beadlet diets can be achieved successfully at small and large-scale using a 250 mL hand-held glass atomiser through to a 1.35 L cartridge atomiser and the curing bath designs shown in Figs. 3.5 and 3.6. Whereas the viscosity of the alginate and dietary material must be very low when using glass atomisers, the industrial spraying combination doesn't require as low viscosity fluids due to the higher pressures which may be applied during spraying. The large-scale curing bath design with a "circular waterfall" largely decreased collisions between partially gelled sprayed beadlets and prevented the clumping often experienced with other available methods. The production method was further enhanced by the use of sequential sieves that allowed rapid collection of specific particle size ranges.

Incorporation of industrial-grade $CaCl_2$ significantly reduced the cost associated with the curing bath. Analytical grade $CaCl_2$ costs approximately \$108 $kg^{-1}$ (Sigma Chemical Co), while the industrial $CaCl_2$ flake is \$1.20 $kg^{-1}$ and is only available in 50 kg bags.

Table 3.2 The mean (± SE, n=3) proportional weight of alginate sprayed beadlets prepared using two atomisation devices

| Atomisation device | Recovered weight (%) of beadlets after curing | Beadlet size range |
|---|---|---|
| Syringe dropper (5 mL) | 77.2 ± 3.1 % | 3-4 mm |
| Glass atomiser | 41.1 ± 2.9 % | 100-850 μm |

### 3.3.2 Yield of sprayed beadlets associated with chemical curing process

Only a proportion of the atomised liquid sodium alginate droplets cured into sprayed beadlets as there is dilution at the molecular interface of the beadlet. The higher the surface area to volume ratio of the droplet then the greater the amount of sodium alginate which dilutes into the curing bath to below gelling concentration. For example, when larger particles were produced using a syringe dropper the recovery of beadlets from the original sodium alginate solution was 77.2 ± 3.09 % (Table 3.1). This was significantly greater ($P<0.005$) to that recovered using the glass atomiser, which yielded 41.11 ± 2.94 %. On average approximately 50-60 % retrieval was possible using the large-scale cylinder production system.

### 3.3.3 The effect of spraying head and nozzle combinations on yield of sprayed beadlets for the associated flow rates and pressures

#### 3.3.3.1 *Yield of sprayed beadlets using combination of flow rates and pressures for 2 mm bore head and 3.5 mm nozzle cap*

The effect of two different flow rates on the sprayed beadlet size frequency distribution and total yields are shown in Fig. 3.11. The size frequency distribution of beadlets produced using this apparatus combination was significantly ($P<0.05$) skewed towards larger sized beadlets, where greater than 40% of beadlets were >850μm. The high diet-alginate solution flow rate of 0.5 L/m and a flow-rate of

atomisation gas of 15 L/m had a greater tendency to produce large sprayed beadlets, 55% exceeding >850 μm in diameter.

The effect of high and low operating pressures on the production efficiency and size frequency distribution of sprayed beadlets is shown in Fig. 3.12. The differences between the proportions of beadlets on particular sized sieves were not significant ($P>0.05$) for high and low pressure combinations. However greater than approximately 35-40% of beadlets were >850 μm in diameter.

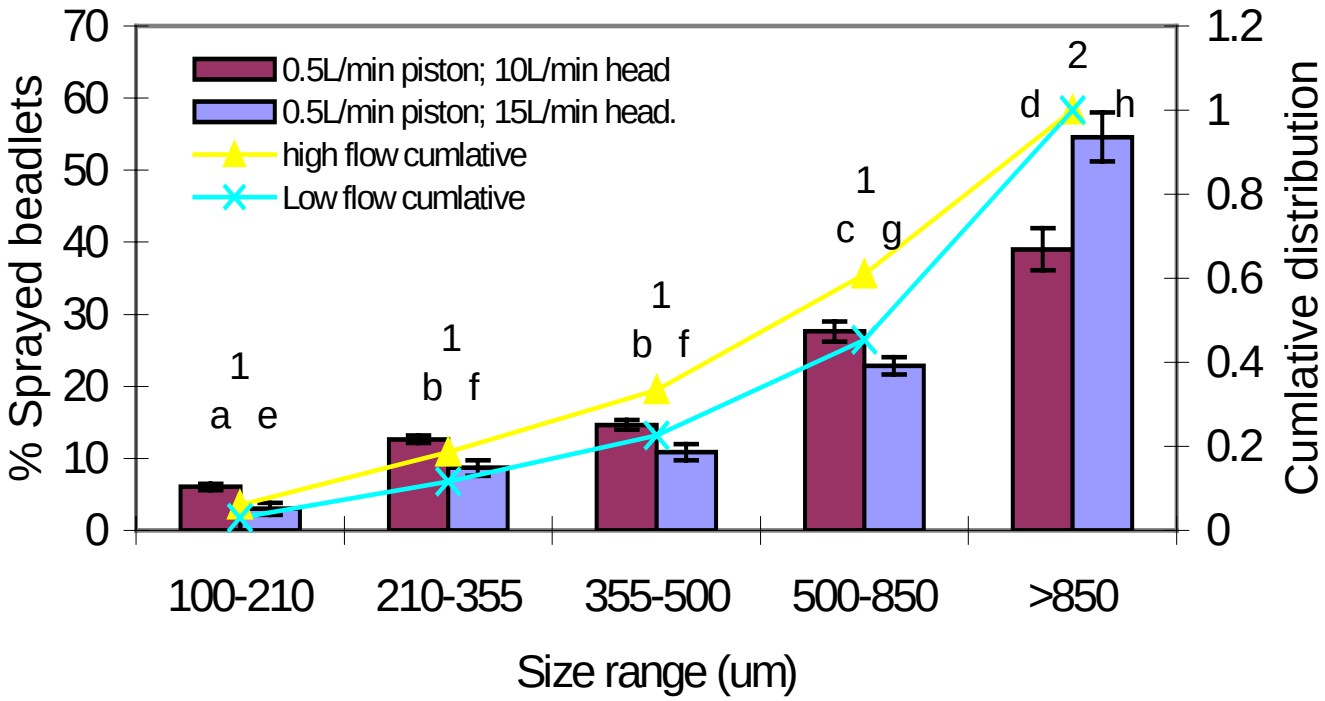



**Fig. 3.11.** The mean (± SE) size frequency distribution of sodium alginate sprayed beadlets produced using high and low operating flow rates. In both cases a constant pressure of 10 psi was applied to the piston and 20 psi of feeder gas to the spray head. The spray head comprised of 2 mm bore head and 3.5 mm bore nozzle cap. The yield of beadlets present on the sieves compared to initial alginate sprayed was 55.84 ± 2.7 % and 56.40 ± 0.8 % for 0.5 L/min piston and 10 L/min feeder gas 0.5 L/min piston and 15 L/min feeder gas combinations, respectively.
The number indicates there were significant differences ($P<0.05$) between high and low pressures at each size range, letters indicate significant differences ($P<0.05$) between size ranges for each high and low pressure.

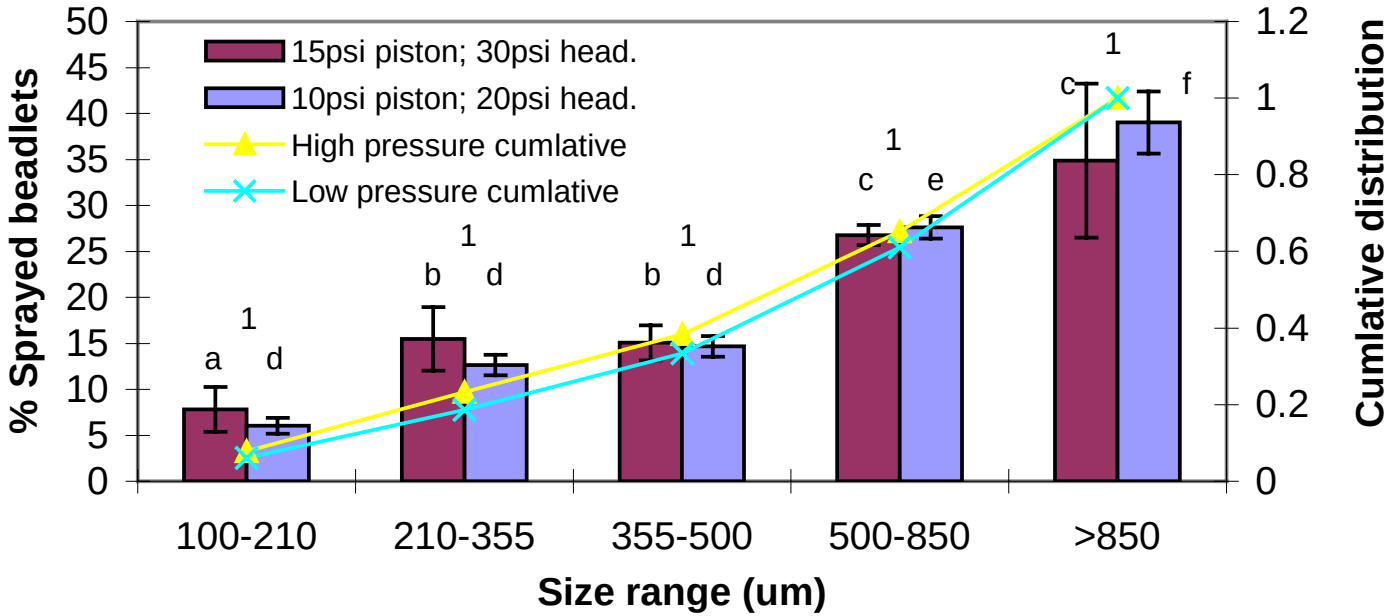



**Fig. 3.12.** The mean (± SE) size frequency distribution of sodium alginate sprayed beadlets, at a constant flow rate of diet fluid at 0.5 L/min and 15 L/min feeder gas to the spraying nozzle. The spray was comprised of 2 mm bore head and 3.5 mm bore nozzle cap. The beadlets yield present on the sieves in comparison to initial alginate loaded into dietary cylinder is 60.6 ± 2.5 % and 56.4 ± 0.8 % for pressure combination 30 psi and 15 psi, and 20 psi and 10 psi for piston and feeder gas combinations respectively. The number represents significant differences between high and low flow rates at each size range ($P<0.05$), while different letters indicates differences between size ranges for each treatment ($P<0.05$).

Slightly higher yields of sprayed beadlets were obtained using higher pressure operating combinations, under conditions of 10 psi; 0.5 L/min diet fluid and 30 psi; 15 L/min atomisation gas, where the yield reached 60.81 ± 2.54 % and 56.40 ± 0.083 % for the high and low pressure combinations, respectively.

3.3.3.2 *Yield of sprayed beadlets using a combination of flow rates and pressures for 2 mm bore head and 3.5 mm bore/ 0.95 mm split nozzle cap*

The effect of the split nozzle cap with the different flow-rate and pressure combinations on size frequency distribution of sprayed beadlets is shown in Fig. 3.13. The majority of the beadlets deposited on the sieves varied between 210 μm to 850 μm. The low flow rate of 0.5 L/min with 10 L/min feeder gas in combination with the split nozzle cap was not able produce sprayed beadlets. Operating pressures influenced the size distribution of sprayed beadlets, although not significantly. High pressure operating conditions had a greater tendency to produce beadlets with a distribution skewed towards the smaller size range, 33% of beadlets were between 210-355 μm. Low pressures produced slightly larger beadlets of which 32% were in the 500-850 μm size range. The size distribution of sprayed beadlets obtained was similar for both operating pressure trial.

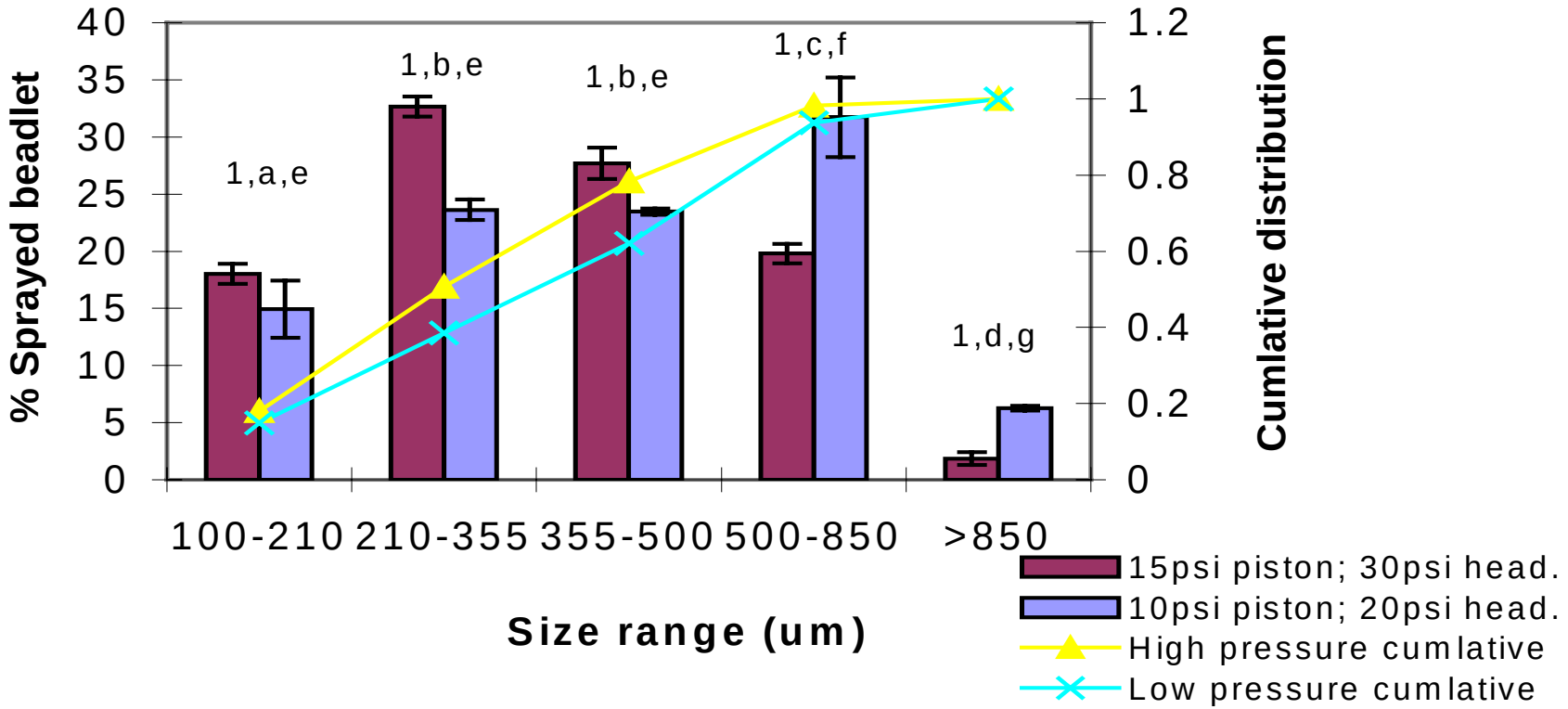



**Fig. 3.13.** The mean (± SE) size frequency distribution of sodium alginate sprayed beadlets deposited on particular sized sieves at high (15 psi piston; 30 psi atomisation) and low (10 psi piston; 20 psi atomisation) pressures. The spraying head was comprised of a 2 mm bore head and 3.5 mm bore/ 0.95 mm split nozzle cap. Constant flow rate of 0.5 L/min and 15 L/min feeder gas to the spraying nozzle (atomisation). The beadlet yield present on sieves compared to initial alginate sprayed was 60.62 ± 1.53 % and 58.03 ± 3.99 % for the high and low pressure treatments, respectively. The number represents significant differences between high and low pressures at each size range ($P<0.05$), while different letters indicates differences between size ranges for each treatment ($P<0.05$).

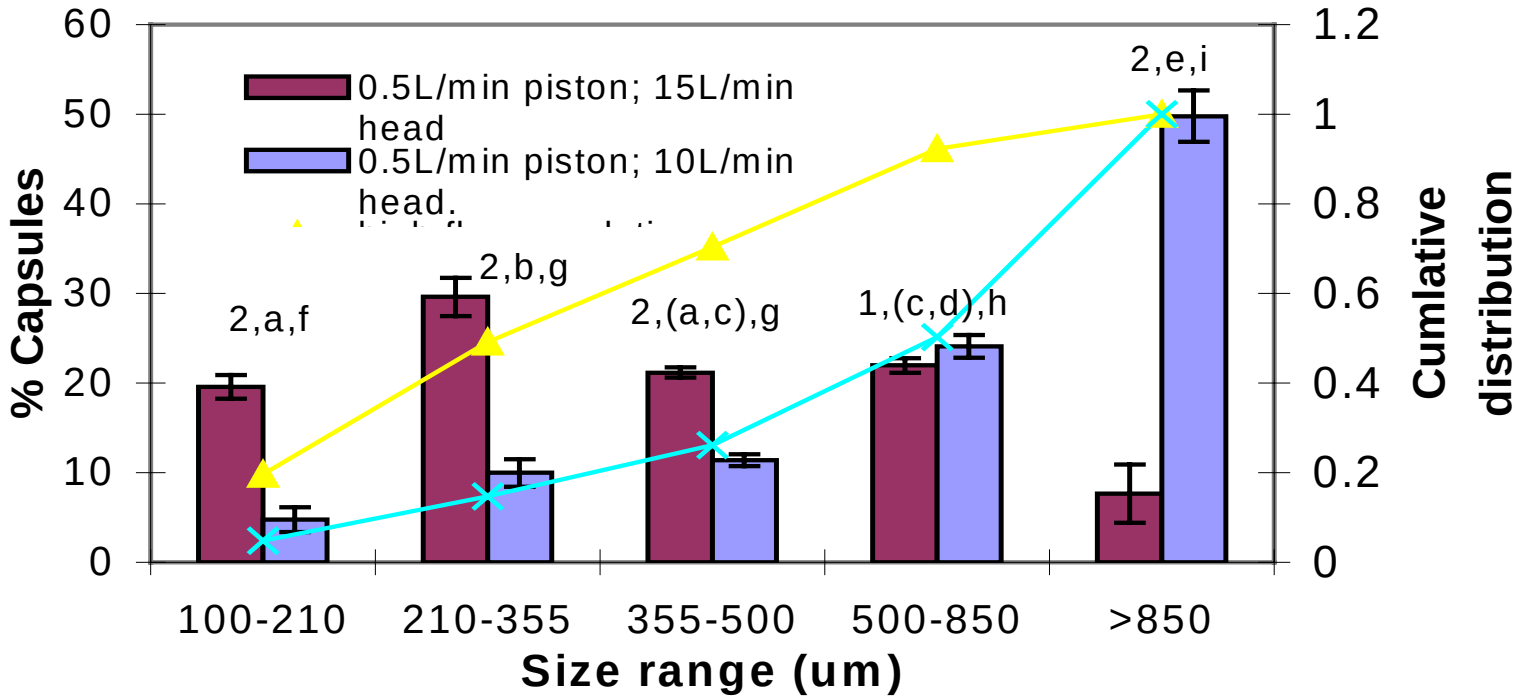



**Fig. 3.14.** The mean (± SE) size frequency distribution of sodium alginate sprayed beadlet deposited on sieves at two flow rates. The spray head was comprised of 2 mm bore head and 3.5 mm bore/ 2.75 mm step nozzle cap. While maintained at a constant pressure of 20 psi spray head and 10 psi piston. The beadlet yield was 51.69 ± 3.57 % and 54.07 ± 0.41 % for atomisation gas flow rates of 15 L/min and 10 L/min, respectively. The number represents significant differences between high and low flow rates at each size range ($P<0.05$), while different letters indicates differences between size ranges for each treatment ($P<0.05$).

