

**Reproduction of the zooplankton, *Daphnia carinata* and *Moina australiensis*:  
Implications as live food for aquaculture and  
utilization of nutrient loads in effluent**

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# Table of Contents

<b>ABSTRACT</b> .....	<b>vii</b>
<b>DECLARATION</b> .....	<b>x</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>xi</b>
<b>FOREWORD</b> .....	<b>xii</b>
<b>1. GENERAL INTRODUCTION</b> .....	<b>1</b>
<b>1.1. Introduction</b> .....	<b>1</b>
<b>1.2. Thesis structure</b> .....	<b>5</b>
1.2.1. Reproduction and potential commercial application of zooplankton (Chapter 2) .....	5
1.2.2. Culture conditions to maintain asexual reproduction of <i>D. carinata</i> and <i>M. australiensis</i> (Chapter 3).....	5
1.2.3. Induction, storage and hatching of ephippia of <i>D. carinata</i> and <i>M. australiensis</i> (Chapter 4) .....	6
1.2.4. The influence of feeding status on use of nutrients in piggery effluent (Chapter 5).....	6
1.2.5. Nutritional profiles of <i>D. carinata</i> and <i>M. australiensis</i> (Chapter 6).....	7
1.2.6. General discussion (Chapter 7) .....	7
<b>2. REPRODUCTION AND POTENTIAL COMMERCIAL APPLICATION OF ZOOPLANKTON</b> .....	<b>8</b>
<b>2.1. Introduction</b> .....	<b>8</b>
<b>2.2. Reproduction of zooplankton</b> .....	<b>9</b>
2.2.1. Morphology of <i>D. carinata</i> and <i>M. australiensis</i> .....	9
2.2.2. Zooplankton habitat and populations.....	10
2.2.3. Reproduction .....	12
2.2.4. Chemical and Physical impacts on wastewater-grown zooplankton .....	15
2.2.4.1. Impact of un-ionised ammonia .....	15
2.2.4.2. Impact of temperature .....	16
2.2.4.3. Impact of photoperiod .....	17
2.2.4.4. Impact of other factors.....	18
2.2.5. Induction of ephippia .....	18
2.2.6. Storage parameters affecting the hatchability of ephippia.....	21
2.2.7. Stimuli required to induce the hatching of ephippia.....	22
<b>2.3. Implication of zooplankton in nutrient removal from effluent</b> .....	<b>23</b>
2.3.1. Wastewater from intensive farming of animals is a concern .....	23
2.3.2. Bio-remediation efficiency.....	23
<b>2.4. Utilization of zooplankton in aquaculture</b> .....	<b>24</b>
<b>2.5. Review summary</b> .....	<b>26</b>
<b>2.6. Research objective</b> .....	<b>28</b>

### **3. CULTURE CONDITIONS TO MAINTAIN ASEXUAL REPRODUCTION OF *D. CARINATA* AND *M. AUSTRALIENSIS* ----- 30**

<b>3.1. Impacts of un-ionized ammonia in digested piggery effluent on reproductive performance and longevity of <i>Daphnia carinata</i> and <i>Moina australiensis</i> -----</b>	<b>30</b>
3.1.1. Abstract -----	30
3.1.2. Introduction -----	31
3.1.3. Methods -----	33
3.1.3.1. Collection and husbandry of stock zooplankton -----	33
3.1.3.2. Preparation of algae -----	34
3.1.3.3. Lethal concentrations (24 hrs LC50 value) of un-ionized ammonia -----	34
3.1.3.4. Impact of un-ionized ammonia on reproductive performances and longevity -----	37
3.1.4. Results -----	39
3.1.4.1. Lethal limits (24 hrs LC50 value) of un-ionized ammonia -----	39
3.1.4.2. Reproductive performance and longevity of <i>Daphnia carinata</i> -----	42
3.1.4.3. Reproductive performance and longevity of <i>Moina australiensis</i> -----	46
3.1.5. Discussion -----	50
3.1.6. Conclusion -----	56

### **3.2. Influences of temperature and photoperiod on reproduction of zooplankton (*Daphnia carinata* and *Moina australiensis*) cultured in anaerobically digested piggery effluent ----- 57**

3.2.1. Abstract -----	57
3.2.2. Introduction -----	58
3.2.3. Methods -----	60
3.2.3.1. Collection and husbandry of stock zooplankton -----	60
3.2.3.2. Preparation of culture medium for experiment -----	61
3.2.3.3. Preparation of algae -----	62
3.2.3.4. Preparation of test animals -----	63
3.2.3.5. Influence of temperature on reproductive performance -----	63
3.2.3.6. Morphology and histology examination of the embryonic development stage -----	66
3.2.3.7. Effects of photoperiod on the time of first reproduction -----	67
3.2.3.8. Statistical analysis -----	68
3.2.4. Results -----	68
3.2.4.1. Influence of temperature on reproductive performance of <i>Daphnia carinata</i> -----	68
3.2.4.2. Influence of temperature on reproductive performance of <i>Moina australiensis</i> -----	71
3.2.4.3. Influence of temperature on the embryonic development -----	74
3.2.4.4. Effects of photoperiod on the time of first reproduction of <i>Daphnia carinata</i> -----	81
3.2.4.5. Effects of photoperiod on the time of maturity of <i>Moina australiensis</i> --	82
3.2.5. Discussion -----	82
3.2.6. Conclusion -----	86

### **4. INDUCTION, STORAGE AND HATCHING THE EPHIPPIA OF *DAPHNIA CARINATA* AND *MOINA AUSTRALIENSIS* ----- 87**

<b>4.1. Abstract -----</b>	<b>87</b>
<b>4.2. Introduction -----</b>	<b>88</b>
<b>4.3. Methods -----</b>	<b>91</b>
4.3.1. Collection and husbandry of stock zooplankton -----	91

4.3.2. Preparation of culture medium-----	91
4.3.3. Preparation of algae-----	92
4.3.4. Preparation of test animals-----	94
4.3.5. Effects of photoperiod, temperature and density on the induction of ehippia -----	94
4.3.6. Influence of sex ratio on the induction of ehippia-----	96
4.3.7. The survival and reproduction after release of ehippia-----	96
4.3.8. Hatching of ehippia and development of the embryo-----	97
4.3.9. Statistical analysis-----	98
<b>4.4. Results-----</b>	<b>99</b>
4.4.1. Effects of photoperiod, temperature and density on the induction of ehippia -----	99
4.4.2. Influence of sex ratio on the formation of ehippia-----	103
4.4.3. The survival and reproduction after release of ehippia-----	104
4.4.4. The hatching process and development of the embryo-----	105
4.4.5. The influences of storage conditions and duration on hatching of ehippia-----	109
<b>4.5. Discussion-----</b>	<b>112</b>
<b>4.6. Conclusion-----</b>	<b>116</b>
<b>5. THE INFLUENCE OF FEEDING STATUS OF <i>DAPHNIA CARINATA</i> AND <i>MOINA AUSTRALIENSIS</i> ON USE OF NUTRIENTS IN PIGGERY EFFLUENT-----</b>	<b>118</b>
<b>5.1. Abstract-----</b>	<b>118</b>
<b>5.2. Introduction-----</b>	<b>119</b>
<b>5.3. Method-----</b>	<b>121</b>
5.3.1. Collection and husbandry of stock zooplankton-----	121
5.3.2. Preparation of culture medium-----	121
5.3.3. Preparation of algae-----	122
5.3.4. Preparation of treatments-----	123
5.3.5. Measurements-----	124
5.3.6. Statistical analysis-----	125
<b>5.4. Results-----</b>	<b>125</b>
5.4.1. Efficiency of nutrient utilization by <i>D. carinata</i> -----	125
5.4.2. Efficiency of nutrient utilization by <i>M. australiensis</i> -----	128
<b>5.5. Discussion-----</b>	<b>130</b>
<b>5.6. Conclusion-----</b>	<b>133</b>
<b>6. NUTRITIONAL PROFILE OF TWO EFFLUENT-GROWN ZOOPLANKTON (<i>DAPHNIA CARINATA</i> AND <i>MOINA AUSTRALIENSIS</i>) FED WITH ALGAE GROWN ON PIGGERY EFFLUENT: IMPLICATIONS FOR USE AS LIVE FEED IN AQUACULTURE-----</b>	<b>135</b>
<b>6.1. Abstract-----</b>	<b>135</b>
<b>6.2. Introduction-----</b>	<b>136</b>

<b>6.3. Methods</b> .....	<b>139</b>
6.3.1. Preparation of culture medium for zooplankton .....	139
6.3.2. Preparation of algae.....	140
6.3.3. Zooplankton culture.....	140
6.3.4. Moisture content .....	141
6.3.5. Protein analysis.....	142
6.3.6. Crude lipid analysis .....	142
6.3.7. Crude ash analysis .....	142
6.3.8. Carbohydrate content .....	143
6.3.9. Gross energy .....	143
6.3.10. Amino acid analysis .....	143
6.3.11. Fatty acid analysis .....	144
6.3.12. Statistic analysis .....	146
<b>6.4. Results</b> .....	<b>146</b>
6.4.1. Proximate composition.....	146
6.4.2. Amino acid content.....	148
6.4.3. Fatty acid composition.....	150
<b>6.5. Discussion</b> .....	<b>153</b>
<b>6.6. Conclusion</b> .....	<b>156</b>
<b>7. GENERAL DISCUSSION</b> .....	<b>158</b>
<b>7.1. Research Issues and Key Findings</b> .....	<b>158</b>
<b>7.2. Reproduction of <i>D. carinata</i> and <i>M. australiensis</i></b> .....	<b>161</b>
7.2.1 Key factors influencing culture of <i>D. carinata</i> and <i>M. australiensis</i> .....	161
7.2.2 Induction, hatching and storage of ephippia .....	162
<b>7.3. Efficiency of nutrient utilization from effluent</b> .....	<b>164</b>
<b>7.4. Implications of using zooplankton as live feed for aquaculture</b> .....	<b>165</b>
<b>7.5. Conclusions and future considerations</b> .....	<b>166</b>
<b>8. REFERENCES</b> .....	<b>168</b>
<b>9. APPENDICES</b> .....	<b>189</b>
<b>Appendix 1. Growth curve</b> .....	<b>189</b>

## Abstract

At present, recycle of resources from agricultural waste is in priority. In order to mitigate impacts cause by the high nutrient loads (N and P) in the agricultural effluent, the utilization of nutrient loads from the effluent is essential. Two South Australian species of zooplankton, *Daphnia carinata* and *Moina australiensis* are commonly found in agricultural effluent ponds in the Roseworthy district of South Australia. These studies compared the ability of both zooplankton species to utilise nutrients when cultured in anaerobically digested piggery effluent and their subsequent suitability for use as live feed for aquaculture.

In order to understand the start-up and maintenance of both species in culture, their reproduction was investigated. The study was carried out from Mar 2007 to Dec 2009 and designed to;

- a. determine the optimal culture conditions for asexual reproduction, based on nutrient tolerance (LC50 of un-ionized ammonia level), optimal culture temperature and photoperiod effects;
- b. identify conditions suited for sexual reproduction by focusing on stimuli to induce and hatch ephippia (resting egg), storage conditions for ephippia, sex ratio and embryonic development of the resting egg;
- c. determine the nutrient utilization efficacy of both zooplankton species; and

- d. quantify the nutritional profile of both species raised on effluent-grown algae to used be as live feed in aquaculture.

*M. australiensis* had greater ( $P < 0.05$ ) tolerance at higher levels of un-ionized ammonia ( $\text{NH}_3\text{-N}$ ) than *D. carinata*. Moreover, older neonates (<48 hrs old) could survive at higher concentrations of un-ionized ammonia nitrogen than younger neonates. Maximum reproductive performance for *D. carinata* and *M. australiensis* occurred at 0.5 and 4.5 mg/l  $\text{NH}_3\text{-N}$  respectively. Both species had an optimum reproductive performance at 23°C. *M. australiensis* had a remarkably higher net reproductive rate (112.05) and intrinsic rate (0.62) than *D. carinata*. Hence *M. australiensis* is a faster growing species compared to *D. carinata*.

*M. australiensis* had a higher percentage of induced ephippial brood and cumulative hatching of ephippia compared to *D. carinata* under multiple stimuli of photoperiod, temperature and density. However, the survival percentage of *M. australiensis* was lower and the time to resume asexual reproduction was longer after release of ephippia. The male to female ratio of 5:5 (1:1) or 4:5 maximized the induction of ephippial brood of both species. The ephippia of both species preferred storage in wet conditions.

The zooplankton candidates (non-fed with algae) utilized higher levels of nutrients (TN and SP) compared to the fed group. In the case of *D. carinata* (non-fed), the reduction of TN, SP and  $\text{BOD}_5$  was 18.8%, 18% and 60%, while in the case of *M. australiensis* (non-fed), the reduction of TN, SP

and BOD<sub>5</sub> was 16.9%, 12.3% and 64.5%. The mean reduction in BOD<sub>5</sub> provided further evidence that both species could utilize nutrients.

Both species contained an appreciable quantity of protein and lipid after being fed with effluent-grown algae. The essential amino acid content of *M. australiensis* met the requirements of both omnivorous and carnivorous fish fry, but *D. carinata* only met the requirements of omnivorous fish fry. Both species had over 50% unsaturated fatty acids which were mainly (n-6) poly-unsaturated fatty acids. Both zooplankton species have the capability of converting mono-unsaturated fatty acids (n-6) to poly-unsaturated fatty acids. The nutritional profile analysis showed that *D. carinata* and *M. australiensis* could be used as live food for freshwater fish larvae.

## **Declaration**

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Yuk Fung Jaime Leung and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

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**Yuk Fung Jaime Leung**

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## **Foreword**

This thesis has been prepared as a series of chapters in a format that will be suitable for future publication in scientific journals. In order to facilitate chapter publication, it was necessary to re-state from time to time some key information details.

The Abstract at the beginning of this thesis is the summary of the “Abstract” section of each of the data chapters (3, 4, 5 and 6). This enables chapters to be published separately without needing extensive cross-references.

# 1. General introduction

## 1.1. Introduction

Currently, increasing levels of nitrogen and phosphorus loading from agricultural waste are the main factors affecting eutrophication of lakes and rivers (Jung, *et al.*, 2009; Leeben, *et al.*, 2008). In order to mitigate such impact, the utilization of the nutrient loads (N and P) from agricultural effluent is essential. The artificial food web manipulation process to treat wastewater is a low cost ecological technology approach in areas without land constraint (Kibria, *et al.*, 1999; Shiny, *et al.*, 2005).

In this process, algae are used to utilize the nutrient loads in effluent and then the algae are fed to zooplankton (Kibria, *et al.*, 1999; Kim, *et al.*, 2003). The cultured zooplankton could be utilized as live feed in aquaculture (Kibria, *et al.*, 1999). Hence zooplankton species are assessed for use in the artificial food web manipulation process. The selection criteria for zooplankton species are based on the adaptation plasticity in effluent, nutrient tolerance, wide distribution in the local area, and ease of culture.

In the current study, two South Australian zooplankton species, *Daphnia carinata* and *Moina australiensis* that are common in the agriculture effluent ponds on the Roseworthy Campus, University of Adelaide, were selected. Whether these local species could be used to

remove nutrient loads from agricultural waste and act as a by-product for aquaculture required assessment.

Zooplankton raised on chemical grown algae or collected from the wild has been widely used as live food in the larval rearing aquaculture industries (Anderson & Silva, 2003; Hertrampf & Piedad-Pascual, 2000; Watanabe, *et al.*, 1983). However, the population of zooplankton that grows in natural aquatic environments or outdoor ponds is affected by seasonal changes (Geddes, 1984; Mitchell & William, 1982a; b). This imposes a commercial imperative to produce not only zooplankton fed with effluent-grown algae but also their ephippia (resting eggs), which can be hatched for mass production in any season.

There are nine different forms of *D. carinata* distributed extensively in south-eastern Australia, most commonly in lakes, reservoirs and weedless ponds (Hebert, 1977). In the case of *M. australiensis*, it can be found around Australia and is a euryhaline and lentic species (Smirnov & Timms, 1983; Vidal, 1973). It is widely distributed in tropical Queensland and suits all types of habitats, including lakes, tropical brackish fishponds, temperate permanent ponds and ephemeral desert pools (Smirnov & Timms, 1983).

Previous research has been conducted mainly on the reduction of nutrient loads by algae and then the feeding of the algae to zooplankton to estimate the removal efficiency. However, research has not been focused on optimal culture of the zooplankton. In addition, the reproduction of

zooplankton when grown in the digested agricultural effluent has been little studied. It is important to understand zooplankton reproduction in order to produce high quality zooplankton that is suitable for use as aquaculture feed

This study is a comparative evaluation of the aforementioned species when cultured in anaerobically digested piggery effluent. In order to understand the start-up and maintenance for culture of both species, their reproduction was investigated. Apart from the culture conditions criteria, the utilization and implication of using both species to utilise nutrients and use them as a food source for aquaculture was discussed.

The study was designed:

- a. To determine the optimal culture conditions for asexual reproduction, based on nutrient tolerance (LC50 of un-ionized ammonia level), optimal culture temperature and photoperiod effects;
- b. To identify conditions for sexual reproduction, which focused on stimuli to induce and hatch ephippia (resting egg), storage conditions for ephippia, sex ratio and embryonic development of the resting egg;
- c. To determine the nutrient utilization efficacy of both zooplankton species; and
- d. To quantify the nutritional profile of both species raised on effluent-grown algae for use as live feed in aquaculture.

The thesis aims to select suitable zooplankton candidates to be produced as a by-product for aquaculture feed and to utilize the nutrient loads in effluent.

## **1.2. Thesis structure**

This thesis consists of seven chapters. The data chapters (Chapter 3 – 6) are presented in manuscript format, allowing for ready preparation and submission to journal publishers. The first section of chapter 3 (3.1) has been submitted to a journal publisher and is under review.

### **1.2.1. Reproduction and potential commercial application of zooplankton (Chapter 2)**

The literature review discusses current knowledge of the reproduction of zooplankton, their implication for use to utilise nutrient loads from effluent and culture of zooplankton as live feed for aquaculture. It focuses on the chemical and physical impacts on effluent-grown zooplankton, the possible stimuli to induce and hatch ephippia and the influence of storage on the hatchability of ephippia. The review also describes the utilization of zooplankton in aquaculture and nutrient utilization from effluent.

### **1.2.2. Culture conditions to maintain asexual reproduction of *D. carinata* and *M. australiensis* (Chapter 3)**

This chapter focuses on the optimal culture conditions required for asexual reproduction of *D. carinata* and *M. australiensis*. The impact of un-ionized ammonia present in digested piggery effluent on the reproduction and survival of both species is evaluated in the first section of chapter 3, while the influence of temperature and photoperiod on reproduction is

investigated in the second section. This study highlights the higher nutrient tolerance and identifies the fast growing zooplankton candidate.

### **1.2.3. Induction, storage and hatching of ehippia of *D. carinata* and *M. australiensis* (Chapter 4)**

This chapter examines sexual reproduction of the selected species, including the conditions for induction, storage and hatching of ehippia (resting eggs) of *D. carinata* and *M. australiensis*. The objectives of this chapter are to determine the interaction effect of stimuli (photoperiod, temperature and density) and influence of sex ratio to induce ehippia including the influence of storage conditions (dry or wet) and duration on hatchability of ehippia. During the hatching process, the sequence of development of the embryo is examined. To further the understanding of the after release of ehippia, the survival rate, the percentage of survivors which can resume sexual reproduction and the time required to release a new clutch of neonates is also investigated. This component of the study aims to understand the complete production and management of ehippia so that indoor culture of zooplankton could be undertaken in any season.

### **1.2.4. The influence of feeding status on use of nutrients in piggery effluent (Chapter 5)**

This chapter evaluates the nutrient utilisation efficacy of *D. carinata* and *M. australiensis* from anaerobically digested piggery effluent. The reduction of total nitrogen, soluble phosphate and biochemical oxygen demand in the effluent is analysed when the both species are supplied with

algae (fed animals) and without algae (non-fed animals). This study aims to determine the influence of feeding status on the use of nutrients in piggery effluent.

#### **1.2.5. Nutritional profiles of *D. carinata* and *M. australiensis* (Chapter 6)**

The nutrition profiles of both zooplankton species raised on effluent-grown algae are assessed for their potential application as a feed source for use in aquaculture. These studies are designed to determine the proximate compositions, amino acid content and fatty acid composition of both zooplankton species raised on algae grown in two stage anaerobically digested piggery effluent. This study also analyzes the capability of both species to synthesise longer chain (n-3) and (n-6) poly-unsaturated fatty acids when raised on effluent-grown and chemical grown algae.

#### **1.2.6. General discussion (Chapter 7)**

This chapter aims to interpret and discuss key findings of this research. In particular future research priorities are highlighted which aim to gain more information about producing quality zooplankton as live feed in aquaculture and further improve the utilization of resources from agricultural waste during the culturing process.

## **2. Reproduction and potential commercial application of zooplankton**

### **2.1. Introduction**

It is essential to utilize nutrient loads from agricultural effluent in order to mitigate eutrophication in lakes and rivers. Zooplankton species are selected for the purpose of utilising nutrients and for use as live food when cultured in effluent. In the current study, two South Australian zooplankton species, *Daphnia carinata* and *Moina australiensis* that are abundant in the agriculture effluent ponds on the Roseworthy Campus, University of Adelaide, are selected. Whether these local species could be used to utilise nutrient loads from agricultural waste and act as a by-product for aquaculture required assessment.

This review outlines the morphology, habitat, population and reproduction of zooplankton. It also discusses in sequence the key issues of nutrient tolerance, ephippia induction, hatching and management, efficacy of nutrient removal and nutritional profile of zooplankton.

## 2.2. Reproduction of zooplankton

### 2.2.1. Morphology of *D. carinata* and *M. australiensis*

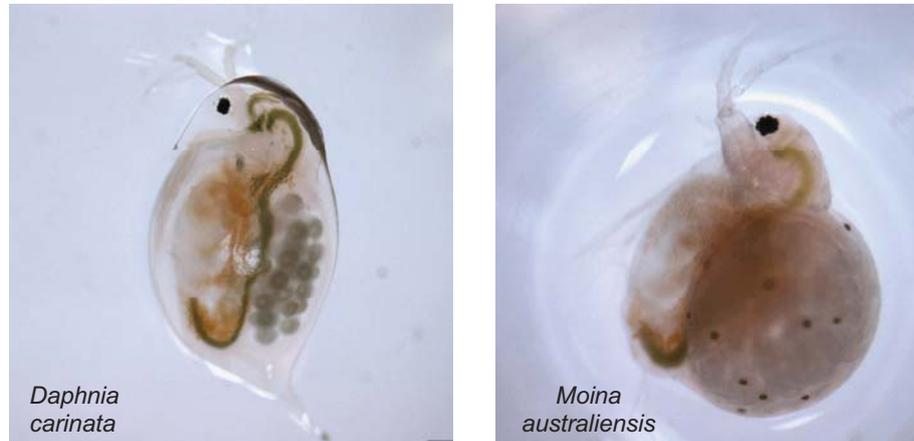


Plate 2.1 Egg-carrying female of *Daphnia carinata* and *Moina australiensis*

*Daphnia carinata* and *Moina australiensis* (Plate 2.1) belong to the family Daphniidae (order Cladocera). They are commonly used as live food in freshwater aquaculture industries (Rottermann, 2001). Their trunks are enclosed by a carapace and the trunk appendages (six pairs) are flattened. The first pair of appendages, the antennules, are rod-shaped and show sexual dimorphism (Plate 2.2); the second pair are antennae and are used mainly for locomotion; while the last four pairs are used for feeding. Large compound eyes are located on either side of the head under skin. The head of the *D. Carinata* is also protected within a helmet. The brood chamber of the female is on the dorsal side where embryos or ephippia (resting eggs) develop. The size of the adult female *D. carinata* (carapace length 4.5 mm) is larger than the male (carapace length 1.2 mm). *M. australiensis* (adult size is 1.9 mm), is a euryhaline and lentic species (Smirnov & Timms, 1983; Vidal, 1973). It

contains ephippia with two resting eggs compared to the morphologically similar *M. micrura* (one resting egg only).

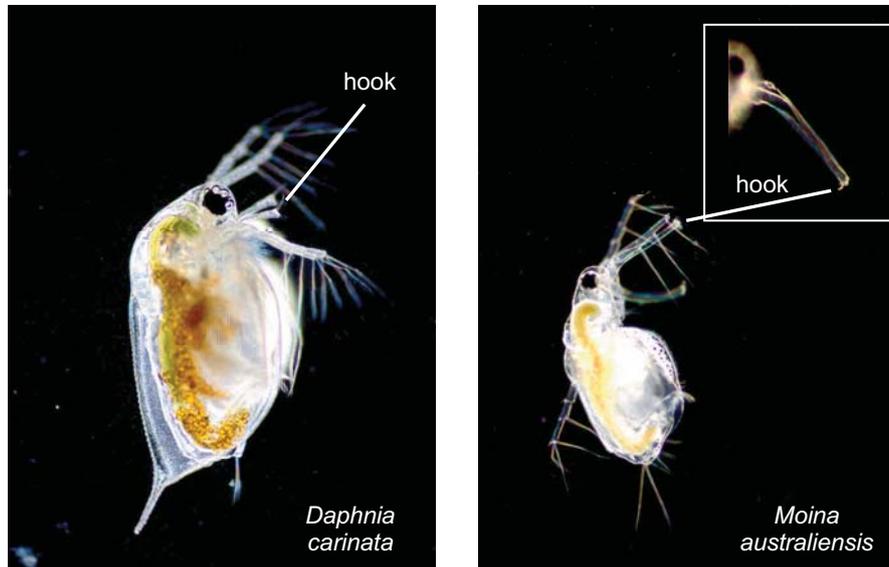


Plate 2.2 Adult male of *Daphnia carinata* and *Moina australiensis* show sexual dimorphism which attached with hooks at the tip of the antennules.

### 2.2.2. Zooplankton habitat and populations

Zooplankton are commonly found in ponds with high organic matter and have a seasonal variation in their population dynamics with the highest density during spring and early summer, when food supply and water temperature are optimum (Brambilla, 1982; Geddes, 1984; 1986; Goldman, *et al.*, 1979; Kobayashi, 1997; Kobayashi, *et al.*, 1998; Threlkeld, 1979; 1980; Winder, *et al.*, 2003). They are mainly hatched from ephippia/resting eggs after winter. Some individuals can survive over winter but do not reproduce neonates during this period (Banta, 1939). Hence for indoor zooplankton culture to be successful at any time, it is necessary to be able to produce and store their ephippia. Zooplankton are sensitive to temperature,

pH, dissolved oxygen, light and suspended particles in the water body (Banta, 1939; Green, 1956). Their survival also depends on the abundance of food and the quantity of predators present such as larvae of macro-invertebrates and fish. Similarly, the longevity of the zooplankton varies with the fluctuation of the favourable abiotic and biotic factors in their environment (MacArthur & Baillie, 1929a; b). Zooplankton have a longer life span at lower temperatures (MacArthur & Baillie, 1929b). Therefore a clear understanding of the optimal culture conditions is required when culturing zooplankton.

Population growth or density in zooplankton culture can be estimated by computing generation time ( $T$ ), net reproductive rate ( $R_0$ ) and intrinsic rate ( $r$ ) of increase according to data from reproduction. Using standard life table methods, the proportion of animals surviving from the start of the life table to age  $x$  ( $l_x$ ) and the number of neonates produced by an average animal of age  $x$  during that age period ( $m_x$ ) can be computed. Then the total number of female neonates produced per adult female during a single generation (the net reproductive rate,  $R_0$ ), average age at which an adult female gives birth to her female neonates (generation time,  $T$ ) and the average number of female neonates produced by an average adult female during its lifetime (intrinsic rate of increase,  $r$ ) are calculated according to the following formulae (Krebs, 1978; Pianka, 1999; Poole, 1974):

$$\text{Net Reproductive rate, } R_0 = \sum_{x=0}^{x \rightarrow \infty} l_x m_x ,$$

$$\text{Mean length of generation, } T = \frac{\sum_{x=0}^{x \rightarrow \infty} l_x m_x x}{R_0} \quad \text{or} \quad T = \frac{\sum_{x=0}^{x \rightarrow \infty} l_x m_x x}{\sum_{x=0}^{x \rightarrow \infty} l_x m_x}$$

$$\text{Intrinsic rate of increase, } r = \frac{\log_e R_0}{T}$$

where  $l_x$  = survivorship at time  $x$ ,

$m_x$  = fertility at time  $x$

The estimation of population growth of zooplankton is of significance when determining the growth rate of species, the feeding regime required throughout the culture process and the harvesting time of the cultured zooplankton.

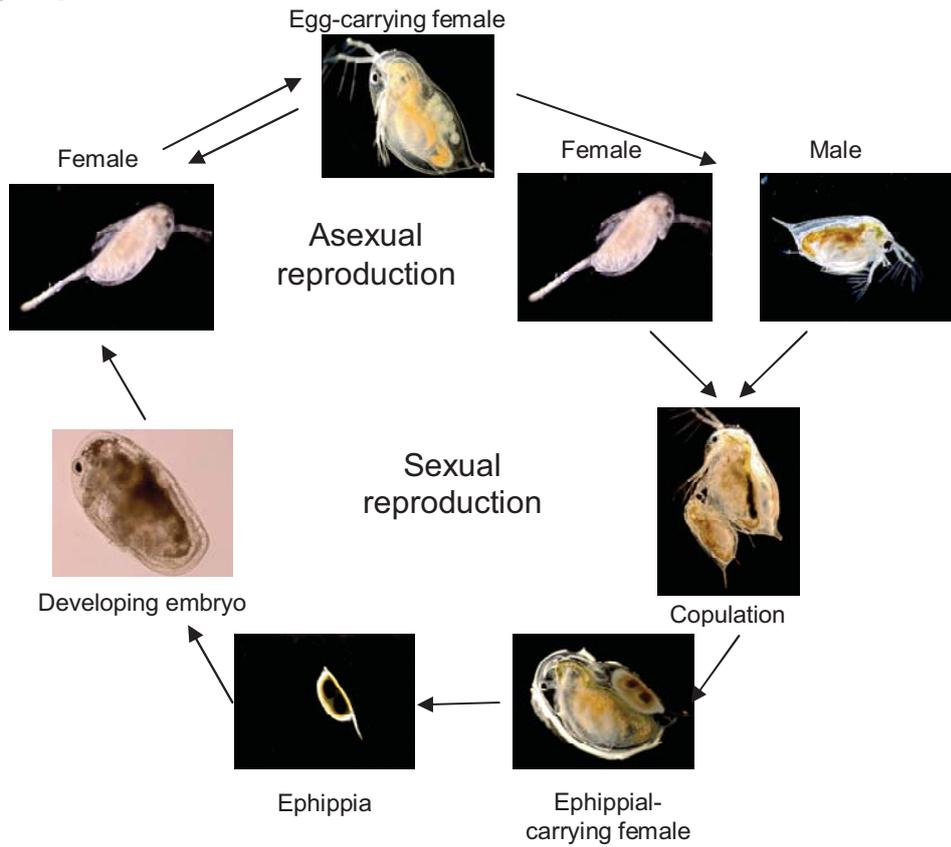
### 2.2.3. Reproduction

The mode of reproduction of both *D. carinata* and *M. australiensis* is similar, both can reproduce either sexually and asexually (Fig. 2.1) (Banta, 1939; Berg, 1934; Hoff & Snell, 2001). Asexual reproduction occurs during favourable environmental conditions including optimum temperature, pH, dissolved oxygen content, food supply, population density, photoperiod and light intensity. Egg-carrying females reproduce neonates that are all females (Banta, 1939). The newly born neonates are miniatures of the adults. However, under stressful or unfavourable conditions, egg-carrying females release either male or female neonates (Banta, 1939; Berg, 1934; 1936; Rottermann, 2001). This allows for sexual reproduction, where adult males

fertilize the eggs in adult females during copulation and produce ephippia (resting eggs). The ephippia are protected in a hardened brood pouch/capsule called an ephippium and can withstand extreme temperature and drought for a lengthy duration (Banta, 1939; Green, 1956; Hoff & Snell, 2001; Mellors, 1975). Hatching of ephippia occurs with favourable environmental conditions and each egg will produce one female neonate (Banta, 1939; Berg, 1934; 1936; Hoff & Snell, 2001).

