EFFECTS OF DIET AND ABIOTIC FACTORS ON THE SURVIVAL AND REPRODUCTION OF CYCLOPOID COPEPOD *Apocyclops dengizicus*

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EFFECTS OF DIET AND ABIOTIC FACTORS ON THE SURVIVAL AND REPRODUCTION OF CYCLOPOID COPEPOD *Apocylops dengizicus*

ABSTRACT

Copepods are one of the essential live feeds in aquaculture and are traditionally raised on algal diets. However, sustainable copepod productions in captivity are still unachievable due to lack of cost-effective feed and culture protocols. Hence, this present study investigated the effects of (1) a biotic factor i.e. marine microheterotrophs (bacteria, yeasts and protists) grown in palm oil mill effluent (POME) and (2) abiotic factors i.e. temperature, salinity, pH, photoperiod and light intensity on survival, reproduction and growth (body size) of brackish water copepod *Apocyclops dengizicus* at a laboratory scale. The aims of this study were to evaluate the feasibility of POME-grown diets for culturing *A. dengizicus* and determine the optimal conditions for sustainable production of *A. dengizicus*. For each experiment, an initial stocking density of fifty copepod nauplii (stage I) with triplicates of each treatment was cultured for twelve days. The scale-up experiment with an initial density of 200 nauplii was then tested on the optimized diet(s) and culture conditions gathered from the initial experiments. Copepods fed on POME-grown *Meyerozyma guilliermondii* (POME-MG) had higher survival (92.0±7.0% by Day 8) ($p < 0.05$) and total F2 nauplii production (445.7±55.4) ($p < 0.05$) compared to copepods fed on other POME-grown diets tested i.e. POME- grown: *Shewanella algae* (POME-SA), *Rhodovulum sulfidophilum* (POME-RS), *Rhodotorula mucilaginosa* (POME-RM), *Aurantiochytrium limacinum* (POME-AL), a mixed diet of POME-(MG+AL) and an instant microalgae diet of *Nannochloropsis oculata* (N). There were no significant body length and width differences in *A. dengizicus* between each diet treatments. The highest survival (58.0±% on Day 6) ($p < 0.05$) and reproduction (101.3±25.4 nauplii) ($p < 0.05$) were achieved

under 10 ppt salinity. The different levels of temperatures (26-36°C), pH (5-9), photoperiod regimes (24:0, 0:24, and 12:12 h light:dark regime) and light intensities (6.08, 14.85 and 23.65 µmol photons m^{-2} s^{-1}) did not produce a significant variation in survival, reproduction and growth of *A. dengizicus*. In a scale-up experiment, *A. dengizicus* fed on POME-MG had significantly higher ($p < 0.05$) adult survival rate (88.5±3.9%) on Day 6 as compared to only POME diet (72.7±2.9%). However, there were no significant differences in the total number of F2 nauplii between *A. dengizicus* fed on POME-MG (212.6±11.3) and only POME (206.6±11.1) throughout the 12-day culture period. The nutritional composition of POME-MG diet consisted of 41.8% protein, 11.0% lipids and 24.8% carbohydrates on dry weight basis. It is rich in polyunsaturated fatty acids (8.0%) and essential amino acids (47.2%). As a conclusion, POME-MG is suitable for culturing *A. dengizicus* with incorporating the optimal culture conditions of 30±1°C, 10±1 ppt, pH 8±0.5, photoperiod regime of 12 hours light and 12 hours dark under low light intensity of 6.08 µmol photons m^{-2} s^{-1}. This study has shown POME grown microhetetrophs as potential feed for mass culturing copepods.

Keywords: *Apocyclops dengizicus*, microheteretrophs, biotic, abiotic, survival.