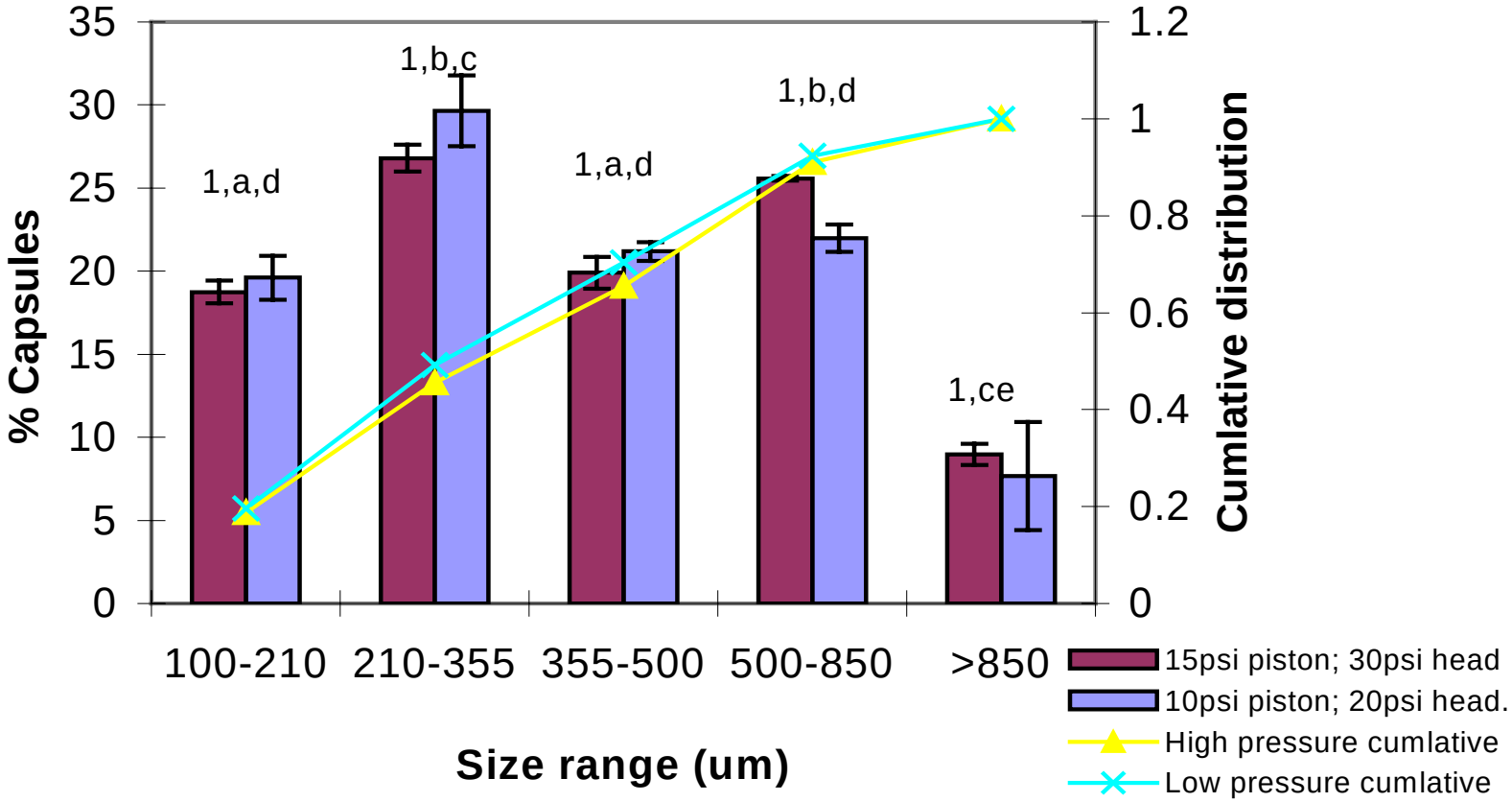



**Fig. 3.15.** The mean (± SE) size frequency distribution of sodium alginate beadlets produced at two feeder gas pressures. Constant flow rate of 0.5 L/min and 15 L/minute at spraying nozzle. The spray head was comprised of 2 mm bore head and 3.5 mm bore/ 2.75 mm step nozzle cap. The beadlets yield deposited on sieves was 57.37 ± 3.85 % and 51.69 ± 3.57 % for 15 psi piston/30 psi atomisation gas and 10 psi piston/20 psi atomisation gas combinations, respectively. The number represents significant differences between high and low pressures at each size range ($P<0.05$), while different letters indicates differences between size ranges for each treatment ($P<0.05$).

### 3.3.3.3 *Yield of sprayed beadlets using a combination of flow rates and pressures for 2 mm bore head and 3.5 mm bore/ 2.75 mm step nozzle cap*

The size frequency distribution of spray beadlets produced using spraying conditions of a 2 mm bore head and 3.5 mm/2.75 mm stepped nozzle cap is shown in Figs. 3.14 and 3.15. The size frequency distribution of sprayed beadlets under the low flow rates was significantly ($P<0.005$) skewed towards the production of larger sized particles, in particular >850 μm size class where 50% beadlet weight was deposited. In contrast, the high flow rate influenced the production of small sized beadlets, particularly the 210-355 μm size class, where 30% of beadlets were deposited. The beadlet yield within the desired sieve ranges >850-100 μm was not significantly different ($P>0.05$) between operating conditions.

The high and low operating pressure trials showed no significant differences in the proportions of sprayed beadlets deposited on the desired sieve sizes ($P>0.05$). The majority of the beadlets produced were deposited in high proportions onto sieve sizes 500-850 μm to 100-210 μm, in particular the size range 210-355 μm (27.4–30.0% of the total). The beadlet yield was similar for high and low pressures using this apparatus combination.

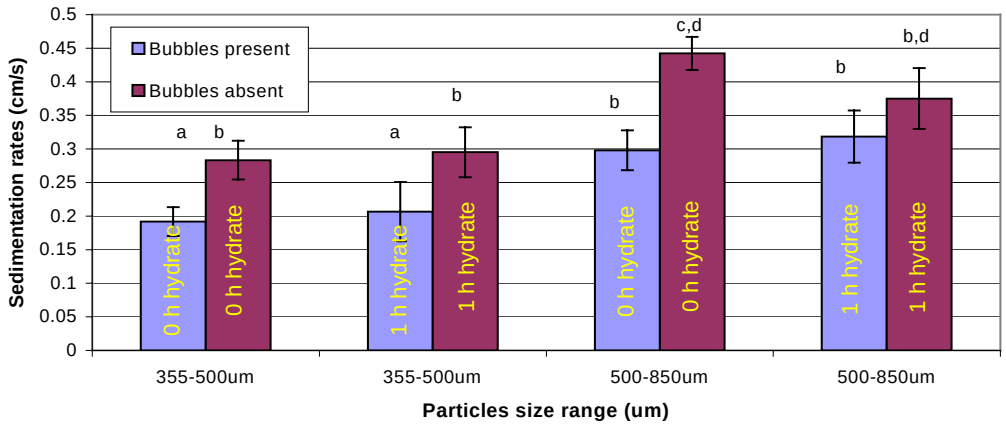



Fig. 3.16. The mean (± SE) sedimentation rate of "normal" sodium alginate sprayed beadlets and those containing bubbles before and after hydration at two size ranges 355-500 µm and 500-850 µm. Means within each size range with a common superscript are not significantly different (P>0.05).

### 3.3.4 Sedimentation rates of sprayed beadlets

The sedimentation rate of artificial food particles described in this study could be manipulated through the encapsulation of nitrogen bubbles within the particles. Sedimentation rates were determined for the two food particle size ranges (355-500 µm and 500-850 µm) prepared as 'normal' or to contain bubbles. The sedimentation rate of these food particles is shown in Fig. 3.16. Sedimentation rates between the two size classes were significantly different (P= <0.001, MANOVA), where the larger sized food particle 500-850 µm had a higher settlement rate with bubbles present (0.298 ± 0.0297 cm/s) and bubbles absent (0.440 ± 0.0246 cm/s). The effect of encapsulating nitrogen bubbles significantly (P= <0.005, MANOVA) affected the sedimentation rate for both size ranges 355-500 µm (0.192 ± 0.0215 cm/s) and 500-850 µm (0.298 ± 0.0297 cm/s), effectively decreasing the sinking velocity. However,

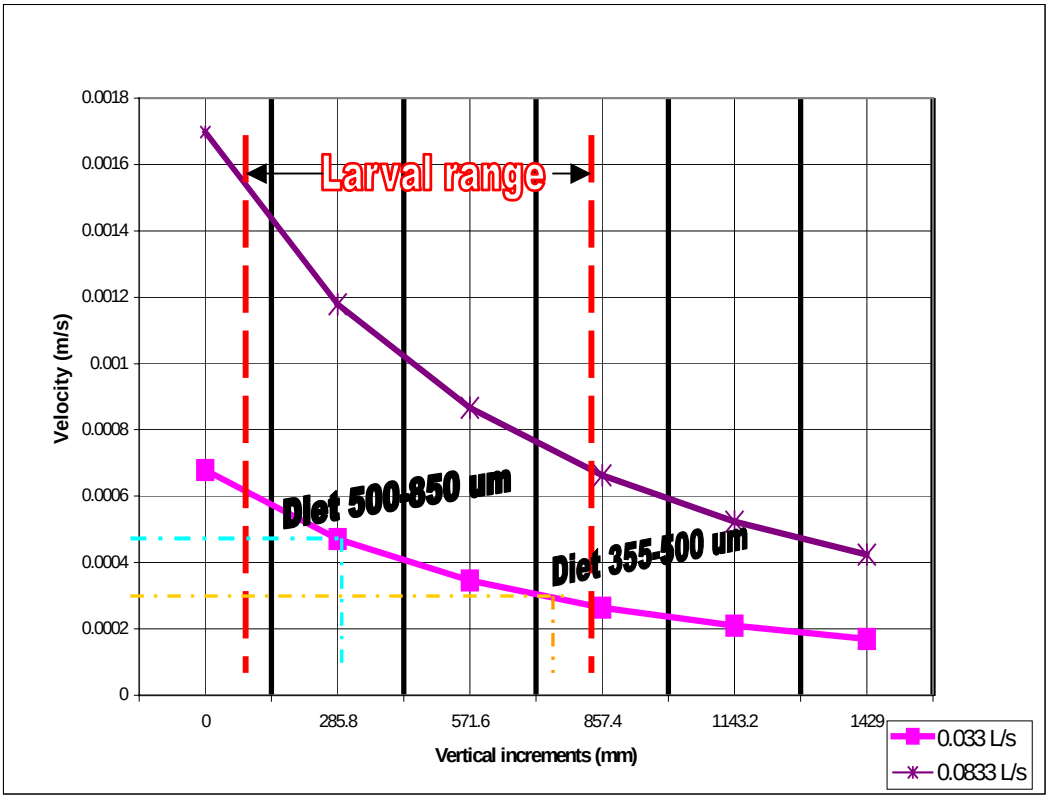
the size class 500-850 μm was only different from the encapsulated bubble treatment and the absent hydration trial, and there were no significant differences between 355-500 μm without bubbles encapsulated and 500-850 μm with bubbles. Also there was no significant difference (P = >>0.05) between hydration times, for either size class.

### 3.3.5 Morphology of sprayed beadlets

The characteristics of the sprayed beadlets varied according to method of preparation. Generally, higher pressures and flow rate combinations resulted in more irregularly shaped sprayed beadlets with rough edges. In contrast low pressures and flow rate combinations produced beadlets with smoother edged but also with irregular shapes.

### 3.3.6 Sedimentation model

The sedimentation model required a number of factors to determine the flow rate required to maintain phyllosoma and diet particles at the same height within culture tanks. The required flow rate for the phyllosoma to remain within the bottom half of the tank is between 0.033 L/sec and 0.0833 L/sec (Fig 3.17). Sedimentation rates recorded for sprayed beadlets in the 355-500 μm and 500-850 μm size ranges were 0.28 cm/s and 0.44 cm/s, respectively. On this basis, at a tank flow rate of 0.033 L/sec they would be expected to be maintained at heights of approximately 285 mm and 700 mm within the water column (Fig. 3.17). At the higher flow rate of 0.083 L/sec the 355-500 size range would be flushed from the phyllosoma culture tank and the larger sprayed beadlets (500-850 μm) would be maintained at a height approximately 1140 mm which is outside the preferred phyllosoma culture range (Fig. 3.17).




Water velocity through the tank at a flow rate of 0.033 L/s
Water velocity through the tank at a flow rate of 0.083 L/s
Sedimentation rate of diet particles 355-500 µm
Sedimentation rate of diet particles 500-850 µm

Fig. 3.17. The sedimentation model. The model incorporates the flow rate of water into the vertical up-welling culture tank, which suspends phyllosoma at specific heights within the tank. If the sedimentation rate of sprayed beadlets is known the height at which it will be maintain in the culture tank at a given flow rate can be determined.

## 3.4 Discussion

Microencapsulated foods have been developed and successfully used for a variety of larval crustaceans but not phyllosoma (Jones *et al*., 1975; Levine *et al*., 1983; Levine and Sulkin, 1984; Kurmaly *et al.,* 1990). The original encapsulated diets gave rise to several microcapsules types with further modifications for specialised feeding

applications (Jones *et al*., 1993). This study assessed modifications for the preparation of microcapsules and has improved production consistency by the addition of the dietary cylinder heated water jacket (Villamar and Langdon, 1993). Furthermore, the advancements in curing bath design allowed a dramatic increase production volume. Through incorporating the 'circular waterfall', a flow of $CaCl_2$ within the curing bath resulted in decreased particle collision and clumping compared to the common method for laboratory scale production of sprayed beadlets (Villamar and Langdon, 1993). Additionally, the continuous recirculation of the curing solution automatically deposited beadlets evenly on the stackable sieves and the use of industrial grade $CaCl_2$ produced large cost savings, with no visible effects on beadlet formation.

Although they are not the natural diet of phyllosoma, the current hatchery feeding protocol is based on *Artemia,* at least for the early stages (Inoue, 1965; Dexter, 1972; Vijayakumaran and Radhakrishnan, 1986). Phyllosoma are thought to change diet with changing nutritional requirements as they develop and it is unlikely that *Artemia* alone could sustain complete larval development (Tong *et al.,* 1997). The alternative to live foods is the manufacture artificial food particles that can be produced in a wide range of sizes to match the changing size of the phyllosoma themselves. Specific microcapule particle sizes can be produced using selected conditions for spraying of the diet-alginate mixture. During the current study the optimal yield of preferred sized particles (210-355 μm and/or 355-500 μm for stage 1 and 2 phyllosoma) was determined. The suitable spraying condition included the use of a 2 mm bore head and 0.95 mm split nozzle cap, under 0.5 L/min diet flow rate by the application 15 psi to the piston and a flow rate of 15 L/min and 30 psi feeder gas to the spraying nozzle cap for atomisation. Particles of 355-500 μm, which could be suitable for phyllosoma

stages 2 to 5, would be most efficiently produced using the 2 mm bore spray head with 0.95 mm split cap and the operating conditions of diet flow rate of 0.5 L/min with 15 psi and 0.95 mm split nozzle cap 15 L/min with 30 psi at the spraying head atomisation gas. In contrast, the larger sized 500-850 μm particles were best obtained with the 2 mm bore head and 3.5 mm/ 2.75 mm step nozzle cap both under the higher pressure of 15 psi and 30 psi. The last few stages of phyllosoma, for example stages 6 to 11, could be fed sizes greater than 850 μm that may be produced by 2 mm bore head with 3.5 mm nozzle cap, under the low pressure and flow rate combination.

A common problem associated with production of artificial food particles is their negative buoyancy within the larval rearing tank. This decreases the diet acceptability, reduces consumption rates and degrades water quality. As phyllosoma are delicate planktonic organisms, individuals reared in captivity must remain in the water column avoiding detrimental swarming bacteria associated with substrates within culture tanks, especially during a long larval duration. Artificial foods can be produced in a range of sizes suitable for phyllosoma and their settlement rate can be manipulated. The encapsulation of nitrogen bubbles was shown to increase buoyancy of sprayed beadlets in the size ranges of 355-500 μm and 500-850 μm. The use of high purity nitrogen has the benefit of reducing oxidation of encapsulated diet ingredients. Although beadlet shapes were observed and noted to be highly irregular and jagged with the higher flow rate and pressure spraying combinations, such particles may benefit the feeding behaviour of the phyllosoma. For example, Levine *et al.* (1983) demonstrated that sodium alginate microcapsules of irregular shapes were easily manipulated by brachyuran crab larvae compared to round microcapsules.

Attempts to culture the southern rock lobsters (*Jasus* spp.*)* largely concentrated on the use of an up-welling tank design (Illingworth *et al*., 1997). The hydraulic design used in this study differs to that used for the southern rock lobster. The tanks used in this study were cone shaped, allowing a range of velocities to be produced within the tank. In contrast the tank design used for southern rock lobster culture had straight vertical walls so only one velocity could be produced. Cone shaped up-welling tanks are successful in maintaining microcapsules at specific levels within a water column, provided the sedimentation rate of the food particle is known. The development of the sedimentation model allows the positioning of the larvae and the suspension of nutritional valuable diets within the same zone of the culture tank. Once the sedimentation rate of individual batches of inert food particles is known, this value can be applied to the model and, at known flow rates within the tank, the position at which they will be maintained can be predicted. The results also show that the buoyancy of sprayed beadlets can be manipulated through the inclusion of bubbles. This development allows greater flexibility in relation to using sprayed beadlets for feeding *P. ornatus* phyllosoma.

# Chapter 4

# Preparation of complex micro-capsules

## 4.1 Introduction

Micro-capsules (MC) are used widely in various fields including the pharmaceutical, food, chemical and biotechnology industries and are made by a variety of methods. Due to potential harsh production conditions only a small number of methods are suitable for food and biological applications. The production of moist sodium alginate MC is commonly used for cell immobilisation including bacterial and cell cultures, microalgae encapsulation, controlled release of biologically active substances and as aquaculture foods (Levine and Sulkin, 1983; Dziezak, 1988; Villamar and Langdon, 1993; Gerbsch and Bucholz, 1995; Jen *et al.,* 1996; Barrows, 2000; Chen, 2001). The permeability of sodium alginate is a favourable characteristic for the encapsulation of living cells since small molecular weight substances can readily move into and out of the capsules. In contrast, this permeability is a non-desirable characteristic for aquaculture foods especially for low molecular weight supplemented ingredients (e.g. amino acids, vitamins and minerals) (Lopez-Alvarado *et al*., 1993; Knauer *et al*., 1993; Lopez-Alvarado *et al*., 1994). The loss of the low molecular weight ingredients is highest during the first few minutes of immersion, with up to 80% of amino acids lost to the surrounding water column (Lopez-Alvarado and Kanazawa, 1993; Lopez-Alvarado, *et al*., 1994). Leaching of nutrients from MC is influenced by binder type and strength, ingredient solubility and the surface area to volume ratio of the MC (Lopez-Alvarado *et al.,* 1994; Barrows, 2000). The loss can be reduced or controlled

by coating the MC (Heinen, 1981; Jackson and Lee, 1991; Lopez-Alvarado, *et al.,* 1994) or by producing complex MC (Prube *et al.*, 2000; Ozkizilcik and Chu, 1996). However, the MC must be ingested and digestible by the target species.

MC are manufactured using a variety of techniques including extrusion (Barrows and Lellis, 2000), dripping (Romo and Perezmartinez, 1997), atomisation (Kwok *et al*., 1991; Halle *et al*., 1994; Poncelet *et al*., 1994; Langdon and Buchal., 1998), emulsification or coacervation (Green *et al*., 1996; Poncelet *et al*., 1992, 1995) and rotating disc atomization (Begin *et al*., 1991; Ogbanna *et al*., 1991). However, depending on the application, the method of production may be limited. In addition most methods of preparing MC are unable to produce a low variation in MC particle diameter, to avoid waste. MC diameter needs to be optimised to each size class of phyllosoma.

The dripping method, where the binder and material is extruded through a syringe into a curing bath, is one of the most common technique used for producing MC (Brandenberger and Widmer, 1998). To obtain smaller MC particles, additional devices are required to "break" the extruded material into smaller droplets. Mechanisms to do this include air-jet (Klein *et al*., 1983; Levee *et al*., 1994; Birouk *et al*., 2002), vibrating nozzles (Hulst *et al*., 1984; Matsumoto *et al*., 1986; Ghosal *et al*., 1993; Brandenberger and Widmer, 1997; Seifert and Phillips, 1997; Serp *et al.,* 2000) electrostatics (Poncelet *et al*., 1994) and mechanical cutting (Prube *et al*., 1998a, b and c; Prube *et al.,* 2000). All of these have been developed in the pharmaceutical industry for particular applications. Some of these techniques have limitations in achieving large-scale production of MC within a narrow size range. The extrusion

method using single or multiple needles in combination with a mechanical cutting device, however, has been shown to have a high production rate (Prube *et al*., 1998 a, b and c; Prube *et al*., 2000).

The main aims of this Chapter were:

(1) To determine the physical parameters required to achieve double encapsulated MC within a narrow size range;

(2) To determine the effect of using a mechanical cutting device to achieve a narrow size range of MC;

## 4.2 Materials and methods

### 4.2.1 The extruding device

A method for mass producing complex syringe extruded and mechanically cut MC to produce specific size particles was first described by Prube *et al*. (2000). This method of preparing MC was further modified to produce larval feeds for this study.

An extruding device was constructed at the AIMS workshop and consisted of a stainless steel chamber (Fig. 4.1 and Fig. 4.2) with an inlet side port for introducing the particle coating solution. This solution exited through a wide bore stainless steel hypodermic tube. Within the wide bore tube was mounted another stainless steel hypodermic tube which passed through the centre of the coating needle. Both needles were level at the point of extrusion. This produced a double barrel hypodermic syringes system, where a solution of dietary material was pumped through the inner tube, and the outer tube contained the coating material of sodium alginate binder. By

varying the flow rates, the degree of coating or encapsulation can be manipulated. This unit was mounted on an adjustable stand above a curing bath, containing $CaCl_2$. The inner and outer wall mixture was pumped separately using a peristaltic pump, producing a vertical stream downwards, which, on exiting the needles, was then cut by a spinning disc of wires (Fig. 4.3). The stream was cut into small cylinders, which formed round beads as they fell from the needles into the curing bath.