Banta (1939) reported that when there are favourable conditions, zooplankton return to asexual reproduction. He reported that *Daphnia sp.* generally need a few days to two weeks to resume reproductive activity if they are still alive, but *Moina sp.* may not be able to resume full vigour until the next generation. Some weak clones could require a longer time or more than one generation to restore normal vigour (Banta, 1939). Hence it is crucial to understand the response to favourable and unfavourable culture conditions of each zooplankton species.

**(a) *Daphnia Carinata***



**(b) *Moina australiensis***

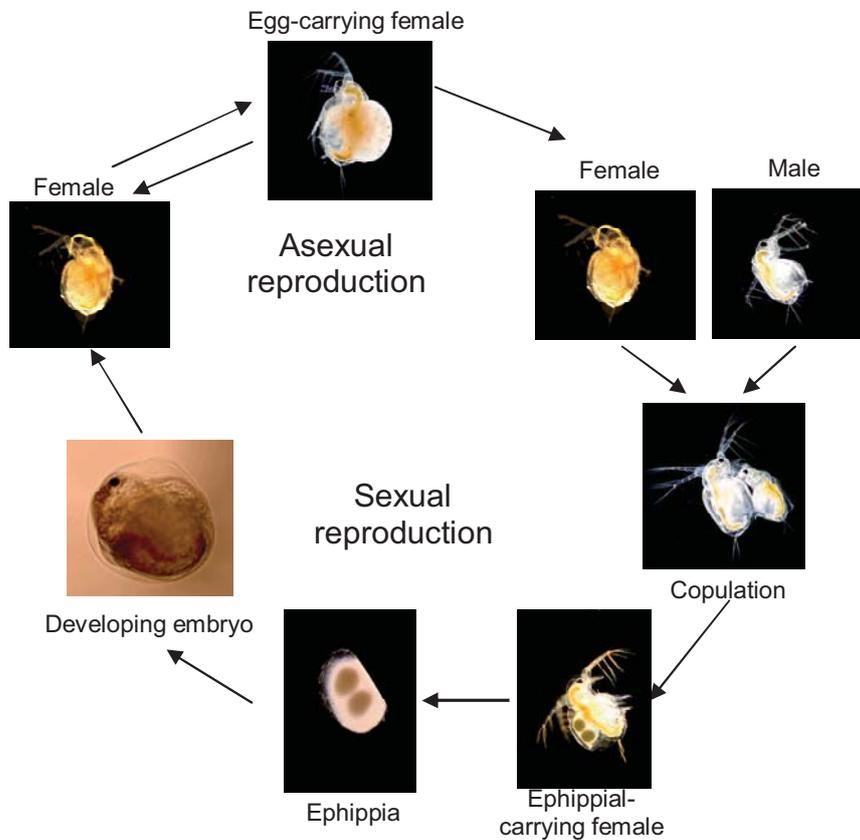


Fig. 2.1 Reproduction cycle of *Daphnia carinata* and *Moina australiensis*

## **2.2.4. Chemical and Physical impacts on wastewater-grown zooplankton**

### *2.2.4.1. Impact of un-ionised ammonia*

The harmful effect of ammonia on aquatic organisms such as the juvenile big bellied seahorse, juvenile greenlip abalone, juvenile turbot, juvenile gilthead sea bream, rainbow trout, *Penaeus chinensis* and *Penaeus monodon* has been extensively studied (Adam, *et al.*, 2001; Chen & Lin, 1995; Harris, *et al.*, 1998; Noor-Hamid, *et al.*, 1994; Rasmussen & Korsgaard, 1996; Smart, 1978; Thurston, *et al.*, 1981; Wajsbrodt, *et al.*, 1993). Ammonia is present in effluent ponds or wastewater treatment systems as a result of biological degradation of organic matter (Arauzo, 2003; Blažka, *et al.*, 1982). Aqueous ammonia exists both in the ionized and un-ionized form. However, the un-ionized form of ammonia is toxic to aquatic life (Alabaster & Lloyd, 1980). Hence it is important to determine the lethal limit of un-ionised ammonia when culturing zooplankton in the effluent medium.

Previous studies have examined the harmful effect of ammonia on the survival of zooplankton (Arauzo, 2003; Arauzo & Valladolid, 2003; Arauzo, *et al.*, 2000). The short term effects (over 90 minutes) of un-ionised ammonia concentration resulted in variable mortality (27-63%) of *Moina micrura* (Arauzo & Valladolid, 2003). The mortality was caused by an increase in pH due to stratification and hence a subsequent increase in un-ionised ammonia levels (Arauzo, 2003; Arauzo & Valladolid, 2003). It may also be due to stress in the zooplankton (Banta, 1939). Zooplankton

populations were found to decrease at a concentration of 2.5 mg/l of un-ionised ammonia (Arauzo, 2003), but Lincoln and Koopman (1983) reported that the lethal concentration limit of un-ionised ammonia (LC<sub>100</sub> value) for rotifers (*Brachionus rubens*) was 17 mg/l and for cladoceran (*Diaphanosoma brachyurum*) was just below 20 mg/l.

Since the lethal limit of un-ionized ammonia is different for different aquatic species, it is crucial to determine the exact value for our selected zooplankton species (*Daphnia Carinata* and *Moina australiensis*) in order to culture zooplankton in the effluent medium. For optimal culture conditions, it is also important to understand the impact of un-ionised ammonia on reproductive and population growth of the chosen zooplankton species.

#### 2.2.4.2. *Impact of temperature*

Optimal temperature is vital for the growth, maturation, production and longevity of zooplankton (Banta, 1939; Bottrell, 1975; Brown, 1929a; b; Burgis, 1970; Hanazato & Yasuno, 1985; MacArthur & Baillie, 1929a; b; Munro & White, 1975; Murugan, 1975). Zooplankton is an oviparous poikilotherm whose body temperature varies along with the ambient temperature (Anderson-Carnahan, 1994; Korpelainen, 1986; Munro & White, 1975). Within the temperature lethal limit, the egg development duration was inversely related to temperature (Bottrell, 1975; Burgis, 1970; Munro & White, 1975). Lower temperature (late autumn temperature or 15-17°C) delays maturation and prolongs egg incubation time (Bottrell, 1975; Schwartz, 1984) hence resulting in a lesser number of clutches and lower

total fertility during their lifetime. However the effect of temperature on egg development is gender specific (Brown & Banta, 1939), species specific and also depends on the size of species (Bottrell, 1975).

Temperature also influences the longevity of zooplankton which is inversely correlated to temperature level (MacArthur & Baillie, 1929a; b). At a low temperature (late autumn temperature or 15-17°C), the life span of zooplankton is longer. However the reproductive organs of the animal are inactivated when the temperature is too low such as below 10°C (Banta, 1939) which subsequently influences their productivity. It is therefore necessary to examine the effect of temperature on reproductive performance, longevity, population growth and egg development of the selected zooplankton candidate.

#### *2.2.4.3. Impact of photoperiod*

The effects of photoperiod on zooplankton have been investigated mainly in relation to sex determination, resting egg formation and survival (Korpelainen, 1986; Stross, 1969a; b). *Daphnia magna* were reported to have produced male neonates at 14°C with photoperiod L:D = 16:8 (Korpelainen, 1986). In general, the effects of temperature and photoperiod are correlated (Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Korpelainen, 1986). Photoperiod manipulation has also been used as a stimulus to break the diapause of the ephippia/resting egg (Schwartz & Herbert, 1987; Stross, 1966). However, a controlled photoperiod has been extensively manipulated in fish farming to alter maturation time as required (Unwin, *et al.*, 2005; Van

Der Meeren & Ivannikov, 2006). Hence, how photoperiod influences the maturation time of zooplankton in culture requires further study.

#### 2.2.4.4. *Impact of other factors*

Other factors which affect zooplankton growth and reproduction are pH and dissolved oxygen. The pH range for optimum growth of *Daphnia carinata* is pH 7.0 – 8.2 (Chandini, 1987; FAO, 1996). Aeration of the culture medium is recommended to avoid mass mortality, improve growth and fertility, and to enhance the suspension of the algal food to improve the feeding regime (Guerguess, 1987; Ovie & Ovie, 2004). Hence, the effects of pH and dissolved oxygen of the culture medium are monitored throughout experiments in this study.

#### 2.2.5. **Induction of ehippia**

Induction of ehippia (resting eggs) has been extensively studied and several factors were reported to successfully induce ehippia. Previous research has mainly focused on the induction of male offspring and ehippia using two or three stimuli by combining either temperature, water quality, photoperiod, density, and food limitation, or maternal/ offspring environment, alarm substances or cues from predators, and shaking of adult females (Alekseev & Lampert, 2001; Banta & Stuart, 1932; Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Slusarczyk, 1999; Stross, 1969a; Stross & Hill, 1965; Stuart & Cooper, 1932; Stuart, *et al.*, 1939). The issues in the induction of male and ehippia are as follows:

- High population density is effective to induce sexual reproduction. However, the drawbacks of crowding for some species are that it can cause changes in carapace morphology, produce animals devoid of tail spines, reduce the size of animals, increase competition for food, produce more metabolic wastes in the environment which increase pH, and increase physiological stresses which depress growth and slow down maturation and first reproduction (Banta, 1939; Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Stuart & Cooper, 1932).
- Poor culture medium quality due to metabolic wastes or excretory by-products in the culture medium has the same effect as crowding, but the effects on induction of ephippia are not very consistent. The associated change in pH and un-ionized ammonia concentration in the culture medium may easily cause a decline of the zooplankton population (Banta, 1939; Stuart & Cooper, 1932).
- A photoperiod with a short day/long night, which simulates winter's daylight duration, helps to produce ephippia. However, the stimuli could be effective for a period of time before the zooplankton could acclimatize to the induced conditions (Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Stross, 1966; Stross & Hill, 1968).
- Decreasing temperature, which also stimulates winter conditions can assist in the production of males and induction of ephippia (Korpelainen, 1986; Stross, 1966). However, a low temperature will damage the reproductive organs of the zooplankton (Banta, 1939).
- Limiting the supply of food, such as algae and bacteria in the effluent, to the populations causes the zooplankton to stop producing new

neonates and change to male and ehippia production. These effects are similar to those of high population density and increased metabolic waste, and it is difficult to separate these impacts (Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Stuart, 1932).

➤ Maternal food and photoperiod effect has been shown by *Daphnia pulicaria*, to transmit information to their neonates and affect the time to reach production of ehippia (Alekseev & Lampert, 2001).

➤ Predation by fish or larger size invertebrates which normally occurs in lakes and open ponds can also influence production of males and ehippia. Zooplankton adopt a defensive mechanism which causes a change to sexual reproduction and production of ehippia when under predation. In the laboratory, fish kairomones and alarm substances or cues, which are derived from injured conspecific or interspecific prey, can be used to induce sexual reproduction. The alarm substances or cues help to reflect the existing predation regimes on conspecifics while fish kairomones act as a signal of which predator is dominant (Slusarczyk, 1999).

➤ Gentle shaking of the container with egg carrying mothers, at periodic intervals of a couple of hours has been tested, and resulted in the production of males but not of resting eggs (Stuart, *et al.*, 1939).

In order to stock some ehippia so that indoor culture of zooplankton could be started during any season, this study investigates the interaction of the stimuli, photoperiod, temperature and density, on induction of ehippia in *Daphnia carinata* and *Moina australiensis*.

### 2.2.6. Storage parameters affecting the hatchability of ehippia

Parameters for storage of ehippia, such as duration and temperature, affect the hatchability. Moreira dos Santos and Persoone (1998) reported that the resting egg of *Daphnia magna* was unaffected by storage duration (12-47 months) and the hatching rate was approximately 65-75%, while the hatching synchrony following 47 months storage (36%) was lower than that following 12 months (76%) and 16 (68%) months storage. However the cumulative hatching response was significantly improved by increasing the storage duration from two to six months. Ehippia stored for six to eight months had a temperature dependent response on hatching, which resulted in higher cumulative hatching and better hatching synchrony (in first 4 days) at 4°C, compared to a 20°C storage temperature. The cumulative hatching increase after storage of up to six months was also species specific; for example, the hatching response for the resting egg of *Daphnia pulex* increased considerably when stored up to four months (Persoone, *et al.*, 1990) while *Daphnia longispina* ehippia exhibited a decreased hatching percentage when stored more than five months (Wood & Banta, 1933). However, some species of zooplankton produce two types of sexual eggs, and one type such as *Moina micrura* did not require a prolonged period of dormancy and may hatch after release from the egg-carrying female (Wood, 1932). Ehippia can survive extremes of dry and frozen conditions (Meester, *et al.*, 1998). However it is not known whether the hatchability and hatching time may vary under the interaction of different storage durations

and conditions (dry and wet). Hence, this issue is investigated in the current study.

### **2.2.7. Stimuli required to induce the hatching of ephippia**

The stimuli required for the hatching of zooplankton from ephippia have been examined over many years (Alekseev & Lampert, 2001; Banta & Stuart, 1932; Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Stross, 1969a; Stross & Hill, 1965; Stuart & Cooper, 1932). Factors affecting hatchability included temperature, photoperiod, pH, ionic concentrations and duration of storage (Doma, 1979; Rojas, *et al.*, 2001; Schwartz & Herbert, 1987; Stross, 1966). The process to hatch ephippia involved decapsulation of the hard casing and a series of heat and photoperiod shocks. For *Daphnia magna*, the low incubated temperature (4°C) could result in higher hatching rate (71.43%) than those incubated at 20°C (7.41%) (Meester & Jager, 1993). Moreira dos Santos and Persoone (1998) have developed methods for decapsulation of the ephippia, which involve washing the ephippia with 10% NaOCL and 1M NaOH (3 : 1) for 15 minutes. During the hatching process, imaging the sequence of development of the embryo could provide further understanding of the hatching process.

## **2.3. Implication of zooplankton in nutrient removal from effluent**

### **2.3.1. Wastewater from intensive farming of animals is a concern**

The pressures on water resources that are available for agriculture are becoming more critical because of global warming. Moreover, eutrophication is the key water quality problem in farming communities (Jung, *et al.*, 2009). Hence it is crucial to make use all resources available including the removal of nutrient loads (N and P) in agricultural effluent, to overcome this problem so that renewed water can be reused in agriculture. Among several wastewater treatment systems, artificial food web manipulation (an integrated wastewater treatment system) is a low cost ecological technology approach in areas without land constraint (Kibria, *et al.*, 1999; Shiny, *et al.*, 2005).

### **2.3.2. Bio-remediation efficiency**

Zooplankton is used to control algal blooms or for grazing on algae in wastewater treatment systems to avoid the expensive harvesting of algae (Kibria, *et al.*, 1999; Matveev, *et al.*, 1994). Zooplankton are found predominantly in wastewater treatment ponds containing high organic matter (Cripp & Kumar, 2003; Jana & Pal, 1983; 1985; Jana & Chakrabarti, 1993; Kibria, *et al.*, 1999; Mitchell & William, 1982b). Nutrient utilization in sewage water by *Daphnia magna* was reported to be 2% in total nitrogen and 4% in total phosphorus in an artificial food web system (Kim, *et al.*, 2003).

Recent research by Jung *et al.* (2009) reported a reduction in total nitrogen (14%) and total phosphorus (13%) from polluted stream water by harvesting the zooplankton (*Daphnia magna*) biomass from the wastewater treatment system. Shiny *et al.* (2005) described a role for *Daphnia magna* in reducing bio-chemical oxygen demand (BOD) by 54.3% in the biotreatment of sewage water. Due to the high adaptability of zooplankton (Petrusek, *et al.*, 2004), they are a highly suitable, inexpensive candidate for the removal of nutrient loads in effluent.

#### **2.4. Utilization of zooplankton in aquaculture**

Live food is commonly used in aquaculture to feed larval fish, crustaceans and molluscs up until these animals can be fed with formulated feeds (De Silva & Anderson, 1995; Hertrampf & Piedad-Pascual, 2000; Southgate, 2003). However, the cost of common live food, such as artemia and rotifers, is expensive (FAO, 1996; Hertrampf & Piedad-Pascual, 2000), and other more economic live food sources are required. Rainbow Trout (*Salmo gairdneri*) larvae have an improved performance when fed on zooplankton rather than aquaculture pelleted feeds especially in terms of their red fins (Dave, 1989) and pigmentation of flesh (Torrissen, 1985), which is enhanced by the carotenoids in *Daphnia magna* (Partali, *et al.*, 1985). Moreover carotenoids may act as an anti-oxidant in eggs and larvae, and increase resistance to oxygen deficiency (Craik, 1985). The composition of zooplankton has been investigated extensively and it has been shown that not only do marine and freshwater zooplankton have significant differences

in the content of biochemical compounds such as amino acids and fatty acids (Hertrampf & Piedad-Pascual, 2000; Kibria, *et al.*, 1999), but their composition also differs inter- and intra-specifically and from cohort to cohort (Hertrampf & Piedad-Pascual, 2000; Ventura, 2006).

Nutritional profiles of live organisms or live food vary with the nutrient composition of the food they are supplied with (Ahlgren, *et al.*, 1990; Mitra, *et al.*, 2007; Mulbury, *et al.*, 2008; Nanton & Castell, 1998; Watanabe, *et al.*, 1983). Zooplankton used to feed larvae should contain the necessary nutritional components, i.e. the fatty acids, amino acids, minerals and vitamins required for the growth, reproduction and other physiological functions of the larvae (Tacon, 1990; Tang & Taal, 2005; Watanabe, *et al.*, 1983). The essential fatty acids (EFA) in live food must be in the form of polyunsaturated fatty acids (PUFAs) or highly unsaturated fatty acids (HUFA), which are important for rearing larvae in aquaculture (Anderson & Silva, 2003; Hertrampf & Piedad-Pascual, 2000). PUFAs and HUFAs are a good source of energy for protein sparing and they also improve growth in larvae (Anderson & Silva, 2003; De Silva & Anderson, 1995; Stottrup, 2003). However the mineral composition of zooplankton varies greatly and the vitamin content is still not very clear (Hertrampf & Piedad-Pascual, 2000). Sometimes if there is a shortage of live zooplankton, frozen and freeze-dried forms are utilized for growing fish larvae (Kibria, *et al.*, 1999) but the nutritional profiles following freezing have not been compared with those of the live form. In freshwater aquaculture, *Daphnia sp.* and *Moina sp.* have been used to substitute for *Artemia* in hatcheries (James & Sherief,

1996), and therefore it is important to develop a comparative nutritional profile of the species as quality live food. In this study, it is also important to assess if zooplankton raised on effluent-grown algae meet the requirements as live food for aquaculture.

## **2.5. Review summary**

Basic knowledge of culture conditions to optimize the population density in zooplankton production is required. The un-ionized form of ammonia present in effluent is harmful to aquatic life (Alabaster & Lloyd, 1980), hence it is vital to evaluate the lethal limit during culture for each species. Temperature is the key factor in growth and reproduction of all aquatic life (Bottrell, 1975; Korpelainen, 1986) and the photoperiod affects maturation in fish (Unwin, *et al.*, 2005; Van Der Meeren & Ivannikov, 2006). In order to estimate the biomass of zooplankton species in a specific population or system, it is necessary to evaluate the relationship between temperature, photoperiod and egg development.

The induction of ephippia can be achieved by two or more stimuli, combining either temperature, water quality, photoperiod, density, and food limitation, or maternal/ offspring environment, alarm substances or cues from predators, or shaking of adult females (Alekseev & Lampert, 2001; Banta & Stuart, 1932; Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Slusarczyk, 1999; Stross, 1969a; Stross & Hill, 1965; Stuart & Cooper, 1932; Stuart, *et al.*, 1939).

Storage temperature and duration significantly affect the hatchability of ephippia (Meester, *et al.*, 1998; Moreira dos Santos & Persoone, 1998; Persoone, *et al.*, 1990). However, some zooplankton species do not require a prolonged period of dormancy and may hatch after release from the egg-carrying female (Wood, 1932).

Hatching of ephippia has been successfully induced by an initial temperature and photoperiod shock, followed by decapsulation. Lastly, the decapsulated ephippia can be stimulated by incubating with long daylight at optimal culture temperature (Doma, 1979; Moreira dos Santos & Persoone, 1998).

Zooplankton, *Daphnia magna* was used to remove nutrient loads from polluted stream water and sewage water in integrated wastewater treatment systems or biowastewater treatment processes (Jung, *et al.*, 2009; Kim, *et al.*, 2003; Shiny, *et al.*, 2005). However, the efficiency of nutrient removal by zooplankton varies in different systems or processes. However, zooplankton have a high adaptability for growth in effluent and show the potential to be employed for nutrient removal from effluent.

Zooplankton have been commonly used to substitute for *Artemia* as an inexpensive live food for larval rearing in aquaculture (FAO, 1996; Hertrampf & Piedad-Pascual, 2000). Nutritional profiles of live organisms or live feeds vary with the dietary composition of the food they are supplied

with (Ahlgren, *et al.*, 1990; Mitra, *et al.*, 2007; Mulbury, *et al.*, 2008; Nanton & Castell, 1998; Watanabe, *et al.*, 1983). Zooplankton used to feed larvae should contain the necessary nutritional components, i.e. the fatty acids, amino acids, minerals and vitamins required for the growth, reproduction and other physiological functions of the larvae (Tacon, 1990; Tang & Taal, 2005; Watanabe, *et al.*, 1983). The essential fatty acids (EFA) in live food must be in the form of polyunsaturated fatty acids (PUFAs) or highly unsaturated fatty acids (HUFA), which are important for rearing larvae in aquaculture (Anderson & Silva, 2003; Hertrampf & Piedad-Pascual, 2000).

## **2.6. Research objective**

This review is the basis for the investigation of the reproduction of *Daphnia carinata* and *Moina australiensis* when cultured in anaerobically digested piggery effluent. The review also provides information on the use of zooplankton as live food in aquaculture and their ability for nutrient removal from effluent. Further understanding of the ability of the selected local zooplankton species to remove nutrients when cultured in effluent medium and to provide a live food for aquaculture is required.

This study has four key objectives:

- a. To determine the optimal culture conditions for asexual and sexual reproduction, based on nutrient tolerance (LC50 of un-ionized ammonia level), optimal culture temperature and the effect of the photoperiod;

- b. To evaluate the impact of stimuli to induce and hatch ephippia (resting egg stage), storage conditions for ephippia, sex ratio and embryonic development of the resting egg;
- c. To determine the nutrient removal efficacy of both zooplankton species; and
- d. To quantify the nutritional profile of both species raised on effluent-grown algae for use as live feed in aquaculture.

A more complete rationale for each objective is provided in the succeeding chapters (chapter 3 – 6).

### **3. Culture conditions to maintain asexual reproduction of *D. carinata* and *M. australiensis***

#### **3.1. Impacts of un-ionized ammonia in digested piggery effluent on reproductive performance and longevity of *Daphnia carinata* and *Moina australiensis***

##### **3.1.1. Abstract**

In effluent treatment systems, a major concern is how to maintain the culture of South Australian zooplankton species (*Daphnia carinata* and *Moina australiensis*) particularly the impact of un-ionized ammonia on their life cycle response. Hence an evaluation was made of the impact of un-ionized ammonia present in digested piggery effluent on the reproduction and survival of *D. carinata* and *M. australiensis*. Both species were cultured in anaerobically digested piggery effluent and fed with algae, *Chlorella vulgaris*. The effects of different un-ionized ammonia concentrations on total fertility, the number of clutches, clutch size and survival were recorded every 24 hours.

The lethal concentration of un-ionized ammonia, with 50% survival after 24 hours exposure (24 hrs LC50 value), was determined for each species. This 24 hrs LC50 value was relative to total ammonia nitrogen, pH and temperature. *M. australiensis* had greater ( $P < 0.05$ ) tolerance at higher levels of un-ionized ammonia than *D. carinata*. However, older neonates (< 48 hrs old) could survive at a higher concentration of un-ionized ammonia nitrogen (up to 2.8 mg/l *D. carinata* and 8.8 mg/l *M. australiensis*) than

younger (< 24 hrs old) neonates (2.2 mg/l *D. carinata* and 7.5 mg/l *M. australiensis*). The net reproduction rates ( $R_0$ ) were derived from fertility and survival while the intrinsic rates of increases ( $r$ ) were calculated using net reproduction rate and generation time. *M. australiensis* has a maximum  $R_0$  (189.84) and  $r$  (0.54) of 4.5 and 6.5 mg/l NH<sub>3</sub>-N respectively, while *D. carinata* has a maximum  $R_0$  (100.46) and  $r$  (0.39) of 0.5 and 1.0 mg/l NH<sub>3</sub>-N. Hence *M. australiensis* is a faster growing species than *D. carinata* when cultured in digested piggery effluent.

### 3.1.2. Introduction

Effluent is commonly treated using algae and zooplankton (Kibria, *et al.*, 1999). Algae are used as a primary bio-converter to utilize the nutrient loads in the effluent. The algae are then used to feed zooplankton, and finally the zooplankton can be used as live feed for fish. Zooplankton has been easily cultured in agricultural effluent (Jana & Pal, 1983; Jana & Chakrabarti, 1993), but there is a question about the toxicity effects of ammonia in effluent on zooplankton.

Ammonia in aquatic systems exists in both ionized (NH<sub>4</sub><sup>+</sup>) and un-ionized forms (NH<sub>3</sub>). Harmful effects of ammonia on aquatic organisms have been extensively studied (Adam, *et al.*, 2001; Alabaster & Lloyd, 1980; Chen & Lin, 1995; Harris, *et al.*, 1998; Noor-Hamid, *et al.*, 1994; Rasmussen & Korsgaard, 1996). However, limited information has been published on the lethal limit of ammonia or ammonia tolerance in digested piggery

effluent for two Australian zooplankton species: *D. carinata* and *M. australiensis* (Julli, *et al.*, 1990). Some studies have reported on the effects of ammonia levels in different diets on the reproduction of *D. carinata* (Jana & Pal, 1983; Jana & Chakrabarti, 1993). In the case of *M. australiensis*, only the acute toxicity caused by potassium dichromate (Krassoi & Julli, 1994), sodium dodecyl sulfate and TIE reagents (Anderson-Carnahan, 1994) has been reported, but no studies have investigated the lethal limit for ammonia.

In an aqueous ammonia equilibrium study, conducted by Emerson *et al.* (1975), the fraction of un-ionized ammonia calculated depended on the total ammonia nitrogen, pH and temperature. Since pH is based on an exponential factor in the calculation, it has a greater influence than temperature at a given concentration of total ammonia nitrogen. The un-ionized form of ammonia present in effluent ponds and systems is toxic to aquatic life (Alabaster & Lloyd, 1980).

During intense photosynthetic activity under strong sunlight, phytoplankton blooms occur in effluent ponds, which increase the pH ( $\geq 8$ ) and hence intensify the fraction of un-ionized ammonia (Arauzo & Valladolid, 2003). Acute toxicity of un-ionized ammonia affects the survival of all aquatic organisms, while long term exposure to un-ionized ammonia induces chronic toxicity effects on growth, reproduction and longevity (Andersen & Buckley, 1998; Arauzo, 2003; Arauzo & Valladolid, 2003;

Banta, 1939; Gersich & Hopkins, 1986; Jana & Chakrabarti, 1993; US EPA, 2002).

It is important to understand the influence of un-ionized ammonia on the reproduction of both species in order to optimize the productivity in up-scale zooplankton culture using digested piggery effluent. It is also critical to control the ammonia levels so as to prevent the collapse of the zooplankton culture and stabilize the trophic structure. Hence an evaluation was made of the impact of un-ionized ammonia present in digested piggery effluent on the reproduction and survival of *D. carinata* and *M. australiensis*. The parameters monitored included: survival rate, mean longevity, total fertility, number of clutches produced, clutch size, net reproductive rate, and intrinsic rate of increase in population of both species.

### **3.1.3. Methods**

#### *3.1.3.1. Collection and husbandry of stock zooplankton*

*Daphnia carinata* and *Moina australiensis* were collected from effluent ponds located near to The University of Adelaide, Roseworthy Campus and reared for six months in the laboratory by the following procedure. The zooplankton (*D. carinata* and *M. australiensis*) were maintained with filtered (30µm nylon mesh) pond water in 70 l tanks and fed with *Chlorella vulgaris* at 700,000 and 300,000 cells/ml for *D. carinata* and *M. australiensis* respectively. Fifty percent of the volume of the culture

medium was changed daily using filtered effluent pond water. Temperature was maintained at  $25 \pm 1^\circ\text{C}$  with a reverse cycle air conditioner and photoperiod was set at 16 hrs light / 8 hrs dark using triphosphor fluorescent lights with a light intensity of  $>2000$  lux. Two batches (70 animals per batch per species) of egg-carrying females ( $\geq 3^{\text{rd}}$  brood) were separated into individual containers with the same culture conditions 24 and 48 hours prior to testing to simplify the ease of collecting  $<24$  and  $<48$  hrs old neonates.