KESAN DIET DAN FAKTOR ABIOTIK KEATAS KADAR KEMANDIRIAN DAN PEMBIAKAN KOPEPOD SIKLOPOID *Apocyclops dengizicus*

ABSTRAK

Kopepod adalah antara zooplankton yang digunakan sebagai makanan hidup dalam akuakultur dan secara tradisinya, ia diberi diet berasaskan alga. Walau bagaimanapun, penghasilan kopepod yang mampan adalah terhad disebabkan oleh kekurangan keberkesanana dalam kos makanan dan protokol kultur. Justeru, kajian ini menyiasat pengaruh (1) faktor biotik iaitu mikroheterotropi marin yang dikultur didalam air kumbahan kilang minyak kelapa sawit (POME) dan (2) faktor-faktor abiotik iaitu suhu, saliniti, pH, tempoh cahaya dan intensiti cahaya terhadap kelangsungan hidup, pembiakan dan pertumbuhan (saiz badan) kopepod air payau *Apocyclops dengizicus* pada skala makmal. Tujuan kajian ini adalah untuk (a) menilai kebolehlaksanaan diet POME dan (b) menentukan persekitaran optimum bagi pengeluaran *A. dengizicus* yang mampan. Lima puluh ekor kopepod nauplii di ternak di bawah keadaan terkawal selama dua belas hari untuk setiap rawatan bagi menguji kesan-kesan diet POME dan pelbagai faktor abiotik. Eksperimen berskala lebih besar telah dijalankan dengan ketumpatan awal sebanyak 200 ekor nauplii untuk menguji keberkesanan diet terpilih dan keadaan optimum yang dicatatkan dari eksperimen awal. Kopepod yang diberi diet *Meyerozyma guilliermondii* yang dikultur dalam POME (POME-MG) mempunyai kelangsungan hidup (92.0 ± 7.0% pada hari ke-8) dan pengeluaran nauplii F2 (445.7±55.4) yang ketara ($p < 0.05$) berbanding dengan kopepod yang diberikan diet lain berasaskan POME iaitu *Shewanella alga* (POME-SA), *Rhodovulum sulfidophilum* (POME-RS), *Rhodotorula mucilaginosa* (POME-RM), *Aurantiochytrium limacinum* (POME-AL), diet campuran POME-(MG+AL) dan diet mikroalga *Nannochloropsis oculata* (N). Tiada perbezaan saiz yang signifikan dalam *A. dengizicus* antara setiap makanan yang

dikaji. Kadar kelangsungan hidup (58.0±% on Day 6) (p < 0.05) dan jumlah pengeluaran nauplii tertinggi (101.3±25.4 individu) (p < 0.05) dicapai pada saliniti 10 ppt. Pelbagai tahap suhu (26-36°C), pH (5-9), tempoh cahaya (24:0, 0:24, and 12:12 jam cahaya:gelap) dan intensiti cahaya (6.08, 14.85 and 23.65 µmol foton m^{-2} s^{-1}) yang diuji tidak mempengaruhi kelangsungan hidup, pembiakan dan pertumbuhan *A. dengizicus*. Dalam eksperimen berskala besar, diet POME-MG (88.5±3.9%) menunjukkan kadar kelangsungan hidup yang lebih tinggi (p < 0.05) pada hari ke-6 berbanding diet POME sahaja (72.7±2.9%), tetapi tiada perbezaan yang ketara dari segi jumlah F2 nauplii antara *A. dengizicus* yang diberi POME-MG (212.6±11.3) dan POME sahaja (206.6±11.1) sepanjang tempoh kultur 12 hari. Diet POME-MG mengandungi 41.8% protein, 11.0% lipid dan 24.8% karbohidrat (berat kering). Ia kaya dengan lemak politaktepu (8.0%) dan asid amino yang penting (47.2%). Oleh itu, diet POME-MG disyorkan untuk kultur *A. dengizicus* dalam persekitaran optimum iaitu 30 ± 1 °C, 10 ± 1 ppt, pH 8 ± 0.5, tempoh 12 jam cahaya dan 12 jam gelap di bawah intensiti cahaya rendah 6.08 µmol foton m^{-2} s^{-1}. Kajian ini menunjukkan diet berasaskan mikroheterotrof yang dikultur dengan POME mempunyai potensi sebagai pemakanan untuk menghasilkan kopepod berskala besar.