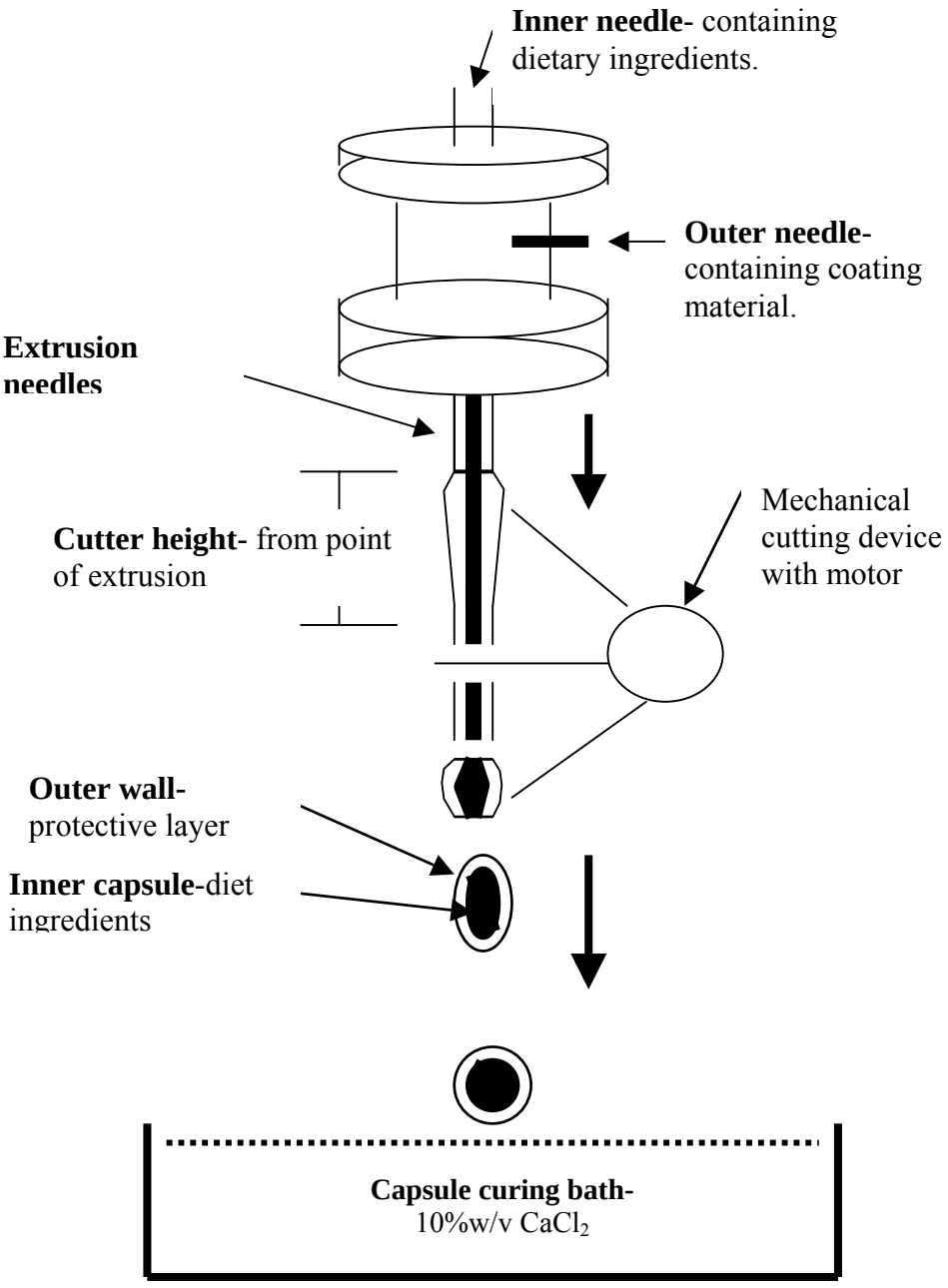



Fig. 4.1. The apparatus used to prepare complex MC, including extrusion device, mechanical cutting device and curing bath of $CaCl_2$ (10%w/v).


Fig. 4.2. The production device used to produced complex MC, including extruder, cutting device and curing bath.

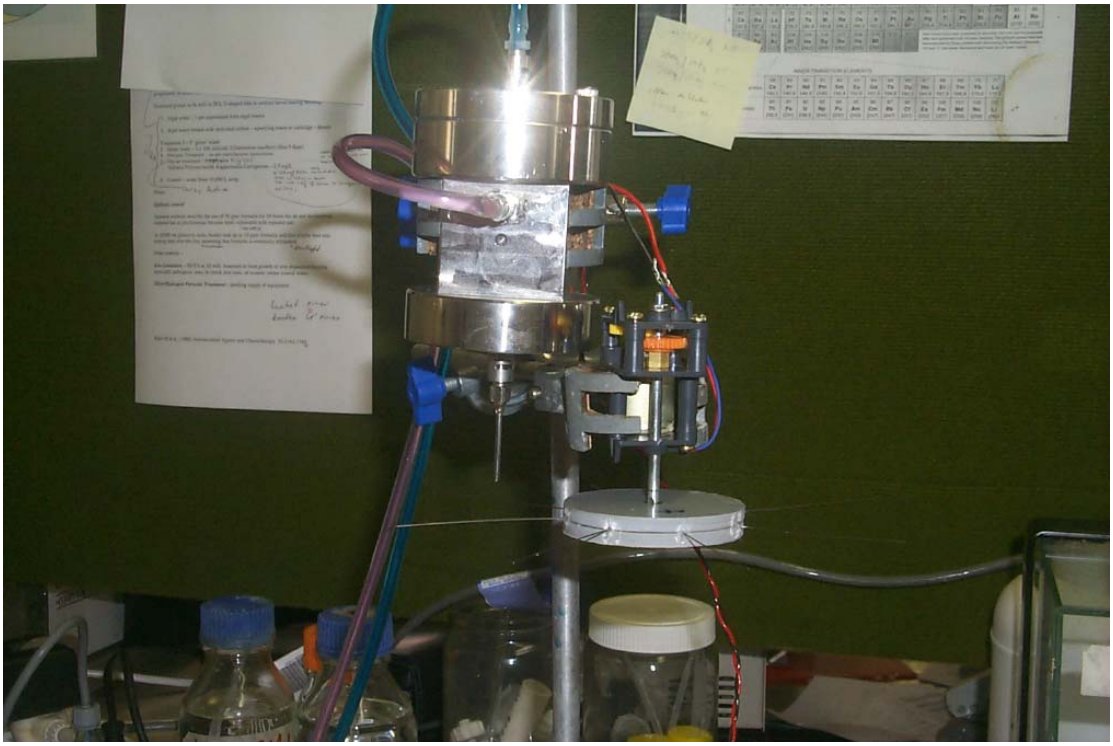


Fig. 4.3. The extruder and mechanical cutting device using to produced complex MC.

The mechanical cutting device (Fig. 4.4) operated using a small electrical motor to drive the disc containing multiple thin wires (Fig. 4.5). The mechanical cutting device could be adjusted to cut the mixture on any angle or rotating speed between 200 to 1000 RPM.


Fig. 4.4. The motor that drives the mechanical cutting discs. (Operating range of 200-1000 RPM).

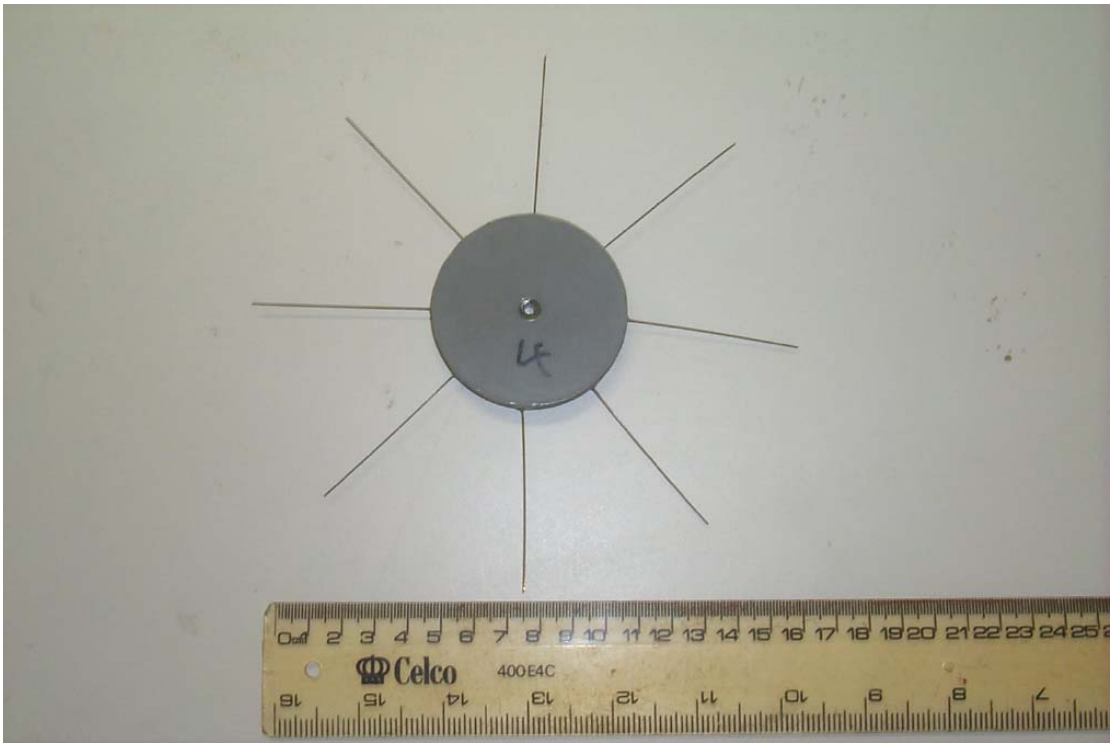



Fig. 4.5. The cutting disc containing 8 wires used to cut the extruding fluid.

### 4.2.2 Preparation of moist MC

The method of preparing the sodium alginate binder mixture is described in Chapter 2 (section 2.4). To optimise operational parameters, complex MC were produced without dietary material, as large volumes were produced to preserve the curing solution.

### 4.2.3 The effect of various physical parameters on MC size range

The physical combinations of extrusion equipment were varied to determine the effects on MC size and size distribution. Seven variations (Table 4.1) of extrusion needle gauge combination, flow rate of extrusion liquid and cutter parameters were

assessed. The position of the cutter device below the extrusion device was 5 cm unless otherwise stated and the rotation speed of the cutter device was maintained at 830-850 RPM during the experiment. The position of the extrusion device was a height of 350 mm from the curing bath.

Table 4.1. The physical parameters examined during the preparation of complex microcapsules.

| Treatment | Physical conditions | Factor |
|---|---|---|
| 1 | No cutter, 21G*: 16G, | Flow rate |
| 2 | Cutter (8 wires) 18G: 13G. Flow rate 13 mL/min | Cutter present |
| 3 | Cutter (8 wires) flow rate 17 mL/min | Needles / cutter |
| 4 | Cutter (8 wires) 21G: 16G. Flow rate 60 mL/min | Cutter heights |
| 5 | Cutter (12 wires) 21G: 16G. Flow rate 50 mL/min | Cutter heights |
| 6 | Cutter (12 wires) 21G: 16G. Flow rate 20 mL/min. | Cutter angle |
| 7 | Cutter (12 wires) 21G: 16G. Flow rate 60 mL/min | Cutter angle |

* G = the standard gauge diameter of the needle (inner:outer needle).

### 4.2.4 The effect of rotating speed of the mechanical device on microcapsule size and size distribution.

The cutting device was mounted on the shaft of a small DC motor and connected to a variable power supply. To determine the speed of the spinning disc under a variety of power inputs a small reflective sticker was attached to the rotating disc and the speed was measured using a tachometer.

## *4.3 Results*

### 4.3.1 Production of complex MC

The results of this experiment to determine the effect of different physical parameters during preparation on the characteristics of resulting complex MC are shown in Figs. 4.6- 4.12. The results show that MC sizes and size frequency distributions are affected by the physical parameters of manufacturing equipment.

### 4.3.2 Production of complex MC using Treatment 1

The size frequency distribution of complex MC prepared using different flow rates is shown in Fig. 4.6. The highest proportion of MC were produced using the lowest flow rate of 17 L/min. Approximately 40% of the MC were produced within the size range of 1.51-1.7 mm and the size distribution was narrow (Fig. 4.6). While the highest flow rate (60 mL/min) appeared to produce larger sized MC, the size distribution was broad. A small proportion of smaller MC was produced in the range of 0.91-1.1 mm, under the low flow-rate treatment.

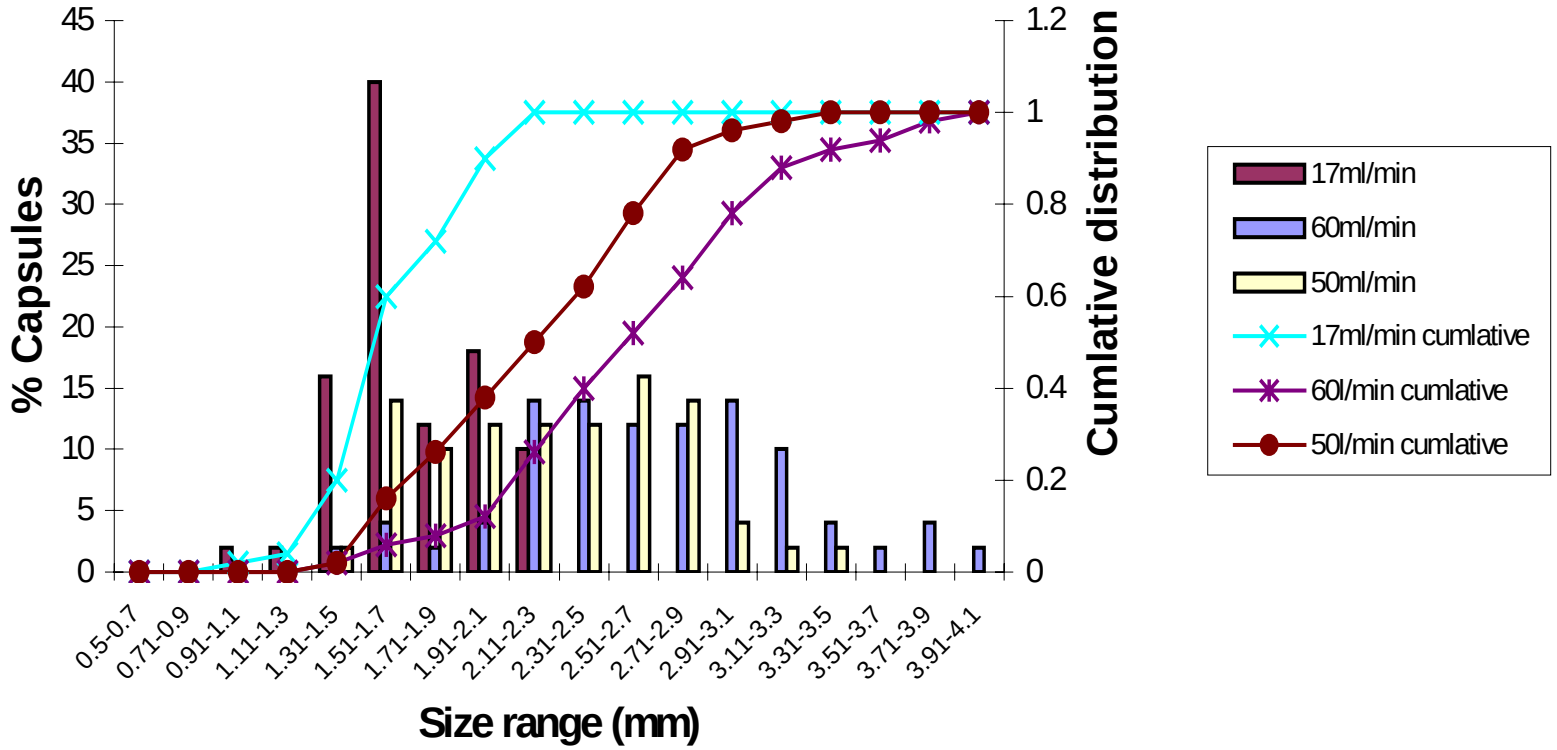



Fig. 4.6. The size frequency distribution of complex MC produced using Treatment 1; using 3 different flow rates of dietary material, without a mechanical device and with needle gauges of 21G inner: 16 G outer.

### 4.3.3 Production of complex MC using Treatment 2

The size frequency distribution of complex MC prepared with a mechanical cutting device present and absent is shown in Fig. 4.7. The presence of the mechanical cutting device resulted in a greater number of MC than without the device and less variation in size.

### 4.3.4 Production of complex MC using Treatment 3

The size frequency distribution of complex MC produced using two different needle sizes, with a mechanical cutting device present and absent is shown in Fig. 4.8. Both the needle diameters with the cutter present produced a high proportion (approx 40%) of MC within the 1.51-1.7 mm size range, although the smaller needle diameter without the cutter also produced an equivalent number of capsules.

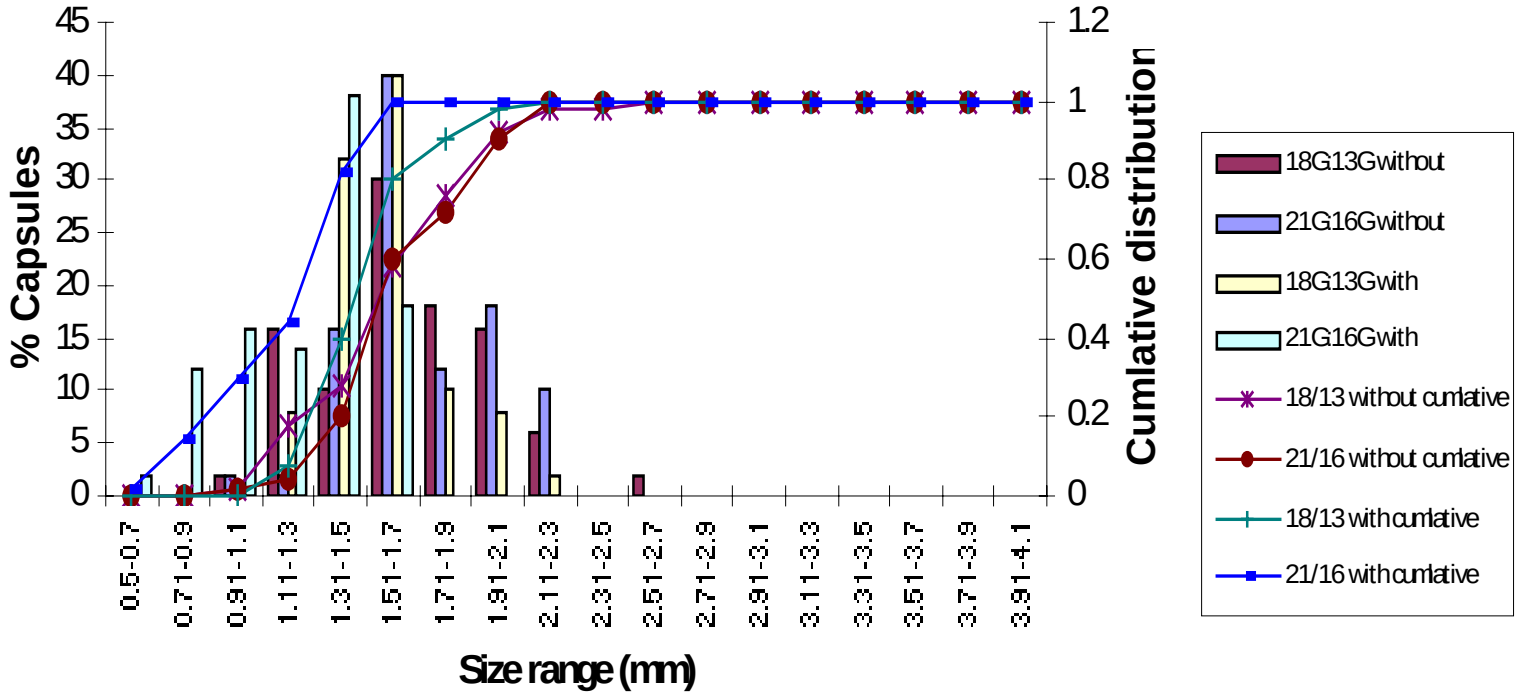



Fig. 4.8. The size frequency distribution of complex MC produced using Treatment 3; using different needle diameter combinations (Flow rate of 17 mL/min).

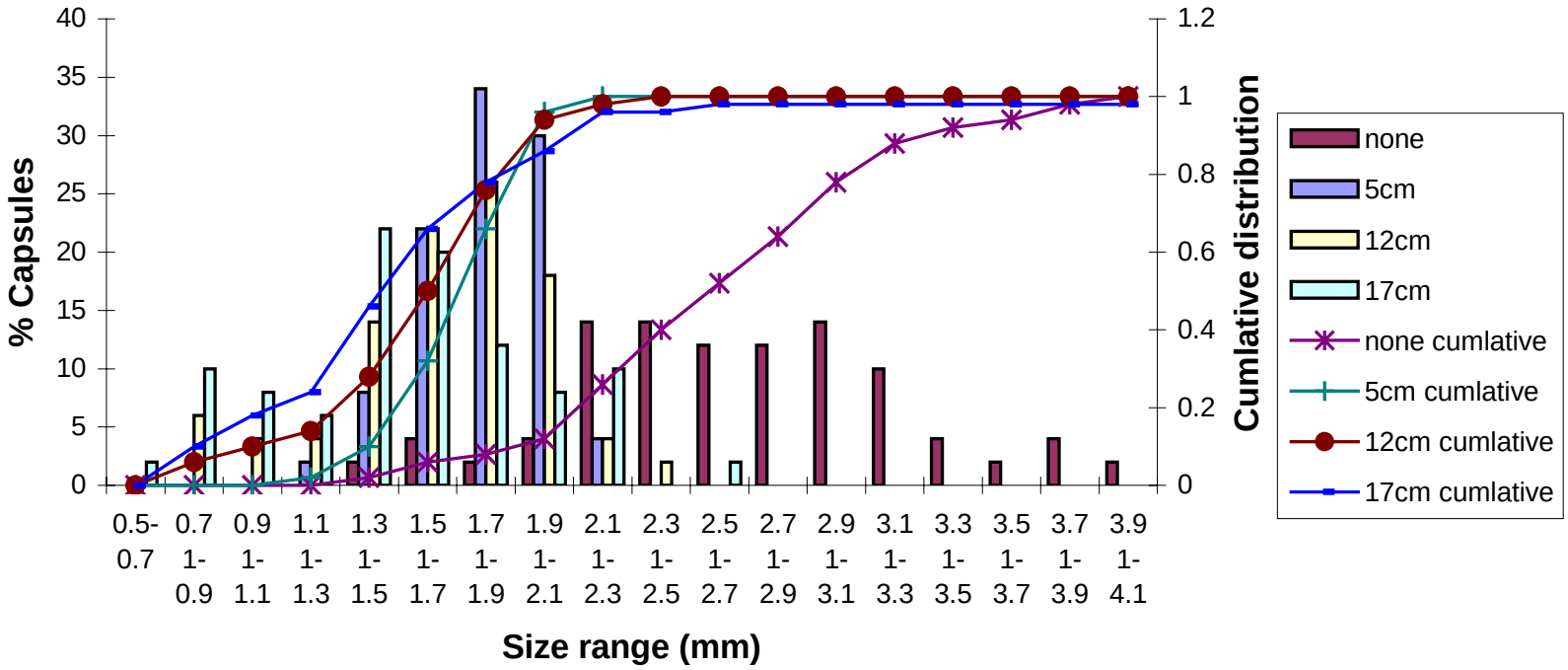



Fig. 4.9. The size frequency distribution of complex MC produced using Treatment 4; using different mechanical cutter heights from curing bath. (cutter disc-8 wires. Needle sizes 21G: 16G. Flow rate of 60 mL/min).