#### 3.1.3.2. Preparation of algae

The algae, *Chlorella vulgaris* were sourced from the Integrated Biosystems Laboratory (IBS) at Roseworthy Campus. These algae originated in and were isolated from the same effluent pond where the zooplankton were collected. *C. vulgaris* was raised on anaerobically digested piggery effluent using a 20 l carboy (Nalgene) in the IBS laboratory. Temperature was maintained at  $25 \pm 1^\circ\text{C}$  with a reverse cycle air conditioner and 24 hrs illumination was provided using triphosphor fluorescent lights with an intensity  $>3000$  lux. The culture was aerated with an electromagnetic air pump (ACO-004 - The Age of Aquarium Pty. Ltd.). Algae were harvested while in the log phase at 6-8 days (approx. 6 million cells/ml) for feeding to both zooplankton species.

#### 3.1.3.3. Lethal concentrations (24 hrs LC50 value) of un-ionized ammonia

The culture medium used in this study containing anaerobically digested piggery effluent, collected from the IBS Laboratory. Effluent was

anaerobically digested in the primary thermophilic acidogenic (55°C) stage digesters for a total of seven days of hydraulic retention time (HRT). The effluent was then transferred through to the tertiary mesophilic (30°C ambient) digester stage, for a total of 20 days HRT. Before experimental use effluent was aerated for 24 hours via an aquarium air stone diffuser and allowed to equilibrate with aerobic atmospheric conditions.

A different range of concentration levels of un-ionized ammonia was used for each species in this experiment, based on preliminary results which indicated that *M. australiensis* was more tolerant to un-ionized ammonia compared with *D. carinata*. The dilution series used were 0, 0.5, 1, 1.5, 2, 2.2, 2.5, 2.8 and 3.0 mg/l un-ionized ammonia (NH<sub>3</sub>-N) for *D. carinata*, and 0, 3.5, 4.5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 8.8 and 9.0 mg/l NH<sub>3</sub>-N for *M. australiensis*.

The total ammonia nitrogen (TAN) of the digested piggery effluent and the culture medium was determined by the phenate method as described in APHA (2005) prior to and at the end of the tests. The respective un-ionized ammonia concentrations (UAN) were calculated from the measured TAN values, pH and temperature as described in the equation:  $UAN = TAN / (10^{pKa-pH + 1})$ , where  $p^{Ka} = 0.09018 + 2729.92/T$ , and T is the temperature (Alabaster & Lloyd, 1980; Emerson, *et al.*, 1975). The correct amount of effluent was measured for each concentration based on a pH of  $8.05 \pm 0.01$  and temperature of  $25 \pm 0.5^\circ\text{C}$ . The effluent pH was adjusted to 8.0 with potassium hydroxide (not more than 1 ppm) during preparation of

culture medium. Milli Q water was used for dilution and control but no pH adjustment was done for the control.

Test animals (*D. carinata* and *M. australiensis*) of < 24 and < 48 hrs old were obtained from stock culture of egg-carrying females ( $\geq 3^{\text{rd}}$  brood). Five replicates of each treatment (concentration) and 2 age groups of <24 hrs old and <48 hrs old of five neonates per replicate were studied. Neonates were carefully inoculated into 70 ml screw capped containers with 50 ml of diluted digested piggery effluent with un-ionized ammonia levels as described above. Neonates in each container were selected from different broods (egg-carrying female), with the same brood composition across treatments within a replicate. Hence at least 50 egg-carrying females were required for two groups for each species.

All containers were placed in incubators at  $25 \pm 0.5^{\circ}\text{C}$  with photoperiod of L:D = 16:8 at >2000 lux. Dissolved oxygen, temperature and pH were measured at the beginning and end point (24 hrs) while survival was counted after 24 hours. The animals were not fed with algae during the test. The total ammonia nitrogen concentration used in the tests ranged from 0 to 155 mg/l, dissolved oxygen was 5.0 – 6.5 mg/l while pH varied within  $\pm 0.02$  and temperature was  $\pm 0.5^{\circ}\text{C}$  [ $\leq 1.4\%$  (based on log unit change) and temperature was varied  $\leq 2.4\%$  as recommended by US EPA (2002)]. The containers were air tight to minimize ammonia loss, carbon dioxide exchange and culture medium evaporation.

The un-ionized ammonia was calculated from the total ammonia nitrogen, pH and temperature as described by Emerson *et al.* (1975) and Alabaster *et al.* (1980). The 24 hrs LC50 values were determined within a 95% confidence interval and significant differences in survival between treatments were analyzed by one way analysis of variance using SPSS 15 statistic software (SPSS Inc. 2006).

#### 3.1.3.4. *Impact of un-ionized ammonia on reproductive performances and longevity*

The impact of un-ionized ammonia in digested piggery effluent on the reproductive performances and longevity was examined in 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5 and 0 (control), 3.5, 4.5, 5.5, 5.5, 6.5, 7.5 mg/l NH<sub>3</sub>-N for *D. carinata* and *M. australiensis* respectively. The culture medium was prepared and test animals (<48 hrs old) were selected as described previously. Twenty replicates with a single neonate were arranged in each treatment (concentration). Same age neonates for each of the 20 replicates were sourced from 20 egg-carrying females.

Food (*Chlorella vulgaris*) was supplied at 30,000 and 15,000 cells/ml to each container of *D. carinata* and *M. australiensis* while culture medium was renewed every 24 hours. All containers were placed in incubators at 25 ± 0.5°C with photoperiod of L: D = 16:8 at light intensity > 2000 lux.

The test animals were observed every 24 hours. Production of neonates and survival was recorded while any newborns were removed from

the test container. Only female neonates were used to conduct the reproduction test but they are fragile (<48 hrs old) animals. Hence gender could only be classified after tests started, resulting in an uneven number of replicates in treatments for reproduction as shown in Table 3.1.

Using standard life table methods (Krebs, 1978; Pianka, 1999; Poole, 1974), the proportion of animals surviving from the start of life table to age  $x$  ( $l_x$ ) and the number of neonates produced by average animals of age  $x$  during that age period ( $m_x$ ) were computed, and then the net reproductive rate ( $R_0$ ), mean length of generation ( $T$ ) and intrinsic rate of increase ( $r$ ) were calculated according to the following formulae (Pianka, 1999):

$$\text{Net Reproductive rate, } R_0 = \sum_{x=0}^{x \rightarrow \infty} l_x m_x ,$$

$$\text{Mean length of generation, } T = \frac{\sum_{x=0}^{x \rightarrow \infty} l_x m_x x}{R_0} \quad \text{or} \quad T = \frac{\sum_{x=0}^{x \rightarrow \infty} l_x m_x x}{\sum_{x=0}^{x \rightarrow \infty} l_x m_x}$$

$$\text{Intrinsic rate of increase, } r = \frac{\log_e R_0}{T}$$

where  $l_x$  = survivorship at time  $x$ ,

$m_x$  = fertility at time  $x$

Different un-ionized ammonia concentration in the culture medium was the influencing factor in this experiment. The reproductive performances

such as fertility, number of clutches, clutches size, net reproduction rate and intrinsic rate of increase at different un-ionized ammonia treatments were analyzed, using Kruskal-Wallis one-way analysis of variance on ranks. Tukey's test was used for all pairwise multiple comparison procedures. All data were checked for normality and homogeneity of variance before analysis. SPSS 15 statistic software for Window (SPSS Inc. 2006) was used for the analysis of data and overall significance level was taken at  $P < 0.05$  or  $P < 0.01$ .

### **3.1.4. Results**

#### *3.1.4.1. Lethal limits (24 hrs LC50 value) of un-ionized ammonia*

The lethal limit of un-ionized ammonia (NH<sub>3</sub>-N) differed ( $P < 0.05$ ) between neonates that were <24 and <48 hours old, for both survival of *D. carinata* and *M. australiensis*. LC50 values were higher for < 48 hrs old neonates, when compared to < 24 hrs old neonates, for both species (Table 3.1).

The 24 hrs LC50 values of *D. carinata* of <24 hrs and <48 hrs age were 2.2 and 2.8 mg/l NH<sub>3</sub>-N respectively, while for *M. australiensis* of <24 hrs and <48 hrs old the 24 hrs LC50 value was 7.5 and 8.8 mg/l NH<sub>3</sub>-N respectively.

The survival percentage for the first 24 hours of exposure was unaffected at 0 - 1.0 mg/l NH<sub>3</sub>-N for *D. carinata* ( $P>0.05$ ), and at 0 – 5.5 mg/l NH<sub>3</sub>-N for *M. australiensis* ( $P>0.05$ ). When the concentration of un-ionized ammonia in the culture medium was increased, the survival of both species gradually declined ( $P<0.01$ ).

Table 3.1. Lethal limits of un-ionized ammonia for survival of *Daphnia carinata* and *Moina australiensis*.

Species	Un-ionized ammonia (mg/l)	Total ammonia nitrogen (mg/l)	pH	Temperature (°C)	Survival % (<24 hrs old)	Survival % (<48 hrs old)
<i>Daphnia carinata</i>	0	0	8.20	24.8	100±0	100±0
	0.5	8	8.06	24.8	100±0	100±0
	1.0	17	8.06	24.8	100±0	100±0
	1.5	25	8.06	24.8	96±4.0	96±4.0
	2.0	33	8.06	24.8	68±4.9	96±4.0
	2.2	37	8.06	24.7	52±4.9	-
	2.5	42	8.05	24.7	36±4.0	88±4.9
	2.8	48	8.05	24.8	-	52±4.9
	3.0	52	8.05	24.7	0	0
<i>Moina australiensis</i>	0	0	8.18	24.8	100±0	100±0
	3.5	59	8.05	24.8	100±0	100±0
	4.5	75	8.05	24.7	100±0	100±0
	5.5	93	8.05	24.8	100±0	100±0
	6.0	103	8.04	24.8	96±4.0	-
	6.5	113	8.04	24.8	88±4.9	96±4.0
	7.0	120	8.05	24.8	76±4.0	-
	7.5	127	8.05	24.8	52±4.9	92±4.0
	8.0	135	8.05	24.8	36±4.0	84±4.0
	8.5	143	8.05	24.8	-	68±4.9
8.8	148	8.05	24.8	-	48±4.9	
9.0	155	8.04	24.8	-	12±4.9	

Values are mean survival ±SEM

#### 3.1.4.2. Reproductive performance and longevity of *Daphnia carinata*

The toxic effect of un-ionized ammonia at different levels of concentration had a significant ( $P < 0.01$ ) impact on the reproductive performance of *D. carinata* (Table 3.2). It was highly productive at 0.5-1.0 mg/l  $\text{NH}_3\text{-N}$ , with mean total fertility (33.73 – 38.83 neonates/animal), mean number of clutches (2.22 - 2.67 clutches/animals) and mean clutch size (14.52 - 15.04 neonates/clutch).

The mean total fertility (1.3 – 19.24 neonates/animals), mean number of clutches (0.2 – 1.53 clutches/animals) and mean clutch size (1.3 – 12.62 neonates/clutch) were reduced ( $P < 0.05$ ) when animals were exposed to 1.5-2.5 mg/l of  $\text{NH}_3\text{-N}$  in the digested piggery effluent.

The mean total fertility and mean number of clutches released by animals at 1.0 mg/l  $\text{NH}_3\text{-N}$  was higher than at 0.5mg/l  $\text{NH}_3\text{-N}$ , but the mean clutch size of the animals was smaller. However there was no significant difference ( $P > 0.1$ ) between these treatments (0.5 and 1.0 mg/l  $\text{NH}_3\text{-N}$ ) on their mean total fertility, mean number of clutches and mean clutch size.

The mean total fertility declined gradually (more than 50%) from 1.5-2.5 mg/l  $\text{NH}_3\text{-N}$  and some animals did not release neonates at this level of  $\text{NH}_3\text{-N}$  (mean no. of clutches  $< 1$  at 2.5mg/l  $\text{NH}_3\text{-N}$ ). However, the reproductive performance of the control animals was poor, with mean total fertility, mean number of clutches and mean clutch size all less than one.

Some of the egg-carrying females in the control group shed the un-developed egg with the old carapace during their moult.

The net reproduction rate ( $R_0$ ) of *D. carinata* was significantly ( $P < 0.01$ ) increased by almost 17 times at 0.5 mg/l  $\text{NH}_3\text{-N}$ , when compared to control (0 mg/l  $\text{NH}_3\text{-N}$ ), but reduced thereafter to nearly zero at 2.5 mg/l  $\text{NH}_3\text{-N}$  (Fig. 3.1a). The intrinsic rate of increase ( $r$ ) of the animal was also significantly ( $P < 0.01$ ) increased by two fold from 0 to 1.0 mg/l  $\text{NH}_3\text{-N}$ , and then declined by 6.5 times from 1.5 to 2.5 mg/l  $\text{NH}_3\text{-N}$  (Fig. 3.1b). The maximum  $R_0$  (100.46) and  $r$  (0.39) occurred at 0.5 and 1.0 mg/l  $\text{NH}_3\text{-N}$  respectively. The  $R_0$  value of the control treatment group was 6, which was the lowest value.

The mean longevity of animals varied from 10 days in the control (0 mg/l  $\text{NH}_3\text{-N}$ ) to 15.6 – 15.9 days in 0.5-1.0 mg/l  $\text{NH}_3\text{-N}$  (Table 3.2). While there was no significant ( $P = 0.5$ ) difference between treatments with un-ionized ammonia levels at 0.5 mg/l (15.6) and 1.0 mg/l (15.9), the mean longevity was greater, when compared to the higher ammonia concentrations. The impact of un-ionized ammonia was indicated by a notable decline ( $P < 0.05$ ) in the mean longevity at 1.5-2.5 mg/l  $\text{NH}_3\text{-N}$  which reduced to 11-13 days.

Table 3.2. Effects of un-ionized ammonia on the reproductive efficacy and longevity of *Daphnia carinata*

NH <sub>3</sub> (mg/l)	Total fertility (neonates/animal)	No. of clutch (clutches/animal)	Clutch size (neonates/clutch)	Longevity (Day)
0 (control)	0.60±0.42 (20) <sup>c</sup>	0.10±0.07 (20) <sup>d</sup>	0.60±0.41 (20) <sup>c</sup>	10.00±0 (20) <sup>c</sup>
0.5	33.73±4.44 (15) <sup>a</sup>	2.20±0.26 (15) <sup>b</sup>	15.04±1.11 (15) <sup>a</sup>	15.60±0.44 (20) <sup>a</sup>
1.0	38.83±3.25 (18) <sup>a</sup>	2.67±0.20 (18) <sup>a</sup>	14.52±0.89 (18) <sup>a</sup>	15.90±0.25 (20) <sup>a</sup>
1.5	19.24±2.59 (17) <sup>b</sup>	1.47±0.13 (17) <sup>c</sup>	12.62±1.12 (17) <sup>a</sup>	13.95±0.38 (20) <sup>b</sup>
2.0	14.00±1.92 (15) <sup>b</sup>	1.53±0.13 (15) <sup>c</sup>	9.33±1.03 (15) <sup>b</sup>	12.60±0.26 (20) <sup>c</sup>
2.5	1.30±0.60 (20) <sup>c</sup>	0.20±0.09 (20) <sup>d</sup>	1.30±0.60 (20) <sup>c</sup>	11.25±0.31 (20) <sup>d</sup>

Values are Mean ± SEM (no. of sample)

Values with different superscript in the same column are significantly different ( $P < 0.05$ )

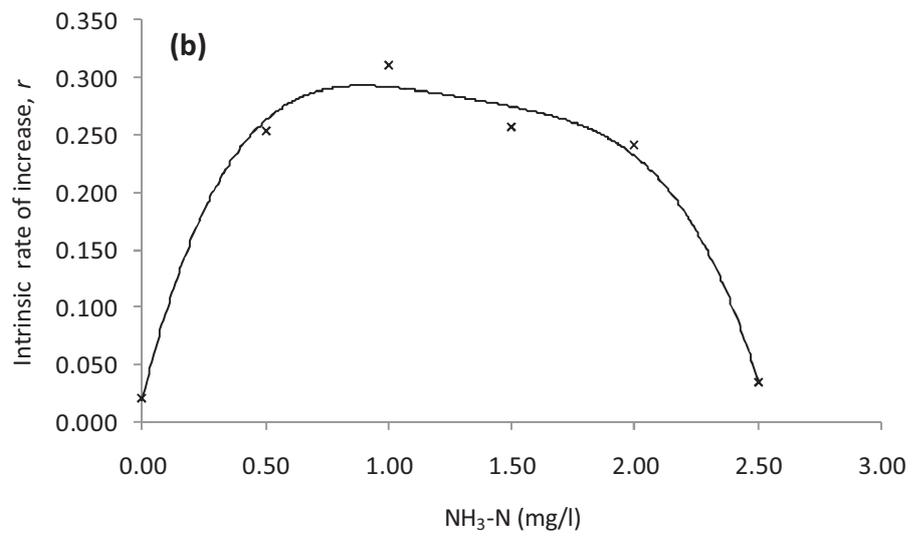
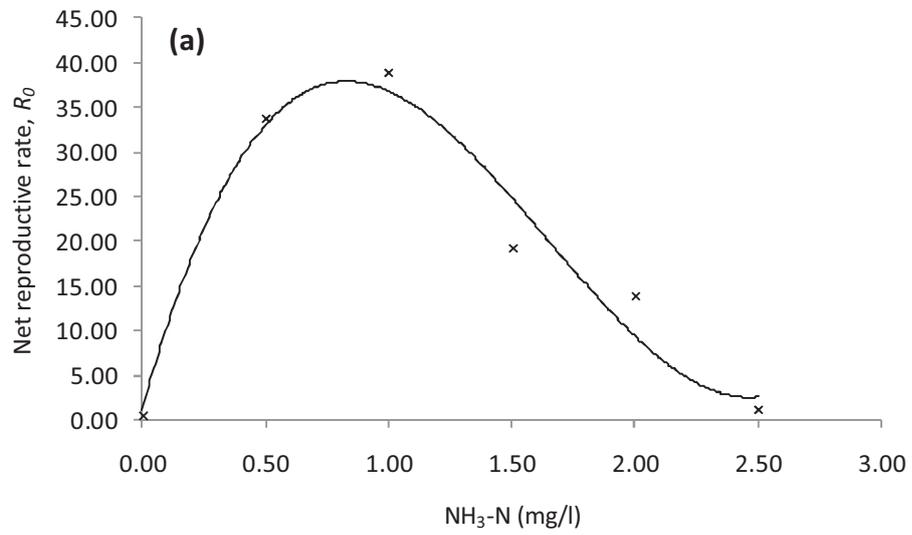


Fig.3.1 Net reproductive rate and intrinsic rate of increase of *Daphnia carinata* at different un-ionized ammonia levels.

### 3.1.4.3. Reproductive performance and longevity of *Moina australiensis*

*Moina australiensis* reproduced efficiently at 3.5-5.5 mg/l NH<sub>3</sub>-N with mean total fertility 102.6-136.75 neonates/animal, mean number of clutches 4.45-5.3 per animal and mean clutch size of 21.72 - 25.07 neonates/clutch (Table 3.3).

Although there were no significant differences ( $P > 0.1$ ) between these treatments (3.5, 4.5 and 5.5 mg/l NH<sub>3</sub>-N) in mean total fertility, mean number of clutches and mean clutch size; the peak mean total fertility, mean number of clutches and mean clutch size released by animals occurred at 4.5 mg/l NH<sub>3</sub>-N.

There was a decline in mean total fertility (86.6 – 88.0 neonates/animals), and mean number of clutches (3.85 – 3.95 clutches/animals) when exposed to 6.5-7.5 mg/l of NH<sub>3</sub>-N in the digested piggery effluent.

The total fertility was reduced (14-16%) from 5.5 to 6.5 and 7.5 mg/l NH<sub>3</sub>-N, while there was no significant ( $P > 0.1$ ) difference in mean total fertility, mean number of clutches and mean clutch size between these treatments (5.5, 6.5 and 7.5 mg/l NH<sub>3</sub>-N). However, there was a poor reproductive performance of the animals in the control (0 mg/l NH<sub>3</sub>-N), in which total fertility and clutch size were less than four, with only one clutch

being released. When some of the egg-carrying females in the control moulted, they shed the un-developed egg with the old carapace which was similar to *D. carinata*.

The net reproduction rate ( $R_0$ ) of *M. australiensis* was significantly ( $P < 0.01$ ) increased by 57 fold at 4.5 mg/l  $\text{NH}_3\text{-N}$ , when compared to control (0 mg/l  $\text{NH}_3\text{-N}$ ), but reduced by 30% at 7.5 mg/l  $\text{NH}_3\text{-N}$  (Fig. 3.2a). The intrinsic rate of increase ( $r$ ) of this animal was also significantly ( $P < 0.01$ ) increased by 3 fold at 4.5 mg/l  $\text{NH}_3\text{-N}$  compared to the control, and was maintained at 0.52 – 0.54 from 4.5 to 7.5 mg/l  $\text{NH}_3\text{-N}$  (Fig. 3.2b). However it obtained a maximum  $R_0$  of 189.84 at 4.5 mg/l  $\text{NH}_3\text{-N}$  and  $r$  of 0.54 at 6.5 mg/l  $\text{NH}_3\text{-N}$ . The  $R_0$  value of the control treatment group was 3.33 which was the lowest between treatments.

The mean longevity of *M. australiensis* varied from 9 days in the control (0 mg/l  $\text{NH}_3\text{-N}$ ) to 13.35 days at 4.5 mg/l  $\text{NH}_3\text{-N}$  (Table 3.3). Although the mean longevity was not significantly ( $P > 0.1$ ) different between 3.5 and 4.5 mg/l  $\text{NH}_3\text{-N}$ , it was relatively higher than other treatments (0, 5.5, 6.5, 7.5 mg/l  $\text{NH}_3\text{-N}$ ). The toxic impact of un-ionized ammonia was indicated by a decline in longevity to 11-12 days at 5.5 -7.5 mg/l  $\text{NH}_3\text{-N}$

Table 3.3. Effects of un-ionized ammonia on the reproductive efficacy and longevity of *Moina australiensis*

NH <sub>3</sub> (mg/l)	Total fertility (neonates/animal)	No. of clutch (clutches/animal)	Clutch size (neonates/clutch)	Longevity (Day)
0 (control)	3.33±0.50 (9) <sup>c</sup>	1.00±0 (9) <sup>c</sup>	3.33±0.50 (9) <sup>c</sup>	9.00±0 (20) <sup>b</sup>
3.5	112.44±15.52 (18) <sup>ab</sup>	4.67±.54 (18) <sup>ab</sup>	22.72±1.09 (18) <sup>ab</sup>	13.30±0.66 (20) <sup>a</sup>
4.5	136.75±11.84 (20) <sup>a</sup>	5.30±0.36 (20) <sup>a</sup>	25.07±0.86 (20) <sup>a</sup>	13.35±0.54 (20) <sup>a</sup>
5.5	102.60±13.19 (20) <sup>b</sup>	4.45±0.46 (20) <sup>ab</sup>	21.72±1.36 (20) <sup>b</sup>	12.85±0.63 (20) <sup>a</sup>
6.5	88.00±10.04 (20) <sup>b</sup>	3.85±0.39 (20) <sup>b</sup>	22.33±0.72 (20) <sup>ab</sup>	11.80±0.54 (20) <sup>a</sup>
7.5	86.60± 9.59(20) <sup>b</sup>	3.95±0.20 (20) <sup>b</sup>	21.17±1.37 (20) <sup>b</sup>	12.35±0.47 (20) <sup>a</sup>

Values are Mean ± SEM (no. of sample)

Values with different superscript in the same column are significantly different ( $P < 0.05$ )

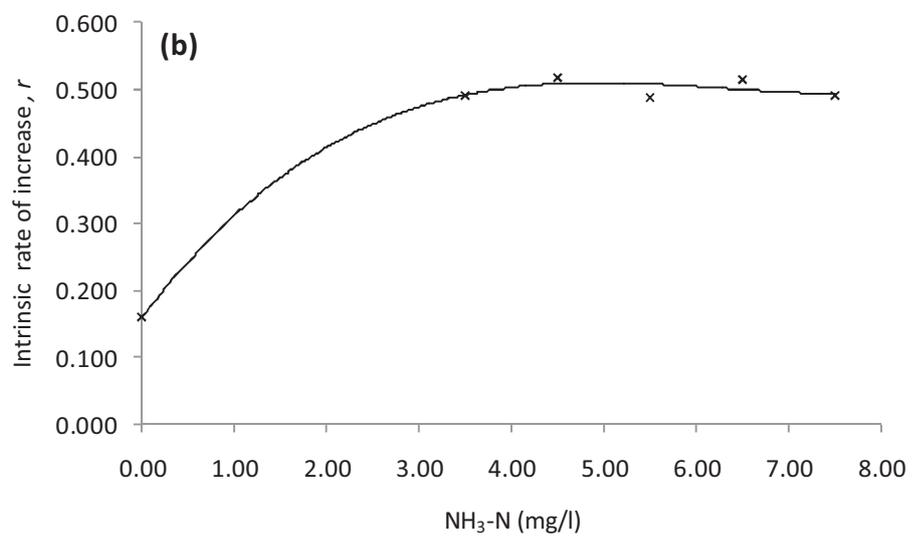
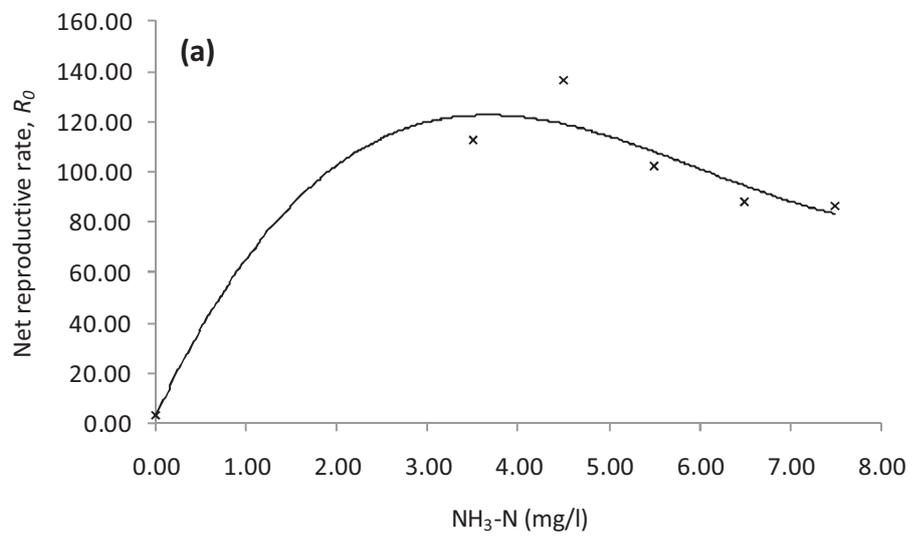


Fig. 3.2 Net reproductive rate and intrinsic rate of increase of *Moina australiensis* at different un-ionized ammonia levels

### 3.1.5. Discussion

The 24 hrs LC 50 value clearly indicated that *M. australiensis* is much more tolerant (3 times) than *D. carinata* to un-ionized ammonia in the digested piggery effluent. The LC 50 values are species specific which could be due to the different genetic makeup and difference in the morphology of these two species. Other species of *Daphnia* are similar; for example the LC 50 value of *Ceriodaphnia dubia* (1.18 mg/l) (Andersen & Buckley, 1998) was different from *Ceriodaphnia acanthine* (0.60 mg/l) (Gersich & Hopkins, 1986).

The neonates (<48 hrs old) of *M. Australiensis* and *D. carinata* also showed higher tolerance than the new born (<24 hrs old). Anderson and Buckley (1998) also reported that older neonates (19 - 24 hrs) of *Ceriodaphnia dubia* survived better than new born (<2.5 hrs old). Hence older neonates of both species were more tolerant to toxicity effects of un-ionized ammonia. Since the diluted effluent culture medium contained bacteria which was a food source for both species (Shiny, *et al.*, 2005), they could tolerate relatively higher ammonia (Mangas-Ramírez, *et al.*, 2001) compared to culture medium made up with pure ammonium chloride as described in US EPA (2002).

The results of this experiment were used as an indicator of the lethal limit of un-ionized ammonia to improve control on longevity and

reproduction performance such as total fertility, number of clutches, clutch size, net reproductive rate and intrinsic rate of increase of the two species while culturing *D. carinata* and *M. australiensis* in digested piggery effluent.

The morphological difference in *D. carinata* and *M. australiensis* could have an impact on their tolerance to external toxic chemicals. The brood chamber of *M. australiensis* is sealed, while it is open in *D. carinata* which allows fluid interchange of dissolved material with the surrounding culture medium (Banta, 1939). Hence *D. carinata* is more sensitive to the external toxicity effects of un-ionized ammonia.

For controls of both species, reproductive performances were lower and longevity was shorter than other treatments. Since food (*Chlorella vulgaris*) was provided for all treatments including the control, the pH could be brought up by the photosynthesis of the algae (Arauzo & Valladolid, 2003). However, the digested piggery effluent could act as a buffer to stabilize the pH of the culture medium which was added to all treatments except for the control. Therefore the pH of control replicates was increased from 8.2 to 8.9 for *Daphnia carinata* and 8.18 to 8.5 for *M. australiensis* in 24 hours. When some of the egg-carrying females moulted, the non-developed eggs were shed with the old carapace. Moreover the control (without effluent) could not supplement the neonates with bacteria and colloidal particles for the both species to enhance growth and reproduction (Curds & Hawkes, 1975; Gellis & Clarke, 1935; Shiny, *et al.*, 2005).

The reproductive performances have shown that *D. carinata* (max.  $r = 0.39$ ) is less adapted to a high un-ionized ammonia level in the digested piggery effluent than *M. australiensis* (max.  $r = 0.54$ ). The productivity of *D. carinata* declined at  $>1.0$  mg/l  $\text{NH}_3\text{-N}$  with a maximum mean total fertility of 38 neonates/animals released in 2-3 clutches (mean clutch size: 14 neonates/clutch) at 1.0 mg/l  $\text{NH}_3\text{-N}$ . However the reproduction of *M. australiensis* was unaffected and high productivity was maintained with mean total fertility at 136 neonates/animals released in an average of about 5 clutches (mean clutch size: 25 neonates/clutch) even at 4.5mg/l  $\text{NH}_3\text{-N}$  which already exceeded the 24 hrs LC50 value for the toxic effect of un-ionized ammonia for *D. carinata* (2.8mg/l  $\text{NH}_3\text{-N}$ ).