Kata kunci: *Apocyclops dengizicus*, mikroheterotropi, biotik, abiotik, kelangsungan hidup.

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et al.	:	And others
ARA	:	Arachidonic acid
AL	:	*Aurantiochytrium limacinum*
cells/mL	:	Cells per millilitre
ºC	:	Degree Celcius
DHA	:	Docosahexaenoic acid
EPA	:	Eicosapentaenoic acid
EFA	:	Essential fatty acids
FAO	:	Food and Agriculture Organization of the United Nations
e.g.	:	For example
g	:	Grams
HUFA	:	Highly unsaturated fatty acids
i.e.	:	In other words
ISO	:	*Isochrysis galbana*
kg	:	Kilograms
M/F	:	Males over females
m^{-2} s^{-1}	:	Metre squared per second
MG	:	*Meyerozyma guillermondii*
µm	:	Micrometers
µmol	:	Micromole
mg/L	:	Milligrams per litre
mL	:	Millilitres
N	:	*Nannochloropsis* sp.
POME	:	Palm oil mill effluent

ppt	:	Parts per thousand (salinity)
day^{-1}	:	Per day
%	:	Percent
PUFA	:	Polyunsaturated fatty acids
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
RM	:	*Rhodotorula mucilaginosa*
RS	:	*Rhodovulum sulfidophilum*
SA	:	*Shewanella algae*
sp.	:	Species
TET	:	*Tetraselmis suecica*
v/v	:	Volume to volume
w/v	:	Weight to volume

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CHAPTER 1

INTRODUCTION

Aquaculture is a significant contributor to food security and this sector supplies the rising demands for fishery products globally. The world aquaculture production increases yearly with the production volume dominated by finfish farming, which is the main aquaculture product in many countries (FAO, 2016). In spite of that, aquaculture industry is still facing several unsolved obstacles in producing aquatic species sustainably and cheaply. Of all the bottlenecks, insufficient production of high quality live feeds for larviculture is one that needs to be addressed (Hixson, 2014).

Growth and survival of farmed fish are largely dependent on the suitability and nutritional quality of their diet. In larval rearing of aquatic species, finding the ideal feed is a challenging task. Larval feeds should be of a suitable size for fish larvae, highly nutritious, palatable and are able to preserve water quality (Sandeep *et al*., 2015).

Larval feeds are divided into two main types – formulated or live diets. Formulated diets (also known as artificial or inert diets) are convenient as it can be easily prepared and are commercially available (Stottrup & McEvoy, 2003). However, inert diets tend to aggregate either to the water surface or sink to the bottom which makes it less available to the larvae compared to live feed (Stottrup & McEvoy, 2008). In contrast, live feed stimulates larval feeding responses due to its swimming movements and has a high water content which makes them easy to digest, accommodating to the lack of feeding appendages and immature digestive system of fish larvae. The success of larval rearing depends on the supply of high quality feeds (Stotrrup & McEvoy, 2008; Rasdi & Qin, 2014; Conceicao *et al*., 2010).

Traditional live feed products in marine fish production are brine shrimp (*Artemia*) and rotifers (*Brachionus* sp.). *Artemia* and rotifers lack essential fatty acids such as eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3) and arachidonic acid (ARA, C20:4n-6) which are important for fish survival, growth and development. Hence, enrichment is needed before feeding (Hansen *et al*., 2017). In contrast, most copepods have high compositions of *n-3* highly unsaturated fatty acids (EPA and DHA) and they are rich in natural sources of antioxidants, astaxanthin and vitamins A and E (van der Meeren *et al*., 2008; Ajiboye *et al*., 2011). Furthermore, their different body sizes are suitable for different developmental stages of fish and their typical zigzag movement is an important visual stimulus for fish larvae (Stottrup & McEvoy, 2008).