4.3.5 Production of complex MC using Treatment 4

The size frequency distribution of complex MC prepared using a variety of cutter device heights with 8 wires, shown Fig. 4.9. The highest yield (35%) of MC was produced at a cutting height of 5 cm from the point of extrusion, within the size range of 1.71-1.9 mm as well as producing the smallest size range of MC of 1.31-1.5 to 2.11 mm. While a cutter height of 17 cm produced smaller sized MC, the size distribution was broad. While with no cutting device present the size of MC produced was widely distributed and large in size.

4.3.6 Production of complex MC using Treatment 5

The size frequency distribution of complex MC produced using different cutting heights with a disc containing 12 wires are shown in Fig. 4.10. The highest proportion of MC was produced at a cutting height of 5 cm from the point of extrusion. At this height, about 75% of the MC were produced within the size range of 1.31-1.5 mm with the smallest size range. A cutter height of 5 cm produced the lowest size range between 1.13-1.3 to 1.71-1.9 mm, followed by cutter heights of 12 cm (0.9-1.1 to 2.11-2.3 mm), 17 cm (1.11-1.3 to 2.91-3.1 mm) and then with no cutter present (1.31-1.5 to 3.31-3.5 mm). A cutter height of 17 cm produced larger sized MC. The largest size of MC 3.31-3.5 mm produced was achieved without using a cutter device.

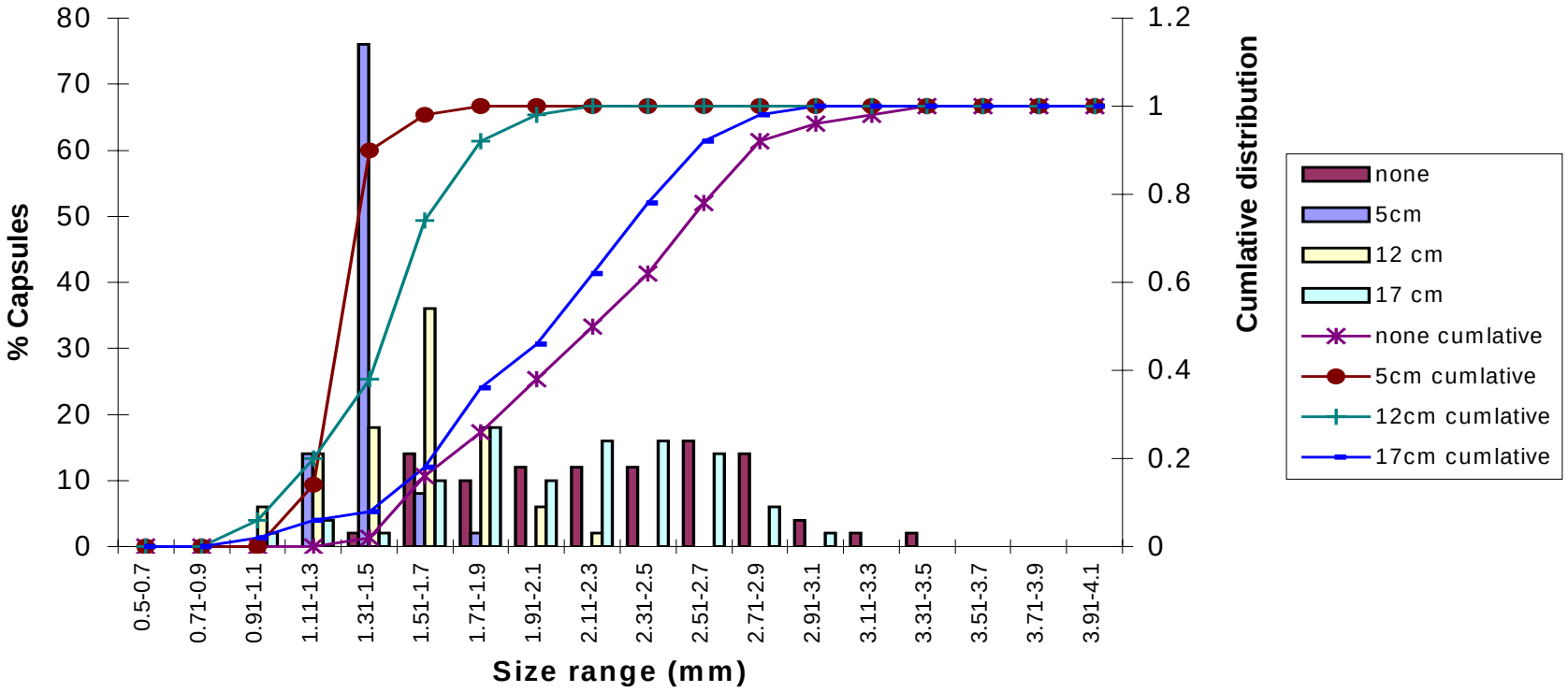



Fig. 4.10. The size frequency distribution of complex MC produced using Treatment 5; using different cutter heights, using cutting disc containing 12 wires. (Flow rate 50 mL/min)

4.3.7 Production of complex MC using Treatment 6

The size frequency distribution of complex MC produce with the mechanical cutting device positioned at $0^{o}$ (horizontal) and $45^{o}$ cutting angles are shown in Fig. 4.11. A slightly higher proportion of 45% of 0.9-1.1mm sized particles was produced using a 45 degree angled cutting device, compared to 41% of 1.11-1.3mm sized particles using the horizontal configuration, also a slightly narrower size distribution were produced with the cutter on the $45^{o}$ angle, under a low flow rate of 20 mL/min (Fig. 4.11).

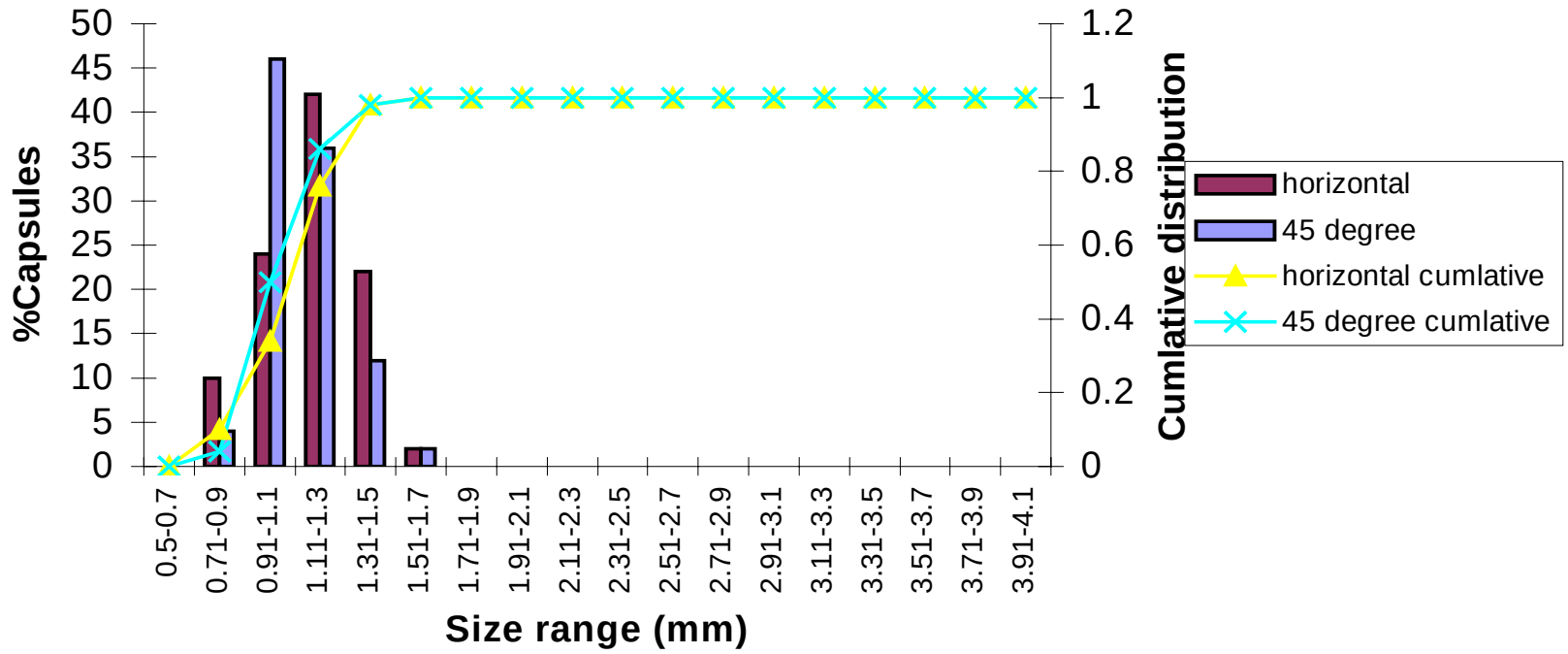



Fig. 4.11. The size frequency distribution of complex MC produced using Treatment 6; using 2 mechanical cutter angles- horizontal and 45$^{o}$ incline. (Flow rate 20 mL/min, cutter disc 12 wires).

4.3.8 Production of complex MC using Treatment 7

The size frequency distribution of complex MC produced with the mechanical cutting device present, at two cutting angles 0 $^{o}$ (horizontal) and 45$^{o}$ angle are shown in Fig. 4.12. The highest proportion and narrowest size distribution of MC was produced with the cutter in the horizontal position, although the MC size was, on average, larger. The MC produced with the cutter at a 45 $^{o}$ were smaller sized, although the size distribution was broad.

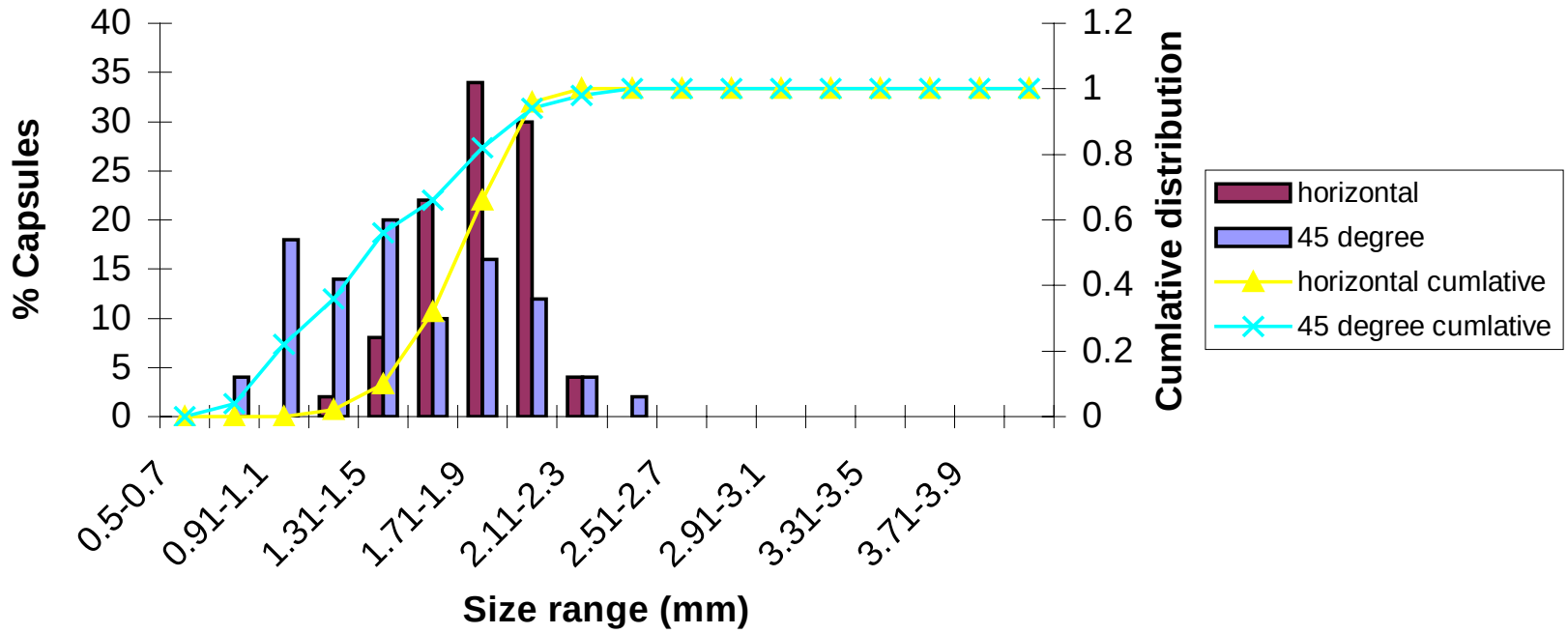



Fig. 4.12. The size frequency distribution of complex MC prepared using two cutter angles at a flow rate 60 mL/min.

### 4.3.9 The rotational speed of the cutting device

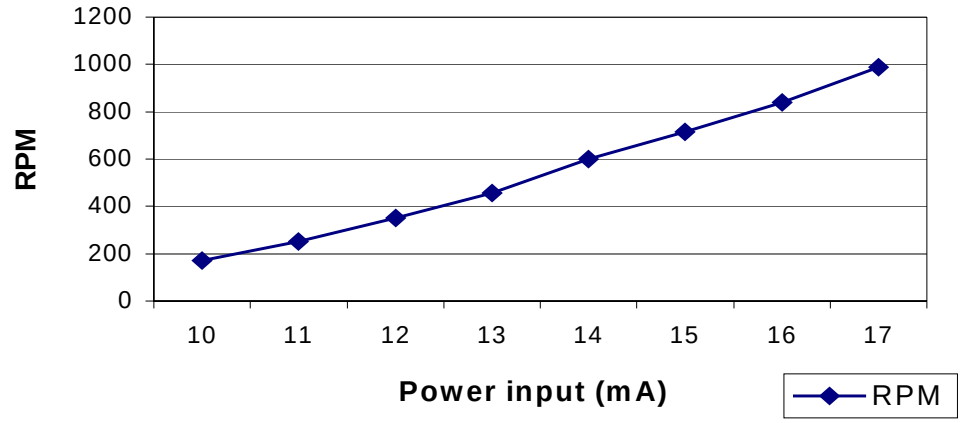



Fig. 4.13. The speed (RPM) of the mechanical cutting device under different power inputs.

The effect of varying the power input on the speed (RPM) of the cutting device is shown in Fig. 4.13. The maximum operating speed reached during the trial was 1000 RPM when supplied with 17 mA, while the lowest speed was just below 200 RPM at a power input of 10 mA.

## 4.4 Discussion

On the basis of the results of these experiments, it is clear that a variety of MC sizes can be obtained by adjusting physical parameters during the encapsulation process. These effects were particularly noticeable with regard to the size frequency distribution of resulting MC. The parameters that proved most important in influencing resultant MC size and size range, were flow rate, the presence and height of a cutter device and needle diameter. The MC size varied between 0.51-0.7 mm through to 4 mm, under the experimental conditions examined. It was not possible to

produce MC within narrow size ranges, although size frequency distribution can be influenced to a degree.

The physical parameters investigated here for their effect on the size frequency distribution of MC were flow rate, cutter height and angle, and the diameter of the extrusion needle. A low-flow rate of 17mL/min, without a cutter device, resulted in approximately 40% of MC within a 1.51-1.7 mm size range. However, smaller MC were produced with the presence of the mechanical cutting device as was found for both needle diameters examined. A small proportion (2% and 12%) of MC were produced within the size ranges of 0.51-0.7 mm and 0.71-0.9 mm, respectively. Without the cutter device the size range of MC was broad, between 1.11-1.3 mm and 2.11-2.3 mm. Narrow diameter (21G inner: 18 G outer) extrusion needles produced smaller MC than larger diameter (18 G inner: 13 G outer) needles, with the cutting device present, although a small quantity of MC were produced between 0.51-0.7 mm. However, it is more difficult to push viscous fluids through small diameter needles. The presence and position of the cutting device has proved to be important component in producing narrow sized MC; the preferred height of the cutting device was 5 cm on an angle of $45^{o}$ from the point of extrusion from the needles. The highest proportion (75% of 1.31-1.5 mm) of MC during the current experiment were produced using the cutter with 12 wires positioned at $45^{o}$ and 5 cm from the point of extrusion under a flow rate of 50 mL/min.

Previous studies investigating the size of MC produced using the extrusion technique in combination with a variety of break-up mechanisms, have shown a range of MC sizes and size distributions can be produced. Prube *et al.* (2000), for example,

produced MC down to 150 μm using a single nozzle extrusion device with a specialised mechanical cutting device similar to that used in the current study. Brandenberger and Widmer (1998) used the vibrating nozzle method to produced MC within a mean size of 340 μm. Devos *et al.* (1997) used air-generated droplet production to produce MC with a mean size of 450 μm, while a similar multi-nozzle system provided a mean MC diameter of 0.8 mm (Seifert and Philips, 1997). Prube *et al*. (1998a) produce a high proportion of MC within the 0.8-1 mm size range, with the smallest being 0.3 mm, using a similar method. Poncelet *et al*. (1994) used a low flow-rate electrostatic break-up method to produce MC with a mean diameter of 750 μm, although the size range was broad, and the smallest MC produced were 450 μm in diameter. The size of MC produced using the extrusion and mechanical cutting method can be adjusted from 150 μm to several mm in size; the size is determined by the nozzle diameter, flow-rate through the nozzle, the number of wires and the rotational speed of the cutting device (Prube *et al.,* 2000).

During the mechanical cutting process, some losses of dietary material occured, due to the action of the wire cutting through the extruded diet stream. These losses can be minimised by inclining the cutting device and reducing the diameter of the cutting wires (Prube *et al.,* 2000). Losses can be minimised by inclining the cutting direction to approximately 45 degrees giving a perpendicular cut. The loss ranges from 15% to 1-2% when the cutting tool is placed on an incline with small diameter wires (0.05 mm) (Prube *et al.,* 2000).

The objective of microencapsulation is to protect the internal ingredients from the external environment (Fig. 4.14). Nutrients vulnerable to leaching from MC include

low molecular weight and water-soluble ingredients, such as vitamins, minerals and amino acids. This method of producing complex MC may reduce nutrient leaching while retaining a digestible protective wall surrounding the diet. This rationale is the basis of U.S Patent 5,776,490 where the pre-encapsulation of the amino acid lysine using lipid-walled MC followed by the incorporation into protein walled MC, resulted in significant retention of the water soluble ingredients, compared to single encapsulation techniques (Lopez-Alvarado, *et al.,* 1994).

Producing complex MC for feeding applications in aquaculture is favourable, especially as valuable ingredients would remain within the particles longer than is possible with current preparation methods. The potential for complex MC to feed phyllosoma would be aimed at mid- to late-staged phyllosoma due to the limited size range (relative large) of the MC produced using the method described here. Previous feeding trials using complex MC to feed the larvae of aquatic animals are few and further investigation is required to determine whether complex MC are ingested and digested by the target species (Villamar and Langdon, 1993). The large-scale manufacturing of these MC would be expensive at present, due to the cost of the equipment required and the MC size and size distribution required; however, in principle, this form of MC is promising.

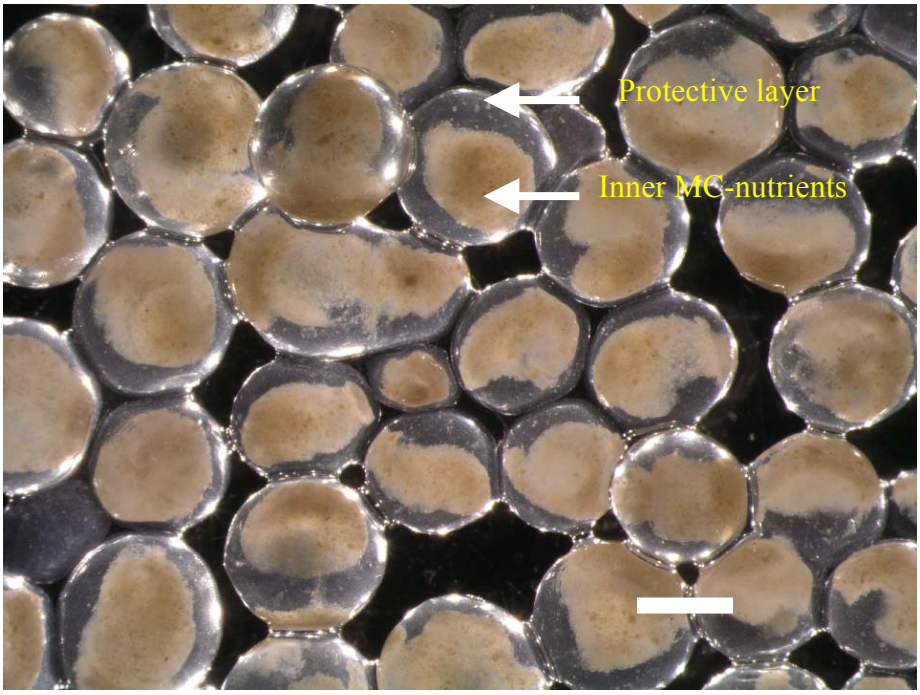



Fig. 4.14. Complex MC showing the protection of internal ingredients from the external environment via an extra MC wall. These MC were prepared using Treatment 1 at a flow rate of 17 mL/min. Scale bar = 1 mm.