At low un-ionized ammonia levels, the effluent culture medium served as a supplementary bacteria meal for the animals, as well as providing algae with a source of bacteria which increased the production of neonates (Anderson-Carnahan, 1994; Langis, *et al.*, 1988). Some fish ponds were fertilized with ammonia at a lower level than the toxic level to fish to enhance the productivity (Alabaster & Lloyd, 1980). However exposure to un-ionized ammonia beyond the threshold level could affect growth and utilization of food in fish (Rasmussen & Korsgaard, 1996). Long term exposure below limits would have adverse physiological and histological effects (Lloyd, 1961) which could be the same for zooplankton.

At high un-ionized ammonia levels (high concentration of effluent), oxygen demand was high for zooplankton (Blažka, *et al.*, 1982), but dissolved oxygen content was low (< 2 ppm). With hypoxic conditions, the haemoglobin synthesis in the animals was increased 10-15 fold (Kring & O'Brien, 1976) which affected the metabolic rate (Wiggins & Frappell, 2000; 2002), the population and their life history (LaBerge & Hann, 1990). Hence the longevities of both species in our experiment were shortened when the un-ionized ammonia level was close to their 24 hrs LC 50 values.

*D. carinata* adapted better at an un-ionized ammonia level of 1.0 mg/l when cultured in digested piggery effluent (intrinsic rate of increase was 0.39 and mean longevity was 16 days at 1.0 mg/l NH<sub>3</sub>-N) compared to studies reported by Jana *et al.* (1993). They used manure culture (mixture of mustard oil cake, cattle and poultry manure) and showed that animal reproduction ( $r = 0$ ) and mean longevity (7 days) was influenced by environmental stress at ammonia concentrations of 0.47-0.48 mg/l NH<sub>3</sub>-N. Moreover the result of the control treatment in their experiment using well water ( $r = 0.31$ ) was different from our findings using milli Q water ( $r = 0.20$ ) on *D. carinata*.

Likewise Zalizniak *et al.* (2004) reported that the intrinsic rate of increase of *D. carinata* could be varied with different algae feed or the same algae but cultured with different media. The authors had shown that *D. carinata* cultured in carbon-filtered tap water and with *Chlorella vulgaris* (grown in Keating medium) had an intrinsic rate of increase -0.045 (after 14

days) and 0.047 (after 21 days) which was lower than the values of the control treatment in this study.

During the experiment the maximum intrinsic rate of increase ( $r$ ) of *D. carinata* was 0.39 (at 1.0 mg/l NH<sub>3</sub>-N), when fed with digested piggery effluent-grown *Chlorella vulgaris*, and cultured in diluted digested piggery effluent medium. An acute effect of un-ionized ammonia levels at 2.5 mg/l caused a notable reduction in the reproductive performances of *D. carinata*. This result was similar to the findings by US EPA (1999) for *Daphnia magna* (2.5 ± 0.2 mg/l NH<sub>3</sub>-N). Maximum  $R_0$  and  $r$  value was at 0.5 and 1.0 mg/l NH<sub>3</sub>-N respectively. This outcome means that the latter concentration of un-ionized ammonia had a higher mortality (Jana & Pal, 1983).

*D. carinata* could be cultured at either concentration of 0.5 and 1.0 mg/l NH<sub>3</sub>-N with the maximum reproduction output. However, it is recommended to use a lower range (0.5 mg/l NH<sub>3</sub>-N) in high density culture of the animals to offset the ammonia accumulated from metabolic waste released by the zooplankton.

*M. australiensis* is a euryhaline and lentic species which dominates both freshwater and brackish environments (Anderson-Carnahan, 1994; Smirnov & Timms, 1983; Vidal, 1973). This could be a reason why they tolerate higher un-ionized ammonia in the digested piggery effluent culture medium. Anderson-Carnahan (1994) conducted experiments using *M. australiensis* investigating different aspects such as water hardness, culture

volume, various food types and concentration. Mean total fertility of *M. australiensis* was 30.3 - 41.8 neonates/ animals in 7 – 10 day periods.

It is clear that *M. australiensis* has a high adaptability to digested piggery effluent and demonstrates extremely good reproductive performances. The maximum  $R_0$  and  $r$  value occurred at 4.5 and 6.5 mg/l  $\text{NH}_3\text{-N}$  respectively. This outcome shows that the latter concentration of un-ionized ammonia has a higher mortality (Jana & Pal, 1983). Hence 4.5 mg/l  $\text{NH}_3\text{-N}$  is the maximum sustainable un-ionized ammonia level to culture the *M. australiensis*.

### 3.1.6. Conclusion

*M. australiensis* was less sensitive to the toxic effect of un-ionized ammonia than *D. carinata* and likewise for the new born neonates (<24 hrs old) of both species. It is clear that *M. australiensis* has appreciably higher productive performances with a higher intrinsic rate of increase ( $r = 0.54$ ). Hence there is a potential to culture *M. australiensis* in digested piggery effluent to utilize the nutrients in the effluent, consume the effluent grown algae and save the cost of harvesting the algae by filtration. However the biomass (dry weight) production per day and nutrient contents of both zooplankton candidates will determine their use in commercial waste management systems.

## **3.2. Influences of temperature and photoperiod on reproduction of zooplankton (*Daphnia carinata* and *Moina australiensis*) cultured in anaerobically digested piggery effluent**

### **3.2.1. Abstract**

Seasonal variation in temperature affects populations of *Daphnia carinata* and *Moina australiensis* in effluent treatment ponds. In this study, the reproduction of both species was investigated at different temperatures (16, 23 and 30°C) and photoperiods (Light: Dark = L: D of 0:24, 10:14, 12:12, 14:10 and 24:0) in incubators using digested piggery effluent. Temperature had a significant effect on reproductive performance including fertility, number of clutches, clutch size (neonates/clutch), time of first reproduction, generation time, net reproductive rate and intrinsic rate. Photoperiod influenced the time of first reproduction only when provided with complete darkness. Both species had optimum reproductive performance at 23°C. However, mean total fertility, number of clutches and clutch size was higher at 30°C than 16°C, while generation time was longer at 16°C than 30°C.

The morphological and histological studies of embryonic development showed that the embryos of both species developed faster with increasing temperature. *M. australiensis* had remarkably higher net reproductive rate (112.05) and intrinsic rate (0.62) compared to *D. carinata*. *M. australiensis* and *D. carinata* could be expected to have the maximum

productive performance and population growth when cultured at 23°C in the anaerobically digested piggery effluent.

### **3.2.2. Introduction**

Bio-treatment of wastewater using zooplankton is commonly employed as a low cost effluent treatment process (Shiny, *et al.*, 2005). However seasonal variation influences population dynamics of the effluent treatment pond. Zooplankton are poikilothermic animals, which derive body heat from the environment, and therefore temperature is a key factor affecting their life history and population (Anderson-Carnahan, 1994; Banta, 1939; Korpelainen, 1986). Moreover day light variation across seasons also has an impact on zooplankton and influences the type of reproduction, either through sexual or asexual reproduction (Korpelainen, 1986; Stross, 1966).

Temperature is known to influence growth, maturation, fertility and longevity of zooplankton (Brown, 1929a; b; Hanazato & Yasuno, 1985; Murugan, 1975). Zooplankton grow faster at a higher temperature and reach final maturation stages earlier (Korpelainen, 1986; Schwartz, 1984). Egg development is also promoted by temperature, but these effects are species specific and also depend on the body size of the species (Bottrell, 1975). The longevity of zooplankton is inversely correlated to temperature (MacArthur & Baillie, 1929a; b), with longer life spans observed in a cold environment. However the reproductive organs of the animals are

inactivated when the ambient temperature is too low which subsequently influences their fertility (Banta, 1939).

The effects of photoperiod on zooplankton have been investigated mainly for sex determination, resting egg formation and survival (Korpelainen, 1986; Stross, 1969a; b). Photoperiod manipulation has also been used as a stimulus to break the diapause of the resting egg (Schwartz & Herbert, 1987; Stross, 1966). However, a controlled photoperiod has been extensively manipulated in fish farming to alter maturation time as required (Unwin, *et al.*, 2005; Van Der Meeren & Ivannikov, 2006). Hence, how photoperiod influences the maturation time of zooplankton in culture requires further study.

The South Australian Research and Development Institute (SARDI) has an integrated bio-treatment system (IBS) where nutrients are removed from the anaerobically digested piggery effluent by an artificial aquatic food web manipulation using algae and zooplankton.

Two South Australian local zooplankton species (*Daphnia carinata* and *Moina australiensis*) are being tested in our system as the bio-remediation tool for nutrient removal. It is therefore crucial to understand the influences of temperature and photoperiod on the reproduction of these species when cultured in diluted digested piggery effluent. Early studies on the effects of temperature on zooplankton mostly focused on growth, longevity, metabolic rate, embryonic development and duration of egg

development, resting egg formation and hatching (Bottrell, 1975; Korpelainen, 1986; MacArthur & Baillie, 1929a; b; Munro & White, 1975; Schwartz & Herbert, 1987; Stross, 1966; Venkataraman & Job, 1980), while research on photoperiod effects was mainly based on male production, resting egg production and hatching.

However, there is a lack of information on the reproductive performances of *D. carinata* and *M. australiensis* under the effects of temperature and photoperiod. Hence in this study, the influence of temperature and photoperiod on reproduction of both species was investigated. The reproductive performance of both species was assessed in terms of fertility, number of clutches, clutch size and time of first reproduction. To further the understanding of the influence of temperature on embryonic development, the morphology and histology of both species was examined following culture under different temperatures. The long term temperature effect on population growth was estimated from generation time, net reproductive rate and intrinsic rate of increase. This study also included the influences of photoperiod on the first time of reproduction (time of the 1<sup>st</sup> clutch release) for both species.

### **3.2.3. Methods**

#### *3.2.3.1. Collection and husbandry of stock zooplankton*

*Daphnia carinata* and *Moina australiensis* were collected from effluent ponds at The University of Adelaide, Roseworthy Campus and

reared for six months in the laboratory by the following procedure. Temperature of the pond was recorded from winter to autumn to decide the temperatures to use in the experiment. The zooplankton (*D. carinata* and *M. australiensis*) were maintained with effluent culture medium in 70 l tanks. Algae (*Chlorella vulgaris*) were supplied at 700,000 and 300,000 cells/ml (based on feeding to satiation) for *D. carinata* and *M. australiensis* respectively. Fifty percent of the culture medium was changed every two days and twenty percent of biomass was harvested twice weekly. Temperature was maintained at  $25 \pm 1^\circ\text{C}$  with a reverse cycle air conditioner and photoperiod was L :D = 16 :8 using triphosphor fluorescent lights with an intensity >2000 lux.

#### 3.2.3.2. Preparation of culture medium for experiment

The culture medium containing anaerobically digested piggery effluent was collected from the Integrated Biosystems (IBS) Laboratory's Bio-reactors at Roseworthy Campus. The effluent was anaerobically digested in the primary thermophilic and acidogenic ( $55^\circ\text{C}$ ) stage digesters for a total of seven days of hydraulic retention time (HRT). It was then transferred through to the tertiary mesophilic ( $30^\circ\text{C}$  ambient) digester stage, for a total of 20 days HRT. Before its experimental use, effluent was aerated for 24 hrs via an aquarium air stone diffuser and allowed to equilibrate with aerobic atmospheric conditions. The effluent was diluted with milli-Q water (ultra de-ionised water) to un-ionized ammonia concentrations of 0.5 and 4.5 mg/l for the culture medium of *D. carinata* and *M. australiensis* respectively,

as these were the optimum conditions for their reproduction previously obtained in a nutrient tolerance experiment (Leung, unpublished data).

The effluent was made up to the required un-ionized ammonia concentrations (UAN) based on the total nitrogen ammonia level, temperature and pH of the effluent as described in the equation:  $UAN = TAN / (10^{pKa - pH + 1})$ , where  $p^{Ka} = 0.09018 + 2729.92/T$ , T and is the temperature (Alabaster & Lloyd, 1980; Emerson, *et al.*, 1975). The total ammonia nitrogen (TAN) of the digested piggery effluent and the culture medium was determined by the phenate method as described in APHA (2005) prior to use. The culture medium was changed daily to avoid a build up of metabolic waste.

#### 3.2.3.3. Preparation of algae

The algae, *Chlorella vulgaris* were obtained from the IBS laboratory at Roseworthy Campus. These algae originated in and were isolated from the same effluent pond from which the zooplankton were collected. *C. vulgaris* was raised on anaerobically digested piggery effluent using a 20L carboy (Nalgene) in the laboratory at the IBS. The temperature was maintained at  $25 \pm 1^\circ\text{C}$  with a reverse cycle air conditioner and constant illumination was provided using triphosphor fluorescent lights with an intensity  $>3000$  lux. The culture was aerated with an electromagnetic air pump (ACO-004 - The Age of Aquarium Pty. Ltd.). Algae were harvested

while in the log phase at 6-8 days (approx. 6 million cells/ml) for feeding to both zooplankton species.

#### *3.2.3.4. Preparation of test animals*

Three days before the start of the experiment, two batches (25 animals per batch for each species) of egg-carrying females ( $\geq$  3rd brood) from the stock culture were inoculated into individual containers for culture. In order to allow the animal to acclimatize to the temperature and photoperiod conditions (Bullock, 1955), these egg-carrying females were cultured in the experimental conditions as described in the following sections. Newly born neonates (< 24 hrs old) were separately isolated using a glass pasture pipette into each container. Only female neonates, identified under a dissecting microscope (Olympus SZ61 microscope with Color View I digital camera), were used to conduct this experiment. Algae (*Chlorella vulgaris*) at the rate of 30,000 and 15,000 cells/ml were supplied daily in the freshly prepared culture medium for *D. carinata* and *M. australiensis* respectively.

#### *3.2.3.5. Influence of temperature on reproductive performance*

Newly born neonates (< 24 hrs old) of each species were separately isolated into the well of each container (NUNC 6-well flat bottom culture plate) with 10 ml of culture medium in each well. Two sets of six neonates per replicate and three replicates of each treatment at 16, 23 and 30°C were

studied. One set of neonates was prepared for the histology study of embryonic development in the brood chamber as described in section 3.2.3.6. The high and low temperature setting was based on the late autumn and early summer average temperature (before winter and summer diapauses) recorded during sample collection from the effluent ponds.

In order to prevent influences of parentage on survival and reproduction of the test animals, neonates in each container were selected from different broods (egg-carrying females), with the same brood composition across treatments within a replicate. Hence at least 18 egg-carrying females were required for each species.

All containers were placed randomly into incubators (Thermoline - L-094 refrigerated incubator with diurnal light and temperature control) with photoperiod of L: D = 16:8 at a light intensity >2000 lux. Dissolved oxygen (TPS - WP-82 dissolved oxygen and temperature meter), temperature and pH (Eutech instruments - pH510 pH and temperature meter) were measured twice daily while survival was counted every 24 hours. The containers were air tight to minimize ammonia loss, carbon dioxide exchange and culture medium evaporation.

The test animals were observed and counted every 24 hours for reproduction parameters, including fertility, number of clutches, clutch size and survival of neonates. Any newborns were removed from the container.

The long term effects of temperature on population growth were estimated from generation time, net reproductive rate and intrinsic rate of increase.

Using standard life table methods, the proportion of animals surviving from the start of the life table to age  $x$  ( $l_x$ ) and the number of neonates produced by an average animal of age  $x$  during that age period ( $m_x$ ) were computed. Then the total number of female neonates produced per mature female during a single generation (the net reproductive rate,  $R_0$ ), average age at which a mature female gave birth to her female neonates (generation time,  $T$ ) and the average number of female neonates produced by an average mature female during its lifetime (intrinsic rate of increase,  $r$ ) were calculated according to the following formulae (Krebs, 1978; Pianka, 1999; Poole, 1974):

$$\text{Net Reproductive rate, } R_0 = \sum_{x=0}^{x \rightarrow \infty} l_x m_x,$$

$$\text{Mean length of generation, } T = \frac{\sum_{x=0}^{x \rightarrow \infty} l_x m_x x}{R_0} \quad \text{or} \quad T = \frac{\sum_{x=0}^{x \rightarrow \infty} l_x m_x x}{\sum_{x=0}^{x \rightarrow \infty} l_x m_x}$$

$$\text{Intrinsic rate of increase, } r = \frac{\log_e R_0}{T}$$

where  $l_x$  = survivorship at time  $x$ ,

$m_x$  = fertility at time  $x$

#### *3.2.3.6. Morphology and histology examination of the embryonic development stage*

A subset of adult female *M. australiensis* (18 hours after release of the first brood) and *D. carinata* (36 hours after release of the brood) treated as described above were preserved by fixing in 10% formalin for 10 minutes. The morphology of the brood chamber was examined under a dissecting microscope and photos were taken (Olympus CZ61 microscope with ColorView I digital camera) before preparation of the histology slides. The specimens were embedded in paraffin wax individually; sections were cut at 7 microns, and stained with Haematoxylin and Eosin. The sections were examined and photos were taken (Olympus CX41 microscope with DP25 digital camera).

### *3.2.3.7. Effects of photoperiod on the time of first reproduction*

Newly born neonates (< 24 hrs old) of each species were separately isolated into each container (Techno Plastic 10 ml flat bottom tube) with 10 ml of culture medium. A single neonate per replicate and 20 replicates of each treatment with photoperiod of L: D = 0:24, 10:14, 12:12, 14:10 and 24:0 at a light intensity >2000 lux were studied. In order to prevent the influence of parentage on survival and reproduction of the test animals, neonates in each container were selected from different broods (egg-carrying females), with the same brood composition across treatments within a replicate. Hence at least 20 egg-carrying females were required for each species.

All containers were placed randomly into incubators (Thermoline - L-094 refrigerated incubator with diurnal light and temperature control) at  $23 \pm 0.5^{\circ}\text{C}$  which was the optimum culture temperature derived from the previous test. Dissolved oxygen, temperature and pH were measured twice daily while survival was counted after 24 hours. The containers were air tight to minimize ammonia loss, carbon dioxide exchange and culture medium evaporation. The test animals were observed twice daily until eggs being carried were noticed in their brood chamber. Following this, animals were monitored every hour, the time of first reproduction was recorded when the first clutch was released from the brood chamber and the experiment was

then ceased. The time was recorded in hours in the reproduction period between different photoperiod treatments.

#### 3.2.3.8. *Statistical analysis*

Different culture temperature and photoperiod were the main factors in this experiment. The reproductive performances such as fertility, number of clutches, clutch size, generation time, net reproduction rate, intrinsic rate of increase and time of first reproduction at different temperature and photoperiod treatments were analyzed, using one-way analysis of variance (ANOVA). Tukey's test was used for all pairwise multiple comparison procedures. All data were checked for normality and homogeneity of variance before analysis. SPSS 15 statistic software (SPSS Inc. 2006) was used for the analysis of data and overall significance level was taken at  $P < 0.05$ .

### 3.2.4. Results

#### 3.2.4.1. *Influence of temperature on reproductive performance of *Daphnia carinata**

Reproductive performance of *Daphnia carinata* was significantly different ( $P < 0.05$ ) at different temperatures. *D. carinata* showed the highest ( $P < 0.05$ ) mean total fertility, mean number of clutches and mean clutch size at 23°C (Table 3.4). At 23°C, the mean total fertility of *D. carinata* was 3

fold and 48 fold higher ( $P<0.05$ ) than at 30 and at 16°C respectively. Productivity of *D. carinata* was better ( $P<0.05$ ) at 30 °C than 16°C. *Daphnia* cultured at 16°C either reproduced one clutch or none during their life spans in this study and had the smallest ( $P<0.01$ ) clutch size. At 30°C, the mean number of clutches and clutch size of *Daphnia* was 28% and 57% lower than at 23°C. The maximum clutch size of the animal at 23°C was 47 neonates released on day 13 (3<sup>rd</sup> clutch) while the maximum clutch size of the animal at 30°C was only 19 neonates released on day 9 (2<sup>nd</sup> clutch).

The time of first reproduction of *D. carinata* at 16°C was 12 days which was notably the longest ( $P<0.01$ ) but there was no significant ( $P=0.6$ ) difference between 23°C which was 8 days, and 30°C which was 7 days (Table 3.4). The animals cultured at 30°C had shed the un-developed egg when they moulted on the fourth and fifth day of the experiment. Hence there were no neonates produced at the beginning and the first time of reproduction recorded was the second or the third clutch.

The mean generation time ( $T$ ) of *D. carinata* was the shortest ( $P<0.01$ ) at 30°C (8.66 days) (Table 3.4). However, similar values of generation time occurred ( $P=0.45$ ) at 16°C and 23°C. The net reproductive rate ( $R_0$ ) of the animals was the highest ( $P<0.01$ ) at 23°C, while it was less than one at 16°C. The intrinsic rate of increase ( $r$ ) of *Daphnia* was also similar ( $P=0.52$ ) at 23 (0.34) and 30°C (0.3). However  $r$  was negative at 16°C and some animals showed ephippia in their brood chambers before they died.

Table 3.4. Influence of temperature on the reproductive performance of *Daphnia carinata*

Parameters	16°C	23 °C	30 °C
Mean total fertility (neonates/animal)	0.94±0.36 <sup>c</sup>	47.67±4.67 <sup>a</sup>	14.94±2.44 <sup>b</sup>
Mean number of clutches (Clutches/animal)	0.44±0.06 <sup>c</sup>	3.17±0.09 <sup>a</sup>	2.28±0.20 <sup>b</sup>
Clutch size (neonates/clutch)	0.94±0.36 <sup>c</sup>	14.96±2.01 <sup>a</sup>	6.48±0.59 <sup>b</sup>
Mean time to release 1 <sup>st</sup> clutch (days)	11.83±0.10 <sup>a</sup>	7.83±0.42 <sup>b</sup>	7.22±0.61 <sup>b</sup>
Mean generation time, T	11.63±0.32 <sup>a</sup>	11.12±0.14 <sup>a</sup>	8.66±0.34 <sup>b</sup>
Mean net reproductive rate, R <sub>0</sub>	0.41±0.12 <sup>c</sup>	44.10±3.03 <sup>a</sup>	14.27±2.42 <sup>b</sup>
Mean intrinsic rate, r	-0.08±0.02 <sup>b</sup>	0.34±0.01 <sup>a</sup>	0.30±0.03 <sup>a</sup>

Values (mean±SEM) with different superscript letters in the same row were significantly different ( $P<0.05$ ); no. of replicates was 3 for each treatment

#### 3.2.4.2. Influence of temperature on reproductive performance of *Moina australiensis*

Reproductive performance of *M. australiensis* was significantly affected ( $P < 0.05$ ) by temperature. The animals cultured at 23°C showed the highest ( $P < 0.01$ ) mean total fertility, which was more than two fold higher than values obtained at 16°C and 30°C (Table 3.5). The mean number of clutches released at 16°C was 50% less ( $P < 0.01$ ) than other treatments. The mean clutch size of *M. australiensis* at 30°C was the lowest ( $P < 0.01$ ) between treatments. The maximum clutch size of the animals was 39 neonates released on day 10 (4<sup>th</sup> clutch) and day 13 (10<sup>th</sup> clutch) at 23°C, while the same number of neonates was also produced on day 11 (3<sup>rd</sup> clutch) at 16°C. However the maximum clutch size of the animal at 30°C was only 22 neonates released on day 3 (2<sup>nd</sup> clutch).

The time of first reproduction of *M. australiensis* was inversely ( $P < 0.05$ ) related to temperature. It was notably the shortest ( $P < 0.05$ ) at 30°C while the time increased ( $P < 0.01$ ) by 39% and 300% at 23°C and 16°C respectively (Table 3.5).

The mean net reproductive rate ( $R_0$ ) of *M. australiensis* was the highest ( $P < 0.05$ ) at 23°C (Table 3.5). However, a similar ( $P > 0.05$ ) value of  $R_0$  occurred at 16 and 30°C. At 30°C, the animal had the shortest ( $P < 0.05$ ) mean generation time but the highest ( $P < 0.01$ ) intrinsic rate of increase. The

survival of *M. australiensis* at 30°C declined to 50% on day 6 and 17% on day 7 while other treatments had 100% survival.

Table 3.5. Influence of temperature on the reproductive performance of *Moina australiensis*

Parameters	16°C	23 °C	30 °C
Mean total fertility (neonates/animal)	44.5±7.0 <sup>b</sup>	121.8±12.75 <sup>a</sup>	56.22±0.72 <sup>b</sup>
Mean number of clutches (Clutches/animal)	2.5±0.19 <sup>b</sup>	5.80±0.60 <sup>a</sup>	5.00±0.17 <sup>a</sup>
Mean clutch size (neonates/clutch)	17.39±1.53 <sup>a</sup>	20.59±0.36 <sup>a</sup>	11.46±0.43 <sup>b</sup>
Mean time to release 1 <sup>st</sup> clutch (days)	6.56±0.44 <sup>a</sup>	3.53±0.29 <sup>b</sup>	2.16±0.17 <sup>c</sup>
Mean generation time, T	8.67±0.36 <sup>a</sup>	7.59±0.15 <sup>b</sup>	4.08±0.09 <sup>c</sup>
Mean net reproductive rate, R <sub>0</sub>	40.05±7.46 <sup>b</sup>	112.05±12.78 <sup>a</sup>	52.27±1.06 <sup>b</sup>
Mean intrinsic rate, r	0.42±0.02 <sup>c</sup>	0.62±0.02 <sup>b</sup>	0.97±0.02 <sup>a</sup>

Values (mean±SEM) with different superscript letters in the same row were significantly different ( $P<0.05$ ); no. of replicates was 3 for each treatment

#### 3.2.4.3. Influence of temperature on the embryonic development

In the case of *D. carinata*, the embryonic development at 30 hours after release of the first brood had notable differences at different culture temperatures (Plate 3.1, 3.2 and 3.3). For animals cultured at 16°C, the embryo attained an oval shape and was surrounded with fat globules (Plate 3.1) (Murugan & Venkataraman, 1977). When the animals were cultured at 23°C, the embryos were elongated and showed a developed head and trunk, with some of the embryos also showing a head, trunk and antennary bud (Plate 3.2). For the animals cultured at 30°C, the embryo showed distinct eye, head, trunk and antennary (Plate 3.3).

In the case of *M. australiensis*, the results were similar to *D. carinata*. The embryonic development at 18 hours after release of the first brood differed when embryos were raised at different temperatures. At 16°C, the subitaneous eggs of the animal were packed inside the brood chamber and some embryos had attained an oval shape (Plate 3.4). When culture temperature was at 23°C, the embryo was elongated and was tightly packed with fat globules (Plate 3.5). For the animals cultured at 30°C, the embryo had a distinct eye, head and body which were tightly packed with fat globules inside the brood chamber (Plate 3.6).



Plate 3.1. *Daphnia carinata* cultured at 16°C with developing embryos in the brood chamber. (Above) The subitaneous eggs in the brood chamber. (Below) The oval shaped embryo was surrounded by fat globules.

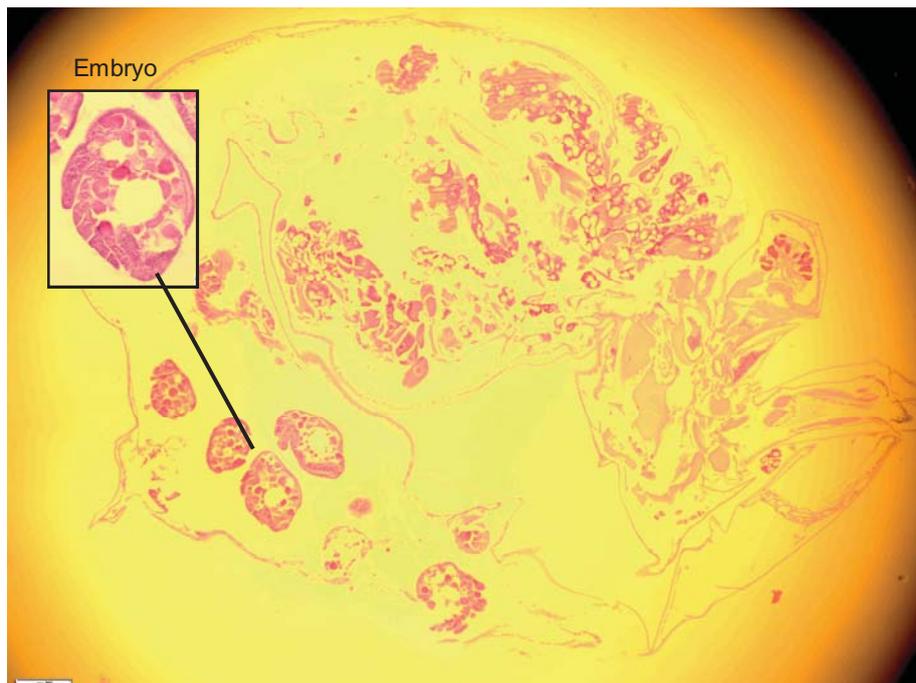
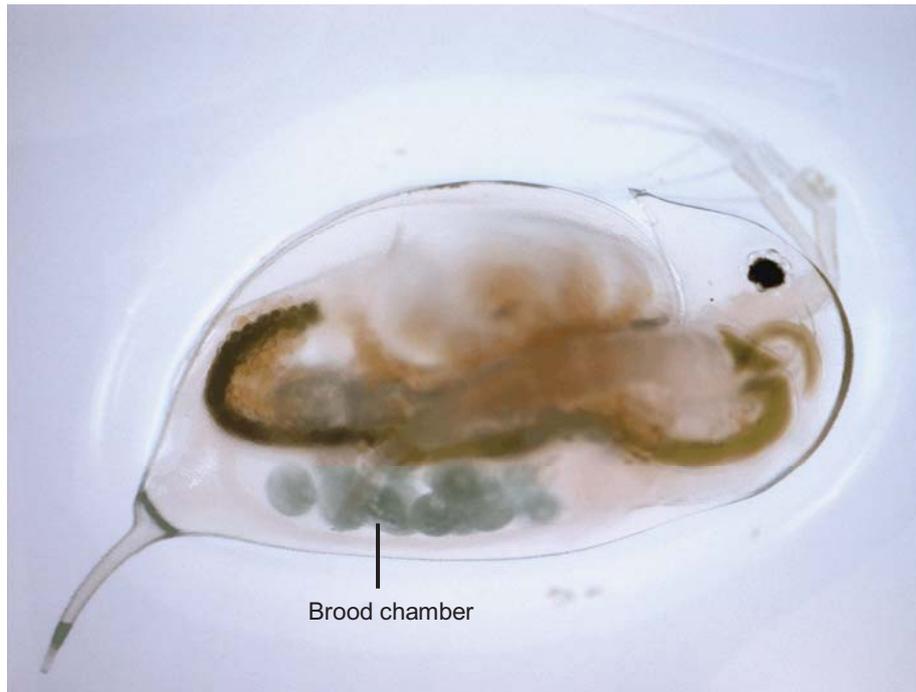


Plate 3.2. *Daphnia carinata* cultured at 23°C with developing embryos in the brood chamber. (Above) A few embryos had elongated and some had developed a rudimentary head lobe and body in the brood chamber. (Below) Some embryos had developed head lobe, body and antennary buds.