When compared to *Artemia* and/or rotifers, considerably better results of larval performance and quality were generally achieved with the use of copepods as live feeds in marine fish larvae of Atlantic halibut and Atlantic cod (Conceicao *et al*., 2010). Copepod *Acartia tonsa* nauplii used in first-feeding of Atlantic cod and Ballan wrasse resulted in positive long-term effects of growth and viability of the fish larvae compared to those fed with rotifers (Oie *et al*., 2017). Traditionally, copepods used in fish production are caught from the natural environment and are either fed directly to fish larvae or cultured in semi-extensive tanks (Drillet *et al*., 2011). Many copepod species such as *A. tonsa*, *Pseudodiaptomus annandalei*, *Paracylopina nana* and *Apocyclops royi* have been identified as potential candidates for mass culture (Rasdi & Qin, 2014) but establishing cost-effective protocols for mass production remain a challenge (Ajiboye *et al*., 2011). The problems related to culturing copepods include long generation time, limited culture density, seasonal variations of yields and high costs (Conceicao *et al*., 2010; Ajiboye *et al*., 2011). The success of copepod cultures depends not only on the diet but also on abiotic factors such as temperature, salinity, dissolved oxygen and light.

There is a knowledge-gap in regards to species-specific environmental parameters and its effects which important in improving protocols for copepod culture systems especially for intensive cultivation. These parameters affect multiple stages of a copepod's life cycle including its development, survival, egg production and egg hatching success (Drillet *et al*., 2011).

Copepod feeding studies have mostly evaluated microalgae diets as a source of EPA and DHA in cyclopoid copepods *Thermocyclops hyalinus*, *Mesocyclops aspericornis* (Vidhya *et al*., 2014), *Apocyclops royi* (Pan *et al*., 2018) and *Apocyclops borneoensis* (Wang *et al*., 2017). However, the high production costs of microalgae biomass remain a limiting factor in the aquaculture industry (Hemaiswarya *et al*., 2011; Chauton *et al*., 2015; Acien *et al*., 2018). A proposed alternative to microalgae in this study is marine microheterotrophs as feed for copepods. Microheterotrophs consumed by live prey can be used as a vector to transfer beneficial amino acids or fatty acids to fish larvae in early feeding stages (Sayes *et al*., 2018).

A few studies have evaluated the use of marine microorganisms as diet for zooplankton: rotifers (Rieper, 1978; James *et al*., 1987; Loo *et al*., 2013) and copepod *Paracyclopina nana* (Lee *et al*., 2006), with positive results in terms of growth and survival, and its nutritional composition compared to those fed with traditional diets. The culture of heterotrophs in lipid rich wastewaters such as palm oil mill effluent (POME) may improve its highly unsaturated fatty acid (HUFA) and polyunsaturated fatty acid (PUFA) content (Shah *et al*., 2016).

In Malaysia, the palm oil industry has become one of the world's largest producers of palm oil and its derivatives. The wet process of palm oil milling discharges wastewater known as POME which could become one of the major sources of water pollution if discharged prior to proper treatments (Madaki & Seng, 2013). However, the nutritional

composition of POME makes it possible for the reuse of treated effluent as fermentation media for single-cell protein production, fertilizer and live feed for animals and aquaculture organisms (Wu *et al*., 2009). Some examples of POME usage in aquaculture include effluent fed to chironomid larvae (also known as "bloodworms") which is a food source for aquatic species (Habib *et al*., 1997) and culturing a marine microalga *Isochrysis* sp. in POME as supplementary feed for rotifer culture (Vairrapan & Yen, 2008). Alternatively, POME grown *Rhodovulum sulphidophilum* was shown to be an effective bacterial diet for mass culture of rotifers (Loo *et al*., 2013). Such production method had provided an alternative waste management solution to oil palm by-products (Madaki & Seng, 2013).

In this study, a brackish water cyclopoid copepod *Apocyclops dengizicus* (Lepeshkin, 1900) was used as a research candidate because most cyclopoids have a short maturation time of four to five days and are planktonic which is advantageous during the harvest of nauplii as it is easier to separate them from debris compared to benthic harpacticoids (Stottrup, 2006). Previous studies showed that *Apocyclops dengizicus* can tolerate a wide range of environmental parameters (Farhadian *et al*., 2014; Altaff & Janakiram, 2015) and high densities and growth rates were achieved using the diet of microalga *Tetraselmis tetrathele* (Farhadian *et al*., 2008a). The lipid content and fatty acid composition of *A. dengizicus* met the nutritive requirements of larval fish and shrimp rearing (Farhadian *et al*., 2008b; 2009). *A. dengizicus* has been acknowledged as good live feed candidate for larval fish and shrimp but their cultures were mostly limited to diets of microalgae.