# Chapter 5

# Ingestion of $^{14}$C-labelled artificial diets by early phyllosoma stages

## 5.1 Introduction

Phyllosoma culture has proved difficult due to the length of the larval phase, inadequate culture techniques and, in particular, lack of suitable nutritionally adequate foods. Due to the relatively long larval phase of rock lobster phyllosoma and the high cost of live food production, development of artificial alternatives, is potentially economically favourable. To date, however, there is limited information on the use of artificial diets for carnivorous crustacean larvae, in particular, phyllosoma.

Ingestion of diets by aquatic larvae have been investigated using several methods including survival, growth, use of fluorescent or radio-labelled tracers ($^{14}$C $^{3}$H and $^{15}$N), measurement of intestinal swelling and hydrocarbons tracers (cholestane or dotriacontane) (Weinhart *et al.,* 1991; Jones, 1998; Teshima *et al*., 2000). To fully understand feeding biology of larvae the level of feed intake must be accurately measured (Teshima *et al*., 2000). The use of dietary radiolabelled tracers ($^{14}$C, $^{3}$H) as a means of measuring ingestion of aquatic larvae has been reported in a number of studies that provided useful information on ingestion and assimilation of artificial food particles (Kolkovski *et al*., 1993b, c, d; Kolkovski *et al*., 1997a, b; Partridge and Southgate, 1999; Genodepa *et al.,* 2004a).

Phyllosoma are suspected of mainly consuming gelatinous zooplankton, possibly ctenophores in the wild (Kittaka, 1997; Cox and Johnston, 2003). Although speculative, the objective of this study was to examine the possibly of producing a soft jellyfish-like artificial food. The most appropriate method of dietary entrapment for this is a gelled hydrocolloid matrix. Appropriate binders to form the matrix include alginate, carrageenan, gelatine, zein and pectins (Meyer *et al.,* 1972; Miller 1973; Heinen, 1981). MBD particles have proven successful for larval culture of finfish and crustacean, primarily due to the lack of an indigestible capsule wall (Southgate and Partridge, 1998). Despite encapsulation of dietary ingredients within a matrix, the loss of nutrients from the particles during immersion remains relatively high (Lopez-Alvarado *et al*., 1993).

The main aims of this Chapter were:

(1) To determine the ingestion rate of MBDs containing $^{14}$C-labelled nutrients by stage 1, 2, 3 and 4 *P. ornatus*, phyllosoma; and

(2) To determine the leaching rates of $^{14}$C-labelled nutrients from sodium alginate and carrageenan bound MBD.

## 5.2 Materials and methods

### 5.2.1 Larval production

Phyllosoma were obtained from artificially induced, captive broodstock and reared at the AIMS mariculture facility detailed in Chapter 2 (section 2.3.1). Phyllosoma were carefully transported in aquarium bags to the MARFU facility at JCU. Phyllosoma

were acclimatized and starved in 1 L beakers containing 1 μm filtered seawater with gentle aeration for a 12 h period.

### 5.2.2 Method of $^{14}$C-labelling *Artemia*

The method used for labelling the MBD with $^{14}$C was based on techniques described by Kolkovski *et al.* (1993). Briefly, the microalga (T- *Isochrysis* spp.) was incubated in seawater containing [$^{14}$C]-sodium bicarbonate (Amersham Biosciences). *Artemia* were then allowed to graze on the labelled algae for 22 h (Chapter 2, section 2.4.2). The $^{14}$C-labelled *Artemia* were harvested, dried at 45$^{o}$C for 24 h, ground with mortar and pestle and added to MBD experimental diets of the composition shown in Table 5.1. The MBD mixture was thoroughly stirred by hand and place onto a tray at a thickness of 3mm prior to being oven dried at 45$^{o}$C for 24 h. The dried mixture was then ground and sieved to a particle size range of 355-500 μm for stage 1 and 2 phyllosoma, and 500-850 μm for stage 3 and 4.

Table 5.1. The composition (% dry weight) of the $^{14}$C-labelled MBD used to feed *P. ornatus*, phyllosoma.

| **Ingredients of $^{14}$C labelled MBD diets** | **% Weight** |
|---|---|
| Radioactive *Artemia* | 16.6 |
| Squid meal (<100 μm) | 16.6 |
| Binder (alginate or carrageenan) | 1.33 |
| Distilled water | 65.33 |
| **Total** | **100** |

### 5.2.3 Experiment 1 - Ingestion rate of MBD by phyllosoma

Experiments to investigate ingestion of $^{14}$C-labelled MBD were conducted within the MARFU feeding facility. Each beaker contained 1 L of 1 μm filtered 35 ppt seawater to which 20 phyllosoma were added. The container was gently aerated to maintain the diet particles in suspension. Each beaker was randomly assigned to a treatment and each treatment was replicated five times (n=5). The first experiment investigated ingestion of $^{14}$C-labelled MBD after 1, 2, 3 and 4 h ingestion times. The control treatments included dead phyllosoma which were 'fed' $^{14}$C-labelled diets and live phyllosoma exposed to 20 μm filtered water in which $^{14}$C-labelled MBDs had been immersed for 1 h. Each beaker was harvested after the appropriate feeding time into a cone shaped sieve and larvae were gently rinsed while submerged, to remove excess diet material from the phyllosoma body and shell. Washed phyllosoma were then rinsed into scintillation vials with 10-15 mL of 1 μm filtered seawater and then placed directly on ice slurry, then into a freezer shortly after.

### 5.2.4 Experiment 2 - Leaching of $^{14}$C from experimental MBD

Leaching of $^{14}$C material from $^{14}$C-labelled MBD was determined using two methods. In the first, $^{14}$C-labelled MBD was added at hourly intervals over a 4 h period and radioactive content of the water was measured during the first hour and then every hour. In the second, measurement of the $^{14}$C content of the water was measured conducted as above following a single addition of radioactive MBD. The experimental $^{14}$C-labelled MBD were added to 1 L beakers containing 1 μm filtered 35 ppt gently aerated seawater, all treatments were sampled from each of the 5

replicate tanks during the ingestion trial. One mL samples were taken every 15 min for the first hour and then every hour for 4 h. The samples were passed through a 0.45 μm cellulose acetate filter to remove diet particles and placed into scintillation vials. Five mL of scintillation cocktail was then added to each vial and the radioactivity of the water samples was determined and corrected for background radiation (Chapter 2, section 2.4.3). The control treatments were: (1) dead phyllosoma 'fed' $^{14}$C-labelled MBDs; and (2) live phyllosoma within 1 h $^{14}$C-labelled water from MBD leachate. Both controls were subtracted from the ingested diet to remove the influence from diets, water and background radiation.

### 5.2.5 Statistical analyses

Data were tested for homogeneity of variances using Levines test and for normal distribution with a Q-Q plot, before conducting a one-way ANOVA on each of the trials, for both ingestion rate of the individual stages and leaching rates of MBDs.

## 5.3 Results

### 5.3.1 Experiment 1 - Ingestion rate of MBDs by phyllosoma

The results of this experiment are shown in Fig. 5.1 to Fig. 5.4.

The rates of ingestion of both alginate and carrageenan bound MBDs by stage 1 phyllosoma over a 4 h feeding duration are shown in Fig. 5.1. The MBD bound with sodium alginate had the highest ingestion rate (0.177 ± 0.448 μg mg$^{-1}$ larvae$^{-1}$) after 4 h, although this was not significantly greater than that for other ingestion times. The ingestion rates of sodium alginate and carrageenan bound MBDs were not

significantly different from each other or from the controls, over the 4 h experimental period.

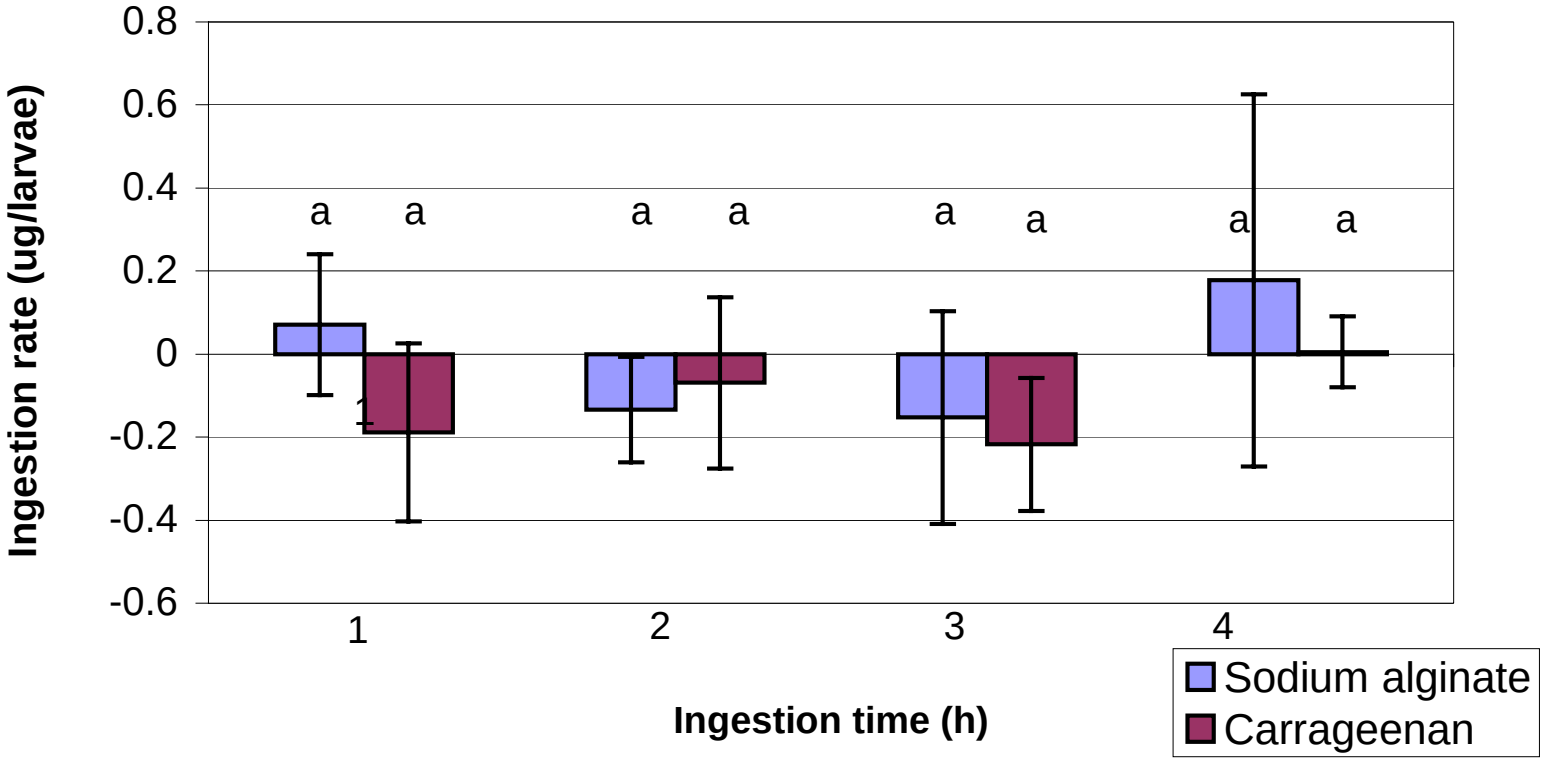



Fig. 5.1. The mean (± SE) ingestion rate (µg of MBD $mg^{-1}$ larval dry weight) of $^{14}C$-labelled MBD bound with sodium alginate and carrageenan by newly hatched stage 1 phyllosoma. Means sharing the same letter were not significantly different between feeds or ingestion periods ($P>0.05$).

The ingestion rates of stage 2 phyllosoma fed sodium alginate and carrageenan bound MBDs are shown in Fig. 5.2. The MBD bound with carrageenan after 4 h had the highest ingestion of 0.325 ± 0.112 μg $mg^{-1}$ larvae, although this was not significantly different from other ingestion times. There was no significant difference ($P>0.05$) between binder types for ingestion rates at any time, over the 4 h experimental period.

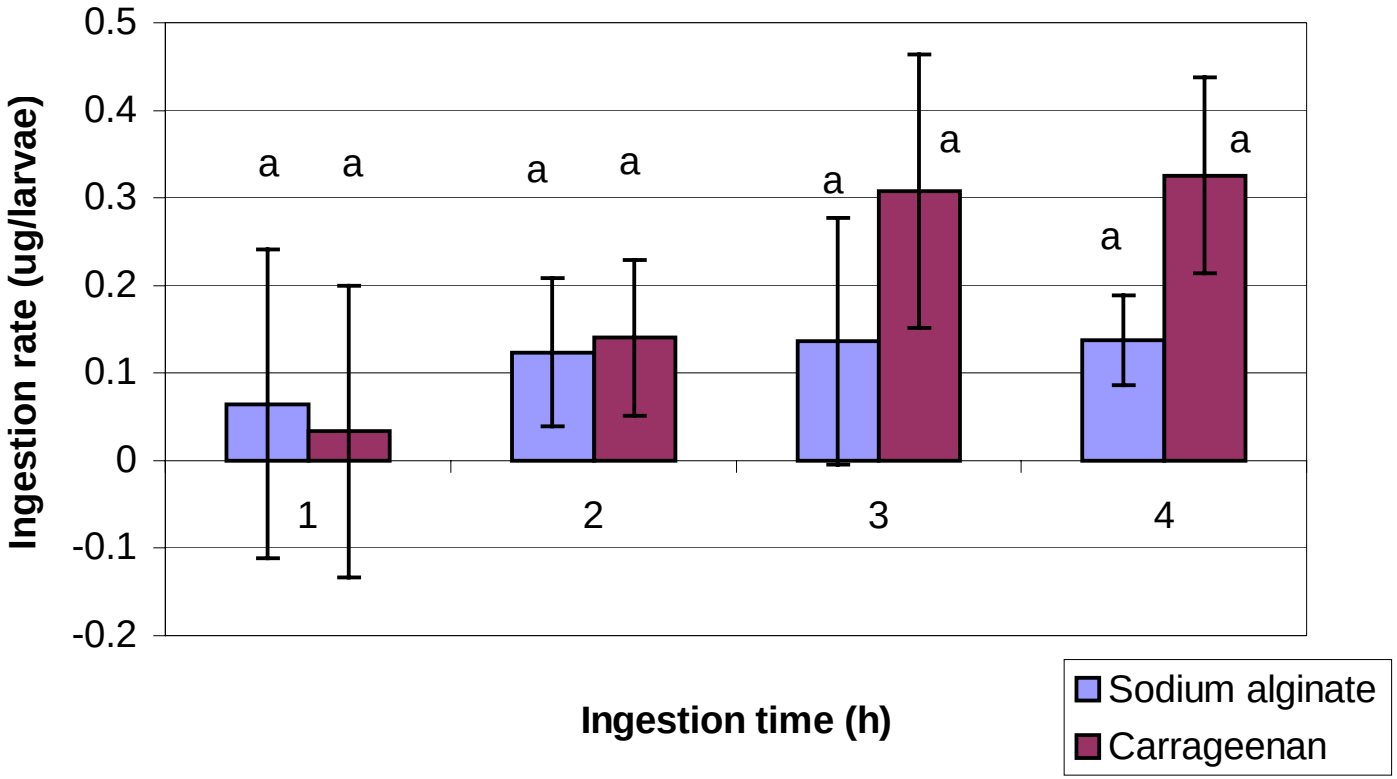



Fig. 5.2. The mean (± SE) ingestion rate (µg of MBD $mg^{-1}$ larval dry weight) of $^{14}C$-labelled MBD bound with sodium alginate and carrageenan by stage 2 phyllosoma. Means sharing the same letter were not significantly different between feeds or ingestion periods ($P>0.05$).

The rates of ingestion of MBDs by stage 3 phyllosoma over a 4 h feeding period are shown in Fig. 5.3. The MBD bound with sodium alginate after 3 and 4 h had the highest ingestion rate of 3.615 ± 0.501 and 3.681 ± 0.658 μg $mg^{-1}$ larvae, respectively. The ingestion rate of the sodium alginate bound MBD was significantly higher than background radiation. There was a significant ($P<0.05$) increase in the amount of ingested MBD after 1 h however, there was no further significant increase in ingestion rate between 2 h and 4 h (Fig. 5.3).

The rates of ingestion of MBD bound with sodium alginate by stage 4 phyllosoma over a 4 h feeding duration are shown in Fig. 5.4. The MBD had the highest ingestion of 2.742 ± 0.766 μg $mg^{-1}$ larvae after 4 h, which was significantly greater than that of 1 and 2 h ($P<0.05$). There were no significant difference ($P>0.05$) in ingestion rate between 1, 2, 3 and 4 h feeding durations.

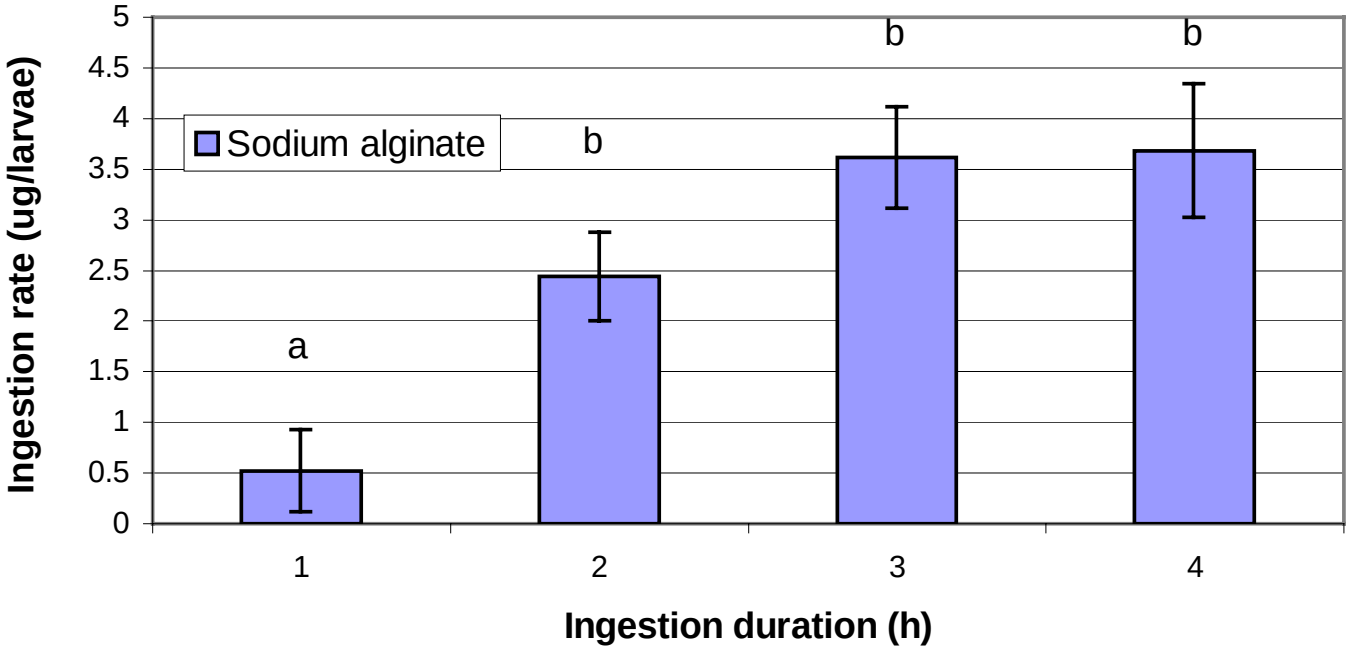



Fig. 5.3. The mean (± SE) ingestion rate (µg of MBD $mg^{-1}$ larval dry weight) of fed $^{14}C$-labelled sodium alginate bound MBD by stage 3 phyllosoma. Means sharing the same letter were not significantly different between ingestion periods ($P>0.05$).

Ingestion of $^{14}C$-labelled sodium alginate bound MBD after 1 h feeding was significantly different between phyllosoma stages 1 and 3 ($P<0.05$), between stages 2 and 3 ($P<0.005$) and also between stages 3 and 4 ($P<0.05$). The level of ingestion appeared to increase with progressive phyllosoma stages, between 1 and 4 (0.008 - 0.5 μg/larvae, respectively) ($P<0.005$). The ingestion rate after a 2 h feeding period was significantly greater than between phyllosoma stage 1 and 3 (0 - 2.5 μg/larvae, respectively) ($P<0.005$), additionally between phyllosoma stages 2 and 3 ($P<0.005$), 2 and 4 ($P<0.05$) and 3 and 4 ($P<0.005$). After a 3 h feeding duration the significant differences in ingestion rate were achieved between phyllosoma stages 1 and 3 (0 - 3.6 μg/larvae, respectively) ($P<0.005$), 1 and 4 ($P<0.006$), 2 and 3 ($P<0.005$), 2 and 4 ($P<0.01$) and 3 and 4 ($P<0.005$). While after 4 h feeding the significant increase were between phyllosoma stages 1 and 3 (0.19 - 3.7 μg/larvae, respectively) ($P<0.005$), 1 and 4 ($P<0.005$), 2 and 3 ($P<0.005$) and 2 and 4 (0.19 - 2.75 μg/larvae, respectively) ($P<0.001$).