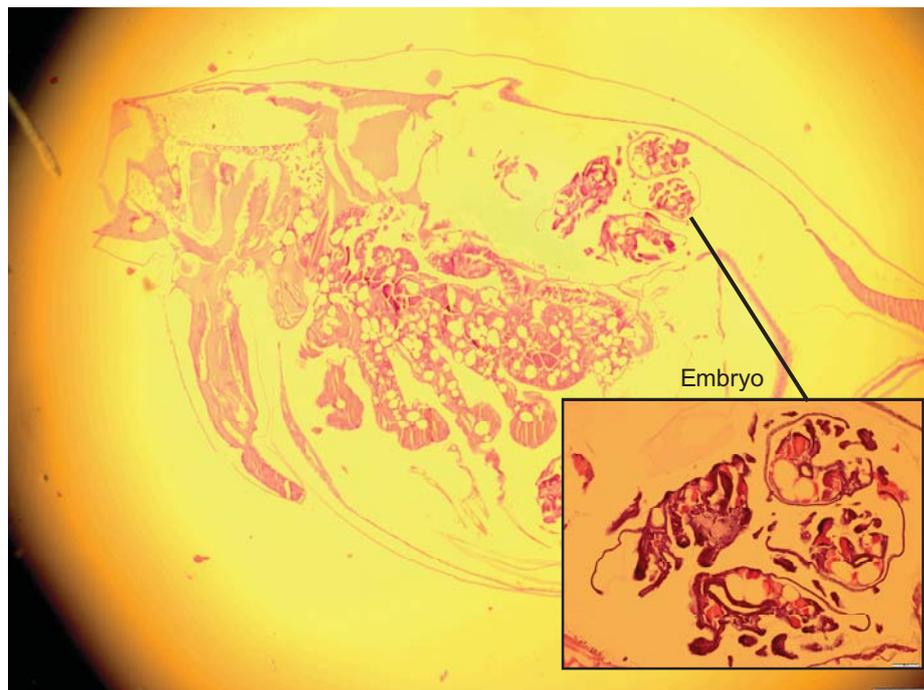
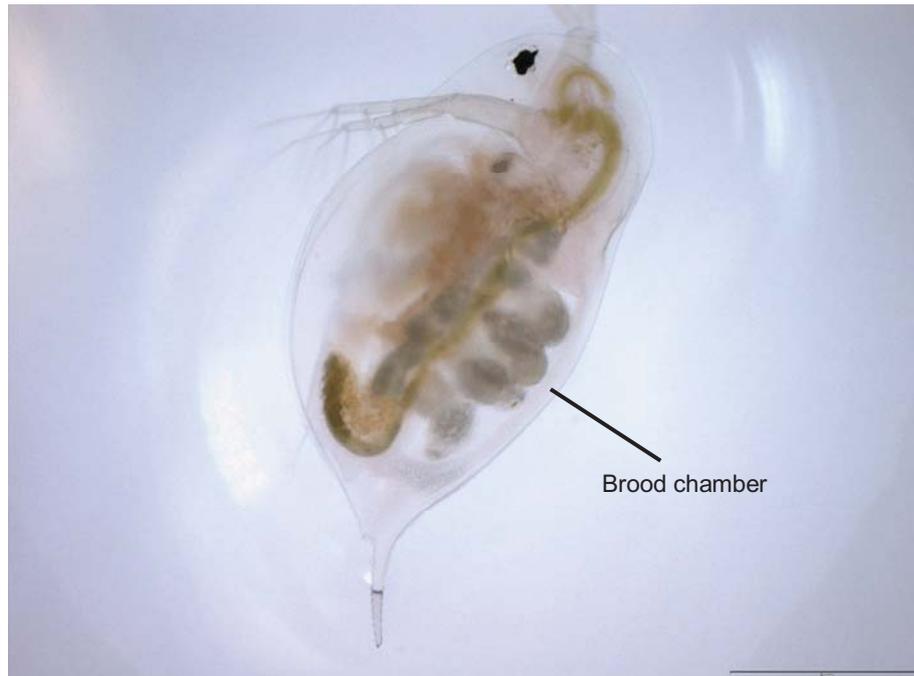


Plate 3.3. *Daphnia carinata* cultured at 30°C with developing embryos in the brood chamber. (Above) Embryos had distinct eye, head lobe and body in the brood chamber. (Below) Embryos had distinct head lobe, body and antennary buds.

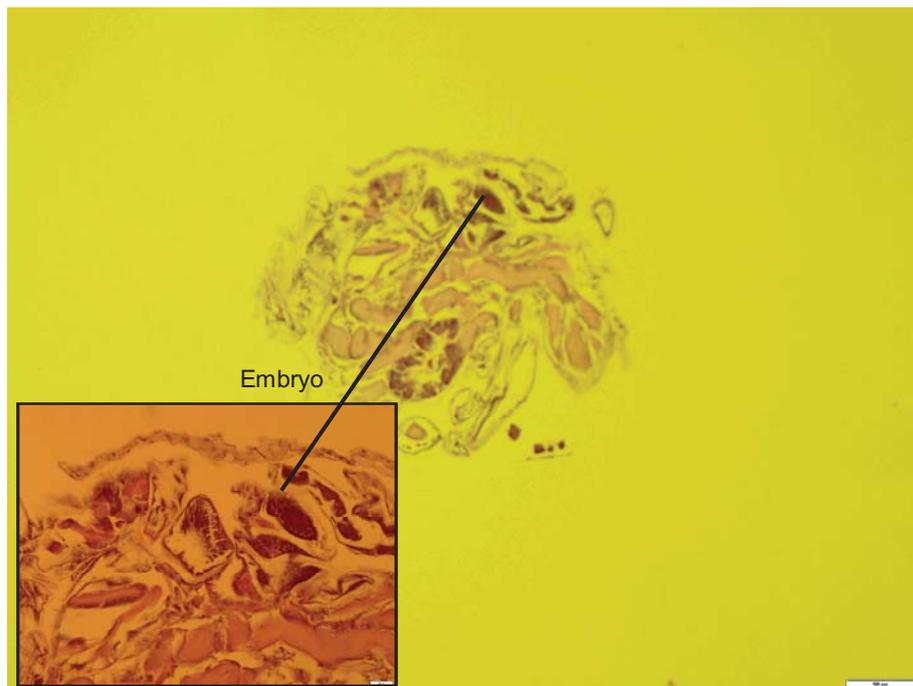


Plate 3.4. *Moina australiensis* cultured at 16°C with developing embryos in the brood chamber. (Above) The subitaneous eggs in the brood chamber. (Below) The embryo had attained an oval shape.

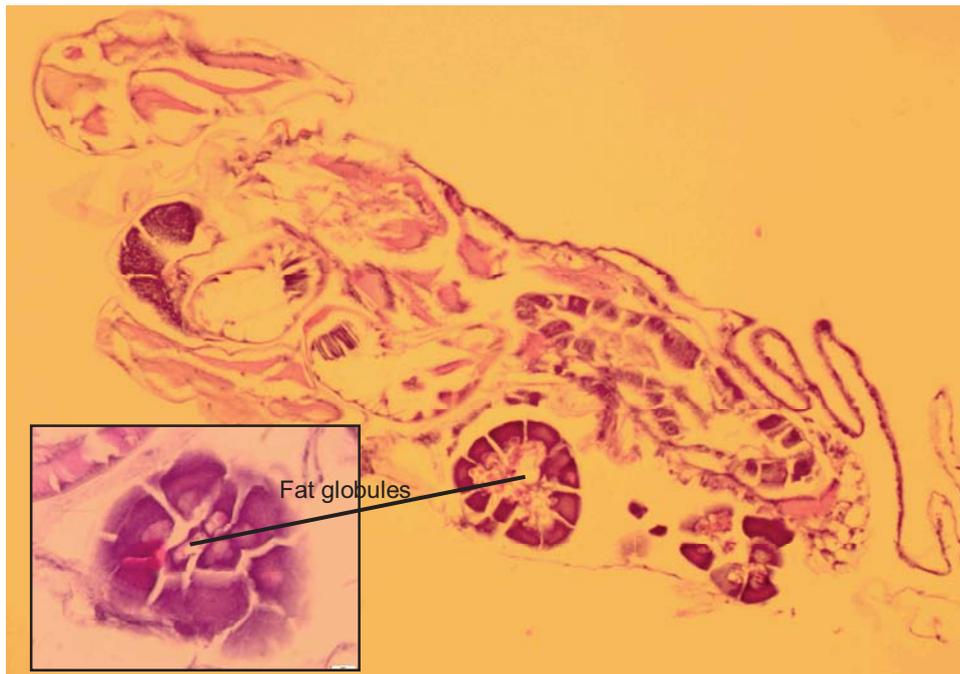


Plate 3.5. *Moina australiensis* cultured at 23°C with developing embryos in the brood chamber. (Above) Embryos had elongated in the brood chamber. (Below) Embryos had tightly packed with fat globules.

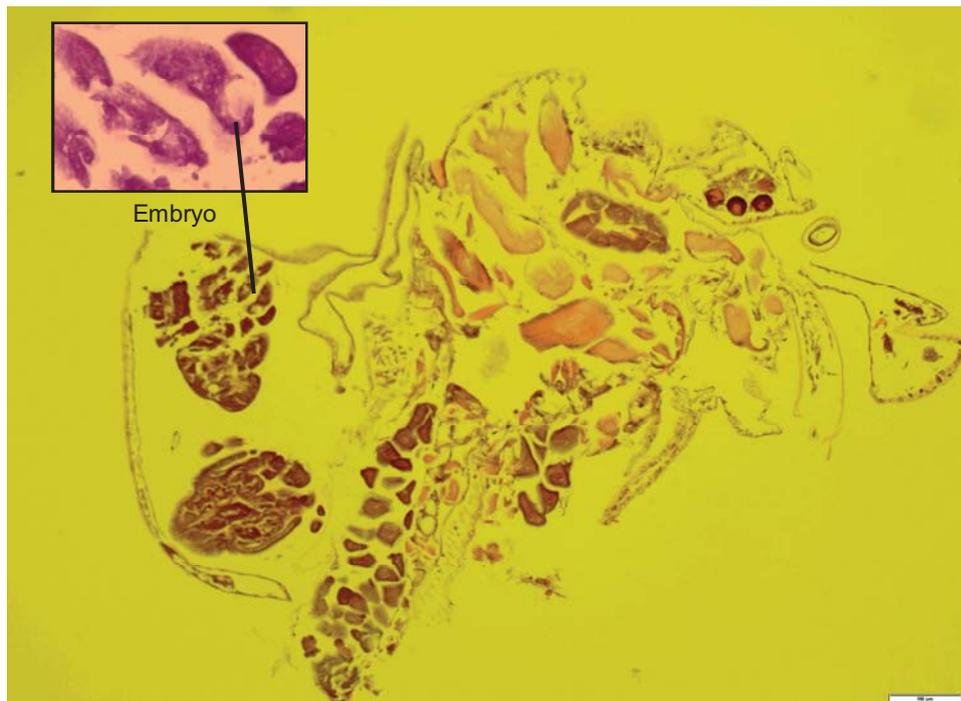


Plate 3.6. *Moina australiensis* cultured at 30°C with developing embryo in the brood chamber. (Above) The embryo had distinct eye, head lobe and body in the brood chamber. (Below) The embryo showed head lobe and body, and had tightly packed with fat globules.

#### 3.2.4.4. Effects of photoperiod on the time of first reproduction of *Daphnia carinata*

The time of first reproduction of *Daphnia carinata* was notably the shortest ( $P < 0.01$ ) with complete darkness (L:D = 0:24) (Table 3.6) which was about 14 hours earlier than other light treatments. However, there was no significant difference in time of first reproduction ( $P > 0.05$ ) between other light treatments (L: D = 10:14, 12:12, 14:10 and 24:0). The *D. carinata* with continuous illumination (L: D = 24:0) had shed their under developed eggs with the moulted carapace and died before releasing any neonate.

Table 3.6. Influence of photoperiod on time of first reproduction of *Daphnia carinata* and *Moina australiensis*

Photoperiod (L: D)	Time of first reproduction (hrs)	
	<i>Daphnia carinata</i>	<i>Moina australiensis</i>
0:24	188.20±4.37(20) <sup>b</sup>	69.60±0.97(20) <sup>b</sup>
10:14	202.68±2.97(19) <sup>a</sup>	72.50±1.31(20) <sup>a,b</sup>
12:12	205.39±2.90(18) <sup>a</sup>	73.05±0.30(20) <sup>a,b</sup>
14:10	203.72±3.60(18) <sup>a</sup>	74.00±1.47(20) <sup>a</sup>
24:0	No reproduction of egg	74.55±0.28(20) <sup>a</sup>

Values (mean±SEM) (no. of replicates) with different superscript letters in the same column were significantly different ( $P < 0.05$ )

#### 3.2.4.5. Effects of photoperiod on the time of maturity of *Moina australiensis*

There was a significant difference ( $P < 0.05$ ) between treatments in the time of maturity of *Moina australiensis* (Table 3.6). The complete darkness treatment (L: D = 0:24) had a shorter time required to achieve maturity than treatment with a constant photoperiod (L: D = 24:0) and L: D = 14:10. However the time required for maturity in treatment L: D = 10:14 and L: D = 12:12 was slightly ( $P > 0.05$ ) higher than the treatment with a constant dark period.

#### 3.2.5. Discussion

*D. carinata* and *M. australiensis* had better reproductive performance at 23°C in the anaerobic digested piggery effluent. Temperature influenced the reproduction performance of both species. The lower temperature (16°C, late autumn) and higher temperature (30°C, early summer) affected both species with lower ( $P < 0.05$ ) mean total fertility and number of clutches.

Zooplankton are an oviparous poikilotherm whose body temperature varies along with the ambient temperature (Anderson-Carnahan, 1994; Korpelainen, 1986; Munro & White, 1975). Within the temperature lethal limit, the egg development duration was inversely related to temperature (Bottrell, 1975; Burgis, 1970; Munro & White, 1975). Lower temperature delays maturation and prolongs egg incubation time (Bottrell, 1975;

Schwartz, 1984) hence resulting in lower number of clutches and total fertility during their lifetime.

The life duration of zooplankton was an inverse function of temperature (MacArthur & Baillie, 1929a; b). At high temperature, longevity of both species was reduced (Korpelainen, 1986; MacArthur & Baillie, 1929b) and alternatively the reproduction period was shortened. Therefore at high temperature (30°C) the total fertility and number of clutches of both species were lower ( $P < 0.05$ ) than at 23°C which could be considered the optimum temperature. *M. Australiensis* had higher productivity ( $P < 0.05$ ) than *D. carinata*. Temperature also influences zooplankton to produce resting eggs (Banta, 1939; Stross, 1969b). When the culture temperature was similar to late spring (30°C) or late autumn (16°C), some animals of both species carried ephippia in their brood chamber.

The time required to reach maturity of both species was remarkably lengthened ( $P < 0.01$ ) at low temperature (16°C) while the time of first reproduction was shortened ( $P < 0.05$ ) at high temperature (30°C) in *M. australiensis*. The embryonic development of both species also showed a delay at low temperature (16°C) but embryos had nearly formed the miniature of the adult at high temperature (30°C). The metabolic rate of *D. carinata* was doubled for every 10°C increase (MacArthur & Baillie, 1929a). Hence the growth rate of the animal increases with temperature (Korpelainen, 1986; MacArthur & Baillie, 1929a). Eventually earlier maturation, faster embryonic development and the shortened first time of

reproduction occurred at high temperature for both species in this study (Schwartz, 1984).

Moreover previous research showed that the duration of embryonic development was inversely proportional to temperature (Venkataraman & Job, 1980). Considerable variation in genetic constitution of different species would affect reproduction (MacArthur & Baillie, 1929b). This genetic variation could be attributed to the better reproductive performance of *M. australiensis* compared to *D. carinata*. Furthermore the small body size of *M. australiensis* is associated with a shorter time for egg development compared to the larger *D. carinata* (Bottrell, 1975).

The generation time ( $T$ ) of *D. carinata* and *M. australiensis* was notably shortened ( $P < 0.01$ ) at high temperature. Generation time was affected by survival ( $l_x$ ) of animals to age  $x$  as illustrated in the equation in the methods section (Pianka, 1999). Therefore survival was lowered when longevity of animals was reduced at high temperature (MacArthur & Baillie, 1929a; b). The maximum net reproductive rate ( $R_0$ ) of both species occurred at 23°C and was lower ( $P < 0.01$ ) at 16°C and higher at 30°C.

Similarly generation time is also related to the survival ( $l_x$ ) of animals to age  $x$  in determining the value. The intrinsic rate of increase ( $r$ ) of *M. australiensis* was high ( $P < 0.01$ ) at 30 °C but net reproductive rate was low ( $P < 0.01$ ). This was due to the short longevity and hence low survival rate in this treatment. In a previous study, data was collected at 25°C (Leung ,

unpublished) with intrinsic rates of increase observed for *D. carinata* being  $r = 0.25$  and *M. australiensis* being  $r = 0.52$ . These rates are lower than those observed at 23°C in the current study. Hence the long term effect on population growth of both species would be expected to be optimum at 23°C, which was equivalent to the temperature in late summer, early autumn and spring in effluent ponds in South Australia.

The time of first reproduction of *D. carinata* and *M. australiensis* was notably shorter during a constant dark period (L: D = 0:24). Longer daylight exposure lengthened the time of first reproduction of both species. However, the pH of the *D. carinata* culture with continued illumination was so high (pH > 9.2) that those eggs could not develop and shed with the exuviae. Since the animals were fed with green algae, photosynthesis of the non-consumed algae with continued illumination increased the pH of the culture medium (Kawasaki, *et al.*, 1982; Mulbry, *et al.*, 2008). At high pH in the effluent culture medium, the equilibrium of aqueous ammonia shifted to the right and increased the toxic un-ionized ammonia level (Proulx & de la Noue, 1985). Moreover, light also affected the metabolism of algae; thus it could be chemically different when used as a food source in the dark, compared to light (Green, 1956).

Previous research showed that photoperiod mainly affected survival, sex determination and resting egg production of zooplankton (Anderson-Carnahan, 1994; Korpelainen, 1986; Stross, 1969b; Stross & Hill, 1968). In some cases, photoperiod only influenced reproduction within a certain

temperature range (Stross, 1969b; Stross & Hill, 1968). Hence photoperiod effects on the time of first reproduction for both species were observed only when exposed to complete darkness in the current study.

### **3.2.6. Conclusion**

*M. australiensis* had higher productivity compared to *D. carinata*. In the piggery effluent bio-treatment process, *D. carinata* and *M. australiensis* could be expected to have the maximum productive performance and population growth when cultured at 23°C. Photoperiod had notable influences on the time of first reproduction of both species only with constant darkness. Hence further research is required to understand the interaction of photoperiod and temperature on sex determination and resting egg formation of both zooplankton species.

## **4. Induction, storage and hatching the ephippia of *Daphnia carinata* and *Moina australiensis***

### **4.1. Abstract**

The ephippia/resting egg of zooplankton could be used to initiate a population for indoor culture, for use as live feed for fish larvae or used as a bio-remediation tool to remove nutrients in effluent. In order to retain a stock of ephippia to provide starter cultures as required, it is important to understand the techniques required to induce ephippia (resting egg) production, storage and hatching. Induction of ephippia of *Daphnia carinata* and *Moina australiensis* was assessed by conducting studies with different photoperiods, temperatures and densities in a laboratory scale facility. In the case of *D. carinata* a short daylight (L:D = 8:16) at low temperature (20°C) and 8 -10 animals/20ml optimised the formation of ephippia. The same photoperiod and temperature (but a different density of 2 - 6 animals/20ml) also optimized the formation of ephippia in *M. australiensis*.

However, due to the high adaptability of these species, stimuli (photoperiod, temperature and density) could only be employed periodically. Sex ratio also influenced the formation of ephippia of both species, with a male to female ratio of 5:5 (i.e. 1:1) or 4:5 maximizing the induction of the ephippial brood. A reduction in the proportion of males decreased the percentage of ephippia formation. After release of ephippia, surviving *D.*

*carinata* (92%) and *M. australiensis* (70%) females took an average of 3.1 and 4 days to resume asexual reproduction and release the first clutch of asexual eggs. The ephippia of both species preferred storage in wet conditions. During embryonic development, the time required for a neonate to hatch after rupture of the outer member was 30 - 36 and 20 - 24 hours for *D. carinata* and *M. australiensis* respectively. In the current study, *M. australiensis* had a higher percentage of induced ephippial brood and cumulative hatching of ephippia compared to *D. carinata*. However, the survival percentage of *M. australiensis* was lower and the time to resume asexual reproduction was longer after release of ephippia.

## **4.2. Introduction**

Cyclic parthenogenesis can be employed by most cladoceran to maintain reproduction in an aquatic environment (Bell, 1982). Alternation between asexual and sexual reproduction allows the animal to restart its population from either a single female or one ephippium (resting eggs) (Kleiven, *et al.*, 1992). Asexual reproduction of zooplankton produces asexual (subitaneous) eggs which are incubated inside the brood chamber of the egg-carrying female and then develop into miniature adults by the time of release from the chamber.

Genders of the young are dependent on environmental factors. Sexual reproduction involves fertilisation of the ephippium / resting (recombinant) egg, which then usually enters embryonic diapause after release from the

ephippial carrying female (Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992). Sex ratio, or the presence of males in the population, is one of the main factors controlling the rate of sexual reproduction.

Two South Australian zooplankton species *Daphnia carinata* and *Moina australiensis*, which originated from local effluent ponds, were selected for this study. These species could be used as live feed for fish larvae or as bio-remediation tools to remove nutrients in the anaerobically digested piggery effluent (Leung, unpublished data). Although both species could be collected from the wild, the population is affected by seasonal change (Brambilla, 1982; Kobayashi, 1997). Hence mono-culture of each species is preferred. Culture can be started by hatching the animals directly from ephippia.

In order to retain a stock of ephippia to provide starter cultures as required, it is important to understand the conditions that will result in production of ephippia. Moreover, fish such as yellow perch and pumpkinseed sunfish are likely to select ephippial carrying females as food, which may be due to the visibility of the dark-pigmented ephippium (Mellors, 1975). Hence it is also useful to induce ephippial carrying females as live fish feed. After induction of ephippia, storage parameters such as temperature, moisture content and duration could also affect the hatchability of the ephippia.

No studies have investigated the interaction of the stimuli, photoperiod, temperature and density, on induction of ephippia or male formation in *Daphnia carinata* and *Moina australiensis*. Previous research mainly focused on the induction of ephippia using two or three stimuli by combining either temperature, water quality, photoperiod, density, food limitation, or maternal/ offspring environment (Alekseev & Lampert, 2001; Banta & Stuart, 1932; Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992; Stross, 1969a; Stross & Hill, 1965; Stuart & Cooper, 1932). Other research focused on the stimuli and techniques required to release the ephippia from diapause (Doma, 1979; Schwartz & Herbert, 1987; Stross, 1966); or the influence of storage temperature and duration on hatchability and hatching synchrony (Moreira dos Santos & Persoone, 1998). Again there has been no examination of the effects of interactions between storage conditions (dry or wet) and storage duration on the hatchability of ephippia. The objectives of this study were:

- a. To determine the effect of stimuli (photoperiod, temperature and density and their interaction) on the induction of ephippial brood,
- b. To find the influence of sex ratio on induction of ephippia,
- c. To evaluate the impact of storage conditions (dry or wet),
- d. To determine the effect of storage durations on hatchability of ephippia.
- e. To examine the sequence of development of the embryo during the hatching process,
- f. To further the understanding of reproduction after release of ephippia, such as the survival rate, the percentage of survivors which

can resume asexual reproduction, and the time required to release a new clutch of neonates were also investigated.

### **4.3. Methods**

#### **4.3.1. Collection and husbandry of stock zooplankton**

*D. carinata* and *M. australiensis* were collected from effluent ponds at The University of Adelaide, Roseworthy Campus, in South Australia and reared for six months in the laboratory using the following procedure. Temperature of the outdoor ponds at the Campus was recorded from winter to autumn to decide the temperatures to use in the laboratory experiment. The zooplankton (*D. carinata* and *M. australiensis*) were maintained in 70 l tanks with culture medium which was prepared as follows. Algae (*Chlorella vulgaris*) were supplied at 700,000 and 300,000 cells/ml (based on feeding to satiation) for *D. carinata* and *M. australiensis* respectively. 50% of the culture medium was changed every two days and 25% of biomass was harvested twice weekly. Temperature was maintained at  $23 \pm 1^{\circ}\text{C}$  with a reverse cycle air conditioner and photoperiod was set at L:D = 12:12 using triphosphor fluorescent lights with an light intensity of  $>2000$  lux.

#### **4.3.2. Preparation of culture medium**

The culture medium containing anaerobically digested piggery effluent was collected from the Integrated Biosystems (IBS) Laboratory's Bio-reactors at Roseworthy Campus. The effluent was anaerobically

digested in the primary thermophilic and acidogenic (55°C) stage digesters for a total of seven days of hydraulic retention time (HRT). It was then transferred through to the tertiary mesophilic (30°C ambient) digester stage, for a total of 20 days HRT. Before its experimental use, effluent was aerated for 24 hrs via an aquarium air stone diffuser and allowed to equilibrate with aerobic atmospheric conditions. The effluent was diluted with milli-Q water to un-ionized ammonia concentrations of 0.5 and 4.5 mg/L for the culture medium of *D. carinata* and *M. australiensis* respectively, as these were the optimum conditions for their reproduction previously obtained in a nutrient tolerance experiment (Leung, unpublished data).

The effluent was made up to the required un-ionized ammonia concentrations (UAN) based on the total nitrogen ammonia level, temperature and pH of the effluent as described in the equation:  $UAN = TAN / (10^{pK_a - pH + 1})$ , where  $pK_a = 0.09018 + 2729.92/T$ , and T is the temperature (Alabaster & Lloyd, 1980; Emerson, *et al.*, 1975). The total ammonia nitrogen (TAN) of the digested piggery effluent and the culture medium was determined by the phenate method as described in APHA (2005) prior to use.

#### **4.3.3. Preparation of algae**

The algae, *Chlorella vulgaris* were obtained from the IBS Laboratory Roseworthy Campus. These algae originated in and were isolated from the same effluent pond from which the zooplankton were collected. *C. vulgaris*

was raised on anaerobically digested piggery effluent using a 20 L carboy (Nalgene). The temperature was maintained at  $23 \pm 1^{\circ}\text{C}$  with a reverse cycle air conditioner and 24 hrs illumination using triphosphor fluorescent lights with an intensity  $>3000$  lux. The culture was aerated with an electromagnetic air pump (ACO-004 - The Age of Aquarium Pty. Ltd.). Algae were harvested while in the log phase at 6 - 8 days (approx. 6 million cells/ml) for feeding to both zooplankton species.

#### **4.3.4. Preparation of test animals**

Two batches (25 animals/batch for each species) of cyclic parthenogenetic and egg-carrying females ( $\geq$  3rd brood) from the stock culture were inoculated into individual containers for culture. Newly born neonates (<24 hrs old) of each species were separately isolated into a 30 ml container with 20 ml of culture medium. Female and male neonates were identified under a dissecting microscope (Olympus CZ61 microscope with ColorView I digital camera).

All newly born neonates were placed randomly into the incubators at  $23 \pm 0.5^\circ\text{C}$  and photoperiod was set at L:D = 12:12 using triphosphor fluorescent lights with an light intensity of  $>2000$  lux. After maturation and release of the first two broods, the adult females were inoculated according to each treatment for the induction of ephippia in order to avoid interference by maternal effects. Adult males were also inoculated to conduct the experiment to understand the impact of sex ratio on the formation of ephippia.

#### **4.3.5. Effects of photoperiod, temperature and density on the induction of ephippia**

Combinations of sixteen treatments were set up in the laboratory using different long and short daylight photoperiods (L:D = 16:8 and L:D =

8:16) at a light intensity > 2000 lux; temperature at 20 and  $26 \pm 0.5^{\circ}\text{C}$ ; and densities of 1, 6, 8 and 10 animals/20ml for *D. carinata* and 2, 6, 8 and 10 animals/20ml for *M. australiensis*. Three replicates per treatment were used for *D. carinata* and five replicates per treatment were included for *M. australiensis*. Algae (*Chlorella vulgaris*) at the rate of 350,000 and 150,000 cells/ml were supplied initially for *D. Carinata* and *M. australiensis*. In order to prevent the influence of parentage on survival and reproduction of the test animals, adult females in each container were selected from different broods (egg-carrying female); that is the first adult female of each replicate was from one brood while the second female was from another and so on.

All containers were located at random in the incubators (Thermoline - L-094 refrigerated incubator with diurnal light and temperature control) which were set with the assigned temperature and photoperiod. Dissolved oxygen (TPS - WP-82 dissolved oxygen meter), temperature and pH (Eutech instruments - pH510 – pH and temperature meter) were measured daily.

The test animals were counted every 24 hours for the formation of ephippial or non-ephippial broods and any newborns were removed from the container. The gender of new born neonates was determined by visual examination using a dissecting microscope (Olympus SZ61 microscope with Color View I digital camera).

The containers were air tight to minimize ammonia loss, carbon dioxide exchange and culture medium evaporation. The calculation of

ephippial brood percentage was based on the formation of ehippial brood of the second brood of the replicates (fourth brood of the individual) divided by the total brood released.

#### **4.3.6. Influence of sex ratio on the induction of ehippia**

Four treatments were set up at the Roseworthy IBS laboratory and comprised five replicates for both species with female to male ratio of 5:2, 5:3, 5:4 and 5:5; a photoperiod of short daylight (L:D = 8:16) at light intensity >2000 lux and temperature of  $20 \pm 0.5^{\circ}\text{C}$ . Algae (*Chlorella vulgaris*) at the rate of 350,000 and 150,000 cells/ml were supplied initially for *D. carinata* and *M. australiensis*. The neonates were inoculated into containers and placed randomly in incubators, as described in the previous experiment.

Environmental parameters such as dissolved oxygen, temperature and pH were monitored daily. Adult males were removed from each container after 24 hours. The type of brood (ephippial or non-ephippial) was recorded. The calculation of ehippia % was based on the formation of ehippia of the first brood of the replicates (third brood of the individual).

#### **4.3.7. The survival and reproduction after release of ehippia**

The ehippium carrying females from the previous experiment continued to be monitored to allow assessment of their production after

release of ephippium. The females that had laid ephippium were transferred to individual wells (NUNC 6-wells flat bottom culture plate) with 10 ml of culture medium in each well. Five females per replicate and ten replicates of each species were placed randomly into the incubator (Thermoline - L-094 refrigerated incubator with diurnal light and temperature control) at  $23 \pm 0.5^{\circ}\text{C}$  and photoperiod at (L: D = 12:12) with light intensity  $>2000$  lux. The culture medium was changed daily and algae were supplied at the rate of 350,000 and 150,000 cells/ml for *D. carinata* and *M. australiensis* respectively. Percentage survival and time to release the new clutch were counted.

#### **4.3.8. Hatching of ephippia and development of the embryo**

Ephippia of *D. carinata* and *M. australiensis* were collected from stock zooplankton tanks and were either kept wet (moist) or air dried. The dry or wet (moist) ephippia were then stored using a 10 ml light proof container at  $4 \pm 0.5^{\circ}\text{C}$  in the dark. The storage duration was no storage and 6 months storage for *D. Carinata* and no storage, 6 and 12 months storage for *M. Australiensis*.

Decapsulation of the ephippia followed the methods described in Moreira dos Santos and Persoone (1998). The ephippia were transferred into a 10 ml flat-bottomed tube filled with 10% NaOCL and 1M NaOH (3:1) for 15 minutes. The tube was screwed tight and a gently shaken. The

contents were filtered and washed with milli Q water in a 60 µm nylon mesh filter so as to remove trace chemicals on the ephippia.

Two eggs or a semi-opened ephippium (with two eggs inside) were inoculated into individual wells of each container (NUNC 12-wells flat bottom culture plate) with 5 ml of culture medium. Three replicates with 20 eggs were employed for each storage treatment (wet or dry storage) and duration. All replicates were placed randomly into the incubator (Thermoline - L-094 refrigerated incubator with diurnal light and temperature control) at  $23 \pm 0.5^{\circ}\text{C}$  with a photoperiod of (L:D = 16:8) at light intensity  $> 2000$  lux.

The culture medium was changed daily. Incubation of eggs was carried out for 12 days. Embryo development was examined twice daily under a dissecting microscope (Olympus SZ61 microscope with Color View I digital camera). When the outer membrane of the embryo started to stretch or burst, the embryo was monitored hourly and photos were taken (Olympus CX41 microscope with DP25 digital camera). The number of hatched eggs was counted and the 12-day cumulative hatching % was based on the number of hatched neonates divided by the total number of eggs incubated.

#### **4.3.9. Statistical analysis**

SPSS 15 Statistic Software for Windows (SPSS Inc. 2006) was used for the analysis of data. The effect of photoperiod, temperature and density on the percentage formation of ephippia (Section 4.3.5) and the effect of

storage conditions and duration on the mean cumulative hatching % (Section 4.3.8) were analyzed by two-way ANOVA. The effect of sex ratio on the % formation of ephippia (Section 4.3.6) was analyzed using one-way ANOVA. Tukey's test was used for all pairwise multiple comparison procedures. Percentage survival and time to release the new clutch (Section 4.3.7) was derived by descriptive statistics. All data was checked for normality and homogeneity of variance before analysis. The overall significance level was taken at  $P < 0.05$ .

## **4.4. Results**

### **4.4.1. Effects of photoperiod, temperature and density on the induction of ephippia**

There was a significant ( $P < 0.05$ ) interaction between photoperiod, temperature and density which affected the formation of ephippia in *D. carinata* and *M. australiensis* (Table 4.1). In the case of *D. carinata*, the percentage of ephippia was notably higher ( $P < 0.05$ ) at optimum conditions, which was short daylight (L:D = 8:16) and low temperature (20°C, late autumn temperature) with density of 8 or 10 animals/20 ml. In the case of *M. australiensis*, optimum conditions were similar to those of *D. carinata*, but the density was different (2 or 6 animals/20 ml).

Under optimum conditions, *D. carinata* produced 11% or more ephippia compared to other treatments ( $P < 0.05$ ), while *M. australiensis* released 10% or higher ( $P < 0.05$ ) ephippial broods than other treatments. At

short daylight (L:D = 8:16) and low temperature (20°C), induction of ephippia increased at densities of 8 or 10 animals/20ml for *D. carinata* but decreased for *M. australiensis*.

In this study, the maximum induction of ephippial broods was higher ( $P < 0.05$ ) in *M. australiensis* (80%) than *D. carinata* (50%). The induction of ephippia in both species was also higher ( $P < 0.05$ ) at low temperature (20°C) with the same photoperiod and density or at short daylight (L:D = 8:16) with the same temperature and density.

In the case of *M. australiensis*, a few empty ephippial brood carrying females were observed with short daylight (L:D = 8:16) and low temperature (20°C) with high density (10 animals/20 ml). These empty broods were not included in the analyses. During the copulation process, the small sized adult male mounted onto the anterior of the female carapace (Plate 4.1). Mortality of the males during this process was observed, which may be due to a continuous kicking motion of the sharp claw of postabdomen of the female, causing injury to the male. The healthy (undamaged) males remained alive until the end of the experiment.

The pH of treatments on the long daylight (L:D 16:8) and high temperature (26°C) was above 9.3 for both species. Animals acclimatised to the culture conditions in twelve days for *D. carinata* and six days for *M. australiensis* and both species started to carry neonates in their brood chamber.

Table 4.1. Effects of three stimuli on ephippia formation of *D. carinata* and *M. australiensis*

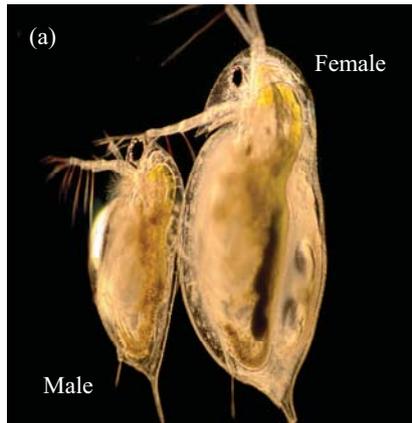
Photoperiod	Temperature (°C)	<i>D. carinata</i>		<i>M. australiensis</i>	
		Density (animals/20ml)	Ephippia (%)	Density (animals/20ml)	Ephippia (%)
Long daylight (L:D = 16:8)	20	1	0	2	0
		6	5.56	6	53.33
		8	20.83	8	45.00
		10	26.67	10	64.00
Long daylight (L:D = 16:8)	26	1	0	2	0
		6	0	6	20.00
		8	8.33	8	45.00
		10	6.67	10	18.00
Short daylight (L:D = 8:16)	20	1	0	2	80.00
		6	38.89	6	80.00
		8	50.00	8	70.00
		10	50.00	10	64.00
Short daylight (L:D = 8:16)	26	1	0	2	0
		6	0	6	20.00
		8	12.50	8	55.00
		10	36.67	10	36.00

*Two-way ANOVA:*

Photoperiod x temperature	$P = 0.025$	$P < 0.001$
Photoperiod x density	$P = 0.015$	$P = 0.010$
Temperature x density	$P = 0.014$	$P = 0.003$
Photoperiod x temperature x density	$P = 0.042$	$P = 0.001$

Ephippia % calculation was based on the formation of ephippia on the total number of brood produced per replicates. Overall significant level = 0.05

*D. carinata*



*M. australiensis*

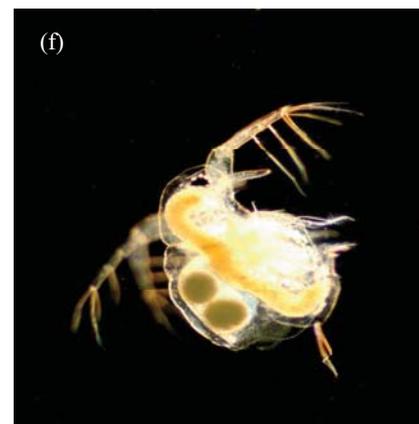
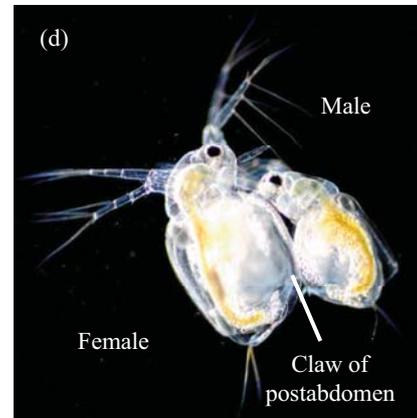
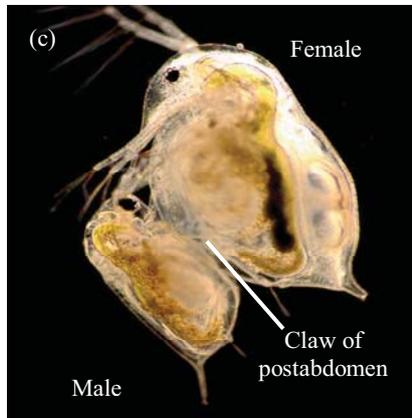
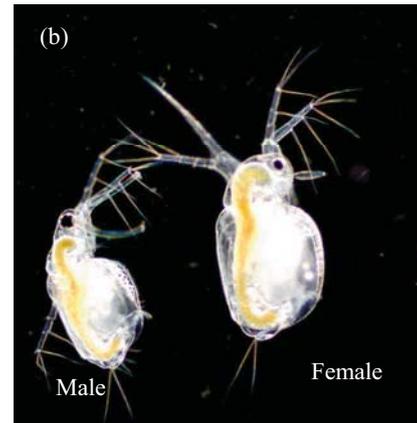


Plate 4.1. (a, c and e) *Daphnia carinata*; (b, d and f) *Moina australiensis*; (a and b) male and female morphology; (c and d) during the process of copulation, the male is mounted on the anterior of the female. (e and f) female carrying ephippium following copulation.

#### 4.4.2. Influence of sex ratio on the formation of ephippia

The formation of ephippial broods in *D. carinata* and *M. australiensis* was significantly ( $P < 0.05$ ) related to the male to female ratio. The production % of ephippia was positively correlated with the male to female ratio ( $P < 0.05$ , Fig 4.1). There was no significant difference ( $P > 0.05$ ) in ephippia production between treatments with a male to female ratio of 4:5 and 5:5. This indicates that the minimum male to female ratio required in the culture to trigger the maximum ephippial brood formation was 4:5. Some empty ephippia of *D. carinata* and *M. australiensis* were released when the male to female ratio was 2:5 and 3:5, and were not included in the analyses.

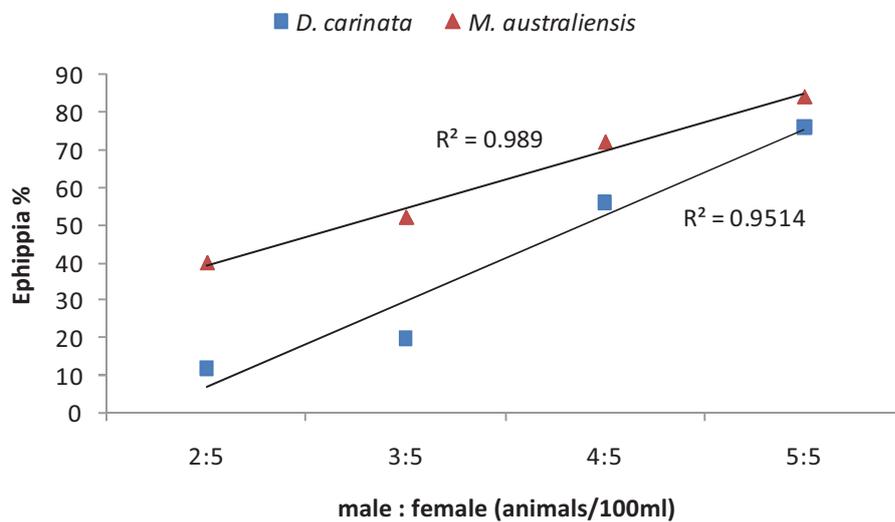


Fig. 4.1 Influence of sex ratio on the formation of ephippia of *Daphnia carinata* and *Moina australiensis*. Ephippia % calculation was based on the formation of ephippia on the total number of brood produced per replicate (5 replicates per treatment)

#### 4.4.3. The survival and reproduction after release of ephippia

There was a high mean survival of *D. carinata* (92%) and *M. australiensis* (70%) after release of ephippia (Table 4.2). All survivors of both species resumed asexual reproduction. It took an average of 3.1 and 4.0 days for *D. carinata* and *M. australiensis* to produce a new clutch of neonates after release of ephippia (either first or second ephippia in *Daphnia*). In the case of *D. carinata*, 10% of the animals laid the second ephippial brood and another 10% moulted the under developed egg before resuming parthenogenetic reproduction.

Table 4.2. Survival and reproduction of *D. carinata* and *M. australiensis* after release of ephippia.

Items	<i>D. carinata</i>		<i>M. australiensis</i>	
	Survival % after release ephippia	Time to release a new clutch (days)	Survival % after release ephippia	Time to release a new clutch (days)
Mean	92.00	3.09	70.00	4.00
SEM	6.11	0.18	7.45	0
SD	19.32	0.58	23.57	0
Minimum	40.00	2.00	20.00	4.00
Maximum	100.00	4.20	100.00	4.00

10 replicates per species; SEM: standard error of mean; SD: standard deviation

#### 4.4.4. The hatching process and development of the embryo

The ephippium of *D. carinata* and *M. australiensis* was carried inside the brood chamber for 2 - 3 and 3 - 4 days respectively before release (Plate 4.1c and 4.1f). Resting eggs released by females of both species could enter the diapause stage as shown in Plate 4.2a and 4.2d. The resting eggs of *D. carinata* were shielded from light by a dark pigmented capsule while the eggs of *M. australiensis* were semi-transparent through the ivory-white coloured capsule.

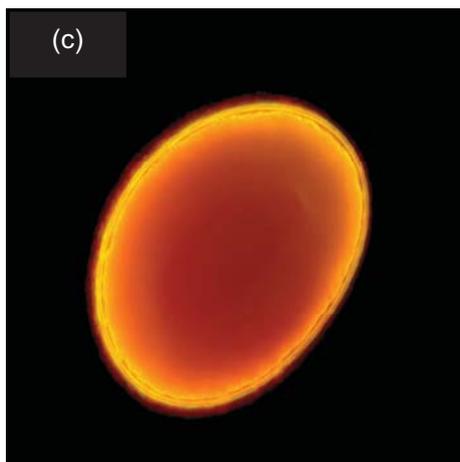
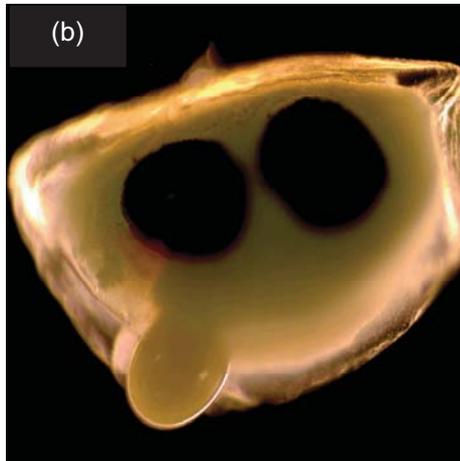
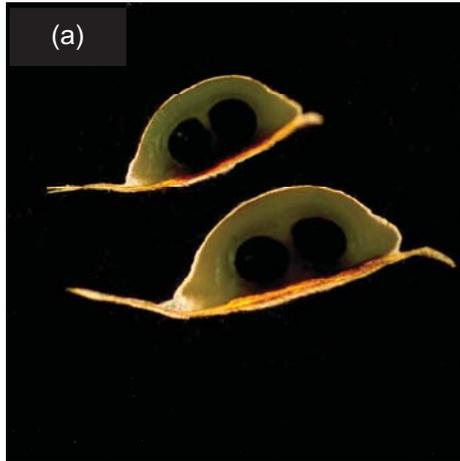
After decapsulation, most of the resting eggs of *D. carinata* and *M. australiensis* were exposed or cast off from the ephippium or remained inside an opened capsule (Plate 4.2b and 4.2e). The embryo of both species was encased inside membrane layers of the oval shaped resting eggs (Plate 4.2c and 4.2f). The flexible inner membrane was continuously stretched when the embryo elongated and started to develop a head and trunk, and later antennary buds (Plate 4.3a and 4.3f). The pressure exerted by the expanding inner membrane pushed the outer member aside on both ends of the resting egg (Plate 4.3f and 4.3g).

The embryo of *D. carinata* and *M. australiensis* continued to develop with distinct antenna and then limbs inside the inner membrane whether the resting eggs were laid at the bottom of the container or remained inside an opened capsule (Plate 4.3b, 4.3c, 4.3g and 4.3h). The developing embryo

regularly twitched and changed position inside the membrane with their antenna and limbs.

Finally the vigorous twitching motion of the mature embryo ruptured the membrane and the new neonates hatched (Plate 4.3d and 4.3i). The new born neonates of *D. carinata* and *M. australiensis* exhibited jerking movements for several minutes before they could stretch their antenna, limbs and caudal spine/setae to become the miniature of the adult and start swimming freely (Plate 4.3e and 4.3k). The time required for a neonate to emerge from the outer membrane was 30 - 36 and 20 - 24 hours for *D. carinata* and *M. australiensis* respectively.

Ephippium of *Daphnia carinata*



Ephippium of *Moina australiensis*

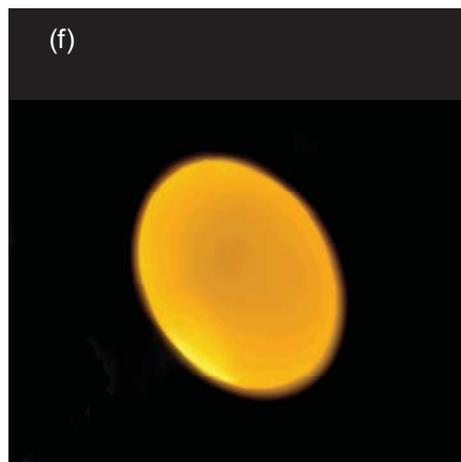
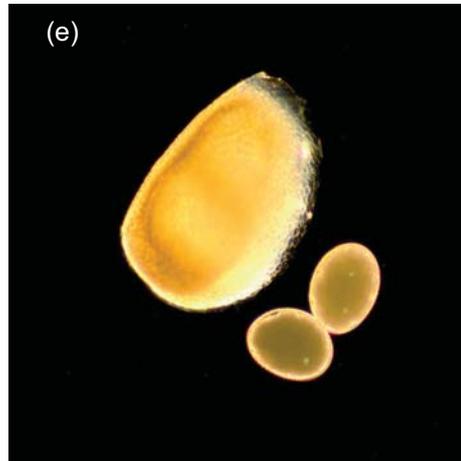
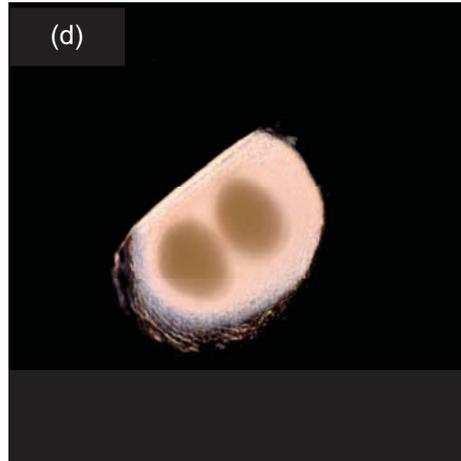
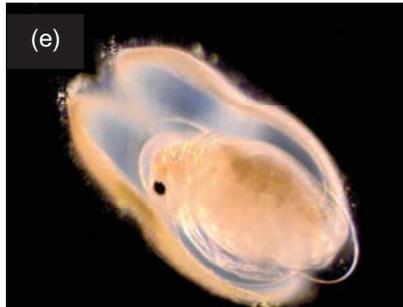
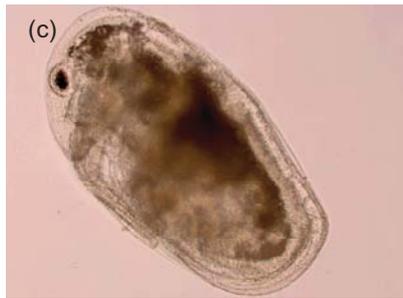


Plate 4.2. Ephippium of *Daphnia carinata* (a, b and c) and *Moina australiensis* (d, e and f). (a) Ephippia of *D. carinata* with anterior spine and dark-pigmented capsule; (d) ephippium of *M. australiensis* with two resting eggs inside the semi-transparent ivory-white capsule; (b and e) resting eggs exposed or casted off from the capsule after decapsulation; (c and f) embryo with deeper shade inside the oval shaped resting egg.

*Daphnia carinata*



*Moina australiensis*

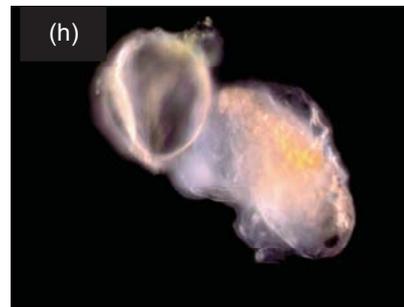


Plate 4.3. Hatching of *Daphnia carinata* (a, c, e, g and i) and *Moina australiensis* (b, d, f, h and k). (a and b) elongated embryo burst from the outer membrane, encased within the inner membrane of the resting egg, showing developed head; (c and d) mature embryo with head, limbs and antennae, inside the inner membrane and ready to hatch; (e and f) mature embryo inside the inner membrane but remaining in an opened capsule and ready to hatch; (g and h) hatching of the new born neonate occurs by rupture of the inner membrane; (i and k) newly hatched female neonate moving freely.

#### **4.4.5. The influences of storage conditions and duration on hatching of ephippia**

In the case of *D. carinata* ephippia, the mean 12-days cumulative hatching percentage (hatchability) was higher ( $P<0.01$ ) with wet storage conditions with either no storage or 6 month storage (Table 4.3). The hatchability under wet storage conditions was increased four fold compared to storage for no storage or 6 months under dry conditions. The hatchability was also higher following 6 months storage under either dry or wet conditions ( $P<0.01$ ). The interaction effect of storage conditions and duration significantly ( $P<0.05$ ) affected the 12-day mean cumulative hatching percentage, but not the mean hatching time ( $P=0.72$ ) of the *D. carinata* ephippia.

In the case of *M. australiensis* ephippia, the 12-days mean cumulative hatching percentage was notably higher ( $P<0.01$ ) for wet storage, when compared to the same storage duration under dry conditions (Table 4.3). The hatchability was two fold higher with wet storage compared to dry conditions for no storage or 6 months storage. The hatchability was also higher ( $P<0.01$ ) following 6 months storage under either dry or wet condition compared to no storage or 12 months storage. Hatchability of ephippia stored for 12 months in wet conditions was higher than 12 months dry storage ( $P<0.01$ ). However, storage for 12 months reduced hatchability, compared to no storage and 6 months storage in both dry and wet conditions. The interaction of storage conditions and duration significantly ( $P<0.01$ )

affected the 12-day mean cumulative hatching percentage, but not ( $P=0.24$ ) the mean hatching time of *M. australiensis* ephippia.

The 12-day mean cumulative hatching percentage for ephippia of *M. australiensis* was higher ( $P<0.01$ ) compared to *D. carinata* under the same storage conditions and duration.

Table 4.3. Influence of storage conditions and durations on the cumulative hatching percentage and hatching time of ephippia of *D. carinata* and *M. australiensis*.

Storage conditions	<i>D. carinata</i>			<i>M. australiensis</i>		
	Storage durations	Cumulative hatching (%)	Hatching time (day)	Storage durations	Cumulative hatching (%)	Hatching time (day)
Dry	0*	1.67±1.67	3.67±3.67	0*	41.67±6.67	8.74±0.19
	6 months	3.53±0.21	6.83±0.33	6 months	43.33±8.82	8.91±0.36
				12 months	23.33±1.67	9.22±1.02
Wet	0*	6.67±1.67	3.00±0.00	0*	80.00±10.41	8.51±0.29
	6 months	15.36±1.58	4.79±0.48	6 months	98.33±1.67	7.15±0.34
				12 months	28.33±1.67	9.15±0.49

The cumulative hatching percentage (mean ±SEM) was based on the number of living hatchings divided by the number of resting eggs incubated in each replicate (3 replicates per treatment).

\*0 storage durations means no storage

## 4.5. Discussion

A photoperiod comprising short daylight (L:D = 8:16), low temperature (20°C) and with different densities were the main factors that induced more than 50% induction of ehippial broods under experimental conditions. In a natural pond system, exposure of zooplankton to seasonal variation in temperature and daylight affects sexual reproduction of ehippia (Banta, 1939). In laboratory culture, the effects of combined stimuli such as photoperiod and density (Stross & Hill, 1965) or photoperiod and temperature (Korpelainen, 1986; Stross, 1966) has been used to induce the formation of males or ehippia in *Daphnia pulex*. Moreover, the combined stimuli of food deficiency and density (Doma, 1979) or food deficiency with chemically mediated crowding and photoperiod (Kleiven, *et al.*, 1992) were successfully used to induce ehippia in *Daphnia magna*.

In the current study, the three stimuli of photoperiod, temperature and density were successfully used to induce ehippia in *D. carinata* and *M. australiensis*. However, the specific effects of density dependent factors, such as food deficiency and high metabolites in culture water, were reported not to be easily separated (Hobæk & Larsson, 1990). Hence, more than three stimuli may be involved in the induction of ehippia in the current study.

However, due to the high adaptability of these animals, stimuli could only be employed periodically. Therefore it is preferable to maintain a number of cultures with populations at different development stages, so that

these stimuli could be imposed on different culture batches to obtain continuous ehippia production.

The treatments with high temperature (26°C) were less effective in inducing ehippia. This could be due to thermal uncoupling of photoperiod control (Stross, 1969b). Similar results were observed in *M. australiensis*. This phenomenon also observed in *Daphnia pulex* (Stross, 1969b) and *Daphnia magna* (Kleiven, *et al.*, 1992). The empty ehippia in *M. australiensis*, observed under certain conditions in the current study, were due to the disintegration of the unfertilized eggs (Wood, 1932). Adult male *D. carinata* and *M. australiensis* were smaller and reached maturity earlier than the female. Males may require less energy to become adult than a female of the same age (MacArthur & Baillie, 1929a).

The role of the males was to fertilize the female egg of ehippial broods, hence males have been reported to have a relatively greater longevity than females when cultured at high density (MacArthur & Baillie, 1929b), and similar results were observed in the current study. Moreover, food limitation in high density culture may affect the formation of sexual eggs in the female, while the influence was relatively low in males as the gametes were small. There was a high chance for the adult males to fertilize the sexual eggs of older females which belonged to the previous generation. Mortality of males after copulation could have been caused by bacterial infection of wounds.

The sex ratio of *D. carinata* and *M. australiensis* in the culture was directly related to the formation of ephippia. In order to trigger more than 50% of their broods to produce ephippia, the culture environments should have a male to female ratio of 4:5 (44.4% male) and 3:5 (37.5% male) for *D. carinata* or *M. australiensis* respectively. The formation of empty ephippia at lower ratios could be attributed to insufficient numbers of males to fertilize the eggs.

In order to trigger the formation of males, timing is critical for sex determination of the neonates which occurs a few hours before the asexual eggs are transferred to the brood chamber (Banta & Brown, 1929). Hence maternal and environmental effects also influence the formation of male neonates (Banta & Stuart, 1932; Kleiven, *et al.*, 1992). The sex ratio adjustment is dependent on the stimulus intensity, and should fluctuate about the threshold value (Hobæk & Larsson, 1990; Kleiven, *et al.*, 1992). This is due to the high adaptability of zooplankton to acclimatize to the culture conditions easily. Although density dependent factors are stimuli known to commonly cause a change from asexual to sexual reproductive mode (Bell, 1982; Stuart & Cooper, 1932) among all other stimuli, photoperiod was the key factor (Kleiven, *et al.*, 1992).

*D. carinata* and *M. australiensis* maintained their vigour and healthy growth after release of the ephippia. Both species required less than 4 days to resume parthenogenetic reproduction when provided with the optimum culture conditions. Some *D. carinata* did take longer to recover which may

be imposed by their genetic differences (Murugan & Sivaramakrishnan, 1976).

The ephippium of *D. carinata* and *M. australiensis* consisted of a capsule (outer and inner cuticle layer), outer and inner embryo membrane, and embryo (Kawasaki, *et al.*, 2004). The dark pigmented ephippium of *D. carinata* may imply that the resting eggs inside the capsule could be more sensitive to light than *M. australiensis*. The decapsulation procedure allowed the opening of the capsule. During the process, some of the eggs were washed out from the capsule. However, most of the eggs would remain in the open capsule, ready to be hatched.

After rupture of the outer embryo membrane, the inner embryo membrane became a permeable layer (Davis, 1959). The elongation and physical movement of the developing embryo could stretch the inner membrane. Moreover, it has been reported that there is an osmotic pressure build up inside the cell, such that the permeable inner membrane is considerably expanded due to the osmotic entry of water (Davis, 1959; Marshall & Orr, 1954; Selvaraj, 1979). The increase in osmotic pressure may be caused by the metabolic waste and other secretions from the developing embryo (Davis, 1959).

The caudal spine of *D. carinata* was bent along the ventral side of the embryo inside the inner membrane and straightened only after hatching, which resembles the developing embryo in the parthenogenetic egg

(Murugan & Venkataraman, 1977). After hatching, the newly born neonates exhibited jerking movements that removed the ruptured membrane, and set the antennae and limbs free for swimming (Selvaraj, 1979).

In the current study, ephippia of *D. carinata* and *M. australiensis* preferred storage in wet conditions. The hatchability of both species was notably higher when kept in wet conditions with the same storage duration. Similar results were reported by Wood and Banta (1937). Both species could be hatched with no storage (0 storage), but needed to be maintained in wet conditions after release. The hatchability of *M. australiensis* ephippia decreased with prolonged storage (12 months) in either dry or wet conditions. Similar results were reported by Moreira dos Santos and Persoone (1998) and Wood and Banta (1937).

#### **4.6. Conclusion**

The stimuli, photoperiod, temperature and density had an interactive effect on induction of ephippia by *D. carinata* and *M. australiensis*. However, due to the high adaptability of these animals, stimuli could only be employed periodically and also kept fluctuating around the lethal limit to prevent the zooplankton acclimatising to the culture conditions.