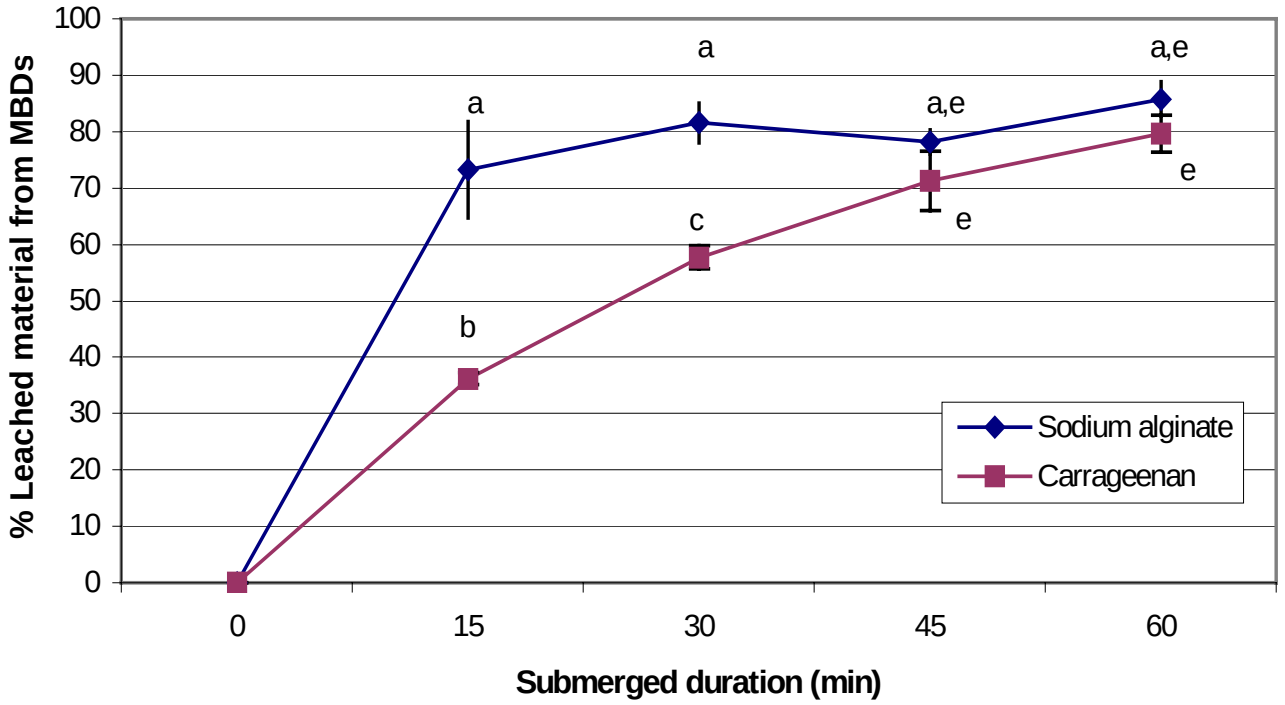



Fig. 5.5. The mean (± SE) estimated total leaching of $^{14}C$ from sodium alginate and carrageenan bound MBDs over the first 1 h of immersion. Means sharing the same letter were not significantly different between feeds or leaching periods ($P > 0.05$).

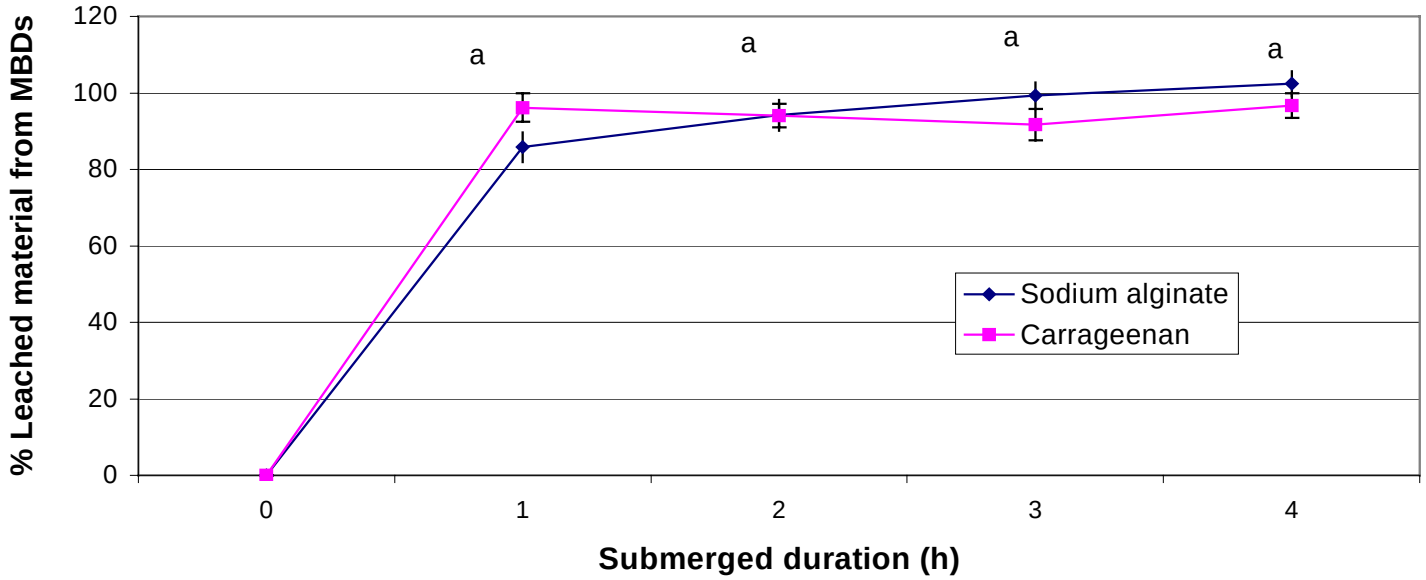



Fig. 5.6. The mean (± SE) estimated total leaching of $^{14}C$ from carrageenan and sodium alginate bound MBDs immersed for a 4 hour period when the MBDs were added initially.

### 5.3.2 Experiment 2 - The leaching rate of $C^{14}$-labelled material from the MBDs following different immersion times

The amount of leachate from each of the $^{14}C$-labeled diets following different immersion times during the feeding trial is shown in Fig 5.5. Leaching losses from sodium alginate MBD were rapid for the first 15 min. and did not increase significantly ($P< 0.05$) beyond this time up to the 60 min. duration.

Leaching loss during the first hour was significantly higher ($P< 0.05$) for sodium alginate bound MBDs than for carrageenan bound MBD (Fig. 5.6). The amount of leachate material from one inoculation of $^{14}C$-labelled diets over the 4 h is shown in Fig 5.6. The amount of $^{14}C$ leachate from the two MBD types was not significantly different after 4 h of immersion (Fig 5.6.). The amount of leachate from $^{14}C$-labelled MBD over the 4 h with hourly inoculations of labelled diets were measured and results are shown in Fig. 5.7. The leaching losses from MBD were rapid and, although not significantly different ($P > 0.05$) during the first hour, although leaching from the sodium alginate MBD apparently decreased between 1 h and 4 h.

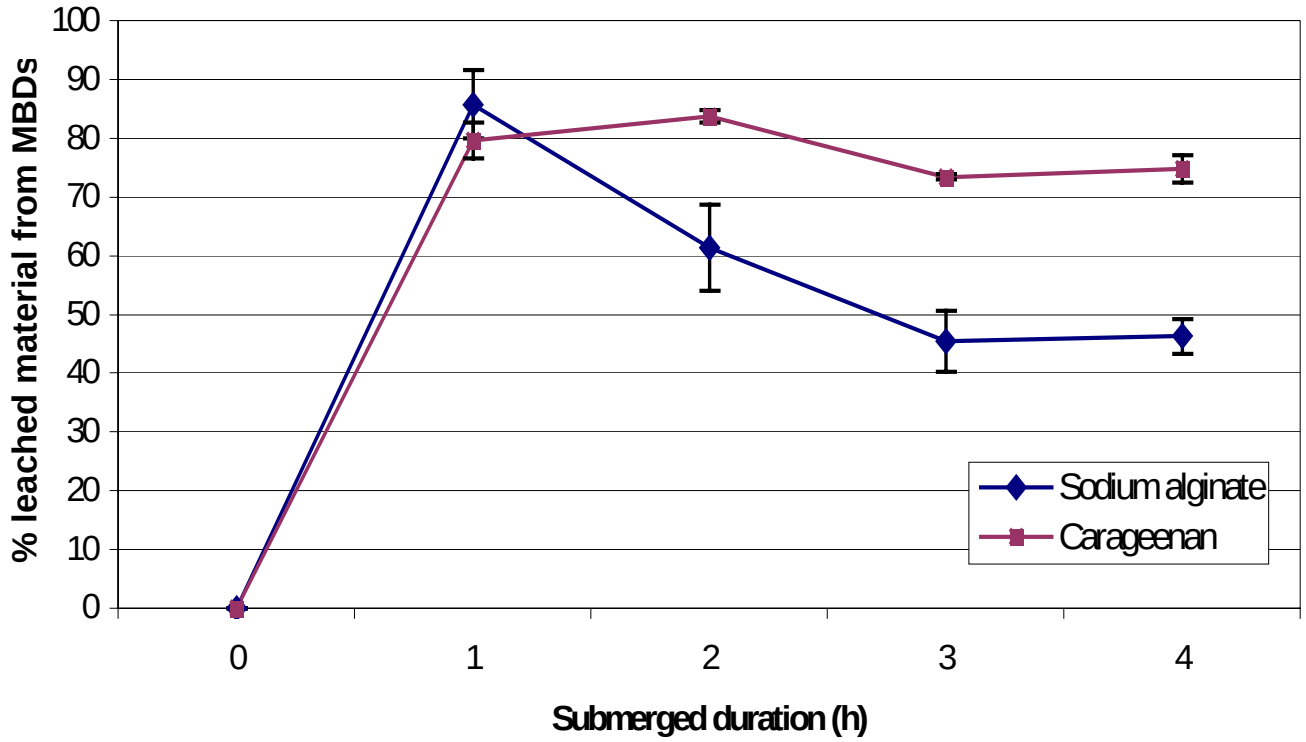



Fig. 5.7. The mean (± SE) estimated total leaching of $^{14}C$ from carrageenan and sodium alginate bound MBD immersed over a 4 hour period, the MBDs were added every hour.

The amount (%) of $^{14}C$ leachate material from diets added hourly appeared to remain constant from carrageenan-bound MBD, although the percentage lost from sodium alginate bound MBD decreased over time (Fig 5.7).

## 5.4 Discussion

Ingestion of MBD varied significantly between the phyllosoma stages tested. The MBD did not appear to be consumed by earlier phyllosoma stages, in particular stage 1. The amount of ingested $^{14}C$-labelled MBD was significantly higher for stages 3 and 4 compared to earlier stages, under these conditions. The relatively low ingestion rate measured during the first 4 h of feeding for stage 1 and 2 phyllosoma in particular, may be due to factors including:

(1) the short feeding duration, although low consumption of MC is characteristic of early staged carnivorous larvae (Jones, 1998);

(2) their feeding strategies require a series of stimuli (Mechano-, chemo- and/or visual reception) in order to detect and capture prey, as opposed to filter feeding;

(3) food consumption by carnivorous crustacean larvae has shown to be significantly lower than in herbivorous larvae, where freshwater prawn (*M. rosenbergii)* and lobster (*H. americanus*) larvae consumed 1.69 and 0.3-0.4 times their dry body weight per day, respectively (Jones, 1998).

In this study the level of ingested $^{14}C$-labelled MBD were significantly higher for stages 3 and 4 phyllosoma compared to earlier stages. In a study on 18-day old finfish

(barramundi, *Lates calcarifer*) larvae, fed $^{14}$C-labelled MBDs had ingestion rates of 20.63 ± 4.75 ug mg$^{-1}$ h$^{-1}$ using sodium alginate bound and 27.44 ± 4.71 ug mg$^{-1}$ h$^{-1}$ zein bound MBDs (Partridge and Southgate, 1998). Even though ingestion of the carrageenan MBD was significantly lower (7.94 ± 2.02 ug mg$^{-1}$ h$^{-1}$) it was more efficiently digested (Partridge and Southgate, 1998). Kolkovski *et al.* (1997) fed similar MBDs to larvae of the gilthead seabream, *D. labrax* and reported and ingestion rate of 9.5 ug mg$^{-1}$ h$^{-1}$ for 22 and 27 day old larvae. Kolkovski *et al.* (1997) also demonstrated that live food present in the rearing tank positively affected assimilation rates of MBD. It is generally accepted that the ingestion of live food is significantly higher than that of MBD (Dabrowski, 1984; Kolkovski *et al.,* 1993b; Kolkovski *et al.,* 1997a, b).

The amount of $^{14}$C-labelled material that leached from the MBDs in this study was shown to be higher during the first hour and, in particular, in the first 15 min. after immersion, for both binder types. The leaching rate of $^{14}$C material from carrageenan-bound MBD was significantly lower during the first hour when compared to sodium alginate-bound MBD. The high leaching rates could influence the accuracy of ingested values for material. The data indicated that up to 80-90% of valuable low molecular weight ingredients (e.g. amino acids and vitamins) would be lost from these MBDs if not consumed within the first 30 min of feeding. Although leaching of attractant molecules (such as amino acids) from micro-diets have been shown to increase the attractiveness of MBD and influence their ingestion rate (Jones *et al.,* 1993; Lopez-Alvarado *et al.,* 1994). This potential benefit must be balanced because of potential negatives, which include reduced nutritional value of the diet and the potential for deteriorating water quality (Muir and Sutton, 1994).

The quantity of ingested MBD was considerably higher for the more advanced phyllosoma stages, in particular stages 3 and 4. This suggests that future research towards development of artificial foods for phyllosoma should be focused on more advanced larval stages which would allow more accurate determination of when artificial food particles are consumed.

# Chapter 6

# Ingestion and assimilation of live $^{14}$C-labelled *Artemia* by stage 1 phyllosoma

## 6.1 Introduction

Brine shrimp (*Artemia* spp.*)* were first used to feed the larvae of aquatic organisms in the 1930s (Sorgeloos *et al.,* 1986). Since then, *Artemia* has become one of the most valuable live foods for larval fish and crustaceans because of a number of favourable factors: (1) the ease of storage of their dormant cysts; (2) a short time (24 h) to induce hatching; (3) variability in size (nauplii to adult stages) and (4) satisfactory nutritional value either naturally or by enrichment with specific diets (Leger *et al*., 1986; Sorgeloos *et al*., 1998). *Artemia* currently forms part of a standard diet for marine larvae which is used worldwide (Sorgeloos *et al.,* 1986; Southgate, 2003). However, some disadvantages associated with live food production include high cost, reliability of supply, sub-optimal nutritional composition and hence the requirement for costly enrichment diets, and the potential for them to become vectors for disease (Southgate, 2003).

The feeding apparatus of phyllosoma is designed to catch and ingest soft-bodied organisms such as ctenophores, medusae, chaetognaths, fish larvae, salps and other soft organisms (Phillips and Sastry, 1980; Kittaka, 1997; Cox and Johnston, 2003). However, the feeding protocol currently used for phyllosoma during culture is based on *Artemia* because of its ease of use and, in particular, their larger size compared to

other available live foods such as rotifers. Previous studies on the use of *Artemia* for feeding phyllosoma have indicated that phyllosoma have the ability to catch, ingest and extract nutrients from *Artemia* (Ritar *et al.,* 2002). The optimum density and size of *Artemia* fed to phyllosoma has also been investigated (Tong *et al.,* 1997; Tong *et al.,* 2000), and as phyllosoma develop they prefer larger sized *Artemia* and consume a greater number of them (Ritar *et al.,* 2002).

Measuring ingestion of food maybe undertaken by a number of methods as described in Chapter 4. One of the most promising and accurate techniques to measure both ingestion and nutrient assimilation is the use of $^{14}$C-labelled food (e.g. Kolkovski *et al*., 1993). This approach has been investigated previously using $^{14}$C- and $^{3}$H- labelled food particles. This technique was first described by Kolkovski *et al.* (1993b) and involves labelling live foods which are then fed to larvae of the target species (Boehlert and Yoklavich 1984; Nimura *et al*., 1994a, b; Kolkovski *et al.,* 1997b), either directly or as a component of an 'artificial' diet. Subsequent measurement of the radioactive content of the larvae provides an estimate of ingestion and nutrient assimilation (Kolkovski *et al.,* 1993b; Kolkovski *et al.,* 1997a, b).

The main aims of this Chapter were to determine the ingestion and assimilation of $^{14}$C-labelled *Artemia* by stage 1 phyllosoma over a 4 hour feeding period.

## 6.2 Materials and Methods

### 6.2.1 Larval production

Phyllosoma were obtained from artificially induced captive broodstock maintained at the AIMS mariculture facility. The phyllosoma used during the experiment were 1 day old and reared to this point as described in Chapter 2 (section 2.3.1). Prior to the experiment, phyllosoma were starved and acclimatized for 20 h in 1 L beakers containing gently aerated 1 μm filtered seawater (35 ppt) at 28 $^{o}$C.

### 6.2.2 Ingestion rate of $^{14}$C-labelled *Artemia*

The method used for $^{14}$C-labelling of *Artemia* is described in Chapter 2 (section 2.4.2). The phyllosoma were transferred to 1 L beakers containing 1 μm filtered gently aerated seawater and were allowed to acclimatise for 20 h prior to the start of the feeding trial. Each beaker contained 25 larvae and there were five replicates per treatments (n=5). To determine the ingestion rate of phyllosoma over time, feeding trials were conducted over 1, 2, 3 and 4 h intervals. Each treatment was supplied with a density 1 *Artemia* metanauplius mL$^{-1}$. The dry weight of 1 *Artemia* metanauplii was calculated as 8.48 ± 0.06 μg. At the end of each feeding period phyllosoma were harvested and measured for $^{14}$C content as described in Chapter 2 (section 2.4.3).

### 6.2.3 Assimilation of live $^{14}$C-labelled *Artemia*

The rate of assimilation of $^{14}$C from $^{14}$C-labelled *Artemia* for the different feeding periods was determined using a similar method to ingestion (Chapter 2, section 2.4.3). Preliminary investigations showed that the gut evacuation time of phyllosoma was approximately 2-3 h. After the phyllosoma had been fed live $^{14}$C-labeled *Artemia* for each duration, they were harvested into small cone shaped sieves and gently rinsed with 1 μm filtered UV treated seawater to remove excess *Artemia*. A 100% water exchange was then administered to the culture vessel. The phyllosoma were then placed back into the clean vessel and fed a "cold chase" or non-radioactive *Artemia* at the same density for 5 h. This process expelled all non-digested $^{14}$C-labelled material from the gut. The phyllosoma were processed in the same manner as described for ingestion (section 6.2.3), and their measured radioactive content was converted to an equivalent value (μg) of *Artemia* per phyllosoma (Chapter 2, section 2.4.3). The assimilation efficiency (AE) was then calculated using the following formula:

Assimilation efficiency (AE) (%) = (Assimilation / Ingestion) x 100.

### 6.2.4 Statistical analyses

Treatment means in all experiments were compared using a one-way analysis of variance (ANOVA). Data were tested for homogeneity of variance and normal distribution. A x4 transformation was applied to ingestion and assimilation data to correct for homogeneity of variance and normal distribution. Analyses were

performed using SPSS windows, version 9.0, and statements of statistical significance refer to the 0.05 level.

## 6.3 Results

The rates of ingestion and assimilation of live $^{14}$C-labeled *Artemia* by stage 1 phyllosoma are shown in Fig. 6.1. The feeding duration that showed the highest (mean ± SE) ingestion rate of 0.976 ± 0.059 individual A*rtemia* or 8.282 ± 0.501 μg larvae$^{-1}$ was 4 h, under these experimental conditions. This rate of ingestion was significantly greater than that after 2 h feeding ($P<0.05$). The highest proportion of *Artemia* ingested was 0.792 ± 0.155 *Artemia* or 6.72 ± 1.31 μg larvae$^{-1}$ (mean ± SE) during the first hour, although this did not differ significantly from that at other feeding times.

Rates of assimilation of live $^{14}$C-labeled *Artemia* during the experiment showed that the highest assimilation of individual *Artemia* was 0.626 ± 0.217 or 5.31 ± 1.84 μg larvae$^{-1}$ and 0.631 ± 0.343 or 5.35 ± 2.9 μg larvae$^{-1}$ (mean ± SE) after 2 and 3 h feeding periods, respectively; however these did not differ significantly ($P>0.05$). The 2 h and 3 h feeding periods also resulted in the highest assimilation efficiencies (AE) of 87.06% and 80.20%, respectively. The lowest assimilation rate of live *Artemia* (0.1579 ± 1.62 μg larvae$^{-1}$) was after 1 h, and was significantly ($P<0.05$) lower than that for 1 h ingestion. The lowest AE was over 4 h, despite having the highest ingestion rate. The highest proportion of *Artemia* assimilated was shown after 2 h.

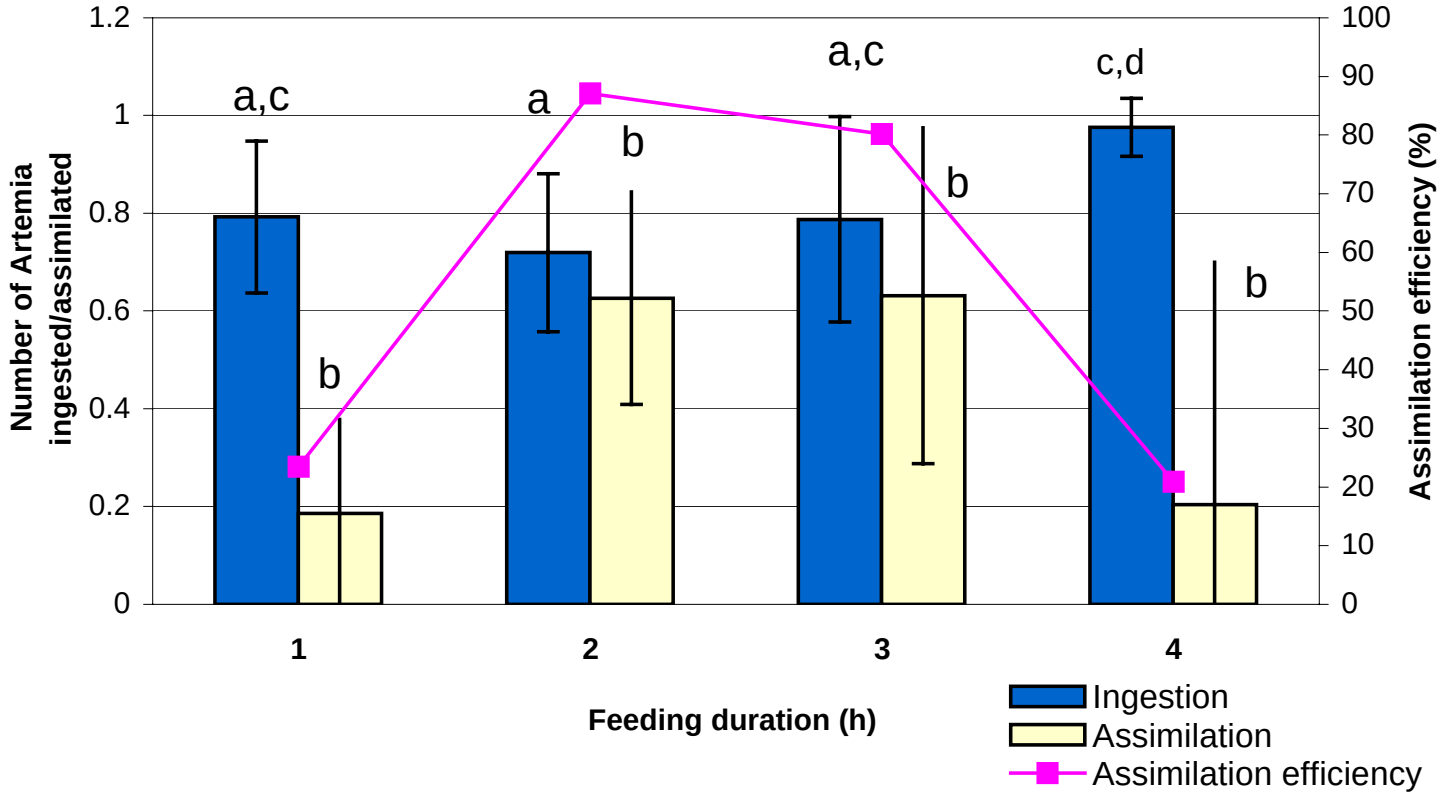



Fig. 6.1. The mean (±SE) number of *Artemia* (1.5 day old) ingested and assimilated by stage 1 phyllosoma over a 4 h period. Mean values for ingestion and assimilation sharing the same superscripts were not significant difference (P>0.05).

## 6.4 Discussion

*Artemia* are used throughout the aquaculture industry and are the most common food given to phyllosoma (Chapter 1, Table 1.2). Ingestion of $^{14}$C-labeled *Artemia* by stage 1 phyllosoma was highest over a 4 h feeding period, when approximately 1 (0.976 ± 0.059) 1.5 day-old *Artemia* metanauplius was consumed, when supplied at a density of 1 $mL^{-1}$. It was possible that after a feeding period of 4 h, some assimilation may have occurred. However, the amount of *Artemia* assimilated after 4 h was shown to be the lowest during the experiment. The highest assimilation rate of *Artemia* (0.626 ± 0.192) was achieved after 2 h and 3 h of feeding, although there were no significant differences between the assimilation times tested. The highest assimilation efficiencies (87.06% and 80.19%) of $^{14}$C-labelled *Artemia* were recorded after 2 h and 3 h feeding.

Phyllosoma have a relatively large gut, which is highly developed from hatch and their ability to consume large fleshy prey increases with age (Cox and Johnston, 2003). The results of this experiment support previous findings that phyllosoma of *P. ornatus* have the ability to ingest and extract nutrients from live *Artemia* under culture conditions (Kittaka, 1997; Tong *et al*., 2000; Ritar *et al*., 2002; Cox and Johnston, 2003), even though their feeding mechanisms appears to be designed for consumption of soft-bodied organism (Cox and Johnston, 2003). The optimum density of *Artemia* for different stages of phyllosoma has been investigated for a variety of species including *J. edwardsii* and *J. verreauxi* (Kittaka, 1997; Tong *et al*., 1997; Ritar *et al*., 2002), and the optimal number, and particularly size, of *Artemia* has been shown to increase with progressive phyllosoma stages (Tong *et al*., 1997; Ritar *et al.,* 2002). Consumption of *Artemia* by stage 1 phyllosoma reported in this study was similar to

that reported for *J. edwardsii*, using phyllosoma survival and numbers of *Artemia* eaten as indicators (Ritar *et al*., 2002). Additionally, Japanese researchers have found that early staged phyllosoma fed at a density of 1 *Artemia* $mL^{-1}$, consumed approximately 0.4 and 0.3 *Artemia* $h^{-1}$ of 0.47 and 1.12 mm length, respectively (Kittaka, 1997). The optimal density appears to be 1-3 *Artemia* $mL^{-1}$ for phyllosoma stages 1-3 (Kittaka, 1997; Tong *et al*., 1997; Ritar *et al.,* 2002). The number of *Artemia* consumed increased to 0.8-1.0 when fed a feeding density of 4 $mL^{-1}$ (Kittaka, 1988; Kittaka *et al*., 1988; Kittaka and Kimura, 1989; Kittaka, 1997). Ritar *et al*. (2002, 2003) concluded that for stage 1 phyllosoma of *J. edwardsii*, the optimum feeding density for *Artemia*, achieving the highest survival (76.4 ± 5.2 %) to stage 2 was 3 *Artemia* metanauplii $mL^{-1}$ (l.5 mm) with lowest survival occurring at a density of 9 *Artemia* $mL^{-1}$ (0.8 mm). Liddy *et al*. (2003) also found that an *Artemia* density of 3 $mL^{-1}$ fed to stage 1 *P. cygnus* phyllosoma, achieved 100% survival to stage 2 (Chapter 1, Table 1.2). The number of *Artemia* consumed by phyllosoma varies depending upon the size of *Artemia* and density and the phyllosma starvation period. For example, Tong *et al*. (2000) investigated the number of *Artemia* eaten by *J. edwardsii* phyllosoma at different water temperatures. At 24$^{o}$C, the highest number of *Artemia* (2.5-3mm) consumed per day was 12.6 ± 3.2.

Because of the ease of *Artemia* culture, their ready acceptance by a range of phyllosoma stages and difficulties associated with feeding alternative diets, *Artemia* will play an important role as a live food for phyllosoma during early stages of culture. Stage 1 phyllosoma are able to consume and digest live *Artemia* under a variety of culture conditions and hence despite the costs associated with *Artemia* culture, their sub-optimal nutritional status and their potential as vectors for disease

(Southgate 2003), they are likely to form the basis for early stage phyllosoma culture until superior alternatives are developed. Alternating feed types may offer a wider range of nutrients and enhance survival and growth of phyllosoma. *Artemia*, mussel gonad and other live zooplankton are suitable foods for culture of phyllosoma (Kittaka, 1994a, 1999). However, some problems have been reported with the use of large fleshy prey (e.g. mussel gonad) for phyllosoma, such as decreased water quality and the tangling of food particle on the spiky pereipods of the larvae (Kittaka, 1997). Potential alternatives to *Artemia*, and of a suitable size to feed phyllosoma, include soft-bodied organisms such as small jellyfish-like organisms (ctenophores and appendicurians), polycheate worms and nematodes. Further research is required to determine the nutritional value of such organisms for phyllosoma and to develop mass culture techniques for them.

# Chapter 7

# Survival of early stage phyllosoma fed *Artemia* substituted with artificial diets

## 7.1 Introduction

Culture of phyllosomes has been carried out over several decades in Japan (Nonaka *et al*., 1958; Nishimura, 1983; Nishimura and Kawai, 1984; Nishimura and Kamiya, 1986; Kamiiya *et al*., 1986; Kittaka, 1994a, b, 1997a, b, 2000). However, extensive culture attempts have so far been relatively unsuccessful. Small numbers of pueruli, however, have been produced under a variety of culture conditions (Silberbauer, 1971; Bardach *et al.,* 1972; Dexter, 1972; Van Olst *et al.,* 1980; Kittaka, 1988, 1994b; Kittaka *et al.,* 1988; Kittaka and Ikegami, 1988; Kittaka and Kimura, 1989; Yamakawa *et al.,* 1989; Booth and Kittaka, 1994; Illingworth *et al.,* 1997; Tong *et al.,* 1997). One of the major impediments to closing the life cycle of spiny lobsters is due to our limited knowledge of biological and technical culture requirements and lack of a nutritionally adequate diet. However, gut content analysis (Phillips and Sastry, 1980; Mikami *et al.,* 1994), fatty acid composition (Smith *et al*., 2003, Jeffs *et al*., 2004; Phleger *et al.,* 2001) and feeding biology (Cox and Johnston, 2003) of wild phyllosoma indicates that their natural prey contains a high proportion of soft-bodied organisms, such as ctenophores, medusae, chaetognaths, fish larvae, salps and other soft organisms (Kittaka, 1997; Cox and Johnston, 2003).

*Artemia*, other live zooplankton and mussel gonad are suitable foods for culture of phyllosoma (Kittaka, 1994a, 1997, 1999). *Artemia* at present are adequate for early stage culture of phyllosoma as they have the ability to catch, eat and extract nutrients from *Artemia* (Kittaka, 1997; Tong *et al.,* 1997; Ritar *et al.,* 2002; Chapter 6). Unfortunately, the production of live feeds still remains expensive and often unpredictable and they are sub-optimal in nutritional composition (Jones *et al*., 1993; Southgate and Kolkovski, 2000). Adequate alternative feeds are required.

Studies investigating the use of alternatives to live foods for crustacean larvae are relatively limited, with exception of a considerable weight of literature on penaeid prawns (Jones, 1993). Artificial diets have been assessed for the larvae of carnivorous decapod crustaceans; *Macrobrachium rosenbergii, Cragon crangon, Palaemon merguiensis, Pagurus bernhardus* (Jones *et al.,* 1975; Mohanta and Rao, 2000; Kovalenko *et al*., 2002), *Eurypanopeus depressus* (Levine and Sulkin, 1984; Levine *et al*., 1983), *Homarus gammarus* (Kurmaly *et al.,* 1990), *Geryon quipuedens, Callinectes sapidus* (Levine *et al*., 1983) *Portunus trituberculatus* (Kanazawa *et al.,* 1983; Jones, 1998) and *Scylla serrata* (Genodepa *et al.,* 2004 a, b). Overall, a low acceptability of the alternative diets is common in many larval crustacean species (Jones *et al.,* 1993). In contrast to penaeid prawns, studies on the performance of artificial diets fed to phyllosoma, have proven relatively unsuccessful. For example, MED containing plant protein, lipid and carbohydrate fed to mid-stage *J. edwardsii* phyllosoma, although readily accepted, supported larval survival for only 110 days and 3 moults (Otawa and Kittaka, unpubl. cited in Kittaka and Booth, 2000).

MBD and MED have been designed and tested for a variety of crustacean species (Jones *et al.,* 1987; Levine and Sulkin, 1983; Jones, 1998) however, MBDs have proved to be the most successful (Teshima *et al.,* 1983; Bautista *et al.,* 1989; Koshio *et al.,* 1989; Kovalenko *et al*., 2002). Calcium alginate particles (spray beadlets) are commonly used today in biomedical industry. They were first used to encapsulate organic solvents and have been adapted further for feeding crustacean larvae (Levine *et al*., 1983; Levine and Sulkin, 1984; Villamar and Brusca, 1987; Villamar and Langdon, 1998; Chapter 3). Encouraging results were recently reported for the replacement of *Artemia* with MBDs fed to larvae of the freshwater prawn, *M. rosenbergii* (Kovalenko *et al*., 2002), and mud crab, *S. serrata* (Genodepa *et al.,* 2004a, b).

The main aims of this Chapter were to determine survival of stage 1 and 3 phyllosoma *P. ornatus*, when fed either *Artemia* alone, an MBD alone or a combination of the two.

## 7.2 Materials and methods

### 7.2.1 Larval production

Phyllosomes were obtained from artificially induced captive broodstock maintained at the AIMS mariculture facility. The phyllosoma were hatched and reared as described in Chapter 2 (section 2.3.1) until they reach the desired developmental stage.

### 7.2.2 Diet preparation

The dietary binders were prepared as described in Chapter 2 (section 2.4.1). The binders used were sodium alginate (GMB), gelatin (Sigma: Type A: Porcine Skin: Bloom: 175) and carrageenan (Sigma: Type 1: kappa). The experimental diet composition is shown in Table 7.1 and dietary ingredient preparation is described in Chapter 2 (section 2.4.1). The astaxanthin (Carophyll pink, Roche) was dissolved in 35$^{o}$C distilled water and added to the diet.

The MBD used in this experiment were made from either of 3 different binders;

- Sodium alginate
- Gelatin
- Carrageenan

Furthermore, diet particles made using sodium alginate were prepared in two forms

- Alginate MBD (dried)
- Alginate sprayed beadlets (moist)

MBDs were exposed to 45$^{o}$C for 24 h. Dried MBDs were ground with mortar and pestle, sieved to 355-500 μm and 500-850 μm particle size, and stored under $N_2$ until required. The moist sprayed beadlet diets were cured in a bath of 10% w/v calcium chloride solution (Industrial grade $CaCl_2$ >>74%); the method of manufacture is described in detail in Chapter 3 (section 3.2.7.1).

Table 7.1. The composition of MBD

| **Diet composition: (500 g batch)** | **Weight (g)** |
|---|---|
| Fresh homogenized pipi mixture | 300 |
| Frippak-larval prawn feed- #2CD (30-90μm) | 100 |
| Cod liver oil | 40 |
| β-glucan | 20 |
| Crustacean mineral mixture[1] | 8.5 |
| Crustacean vitamin mixture[1] | 8.5 |
| Probiotics[2] | 2.2 |
| Astazanthin | 0.8 |
| Binder | 20 |
| **Total** | **500** |

[1] Manufacturer- Rabar Pty Ltd

[2] Manufacturer- Pro-Marine Probiotics

### 7.2.3 Experimental design

This study was divided into two feeding experiments each with two feeding trials. The experiments 1 and 2 were conducted during January 2003.

(1) Experiment 1 determined the survival of newly hatched stage 1 phyllosoma fed MBD. Two separate feeding trials conducted were designated experiments 1a and 1b; and

(2) Experiments 2 determined the survival of stage 3 phyllosoma fed MBD. Two separate feeding trials conducted were designated experiments 2a and 2b.

Each experimental larval rearing tank was stocked with 500 stage 1 or 300 stage 3 phyllosoma. Two replicate tanks per feeding treatment for both of experiments 1 and 2.

Table 7.2. The quantity of MBD (moist sprayed beadlets or dried MBD) added daily to tanks to maintain a density of 1 particle/*Artemia* $mL^{-1}$.

| Diet treatment | Weight (g) | |
|---|---|---|
| | 355-500 μm | 500-850 μm |
| Moist sprayed beadlets | | |
| *100%* | 0.8 | 1.5 |
| 50% MBD:50%*Artemia* | 0.4 | 0.75 |
| Dried MBD | | |
| 100% | 0.25 | 0.5 |
| 50% MBD:50%*Artemia* | 0.125 | 0.25 |

The treatments for each experiment were:

1. 100% *Artemia* ration;
2. 100% MBD ration;
3. 50% MBD : 50% *Artemia*;
4. 50% *Artemia* (control); and,
5. Unfed phyllosoma (control).

All diets were fed on a density basis (number of *Artemia*/MBD particles per mL). The 100% *Artemia* ration was at a density of 1 *Artemia* metanauplius per mL.

Fig. 7.1. The 50 L up-welling larval rearing tanks, under controlled climatic conditions.

### 7.2.4 Culture conditions

The appropriate staged phyllosomes were transferred into 50 L upwelling tanks for feeding trials (Fig. 7.1). Any mortalities during the transfer period were replaced the following day. Lighting was supplied with a single fluorescent light bulb the photoperiod was 10 h:14 h, (L:D). The tanks contained 0.5 μm filtered seawater at 35 ppt salinity and a temperature of 28 ± 1$^{o}$C. The tank bottoms were cleaned on a daily basis by siphon to remove uneaten diets and dead phyllosoma. Daily 50% water exchanges with 0.5 μm seawater were carried out when approximately 500 mL of microalgae (T-*Isochrysis* spp.) were added to each tank. *Artemia* and MBD rations were also added following water exchanges (Table 7.2). The number of dead

phyllosoma was recorded daily. The duration of the feeding trial was 18 days for stage 1 phyllosoma and 15 days for stage 3 phyllosoma.

### 7.2.5 Statistical analysis

The survival of both stage 1 and 3 phyllosoma were analysed using Kaplan-Meier test, which performs chi-square with Log_rank and Wilcoxon analysis. Experiment 1a and 1b, and 2a and 2b, were treated separately as there were significant differences ($P<0.0001$) in mortality between replicate feeding trials for each stage. The acceptance level was $P = 0.01$. Statistical analyses were carried using JMP statistical package (SAS Institute).

## 7.3 Results

### 7.3.1 Experiment 1 - Survival of stage 1 phyllosoma fed dry and moist MBDs.

The results of these two feeding trials (experiment 1a and 1b) are shown in Fig. 7.2 and 7.3. There were significant differences ($P<0.05$) between replicate feeding trials (experiment 1a and 1b) for stage 1 phyllosoma (Appendix 1). There was a significant effect of food type and combination on survival of phyllosoma (Fig. 7.2 and Fig. 7.3). The highest survival of newly hatched phyllosoma was achieved by those fed 100% *Artemia* in experiment 1a, while the survival of those fed 50% *Artemia* was superior in experiment 1b. Survival of larvae fed 100% *Artemia* treatment was significantly higher than that of starved larvae and those fed 50% *Artemia* ($P<0.001$), 50:50% *Artemia*: MBD and 100% MBD ($P<0.0001$), in both feeding experiments. There was

a significant difference between survival in each of the 100% MBD, 50% *Artemia* and 50% MBD and *Artemia* and the starved treatment ($P<0.0001$), in both feeding experiments (Fig. 7.2. and 7.3.). The presence of *Artemia* (50:50 *Artemia*:MBD) significantly increased survival compared to 100% MBD treatments ($P<0.0001$).

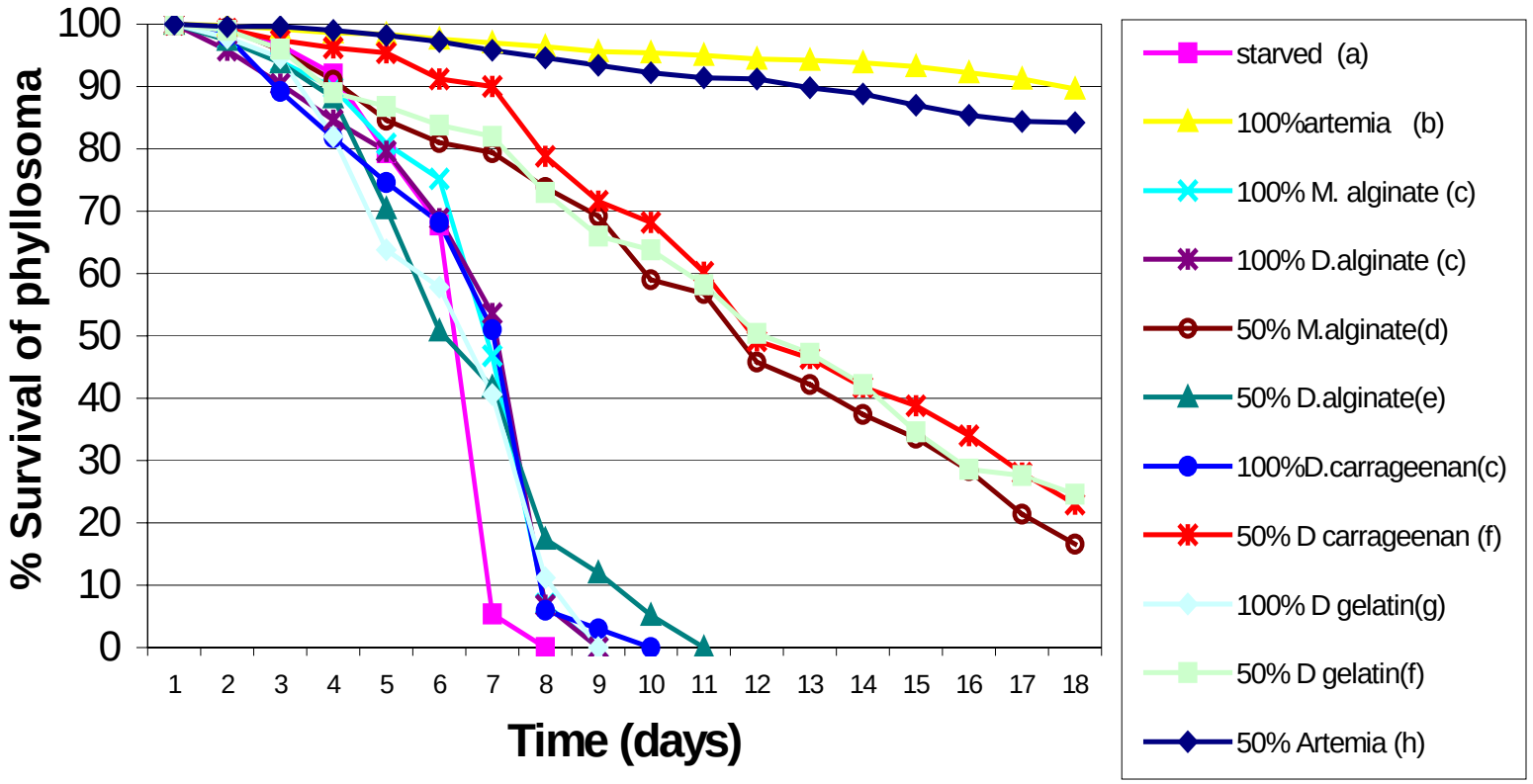



Fig. 7.2. Percent survival of stage 1 phyllosoma fed *Artemia* substituted with 0%, 50% and 100% MBD in experiment 1 a. D = Dried MBD, M = Moist sprayed beadlets. Treatments in legend with different superscripts (a,b,c,d,e,f,g,h etc) are significantly different (P=0.01).