In order to maintain a continuous ephippia production, it is preferable to maintain a number of cultures with populations at different development stages so that these stimuli could be used for a different culture. The male to

female ratio of 5:5 (i.e. 1:1) or 4:5 was ideal to optimize the production of ephippial broods. The increase in male proportion directly increased the percentage of ephippia formation. After release of ephippium, *D. carinata* has a higher survival rate and shorter time to resume sexual reproduction.

Ephippia of both species could be stored, with wet storage conditions preferred as it leads to higher hatchability. In the current study, *M. australiensis* had a higher percentage of induced ephippial brood and cumulative hatching of ephippia compared to *D. carinata*. However, the survival percentage of *M. australiensis* was lower and the time to resume asexual reproduction was longer after release of ephippia.

## **5. The influence of feeding status of *Daphnia carinata* and *Moina australiensis* on use of nutrients in piggery effluent**

### **5.1. Abstract**

Utilization of resources from agricultural waste using zooplankton was evaluated by selecting two South Australian zooplankton species, *Daphnia carinata* and *Moina australiensis*, which have been found in effluent ponds. They were examined for the nutrient utilization potential by culturing in anaerobically digested piggery effluent. The reduction in total nitrogen and soluble phosphate in the effluent during the culture period was measured. In order to determine the efficiency of nutrient utilization by fed and non-fed animals, algae (*Chlorella vulgaris*) was supplied as food. To further understand the ability of the two zooplankton species to utilize nutrient loads, bio-chemical oxygen demand (BOD<sub>5</sub>) was also measured.

*D. carinata* and *M. australiensis* had the potential to utilize nutrients in treated piggery effluent. Moreover, the non-fed animals utilized higher levels of nutrients (TN and SP) compared to fed animals. In the case of *D. carinata* (non-fed), TN, SP and BOD<sub>5</sub> were reduced by 18.8%, 18% and 60%, while in the case of *M. australiensis* (non-fed), TN, SP and BOD<sub>5</sub> were reduced by 16.9%, 12.3% and 64.5% respectively. The mean reduction in BOD<sub>5</sub> provided further evidence that both species could utilize nutrients.

Hence *D. carinata* and *M. australiensis* have the potential to be used for nutrient utilization in digested piggery effluent. The efficiency of nutrient reduction by fed and non-fed animals showed that the feeding status had a notable effect on nutrient utilization.

## 5.2. Introduction

At present, agricultural waste has an increase in levels of nitrogen (N) and phosphorus (P), which are the main factors affecting eutrophication of lakes and rivers (Leeben, *et al.*, 2008). In order to mitigate such impacts, the utilization of the nutrient loads (N and P) from agricultural effluent is essential.

Among several wastewater treatment systems, artificial food web manipulation (an integrated wastewater treatment system) is a low cost ecological technological approach in areas without land constraint (Kibria, *et al.*, 1999; Shiny, *et al.*, 2005). In this treatment process, zooplankton are also employed (Geyer, *et al.*, 1991; Jung, *et al.*, 2009; Kim, *et al.*, 2003). This process is also an effective method to produce a low cost but nutrient rich zooplankton as live feed for fish (Kawasaki, *et al.*, 1982; Kibria, *et al.*, 1999; Kim, *et al.*, 2003).

Although *Daphnia magna* has been shown to utilize nutrient loads and biochemical oxygen demand in treated effluent (Julli, *et al.*, 1990; Kim, *et al.*, 2003; Shiny, *et al.*, 2005), it is not abundant in Australian conditions. Hence it is important to identify a South Australian zooplankton species

which can serve as a suitable tool to utilize nutrients from agricultural effluent. The effluent ponds in South Australia are dominated by *Daphnia carinata* and *Moina australiensis* and these species are highly adaptable to the local weather (Julli, *et al.*, 1990). Therefore, they were assessed for the suitability and efficiency in a nutrient utilization study.

The objectives of this experiment were:

- a. To examine utilization of nutrients in anaerobically digested piggery effluent by both zooplankton species,
- b. To determine the efficiency of nutrient utilization by animals with algae supplied (fed animals) and animals with no algae supplied (non-fed animals), and
- c. To provide further evidence that both zooplankton species are able to reduce nutrient loads in the form of bio-chemical oxygen demand (BOD<sub>5</sub>).

## **5.3. Method**

### **5.3.1. Collection and husbandry of stock zooplankton**

*Daphnia carinata* and *Moina australiensis* were collected from effluent ponds at The University of Adelaide, Roseworthy Campus in South Australia and reared for six months in the laboratory using the following procedure. Temperature of the outdoor ponds at the Campus was recorded from winter to autumn to decide the temperatures to use in the laboratory experiment. The zooplankton (*D. carinata* and *M. australiensis*) were maintained in 70 l tanks with culture medium which was prepared as follows. Algae (*Chlorella vulgaris*) were supplied at 700,000 and 300,000 cells/ml (based on feeding to satiation) for *D. carinata* and *M. australiensis* respectively. 50 % of the culture medium was changed every two days and 25% of biomass was harvested twice weekly. Temperature was maintained at  $23 \pm 1^\circ\text{C}$  with a reverse cycle air conditioner and photoperiod was L:D = 12:12 using triphosphor fluorescent lights with a light intensity of >2000 lux.

### **5.3.2. Preparation of culture medium**

The culture medium containing anaerobically digested piggery effluent was collected from the Integrated Biosystems (IBS) Laboratory's Bio-reactors at Roseworthy Campus. The effluent was anaerobically

digested in the primary thermophilic and acidogenic (55°C) stage digesters for a total of seven days of hydraulic retention time (HRT). It was then transferred through to the tertiary mesophilic (30°C ambient) digester stage, for a total of 20 days HRT. Before its experimental use, effluent was aerated for 24 hours via an aquarium air stone diffuser and allowed to equilibrate with aerobic atmospheric conditions.

The effluent was diluted with milli-Q water (ultra de-ionised water) to un-ionized ammonia concentrations of 0.5 and 4.5 mg/l for the culture medium of *D. carinata* and *M. australiensis* respectively, as these were the optimum conditions for their reproduction previously obtained in a nutrient tolerance experiment (Leung, unpublished data). The effluent was made up to the required un-ionized ammonia concentrations (UAN) based on the total nitrogen ammonia level, temperature and pH of the effluent, as described in the equation:  $UAN = TAN / (10^{pKa-pH+1})$ , where  $p^{Ka} = 0.09018 + 2729.92/T$ , and T is the temperature (Alabaster & Lloyd, 1980; Emerson, *et al.*, 1975). The total ammonia nitrogen (TAN) of the digested piggery effluent and the culture medium was determined by the phenate method, as described in APHA (2005) prior to use.

### **5.3.3. Preparation of algae**

The algae, *Chlorella vulgaris* were obtained from the IBS laboratory at Roseworthy Campus. These algae originated in and were isolated from the same effluent pond from which the zooplankton were collected. *C.*

*vulgaris* was raised on anaerobically digested piggery effluent using a 20 L carboy (Nalgene) in the IBS laboratory. Temperature was maintained at  $23 \pm 1^\circ\text{C}$  with a reverse cycle air conditioner and 24 hrs illumination using triphosphor fluorescent lights with an intensity  $>3000$  lux. The culture was aerated with an electromagnetic air pump (ACO-004 - The Age of Aquarium Pty. Ltd.). Algae were harvested while in the log phase at 6-8 days (approx. 6 million cells/ml) for feeding to both zooplankton species.

#### **5.3.4. Preparation of treatments**

For the experiments, two treatments comprising five replicates were set up using 2 L screw capped containers, and were placed randomly into the incubator (Thermoline L-094 refrigerated incubator with diurnal temperature and light control). Treatments consisted of either animals fed with algae (fed animals) or animals without algae supplied (non-fed animals). The initial inoculated density of *D. carinata* was  $200 \pm 50$  animals/l and for *M. australiensis* was  $700 \pm 50$  animals/l. The densities used were based on the growth curve of animals with algae supplied (fed animals) obtained in preliminary tests which showed optimum density of both species in culture conditions the same as those in this experiment (Appendix 1). Controls without zooplankton were set up for each culture condition to allow comparison of nutrient reduction.

Thirty-six hours before the experiment started, food (algae - *Chlorella vulgaris*) was withdrawn from all animals. One hour before

inoculating the animals into the experimental containers, the fed group of animals was supplied once with algae at 700,000 and 300,000 cells/ml for *D. carinata* and *M. australiensis* respectively. The non-fed group of animals were not supplied with algae throughout the duration of the experiment.

All replicates were maintained in incubators at  $23 \pm 1^\circ\text{C}$  in complete darkness (L:D = 0:24) in order to avoid the increase of pH due to photosynthesis of algae. The sludge at the bottom of the container was removed daily. The zooplankton was harvested at the end of the experiment after 3 days.

#### **5.3.5. Measurements**

In order to determine the efficiency of nutrient utilization from the anaerobically digested piggery effluent, culture medium (10 ml) was sampled from each replicate after inoculation (start) and harvesting (end) of the animals. Total nitrogen (TN) was analysed by the persulphate-hydrazine colorimetric method, soluble orthophosphate (SP) was analysed by the colorimetric ascorbic acid method and biochemical oxygen demand (5 days) ( $\text{BOD}_5$ ) was measured using Winkler's method as described in APHA (2005). The temperature, pH (Eutech instruments - pH510 - pH and temperature meter) and dissolved oxygen (TPS WP-82 dissolved oxygen meter) were monitored daily.

### **5.3.6. Statistical analysis**

The reduction in TN, SP and BOD<sub>5</sub> was compared between treatments of control, fed and non-fed animals using one-way analysis of variance (ANOVA). Tukey's test was used for all pairwise multiple comparison procedures. All data were checked for normality and homogeneity of variance before analysis. SPSS 15 statistic software (SPSS Inc. 2006) was used for the analysis of data and overall significance level was taken at  $P < 0.05$ .

## **5.4. Results**

### **5.4.1. Efficiency of nutrient utilization by *D. carinata***

There was a significant difference ( $P < 0.01$ ) in the reduction of total nitrogen (TN), soluble phosphate (SP) and biochemical oxygen demand (BOD<sub>5</sub>) between control (no animals), fed animals and non-fed animals (Table 5.1). It is clear that the treatment with animals had comparatively more ( $P < 0.01$ ) nutrient reduction than the control. The reduction of TN (18.8%), SP (18%) and BOD<sub>5</sub> (60%) in the effluent was significantly higher ( $P < 0.01$ ) following treatment with non-fed animals. The average reduction in TP, SP and BOD was calculated to be 2.9, 0.1 and 3.0 mg/l following treatment with non-fed animals while the reduction following treatment with fed animals was 1.7, 0.06 and 2.0 mg/l respectively. TN decreased by 0.5%

and SP decreased by 1.53% in the control treatment, however, BOD<sub>5</sub> increased by 31.2%.

Table 5.1. Nutrient reduction, pH, DO and temperature in effluent medium of *D. carinata*

Items	Control		Fed		Non-fed	
	Start	End	start	End	start	End
TN level	15.33±0.78	15.26±0.05	15.41±1.72	13.68±0.47	15.39±1.58	12.50±0.42
TN reduced	0.07±0.05 <sup>c</sup>		1.73±0.47 <sup>b</sup>		2.88±0.42 <sup>a</sup>	
%TN reduced	0.46±0.35 <sup>c</sup>		11.22±3.09 <sup>b</sup>		18.75±2.74 <sup>a</sup>	
SP level	0.52±0.01	0.52±0.00	0.55±0.01	0.48±0.01	0.54±0.01	0.44±0.01
SP reduced	0.01±0.00 <sup>c</sup>		0.06±0.01 <sup>b</sup>		0.10±0.01 <sup>a</sup>	
%SP reduced	1.53±0.38 <sup>c</sup>		11.62±2.62 <sup>b</sup>		18.04±2.50 <sup>a</sup>	
BOD <sub>5</sub> level	4.00±1.00	5.25±0.35	4.00±0.00	2.00±0.00	5.00±1.00	2.00±0.00
BOD <sub>5</sub> reduced	-1.25±0.35 <sup>c</sup>		2.00±0.00 <sup>b</sup>		3.00±0.00 <sup>a</sup>	
%BOD <sub>5</sub> reduced	-31.25±8.84 <sup>c</sup>		40.00±0.00 <sup>b</sup>		60.00±0.00 <sup>a</sup>	
pH	7.10±0.03		7.61±0.07		7.43±0.06	
DO (ppm)	5.51±0.26		5.37±0.29		5.63±0.63	
T	23±0.5		23±0.5		23±0.5	

Values (mean ± SD) with different superscript letters in the same row are significantly different ( $p < 0.05$ ) which represent 5 replicates of each treatment and 3 replicates of control, % of reduction was based on % difference between start and end measured value. Abbreviations: TN, total nitrogen; SP, soluble phosphate; biochemical oxygen demand, BOD<sub>5</sub>, dissolved oxygen, DO, and Temperature, T.

#### 5.4.2. Efficiency of nutrient utilization by *M. australiensis*

There was a significant difference ( $P < 0.01$ ) in the reduction of total nitrogen (TN), soluble phosphate (SP) and biochemical oxygen demand (BOD<sub>5</sub>) between control (no animals), fed animals and non-fed animals (Table 5.2). It is clear that the treatment with animals had comparatively more ( $P < 0.01$ ) nutrient reduction than the control. The reduction of TN (16.9%), SP (12.3%) and BOD<sub>5</sub> (64.5%) in the effluent was significantly higher ( $P < 0.01$ ) following treatment with non-fed animals. The average reduction in TP, SP and BOD was calculated to be 14.7, 0.35 and 14.8 mg/l following treatment with non-fed animals, while the reduction following treatment with fed animals was 6.1, 0.24 and 8.3 mg/l respectively. The observation was similar to *D. carinata*. The control treatment had a decrease in TN by 0.7% and SP by 4.3% but BOD<sub>5</sub> increased by 12.5%.

Table 5.2. Nutrient reduction, pH, DO and temperature in effluent medium of *M. australiensis*

Items	Control		Fed		Non-fed	
	Start	End	start	End	start	End
TN level	86.77±3.05	86.18±0.50	86.70±3.10	80.55±2.38	86.90±1.78	72.26±0.47
TN reduced	0.59±0.50 <sup>c</sup>		6.14±2.38 <sup>b</sup>		14.65±0.47 <sup>a</sup>	
%TN reduced	0.68±0.57 <sup>c</sup>		7.09±2.74 <sup>b</sup>		16.85±0.57 <sup>a</sup>	
SP level	2.83±0.01	2.71±0.01	2.82±0.01	2.57±0.11	2.81±0.02	2.47±0.06
SP reduced	0.12±0.01 <sup>c</sup>		0.24±0.11 <sup>a</sup>		0.35±0.06 <sup>b</sup>	
%SP reduced	4.28±0.29 <sup>c</sup>		8.63±0.37 <sup>c</sup>		12.30±2.14 <sup>a</sup>	
BOD <sub>5</sub> level	20.00±1.00	22.00±1.41	22.00±1.00	13.67±1.21	24.00±1.00	9.17±1.17
BOD <sub>5</sub> reduced	-2.00±1.41 <sup>c</sup>		8.33±1.21 <sup>b</sup>		14.83±1.17 <sup>a</sup>	
%BOD <sub>5</sub> reduced	-12.50±8.84 <sup>c</sup>		37.87±5.50 <sup>b</sup>		64.50±5.08 <sup>a</sup>	
pH	7.89±0.01		8.05±0.04		7.93±0.03	
DO (ppm)	3.88±0.76		3.56±0.45		3.69±0.89	
T	23±0.5		23±0.5		23±0.5	

Values (mean ± SD) with different superscript letters in the same row are significantly different ( $p < 0.05$ ) which represent 5 replicates of each treatment and 3 replicates of control, % of reduction was based on % difference between start and end measured value.

Abbreviations: TN, total nitrogen; SP, soluble phosphate; biochemical oxygen demand, BOD<sub>5</sub>, dissolved oxygen, DO, and Temperature, T.

## 5.5. Discussion

The results of this study clearly indicated that *D. carinata* and *M. australiensis* had the capability for nutrient utilization from anaerobically digested piggery effluent. The non-fed animals of both species had a greater ability to reduce TN and SP. The efficacy of nutrient reduction by fed and non-fed animals showed that the feeding status had a notable effect on nutrient utilization. A reduction in the BOD<sub>5</sub> value provided further evidence of the ability of both species to utilize nutrients in the effluent.

Previous research has shown a reduction in nutrient load, in the form of total nitrogen and total phosphorus (TP), by *D. magna* in sewage water or treated effluent in an artificial food web manipulation process. The utilization of TN and TP was shown to be 2% and 4% in highly polluted domestic sewage water (original TN and TP was 26.9 and 2.6 mg/l) by an artificial food web system (2003). Jung *et al.* (2009) also reported that *D. Magna* could reduce the TN and TP by 14% and 13% in slightly polluted stream water (original TP and TP was 10.6 and 0.65 mg/l), by an artificial aquatic food web system.

In the case of *D. carinata*, the utilization efficiency was 11.2 – 18.8% and 11.6 – 18.0% for TN and SP in anaerobically digested effluent. In the case of *M. australiensis*, the utilization efficiency was 7.1 – 16.9% and 8.6 – 12.3 % for TP and SP in anaerobically digested effluent.

Our results are similar to previous findings which showed that both species had the potential to utilize nutrients in treated effluent. Moreover, *D. carinata* was more suitable for use in treatment of slightly polluted water (original TN was 15 mg/l in the effluent medium), while *M. australiensis* could be employed for highly polluted water (original TN was 87 mg/l in the effluent medium). Studies of un-ionized ammonia tolerance (Chapter 3.1) also indicated that *M. australiensis* had higher nutrient tolerance and reproductive performance when cultured in effluent compared to *D. carinata*.

Bacteria in the medium are a potential food source for zooplankton, and it was expected that the non-fed animals would have a higher grazing rate on bacteria and take up more nutrients. Consistent with what was expected, non-fed animals of both species in the current study had higher utilization of TN and SP compared to fed animals.

The lower reduction in nutrients (TN and SP) following treatment with fed animals may also be due to the release of TN and SP in a form of metabolic wastes such as faecal matter from their digestive system (Kawasaki, *et al.*, 1982; Kim, *et al.*, 2003). In addition, faecal matter is deposited as sludge at the bottom of the container, nutrients could be regenerated into the medium through disintegration of the faecal pellets of both species. Sludge contained a high proportion of TN and SP, and it would increase the nutrients in the effluent medium (Jung, *et al.*, 2009; Kim, *et al.*,

2003). However, sludge was not continuously (24 hrs) removed from the effluent medium in this batch culture, so the regeneration of nutrients mentioned above could lower the efficiency of nutrient reduction when treated with fed animals.

In general, the role of zooplankton in the biotreatment effluent process is to use their cilia to intake bacteria and detritus, use their thoracic appendages to consolidate colloidal particles, and to utilize algae produced in the process (Curds & Hawkes, 1975; Dinges, 1982; Gellis & Clarke, 1935; Kryutchkova, 1968; Langis, *et al.*, 1988; Shiny, *et al.*, 2005; Tarifeno-Silva, *et al.*, 1982). In our observation, the reduction of BOD<sub>5</sub> could be attributed to the utilization of organic particles by both species. Hence in this study, both zooplankton species showed an appreciable efficiency in utilization of nutrients from anaerobically digested piggery effluent. The digested effluent has a relatively higher bacterial population and bioavailable nutrients.

In the control treatment, the decrease in TN could be due to ammonia stripping, while the lowering of SP may be due to the precipitation of the Ca-Mg-PO<sub>4</sub> complex (Kawasaki, *et al.*, 1982). The increased BOD<sub>5</sub> value can confirm that bacteria are active in the effluent, as they demanded oxygen (Shiny, *et al.*, 2005). Therefore it further enhanced the reduction in TN and SP was mainly influenced by the animals themselves.

The current study was performed as a batch culture, as the carrying capacity for zooplankton in 2 L of culture medium was limited by space. A

similar limitation was reported by Jung, *et al.* (2009) and Kim, *et al.* (2003). That means that the density increase was limited by the container size and it is crucial to optimize the time of harvesting to avoid failure of the zooplankton culture. Growth curves were obtained in a preliminary experiment using the same conditions to enable the time of harvesting to be determined. Preliminary tests during the design of this experiment (not reported) indicated that the reduction of TN and SP was not significantly different when varying densities of both species were cultured in the same set up. Therefore the variation in density of the zooplankton culture was not considered as a factor in the statistical analysis in these studies.

## 5.6. Conclusion

The two South Australian zooplankton species, *Daphnia carinata* and *Moina australiensis* have the potential to utilize nutrients in treated piggery effluent. Moreover, non-fed animals utilized higher nutrients amounts (TN and SP) compared to fed animals. The reduction in BOD<sub>5</sub> values provided further evidence that both species could utilize nutrients in the effluent. This study showed that *D. carinata* was more suitable for use in slightly polluted water while *M. australiensis* could be employed for comparatively highly polluted water.

A previous study in this thesis investigating un-ionized ammonia tolerance (Chapter 3.1) indicated that *D. carinata* could not survive at un-ionized ammonia concentration of 2.2 - 2.8 mg/l but *M. australiensis* could

survive at level of un-ionized ammonia up to 7.5 – 8.8 mg/l. *M. australiensis* had a productive performance when cultured in effluent containing a high concentration nutrient load. Further studies could focus on the feeding regime of zooplankton to enhance the efficacy of nutrient reduction in the effluent.

## **6. Nutritional profile of two effluent-grown zooplankton (*Daphnia carinata* and *Moina australiensis*) fed with algae grown on piggery effluent: implications for use as live feed in aquaculture**

### **6.1. Abstract**

Two zooplankton species, *Daphnia carinata* and *Moina australiensis* were cultured as by-products of a wastewater treatment process. Proximate nutrient composition, amino acid and fatty acid composition were analysed in both zooplankton species to evaluate their suitability as live feed in aquaculture.

Algae (*Chlorella vulgaris*) grown in two-stage anaerobically digested piggery effluent was fed to both zooplankton species. Algae were also raised on a chemical culture medium (Woods Hole MBL medium), and algae raised under the two conditions were fed to both species for the assessment of fatty acid composition, to analyze the capability of both zooplankton species to synthesize longer chain (n-3) and (n-6) poly-unsaturated fatty acids.

The crude protein content of *D. carinata* and *M. australiensis* was 43.8% and 37.4 % while their lipid content was 11.7% and 19.1% respectively. The essential amino acid content (Arginine - 3.45; Leucine – 3.44; Lysine - 3.29 g/100g) of *M. australiensis* meets the requirements

(Arginine – 1.81/2.24; Leucine – 2.15/2.26; Lysine – 2.48/3.08 g/100g) of both omnivorous and carnivorous fish fry, but *D. carinata* (Arginine – 2.93; Leucine – 3.4; Lysine – 2.28 g/100g) only meets the requirements (Arginine – 1.81; Leucine – 2.15; Lysine – 2.48 g/100g) of omnivorous fish fry. Both species contained an appreciable quantity (11.7-19.1%) of lipid with over 50% of the total fatty acids being in the unsaturated form. A large proportion of the unsaturated form was (n-6) poly-unsaturated fatty acid. Both zooplankton species have the capability of converting mono-unsaturated fatty acid (n-6) to poly-unsaturated fatty acid. The nutritional profile analysis showed that *D. carinata* and *M. australiensis* could be used as live food for freshwater fish larvae.

## **6.2. Introduction**

Live food is commonly used in aquaculture to feed larval fish, crustacean and molluscs up until these animals can be fed with formulated feeds (De Silva & Anderson, 1995; Hertrampf & Piedad-Pascual, 2000; Southgate, 2003). However, the cost of common live feed, such as artemia and rotifers, is expensive (FAO, 1996; Hertrampf & Piedad-Pascual, 2000), therefore other more economic live feed sources are required. In order to utilize the nutrient loads in agricultural waste to produce useful by-products, algae have been grown in effluent and fed to zooplankton using an artificial food web manipulation of wastewater treatment systems (Kibria, *et al.*, 1999; Kim, *et al.*, 2003; Shiny, *et al.*, 2005).

This ecologically sustainable technology was used in this study, using diluted anaerobically digested piggery effluent to culture algae (*Chlorella vulgaris*). The effluent-grown algae were then fed to two local zooplankton species, *Daphnia carinata* and *Moina australiensis*, which originated from the effluent ponds.

Nutritional profiles of live organisms or live feeds vary with the nutrient composition of the food they are supplied (Watanabe, *et al.*, 1983). The protein content of the live food is important for the growth of cultured fish, while the lipid content is crucial to maintain optimum growth, reproduction, health and flesh quality of finfish species. Since long chain (n-3) and (n-6) poly-unsaturated fatty acids are required to maximize growth, it is necessary to have these components in the live food (Anderson & Silva, 2003; Higgs & Dong, 2000). Carbohydrate is the third most abundant organic compound in the animal body apart from protein and lipid, and is an important source of dietary energy (De Silva & Anderson, 1995; Hertrampf & Piedad-Pascual, 2000; Higgs & Dong, 2000; Li, *et al.*, 2000; Southgate, 2003).

Previous research has studied the nutritional profile of zooplankton raised on culture medium grown algae. However, there has been no study using anaerobically digested piggery effluent-grown algae (*Chlorella vulgaris*) as a nutrient source to feed *D. carinata* and *M. australiensis*. Hence the present study was designed to;

- a. analyze the capability of both species in synthesizing longer chain (n-3) and (n-6) poly-unsaturated fatty acids when raised on effluent-grown and chemical grown algae; and
- b. evaluate the nutritional profile of both zooplankton species raised on algae grown in two stage anaerobically digested piggery effluent.

## 6.3. Methods

### 6.3.1. Preparation of culture medium for zooplankton

The culture medium containing anaerobically digested piggery effluent was collected from the Integrated Biosystems (IBS) Laboratory's Bio-reactors at Roseworthy Campus. The effluent was anaerobically digested in the primary thermophilic and acidogenic (55°C) stage digesters for a total of seven days of hydraulic retention time (HRT). It was then transferred through to the tertiary mesophilic (30°C ambient) digester stage, for a total of 20 days HRT. Before its experimental use, effluent was aerated for 24 hrs via an aquarium air stone diffuser and allowed to equilibrate with aerobic atmospheric conditions. The effluent was diluted with milli-Q water (ultra de-ionised water) to un-ionized ammonia concentrations of 0.5 and 4.5 mg/l for the culture medium of *D. carinata* and *M. australiensis* respectively, as these were the optimum conditions for their reproduction previously obtained in a nutrient tolerance experiment (Leung, unpublished data).

The effluent was made up to the required un-ionized ammonia concentrations (UAN) based on the total nitrogen ammonia level, temperature and pH of the effluent as described in the equation:  $UAN = TAN / (10^{pK_a - pH + 1})$ , where  $p^{K_a} = 0.09018 + 2729.92/T$ , and T is the temperature (Alabaster & Lloyd, 1980; Emerson, *et al.*, 1975). The total ammonia nitrogen (TAN) of the digested piggery effluent and the culture

medium was determined prior to use by the phenate method as described in APHA (2005).

### **6.3.2. Preparation of algae**

The algae, *Chlorella vulgaris* were obtained from the IBS Laboratory, at Roseworthy Campus. These algae originated in and were isolated from the same effluent pond where the zooplankton were collected. The algae was grown either in a chemical medium (Woods Hole MBL medium) (Nichols, 1973) or effluent medium as described below. Anaerobic digested piggery effluent, which was collected from the IBS Laboratory's bio-reactors, Roseworthy Campus, was autoclaved at 121°C for 15 minutes. This autoclaved effluent was checked for TAN level and diluted with milli Q water to TAN 100 mg/l following the calculation and procedure described above.

The cultured algae were maintained at  $23 \pm 1^\circ\text{C}$  and 24 hours illumination using 36W x 4 fluorescent lights with an intensity of >3000 lux. Algae were harvested while in the log phase at 6-8 days (approx. 6 million cells/ml) for feeding to both zooplankton species. The effluent-grown algae were fed to both zooplankton species used in all analysis. The chemical-grown algae were only fed to both species used in fatty acids analysis.

### **6.3.3. Zooplankton culture**

*D. carinata* and *M. australiensis* were collected from effluent ponds at The University of Adelaide, Roseworthy Campus and reared for six months in the laboratory by the following procedure. The temperature of the pond was recorded from winter to autumn to decide the temperatures to use in the experiment. The experiment was set up at the IBS laboratory according to the following procedure. The zooplankton (*D. carinata* and *M. australiensis*) were maintained in 70 l tanks in three replicates. Algae (chemical grown or effluent-grown) were supplied at 700,000 and 300,000 cells/ml (based on feeding to satiation) for *D. carinata* and *M. australiensis* respectively.

Fifty percent of the culture medium was changed every two days. Biomass of both species was harvested twice weekly by screening out on a 200 µm nylon mesh filter. Temperature was maintained at  $23 \pm 1^\circ\text{C}$  and photoperiod was set at L:D = 12:12 using triphosphor fluorescent lights with light intensity of >2000 lux. The harvested biomass was freeze-dried (Christ LOC 2m Freeze Dryer Beta 1-8) and stored in a desiccator until analysis.

Both zooplankton species were analysed for proximate composition, amino acid content and fatty acid compositions.

#### **6.3.4. Moisture content**

Moisture content was determined by weight loss after being freeze-dried (Christ LOC 2m Freeze Dryer – Beta 1-8).

### **6.3.5. Protein analysis**

The nitrogen content of the dry sample was determined by combustion using an Elementar Instrument (Elemental Microanalysis, UK). Crude protein content was calculated from the total nitrogen content as described in AOAC (2005).

$$\text{Crude protein, \% (w/w)} = \% \text{ N} \times 6.25$$

### **6.3.6. Crude lipid analysis**

Crude lipid content was measured in dry samples by the submersion method using boiling hexane (Randall, 1974), adapted for the Soxtec 2050 automatic system (FOSS Analytical).

### **6.3.7. Crude ash analysis**

Samples were dried to constant weight by heating at 105°C under an atmosphere of nitrogen using an automated LECO Thermo gravimetric TGA 701 Analyser (LECO Corporation, St Joseph, Michigan, USA). The ash content was determined by further heating the weighed (*D. carinata* and *M. australiensis*) dry samples in the Thermo gravimetric Analyser at 600°C to constant weight.

### **6.3.8. Carbohydrate content**

The carbohydrate content was calculated by the difference between the dried weight and the sum of crude protein, lipid and ash contents as described by Kibria *et al.*(1999).

### **6.3.9. Gross energy**

Gross energy was determined using a LECO AC-350 Automatic Calorimeter (LECO Corporation, St Joseph Michigan USA) in pelleted (~1000 mg) samples. The instrument was calibrated using AR grade benzoic acid.

### **6.3.10. Amino acid analysis**

Samples of both zooplankton species were separately milled using a Tecator Kniftech mill and lipid was removed via Soxhlet extraction. Samples were then dried in a vacuum desiccator over phosphorous pentoxide. The dried samples were further hydrolysed in 6N HCl at 110°C for 18 hours in sealed tubes, blanketed with nitrogen (Finlayson, 1994). A sub-sample for cysteine and methionine determination was oxidised prior to hydrolysis (as above) with performic acid at 0°C overnight (Moore, 1963). The amino acids were quantitated by pre-column derivatisation with 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, (Accq-Tag method, Waters Corporation) and separated by a reverse phase high pressure liquid chromatography

(HPLC) on a Waters Accq-Tag column, Waters Alliance 2690 separation module and detected on Waters 474 fluorescence detector (Waters Corporation) (Spackman, *et al.*, 1958).

Tryptophan was determined by hydrolysis of a dry sample in 4.2 M NaOH at 110°C for 20 hours in sealed tubes, blanketed with nitrogen (AOAC, 1990). Separation was via reverse phase HPLC (as above) and detected on a Waters 486 variable wavelength detector (Waters Corporation) at 280nm.

#### **6.3.11. Fatty acid analysis**

Lipids were extracted using a modified “Folch” procedure, as described in Christie (1989). The sample (1 g) was homogenized with methanol (10 ml) for 1 minute using an Ultra-Turrax Homogenizer (IKA WERK). Chloroform (20 ml) was then added and the mixture was homogenized for a further 2 minutes. The mixture was filtered and 25% of the total volume of 0.88% potassium chloride was added. The mixture was shaken thoroughly and allowed to settle into two layers. The upper aqueous layer was drawn off by aspiration. The lower layer was centrifuged at 3000 rpm for 10 minutes. The thin upper layer was drawn off by aspiration. The solvent in the bottom layer was removed by a rotary film evaporator (Rotavapor – BÜCHI Switzerland) at 42°C. Color was removed by filtering through florisil.

The fatty acid methyl esters were prepared by dissolving the lipid extract in dry toluene (1 ml) in a test tube, then adding 0.5 M sodium methoxide in anhydrous methanol (2 ml). The solution was heated to 50°C for 10 minutes. Acetic acid (0.1 ml) was added and followed by milli Q water (5 ml). The required methyl esters were extracted twice by adding hexane (5 ml) and the upper layer was removed using a pipette. The hexane layer was dried over sodium sulfate and filtered before the solvent was evaporated at 42°C.

Fatty acid methyl esters were separated by GLC using a Hewlett Packard gas chromatograph (Model 5890A II) fitted with a BPX70 capillary column (25m x 0.25mm; SGE, Melbourne, Australia). Hydrogen was used as the carrier gas. The injector was set at 240°C and detector at 280°C. The temperature program was set at 150°C ramped at 2°C for 10 min. A sample volume of 0.05 µl was injected. The system allowed the identification of *cis/trans* isomers. Methyl esters were identified by comparison with retention times of authentic standards (Supelco and Matreya). Fatty acid concentrations were expressed as the percent of total fatty acids present (normalised values).

### **6.3.12. Statistic analysis**

T-test (SPSS Inc. 2006) was used to compare the means of fatty acid content such as saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acid of algae (effluent-grown and chemically grown) and both zooplankton species raised on the same algae.

## **6.4. Results**

### **6.4.1. Proximate composition**

The proximate nutrient composition of freshwater zooplankton, *D. carinata* and *M. australiensis* fed on effluent-grown algae (*Chlorella vulgaris*) is shown in Table 6.1. The crude protein of *D. carinata* and *M. australiensis* was 43.8% and 37.4%, whereas the lipid level was 11.7% and 19.1% respectively. The protein and lipid level in commercial silver perch dry diet were reported to be 45% and 8.5% (Kibria, *et al.*, 1999). The amount of carbohydrate presence in both species accounted for almost one-third of the nutrient content. The gross energy was 21.8 – 24.0 MJ/kg, similar to that reported in fish pellets by Kibria *et al.* (1999) (Table 6.1). Moisture content of both species was over 93-95%.

Table 6.1. Proximate composition and gross energy value of commercial silver perch dry diet and of freshwater zooplankton (*Daphnia carinata* and *Moina australiensis*) fed with effluent grown *Chlorella vulgaris*.

Component	<i>Daphnia carinata</i>	<i>Moina australiensis</i>	Fish pellets <sup>1</sup>
Carbohydrate (%)	30.9	37.7	36.7
Crude protein (%)	43.8	37.4	45.0
Crude lipid (%)	11.7	19.1	8.5
Crude ash (%)	13.6	5.8	9.8
Moisture (%)	94.9	93.2	9.9
Gross energy (MJ/kg)	21.7	24.0	22.1

Values per cent dry matter.

<sup>1</sup>Kibria et al. (1999)

#### **6.4.2. Amino acid content**

The amino acid composition of both species included a broad range of both essential and non-essential amino acids (Table 6.2). Both species contained similar essential (48.2% in *D. carinata*, 47.3% in *M. australiensis*) and non-essential amino acids. The amino acid content of *M. australiensis* was sufficient to meet the requirements for both omnivorous and carnivorous fish fry (Tacon, 1990). In the case of *D. carinata*, the essential amino acid content was adequate for omnivorous fish fry but did not meet the required lysine and methionine content for carnivorous fish fry.

Table 6.2. Essential and non-essential amino acid compositions of *Daphnia carinata* and *Moina australiensis* fed with effluent grown *Chlorella vulgaris* and the minimum essential amino acids required by finfish fry of 0.05g.

Common name	<i>D. carinata</i>	<i>M. australiensis</i>	Minimum essential amino acid required by finfish fry <sup>1</sup>	
			Omnivorous	Carnivorous
Essential amino acids				
Arginine	2.93	3.45	1.81	2.24
Histidine	1.02	1.27	0.76	0.95
IsoLeucine	1.97	1.91	1.18	1.46
Leucine	3.40	3.44	2.15	2.26
Lysine	2.82	3.29	2.48	3.08
Methionine	0.95	1.05	0.81	1.00
Phenylalanine	2.05	2.21	1.22	1.51
Threonine	2.37	2.57	1.35	1.67
Tryptophan	0.61	0.66	0.25	0.31
Valine	2.46	2.57	1.40	1.73
Non-essential amino acids				
Alanine	2.72	3.22		
AsparticAcid	4.40	4.63		
Cysteine	0.61	0.74		
GlutamicAcid	5.71	6.33		
Glycine	2.25	2.55		
Proline	2.22	2.68		
Serine	2.34	2.56		
Tyrosine	1.83	2.28		

Values expressed as g/100g dry matter.

<sup>1</sup>Tacon (1990)

### 6.4.3. Fatty acid composition

Fatty acid composition, expressed as a percentage of total fatty acids is illustrated in Table 3. There was a significant difference ( $P < 0.05$ ) in the fatty acid dietary composition between both species raised on two dietary different algae (*Chlorella vulgaris*). In the case of *D. carinata*, the saturated fatty acid level (SFA) was 24.2% and 27.9% when fed with effluent-grown (E) and chemically grown algae (M) respectively, while the mono-unsaturated fatty acid (MUFA) level was 46.8% and 29.5% (Table 6.3). However, the poly-unsaturated fatty acid (PUFA) content was 34.2% when fed with chemically grown algae and only 23.7% when fed with effluent-grown algae. They consisted mainly of (n-6) PUFAs. There was a trace amount of (n-3) PUFA present when *D. carinata* was raised on effluent-grown algae.

Table 6.3. Comparative fatty acid compositions of *Daphnia carinata* and *Moina australiensis* fed with *Chlorella vulgaris* raised on effluent and chemical culture medium

Fatty acids x:yn - z	<i>D. carinata</i>		<i>M. australiensis</i>		Algae ( <i>C. vulgaris</i> )	
	E	M	E	M	E	M
SFA						
14:0	2.6	5.0	2.0	3.0	2.0	1.2
15:0	0.7	0.6	0.8	0.7	0.1	0.1
16:0	13.9	16.1	10.0	13.9	14.4	14.1
17:0	3.4	2.9	2.6	3.6	2.0	1.9
18:0	3.6	3.3	1.3	1.4	1.7	1.1
MUFA						
14:1	1.6	1.5	0.7	1.5	0.5	0.3
15:1	0.3	1.4	0.2	0.5	0.4	0.1
16:1n - 5	0.4	5.9	nd	nd	nd	nd
16:1n - 7	13.4	2.8	10.7	4.9	3.7	3.5
17:1	0.7	0.3	0.6	0.5	0.1	0.2
18:1n - 9	30.3	17.6	29.0	22.9	47.8	46.8
PUFA						
18:2n - 6	8.8	8.5	8.4	9.6	8.0	8.9
18:3n - 3	0.3	nd	0.3	nd	nd	nd
18:3n - 6	14.6	25.7	23.5	26.8	13.4	14.9
unidentified	5.3	8.5	9.9	10.8	6.0	6.8
$\Sigma$ SFA	24.2	27.9	16.6	22.5	20.1	18.5
$\Sigma$ MUFA	46.8	29.5	41.2	30.2	52.5	50.9
$\Sigma$ PUFA	23.7	34.2	32.3	36.4	21.4	23.8
$\Sigma$ n-3	0.3	nd	0.3	nd	nd	nd
$\Sigma$ n-6	23.4	34.2	32.0	36.4	21.4	23.8

E – *Chlorella vulgaris* raised on effluent medium, M – *Chlorella vulgaris* raised on chemical medium (Woods Hole MBL medium), SFA – Saturated fatty acid, MUFA – Mono-unsaturated fatty acid, PUFA – Poly-unsaturated fatty acid. The fatty acids (% of total fatty acids) are described by the three numbers x:yn - z, where x is the number of carbon atoms, y is the number of double bond, z is the position of the first double bond, counting from the methyl end of the molecule (“nd“ not detected or <0.2%).

In the case of *M. australiensis*, 41.2% of fatty acids were MUFAs following feeding with effluent-grown algae, but MUFA content was only 30.2% when fed with chemically grown algae (Table 6.3). SFA accounted for 16.6% and 22.5% of fatty acids, while the PUFA content was 32.3% and 36.4% when *M. australiensis* were raised on effluent-grown and chemically grown algae respectively. As observed in *D. carinata*, the PUFA was mainly composed of (n-6) PUFAs with trace amounts of (n-3) PUFA.

The MUFA content of effluent-grown (E) or chemically grown (M) algae was higher ( $P < 0.05$ ) than in *D. carinata* or *M. australiensis* while the PUFA content of the algae was lower ( $P < 0.05$ ) than that in the animals. However, the SFA content of *D. carinata* was higher ( $P < 0.05$ ) than the algae themselves. For *M. australiensis*, the SFA content was lower ( $P < 0.05$ ) in the animals fed with effluent-grown algae than in the algae themselves. However, the SFA was higher ( $P < 0.05$ ) in the animals fed with chemical grown algae than the algae.

In both species, more than 50% of fatty acids were in the form of unsaturated fatty acids when both zooplankton were raised on either effluent-grown (E) or chemically grown (M) algae. *M. australiensis* had a higher ( $P < 0.05$ ) PUFA and lower ( $P < 0.05$ ) SFA value than *D. carinata*. Both species had trace amounts of n-3 fatty acids when fed with effluent-grown algae which were not detected in the algae.

## 6.5. Discussion

The crude protein content of the two zooplankton species in this study was 37 – 44% which satisfies that the requirement and is similar to that in crab meal (30%), shrimp meal (32%) and commercial aquaculture feed pellets for fish and shrimp (37-43%) (Li, *et al.*, 2000; Southgate, 2003). Crude protein content has been reported to be 55-75% in fish meal, 45-50% in meat and bone meal, 42-48% in soybean meal, whereas higher protein content was reported in live foods such as artemia (48 – 71%) and rotifers (60 – 67%) (Hertrampf & Piedad-Pascual, 2000; Li, *et al.*, 2000). However in aquaculture, fish feed needs to contain at least 20% of crude protein, while salmon and trout need to be fed at least 35% protein content (Li, *et al.*, 2000).

Live foods such as rotifers (19 – 30%) contained higher levels of lipid content compared to artemia (5 – 18%) and the two zooplankton species (12 – 19%) investigated in this study (Hertrampf & Piedad-Pascual, 2000). The lipid content of commercial fish feed pellets was 2.8-10%, compared to 6.0-8.5% in fish meal, 3.3% in shrimp meal, 2.0-5.0% in crab meal and 3.3-16.1% in meat and bone meal (Hertrampf & Piedad-Pascual, 2000; Southgate, 2003). A lipid level of 10-20% in the diet is required for optimal growth rates without building up an excessive fatty carcass (Cowey & Sargent, 1979).

Hence both *D. carinata* and *M. australiensis* raised on effluent-grown algae (*Chlorella vulgaris*) in the current study contain sufficient protein and lipid to be used as a fish live foods.

The high carbohydrate content of both zooplankton species (31 – 38%) met the requirements for crustaceans and omnivorous/herbivorous fish. Other live foods, rotifers only contained 5 – 6 % carbohydrate, while artemia had 10 - 33% (Hertrampf & Piedad-Pascual, 2000). Crustaceans require a considerable quantity of carbohydrate for chitin synthesis, non-essential fatty acid synthesis and also protein sparing function (Capuzzo, 1982; Hertrampf & Piedad-Pascual, 2000). Activity of the enzymes required to utilize carbohydrate efficiently is higher in omnivorous and herbivorous fish, when compared to carnivorous fish (Hepher, 1988; New, 1987).

The gross energy content of *D. carinata* and *M. australiensis* (22 -24 MJ/kg) was comparable to commercial feed pellets (22 MJ/kg), rotifiers (22 – 25 MJ/kg), artemia (22 MJ/kg), and copepods (17 – 22 MJ/kg) (Hertrampf & Piedad-Pascual, 2000). However, the high moisture content of both zooplankton species implied that mass culture of both species is necessary to supply the daily nutritional requirements for fish and crustaceans.

The essential amino acid composition of *D. carinata* and *M. australiensis* in the current study (52.3% in *Daphnia* and 50.2% in *Moina*) was greater than that reported in fish feed pellets (49.7%), artemia (46.7%), rotifiers (34.6%) and copepods (33 – 36%) (Kibria, *et al.*, 1999; Watanabe,

*et al.*, 1978). The differences in the essential amino acid content of the zooplankton could be due to the dietary composition of the food (algae) and the culture medium (Allen & Allen, 1981; Watanabe, *et al.*, 1983). Both zooplankton species raised on effluent-grown algae met the amino acid requirements of omnivorous fish fry. In the case of *M. australiensis*, the amino acid composition was also suitable to meet the requirements of carnivorous fish fry.

Since C18:3(n-3) PUFA was not detected in the algae of the current study; both zooplankton species may absorb directly from bacterial biomass in the effluent culture medium (Patil, *et al.*, 2010). Algae grown in effluent or in culture media containing high (n-6) PUFAs are common in freshwater culture (De Silva & Anderson, 1995). Subsequently both zooplankton fed on these freshwater algae also contained a large amount of (n-6) PUFAs. The result is similar to that of rotifers raised by freshwater *Chlorella*, which also contained high (n-6) PUFA and are low in (n-3) HUFA. In contrast, rotifers fed with marine *Chlorella* had low (n-6) PUFA but high (n-3) HUFA (Watanabe, *et al.*, 1983). Marine copepods supplied with *Chaetoceros* were also reported with low (n-6) PUFA and high (n-3) HUFA (Nanton & Castell, 1998).

In general, freshwater fish contain a larger proportion of C18 fatty acids and (n-6) PUFAs than marine fish. Fish require C18:3(n-3) PUFA at about 1-2% of the diet by dry weight which can be replaced by lesser amounts of longer chain PUFAs (De Silva & Anderson, 1995). However,

both zooplankton species contained only trace amounts of C18:3(n-3) PUFA and no longer chain PUFAs for replacement. Hence it would be necessary to supply the fish with both zooplankton species in a large quantity in order to meet the fatty acid requirements.

The MUFAs in either effluent-grown or chemically grown algae could have been converted by the *D. carinata* and *M. australiensis* to PUFA. Similarly, a marine harpacticoid copepod (*Tisbe sp.*) was reported to be able to synthesize highly unsaturated fatty acids (HUFA), EPA and DHA from shorter chain (n-3) PUFAs (Nanton & Castell, 1998). Although fish possess a range of elongase and desaturase enzymes to synthesize fatty acids, crustaceans and marine fish are not able to produce C18:3(n-3) and C18:3(n-6) from C18:3(n-9) (Anderson & Silva, 2003; De Silva & Anderson, 1995). Hence (n-3) and (n-6) PUFAs should be present in the fish diet and considered as essential fatty acids. However, most freshwater fish should be able to elongate and desaturate C18:3(n-3) and C18:3(n-6) to the required HUFAs. Therefore HUFAs are not required in the diet for most of the freshwater fish (Anderson & Silva, 2003).

## **6.6. Conclusion**

The proximate composition of *D. carinata* and *M. australiensis* raised on effluent-grown algae meets the general nutrient requirements used in commercial fish pellets. The fatty acid composition of both species is suitable for use as feed for most freshwater fish. Moreover, the amino acid

content further showed that *M. australiensis* could supply the amino acid requirements for both omnivorous and carnivorous fish fry. However, *D. carinata* may only meet the amino acid requirements for omnivorous fish.

Both zooplankton species may have the capability to synthesize PUFAs from MUFAs, hence further study could investigate the process of such synthesis. Also studies could focus on the effects of culture temperature on the synthesis of fatty acids and the fatty acid composition of both species. *D. carinata* and *M. australiensis* could be fed to an omnivorous freshwater fish for further determination of the growth performance based on their dietary composition.

## 7. General discussion

### 7.1. Research Issues and Key Findings

This study arose from a review of the literature on the reproduction and utilization of zooplankton (Chapter 2). In general, the reproduction of zooplankton raised in effluent medium is affected by the chemical and physical properties of the culture environment, such as the concentration of un-ionized ammonia, the temperature, photoperiod, pH and dissolved oxygen. Similarly, induction, hatching and storage of zooplankton ephippia are also influenced by abiotic factors such as temperature, photoperiod and moisture. The aim of this study is to utilize the nutrient resources in effluent to culture zooplankton, subsequently resulting in nutrient utilization from the effluent and production of a quality live food for aquaculture.

This study has four key objectives:

- a. To determine the optimal culture conditions for asexual and sexual reproduction, based on nutrient tolerance (LC50 of un-ionized ammonia level), optimal culture temperature and effect of photoperiod;
- b. To evaluate the impact of stimuli to induce and hatch ephippia (resting egg stage), storage conditions for ephippia, sex ratio and embryonic development of the resting egg;
- c. To determine the nutrient utilization efficacy of both zooplankton species; and

- d. To quantify the nutritional profile of both species raised on effluent-grown algae for use as live feed in aquaculture.

The key findings were as follows:

- a. *Moina australiensis* was less sensitive to the toxic effects of unionized ammonia in the anaerobically digested piggery effluent culture medium, and had appreciably higher reproductive performances and population growth than *D. carinata* when both species were cultured at optimal conditions (Chapter 3).
- b. *M. australiensis* had a higher percentage of induced ephippial brood and cumulative hatching of ephippia compared to *D. carinata*. However, the survival percentage of *M. australiensis* was lower and the time to resume parthenogenetic reproduction was longer after release of ephippia. The reduction in proportion of males decreased the percentage of ephippia formation.
- c. The ephippia of both species preferred storage in wet conditions (Chapter 4).
- d. There is a potential to produce *D. carinata* and *M. australiensis* in anaerobically digested piggery effluent to utilize the nutrients, consume the effluent-grown algae and save the cost of harvesting the algae by filtration in wastewater treatment systems (Chapter 5).
- e. Both zooplankton species have the capability of converting mono-unsaturated fatty acids (n-6) to poly-unsaturated fatty acids.

The nutritional profile analysis showed that *D. carinata* and *M. australiensis* could be used as live foods for freshwater fish larvae (Chapter 6).

## **7.2. Reproduction of *D. carinata* and *M. australiensis***

### **7.2.1 Key factors influencing culture of *D. carinata* and *M. australiensis***

In effluent culture medium, a major concern is how to maintain zooplankton, particularly with the impact of un-ionized ammonia on their life cycle response. *M. australiensis* had a greater tolerance at higher levels of un-ionized ammonia than *D. carinata* (Chapter 3).

The lethal concentration of unionized ammonia, with 50% survival after 24 hours exposure (24 hr LC50 values) to un-ionized ammonia was higher in *M. australiensis*. However, older neonates (<48 hrs old) of both species could survive at higher concentrations of un-ionized ammonia (24 hr LC50 values was up to 2.8 mg/l *D. carinata* and 8.8 mg/l *M. australiensis*) when compared to younger neonates (<24 hrs old) (24 hrs LC50 values was 2.2 mg/l *D. carinata* and 7.5 mg/l *M. australiensis*).

Maximal reproductive performance was observed at concentrations of un-ionized ammonia in the effluent culture medium of 0.5 and 4.5 mg/l for *D. carinata* and *M. australiensis* respectively. Below 24 hrs LC50 values of un-ionized ammonia levels in both species, the effluent culture medium served as a supplementary bacteria meal for the animals as well as providing algae with a source of bacteria which increased the production of neonates (Anderson-Carnahan, 1994; Langis, *et al.*, 1988).

The optimal temperature of 23°C for culturing both species could enhance their reproduction, giving a maximum population growth. *M. australiensis* was a faster growing species than *D. carinata* when cultured in optimal conditions.

### **7.2.2 Induction, hatching and storage of ephippia**

Populations of zooplankton cultured in outdoor ponds are affected by seasonal changes (Geddes, 1984; Mitchell & William, 1982a; b) while indoor culture may have to overcome technical issues such as contamination, electrical breakdown or aeration pump malfunction. This imposes a commercial imperative to produce not only zooplankton by asexual reproduction (Chapter 3) but also to be able to induce their ephippia (resting eggs) by sexual reproduction (Chapter 4), which can be hatched to re-start a culture in any season.

Induction of ephippia of *D. carinata* and *M. australiensis* was achieved in the current study with the interaction of photoperiod, temperature and density. However, due to the high adaptability of these species, stimuli (photoperiod, temperature and density) could only be employed periodically and also kept fluctuating around the lethal limit to prevent the zooplankton acclimatising to the culture conditions. In order to maintain a continuous ephippia production, it is preferable to maintain a number of cultures with populations at different developmental stages, so that these stimuli could be used for different cultures.

In this study, *M. australiensis* had a higher percentage of induced ephippial brood and cumulative hatching of ephippia compared to *D. carinata*. After release of ephippium, *D. carinata* had a higher survival rate and shorter time to resume asexual reproduction.

Ephippia of both species could be stored, with wet storage conditions preferential for higher subsequent hatchability. An effective hatching process involved temperature and heat shock, followed by decapsulation of the hard capsule of the ephippia, and finally induced hatching by a long daylight at optimal culture temperature. The critical time to separate the newly hatched neonates from the culture medium was 30 - 36 hrs (in the case of *D. carinata*) and 20 - 24 hrs (in the case of *M. australiensis*) after rupture of the outer membrane during embryonic development. It was important to monitor frequently and inoculate the newly hatched neonates into a freshly prepared culture medium with optimal conditions to lower the mortality.

### 7.3. Efficiency of nutrient utilization from effluent

Agricultural waste cause an increase of N and P concentration in freshwater catchments is a key water quality problem. It is a priority to overcome this issue by utilization of the nutrient loads (N and P) in agricultural effluent. *D. carinata* and *M. australiensis* could reduce the total nitrogen and phosphorus levels by utilization of the nutrients in effluent (Chapter 5). Moreover, the non-fed animals had utilized more nutrients (TN and SP) compared to fed animals. The reduction in BOD<sub>5</sub> values provided further evidence that both species could utilize nutrients in the effluent. There is a potential to use either *D. carinata* or *M. australiensis* in the artificial food web manipulation process of the integrated wastewater treatment system, to utilize the resources in the effluent for the production of zooplankton as live feed for aquaculture.

This study shows that *D. carinata* is more suitable for use in slightly polluted water, while *M. australiensis* could be employed for comparatively highly polluted water.

#### **7.4. Implications of using zooplankton as live feed for aquaculture**

In the process of nutrient removal from effluent, production of zooplankton as live feed for aquaculture could further utilize the resources in effluent. *D. carinata* and *M. australiensis* were raised on effluent-grown algae (*Chlorella vulgaris*). Both species contained a sufficient quantity of crude protein and lipids and could be used as fish feed. The high carbohydrate and gross energy content of both species was comparable to commercial fish feed pellets.

However, their high moisture content implied that mass culture of both species is necessary to supply the daily nutritional requirements for aquaculture species. Both zooplankton species raised on effluent-grown algae can meet the amino acid requirements of omnivorous fish fry. In the case of *M. australiensis*, the amino acid composition was also suitable to meet the requirements of carnivorous fish fry. Both species contained over 50% of the total fatty acids in the unsaturated form, which was mainly in the form of (n – 6) poly-unsaturated fatty acid (linoleic and linolenic acid). Both zooplankton species have the capability of converting mono-unsaturated fatty acids (n-6) to poly-unsaturated fatty acids. The nutritional profile analysis showed that *D. carinata* and *M. australiensis* could be used as live food for freshwater fish larvae.

## 7.5. Conclusions and future considerations

In this comparative study of reproduction of two zooplankton species, *Moina australiensis* shows higher tolerance of the toxic effects of un-ionized ammonia in the effluent and has higher reproductive performance. When induction and hatching of ephippia are assessed, *M. australiensis* has a higher percentage of induced ephippial brood and cumulative hatching of ephippia compared to *D. carinata*. Although the survival percentage of *M. australiensis* after release of ephippia is lower than *D. carinata*, the decrease in population could be offset by the high net reproductive rate and intrinsic rate of increase.

Both zooplankton species have the potential to be used in wastewater treatment systems for the utilization of nutrients in effluent. The nutritional profile of both species raised on effluent-grown algae meets the requirements as live feed for freshwater fish.

In summary, *M. australiensis* is the species of choice for culture in anaerobically digested piggery effluent. An enhanced understanding of the reproduction of both species could assist in their mass culture for their use in aquaculture as live feed or as a nutrient utilization tool.

This research achieved the aim of assessing a local zooplankton candidate to utilize the nutrient loads in effluent from local agricultural waste.

A nutritive live feed for aquaculture was also achieved. A recent laboratory study on the bio-conversion of *M. australiensis* has also shown that zooplankton could utilize the bacterial blooms as food sources, which further strengthens the evidence that this zooplankton could be mass produced in effluent as live feed for use in aquaculture (Ward & Kumar, 2010).

This document has described other zooplankton species (e.g. *Daphnia magna*, *Daphnia pulex*) as having the potential to be used for nutrient utilization from effluent. Preliminary observations in studies for this thesis (not reported) indicated that cohorts of *D. carinata* and *M. australiensis* collected from effluent ponds at different South Australian locations could not be successfully cultured in effluent collected at the Roseworthy campus. Only those zooplankton that originated from the local effluent ponds could be used for culture in the same treated effluent.

Hence further research could focus on factors that influence the survival and reproduction of zooplankton collected from different locations when cultured in effluent.

The design of the studies in this thesis research could be applied to assess other local zooplankton species, to utilize nutrient loads in effluent and for production as a live food in aquaculture. Further studies could also examine the efficacy of the use of both zooplankton species as live feed on the reproduction and growth in fish.

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## 9. Appendices

### Appendix 1. Growth curve

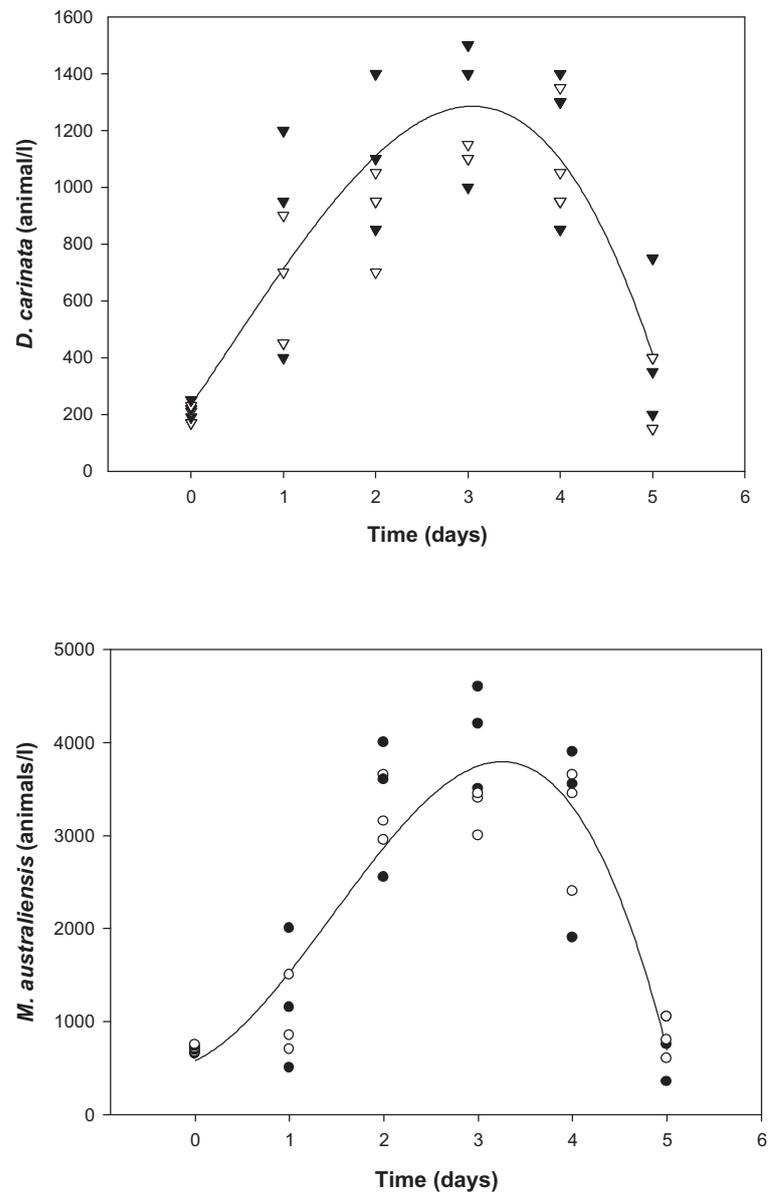


Fig. 9.1 Growth curve of *D. carinata* (above) and *M. australiensis* (below) with non-linear regression line (polynomial to order 3) on daily measurements (6 replicates). Optimum density occurred at day 3 for a batch culture of the both species in 2l effluent culture medium.