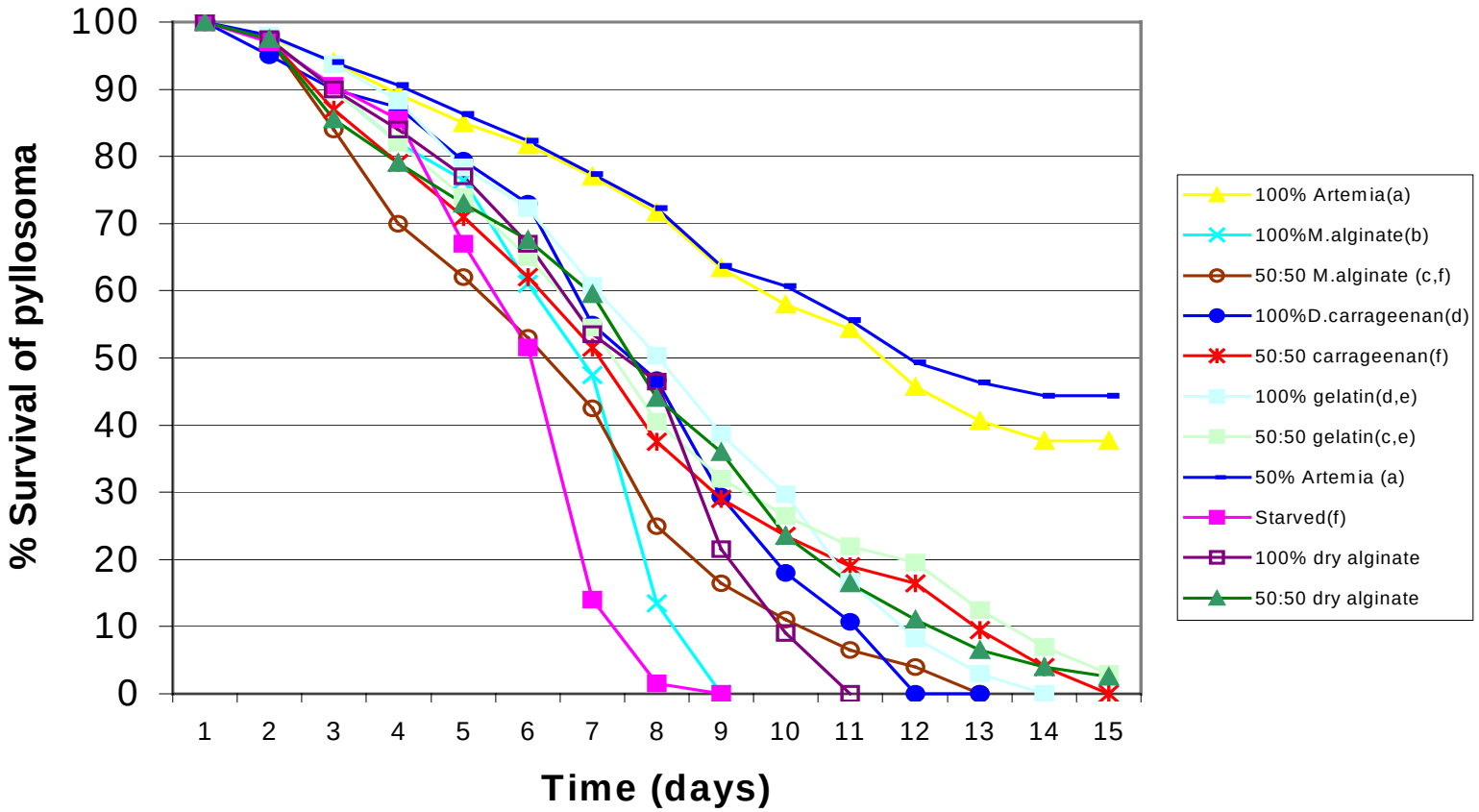



Fig. 7.4. Percent survival of stage 3 phyllosoma fed *Artemia* substituted with 0%, 50% and 100% MBD in experiment 2a. D = Dried MBD, M = Moist sprayed beadlets. Treatments in legend with different superscripts (a,b,c,d,e,f,g,h etc) are significantly different (P=0.01).

Different binders resulted in significant differences in survival of the larvae fed 100% gelatin and 100% carrageenan ($P<0.05$) in experiment 1b, 100% gelatin and 100% moist alginate treatments in both stage 1 feeding trials. Significant differences were shown between survival of larvae fed 50% diet combinations was achieved between dry alginate and carrageenan (Fig. 7.3).

### 7.3.2 Experiment 2 - Survival of stage 3 phyllosoma fed dry and moist MBDs.

The results of these two feeding trials are shown in Fig. 7.4 and Fig. 7.5. As with stage 1 phyllosoma there were significant differences between feeding experiments 2a and b for stage 3 phyllosoma fed artificial diets ($P<0.05$) and therefore trials were analysed individually. There were significant differences in survival of larvae according to food type and combinations of artificial MBD fed to phyllosoma (Fig. 7.4 and 7.5). The highest survival of stage 3 phyllosoma was achieved with the 100% *Artemia* and 50% *Artemia* treatments, which were not significantly different from each other ($P>0.02$) (Fig. 7.4 and 7.5). Survival of larvae fed 100% *Artemia* and those starved were significantly different ($P<0.0001$) to each other and all other diets (including 100% and 50% ratios) in both feeding experiments, with the exception of the 100% dry alginate and starved. There was also a significant difference between all 100% diet treatments ($P<0.0001$), in both feeding trials (Fig. 7.4 and 7.5), with the exception of carrageenan and gelatin treatments in experiment 2a.

The effect of different binder types was significantly different between 100% gelatin and 100% carrageenan ($P<0.05$) in experiment 2a, 100% gelatin and 100% moist

alginate treatments in both in stage 3 feeding experiment. Significant differences between 50% diet combinations were achieved between dry alginate and moist alginate ($P<0.0001$), dry alginate and carrageenan ($P<0.05$) (Fig 7. 4 and 7.5).

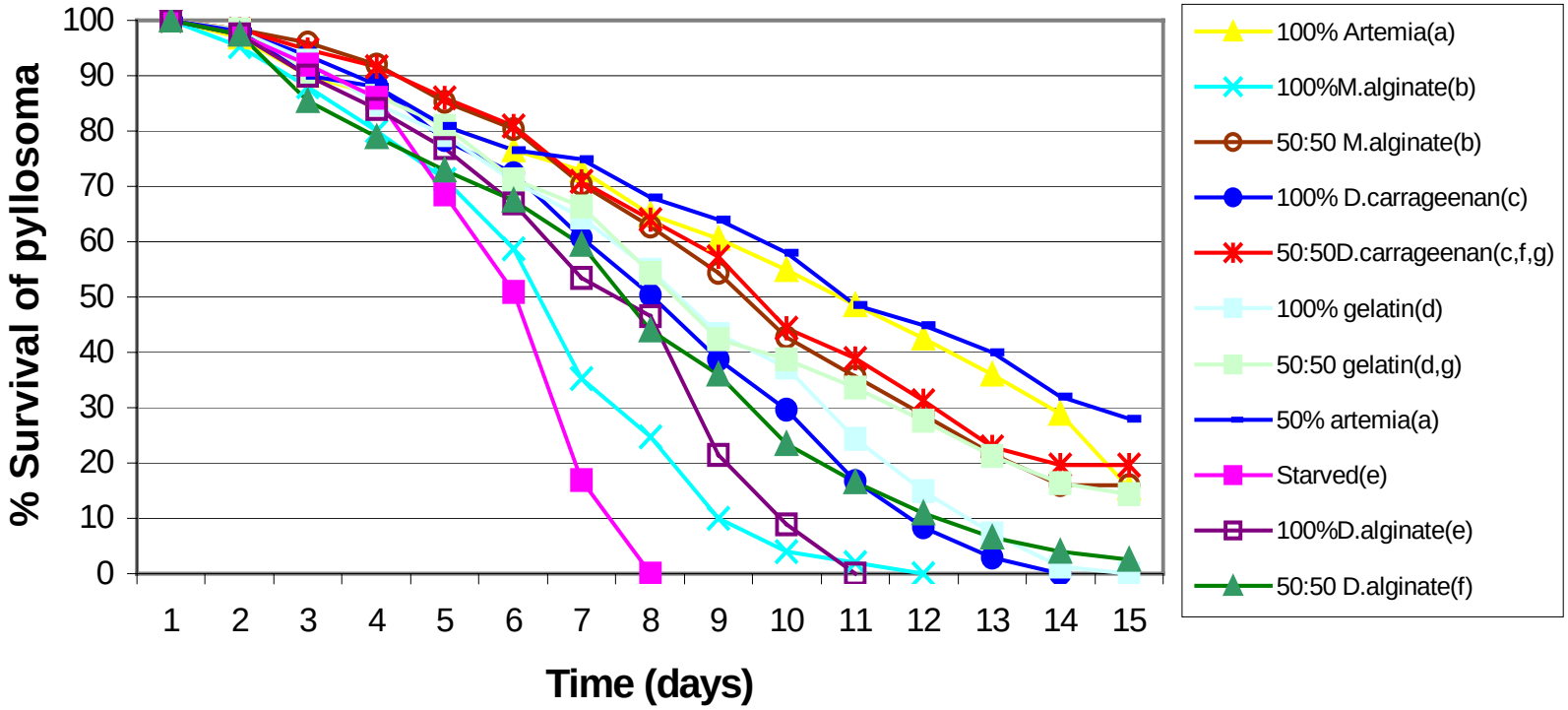



Fig. 7.5. Percent survival of stage 3 phyllosoma fed *Artemia* substituted with 0%, 50% and 100% MBD in experiment 2 b. D = Dried MBD, M = Moist sprayed beadlets. Treatments in legend with different superscripts (a,b,c,d,e,f,g,h etc) are significantly different (P=0.01).

## 7.4 Discussion

Of the feeds tested 100% live *Artemia* was shown to be the most successful for stage 1 and 3 phyllosoma, supporting the highest survival under these experimental conditions. Larvae receiving moist or dried MBDs, as either 100% and 50% of the diet survived significantly longer than unfed larvae in both stages, indicating some nutritional benefit was obtained during the feeding trial. Survival was significantly higher for phyllosoma fed diets of dry carrageenan and gelatin bound MBDs compared to those fed moist alginate sprayed beadlets. Nevertheless, the MBD used in this study supported relatively poor survival of phyllosoma compared to live *Artemia*.

Other studies, which have shown successful use of formulated diets for the culture of larvae of caridean shrimp and crab larvae species, however, still relatively limited (Jones, 1998; Genodepa *et al.*, 2003a, b). Levine *et al.* (1983) and Levine and Sulkin (1984) fed calcium alginate MBD to crab (*Eurypanopeus depressus, Geryon quipuedens, Callinectes sapidus)* larvae and reported that the MBD fed alone supported better larval survival than in unfed treatments, while larvae fed live *Artemia* reached megalopa with relatively high survival. In the majority of treatments, which have combined live foods and MBDs, larvae reached the megalopa stage although development was delayed (Levine *et al*., 1983; Levine and Sulkin, 1983). When *E. depressus* larvae were fed alginate bound *Artemia* in combination with live rotifers 50-53%, survival to megalopa stage similar to that achieved by larvae fed *Artemia* alone (Levine and Sulkin, 1984), while larvae fed live rotifers and 'empty' MC

recorded 20% survival. Similar results with combined feeding regimes were observed for larvae of the crab, *Portunus trituberculatus* (Kanazawa *et al.,* 1983). Survival of crab *Crangon nigricauda* larvae fed calcium alginate MBDs also survived longer than unfed larvae (Villamar and Brusca, 1987). The survival of late staged (stage 5) freshwater prawn *Macrobrachium rosenbegii* larvae fed exclusively on a high moisture content formulated MBD achieved survival nearly equivalent to that of larvae fed live *Artemia* nauplii (Kovalenko *et al*., 2002). Kumlu and Jones (1995) also achieved partial replacement of live foods with a microencapsulated diet, although survival decreased relative to live foods alone.

The use of artificial diets may be influenced by the mode of feeding as penaeid larvae primarily filter feed, until the first protozoeal stage while, in other decapod larvae, the mode of feeding involves a series of detection (visual, chemo- and mechano-reception) and capture techniques to feed. Live *Artemia* metanauplii have been shown to be a superior feed for maintaining a high level of survival of *P. ornatus* phyllosoma, at least during the early stages (Ritar *et al.,* 2002). Low consumption of artificial diets is characteristic of many early staged larval animals, including fish and other carnivorous crustacean larvae (Jones, 1998; Southgate and Kolkovski, 2000; Southgate, 2003). Although consumption of artificial diets was observed in this study MBD did not support high survival.

Prey detection and acceptance by phyllosoma involves several processes including visual, chemical- and mechano-reception (Cox and Johnston, 2003). One or more of these processes maybe required for successful prey capture, which would influence the consumption of inert particles, due to lack of movement. Future research should

focus on weaning strategies and partially introducing nutritionally adequate MBD sources to mid- and later-staged phyllosoma where live feeds are inadequate in size and nutritional composition.

# Chapter 8

# Conclusion

This study was carried out to investigate the possibility of developing of a suitable 'artificial' diet for the culture of early stages of the tropical rock lobster, *P. ornatus*, phyllosoma. As the predicted natural diet of phyllosoma consists primarily of soft-bodied organisms, preparation of a soft, moist micro-bound diet (MBD) was the initial aim. Moist MBD has shown potential in larval and juvenile feeding applications with the freshwater prawn, *M. rosenbergii* (Kovalenko *et al.,* 2000) and crabs (Levine and Sulkin, 1984), the relative ease of ingestion and digestion of soft MBD when compared to the commonly used dry crumble (Partridge and Southgate, 1998), is likely to prove advantageous in presenting artificial diets to crustacean larvae.

Production of a moist MBD using the atomisation method was successfully scaled-up during this study from hand-held glass atomiser to a spraying system capable of producing 1-1.3 kg of moist MBD within 5 min. The range of sizes produced were between 100 μm and >850 μm, under conditions trialed during the study; however, the size distribution of resulting MBD was broad. The curing bath design developed during this study decreased particle collisions and clumping and facilitated automatic deposited of MBD sprayed beadlets onto stackable sieves. Furthermore, significant cost saving was achieved by using industrial grade $CaCl_2$ as the curing agent, with no visible differences in beadlet formation.

The negative buoyancy of MBD within the water column has proven to be a problem in reducing the availability of food particles in larval rearing applications (Southgate, 2003). Encapsulation of nitrogen bubbles into moist MBDs was shown to increase buoyancy, thus potentially improving the acceptability of MBDs by phyllosoma. Additionally, encapsulation of bubbles may increase the digestiblity of MBDs through increasing the surface area of the food particle for enzyme action and therefore absorption of encapsulated nutrients. However this aspect requires further investigation.

This study developed methods for preparing multi-walled microcapsule (MC), which were successful in encapsulating dietary ingredients. However, the particle size range of multi-walled MC was generally large (0.5-4 mm). Further modifications of this technique (to the cutting device in particular) could produce smaller sized MC. Multi-walled MC allow greater retention of valuable dietary material such as low molecular weight water soluble nutrients, and effective delivery of nutrient to the target species. However, the MC must also be ingested and digestible. Large-scale preparation of multi-walled MC at present would be difficult due to expense and achieving the particle size and distribution required, although further investigation is needed.

One of the initial steps in larval feeding experiments is to determine the larval age at which the inert diet particles are accepted and consumed. Using radioisotope tracers the amount of MBD ingested by *P. ornatus* phyllosoma was shown to significantly increase with progressive stages and ingestion period (up to 4 h). Consumption of MBD by stages 1 and 2 phyllosoma was very low; however, consumption by stages 3 and 4 phyllosoma was significantly higher and this could potentially be the age at

which MC are accepted by phyllosoma. Low ingestion rates of inert food particles are characteristic of the larvae of many marine species, with the exception of penaeid prawns (Jones, 1998). This could be due primarily to their mode of feeding, where as filter feeders, prawn larvae consume higher quantities of particles (Jones, 1998). In contrast, feeding in the larvae of other crustaceans including phyllosoma involves detection (chemical, mechanical and/or visual), seeking and capture strategies to obtain prey.

Approximately one *Artemia* (1.5 day old) was consumed by stage 1 phyllosoma, over a 4 h feeding period when supplied at a density of 1 $mL^{-1}$. The highest assimilation efficiency of $^{14}C$-labelled *Artemia* by stage 1 phyllosoma was during the first 2 and 3 h of feeding. Survival of early stage phyllosoma fed *Artemia* (1 metanauplii $ml^{-1}$) was shown to be greater than that of phyllosoma fed combinations of *Artemia* and MC or fed MC alone. However, both stage 1 and stage 3 phyllosoma fed entirely on MC survived longer than unfed phyllosoma. Previous experiments with mid-staged phyllosoma fed artificial diets survived for 110 days and 3 moults when fed a microencapsulated diet composed of plant protein, lipid and carbohydrate ingredients (Otawa and Kittaka, unpubl.; cited in Kittaka and Booth, 2000). MC has been trialed successfully for a variety of larval crustaceans including penaeid prawns, crabs and freshwater prawns (Jones *et al*., 1975; Levine *et al*., 1983; Levine and Sulkin, 1984; Kurmaly *et al.,* 1990; Jones *et al*., 1993; Kovalenko *et al*., 2000; Genodepa *et al.,* 2004a, b).

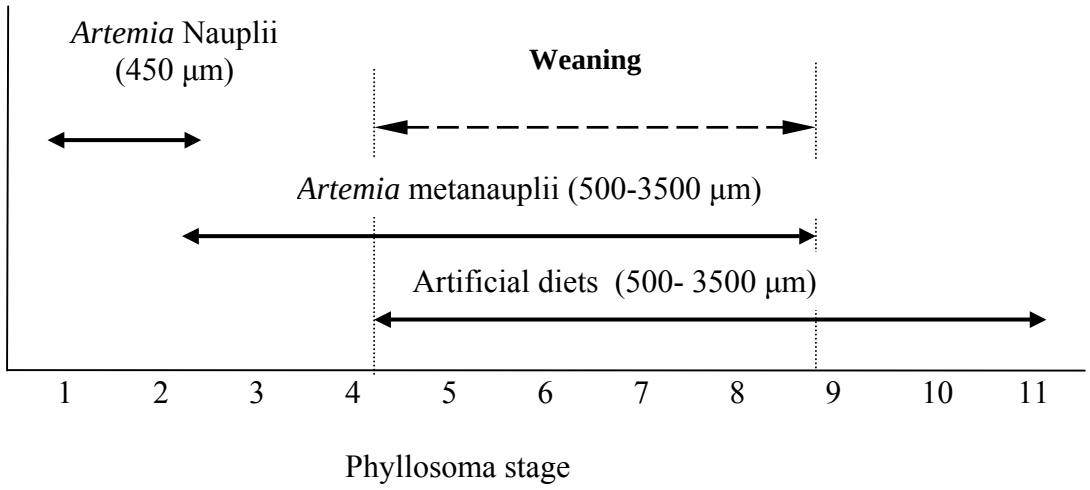



Fig. 8.1. The current and a predicted feeding protocol for phyllosoma culture. First feeding begins with *Artemia* nauplii followed by *Artemia* metanauplii. The phyllosoma are then weaned onto 'artificial' foods.

This study showed that the level of consumption of artificial diet particles by phyllosoma was low during early phyllosoma stages; however, consumption increased with increasing phyllosoma age. Further research is required to determine the acceptability of MBD by older stages of phyllosoma.

A suggested feeding protocol for *P. ornatus* phyllosoma throughout their development is shown in Fig. 8.1. Newly hatched *Artemia* are fed to stage 1 and 2 phyllosoma, while larger metanauplii (500-3500 μm) are suitable in size for older phyllosoma stages. The possible weaning process or introduction of MC could begin at the earliest with stage 4 phyllosoma based on the results of this study. Apart from major cost savings associated with reduced live food production, delivery of an adequate formulated food would increase survival of phyllosoma through their long and relative complex larval life. As the nutritional composition of artificial particles

can accurately be manipulated, the opportunity to determine the nutritional requirements of phyllosoma could then be achieved, at least for phyllosoma stages which consume artificial particles.

The development of an efficient aquaculture industry based on *P. ornatus* depends on the closure of their life cycle. In order to achieve this, future research should be focus on the following issues:

(1) Refine phyllosoma rearing techniques and practices;

(2) Improve delivery of adequate nutrients through live foods for early stage phyllosoma, in particular enrichment of *Artemia*, to maximise survival;

(3) Accurately determine the phyllosoma age at which 'artificial' food particles are accepted. Once this is achieved their specific nutritional requirements can be investigated.

This study has provided valuable new information relating to the feeding biology and nutrition of *P. ornatus* phyllosoma. The results will allow further development towards production of appropriate hatcheries foods for *P. ornatus* phyllosoma and facilitate progress towards more efficient hatchery techniques for this species.

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