Hence, this study was a fundamental step into finding a cheaper alternative to algae-based diets and optimal culture conditions for *A. dengizicus* in hopes of (1) establishing an economically viable copepod-rearing system, (2) producing high-quality waste-grown live feed sustainably and cheaply, and (3) providing a green technology solution

for the local palm oil industry's wastewater treatment. Essentially, this information will serve as a baseline for an indoor commercial-scale production of copepods.

The specific objectives of this study were to:

a. evaluate the feasibility of using POME-grown microheterotrophs as copepod diet;

b. investigate the effects of various salinities, temperatures, pH, photoperiods and light intensities on the survival, growth and reproduction of *Apocyclops dengizicus*;

c. assess the fatty acid composition of POME-grown microheterotroph diets and *Apocyclops dengizicus,* and the nutritional content of the selected diet compared to POME;

d. identify the optimum POME-based diet and abiotic conditions for culturing *Apocyclops dengizicus*.

CHAPTER 2

LITERATURE REVIEW

2.1 World aquaculture industry

Aquaculture is one of the fastest growing food sectors in the world. It is commonly known as the farming or cultivation of fish, crustaceans, molluscs and aquatic plants. The demand for aquatic food products has continued to increase with the rising number of global human population. Fish and other types of aquatic animals are a major source of protein for human consumption. In 2013, 17 percent of the global population's protein intake was fish. Historically, aquatic food production was primarily based on capture fisheries but supply from the aquaculture sector surpassed the supply of wild-caught fish for human consumption in 2014 for the first time. In 2014, 73.8 million tonnes of fish were harvested from aquaculture with an estimated first-sale value of US$160.2 billion with more than 67 percent of harvest consisting of finfish (FAO, 2016).

2.2 Live feeds in marine hatcheries

The most crucial stage for the optimum development in rearing fish larvae is the first feeding phase of the larval cycle. This stage is the transition stage from an endogenous (reliant on yolk) to an exogenous (reliant on external prey) feeding method by the larvae (Olivotto *et al*., 2010). Live feed should have a suitable nutritional composition, appropriate size range and stimulate feeding responses.

One of the main groups of nutrients essential for larval growth is fatty acids. Essential fatty acids (EFA) in aquaculture nutrition in order of importance are docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), arachidonic acid (ARA, 20:4n-6) and their precursors linolenic acid (LNA, 18:3n-3), linoleic acid (ALA, 18:2n-6) and oleic acid (OA, C18:1n-9c). The primary

factor influencing the difference in EFA requirement is its environmental origin (freshwater, estuarine or marine) (Glencross, 2009). Highly unsaturated fatty acids (HUFAs) such as DHA and EPA at the correct ratio significantly affect the survival of marine fish larvae (Ohs *et al*., 2009). Dietary ratio of 2:1 of DHA: EPA was recommended for initial larval feeding to mimic the same ratio of fatty acid content of yolk of many wild marine fish eggs (Parrish *et al*., 1994). Marine aquatic organisms are unable to biosynthesize n-3 and n-6 fatty acids from its precursors, which must be obtained from their diet (Glencross, 2009). Dietary deficiency of EFA will result in poor growth due to higher demands needed for neural development, increased mortality and EFA also plays a diverse role in the physiological and biochemical processes within aquatic animals (Glencross, 2009).

The physical characteristics of prey should also be considered as important factors in larviculture (Ohs *et al*., 2009). The size of live food organisms should be small enough for the mouth gape of fish larvae to consume. In natural habitats, the movement of live prey stimulates fish feeding responses. Accordingly, the larval mouth gape and feeding responses of each specific species should be considered when determining the appropriate live food organism (Ohs *et al*., 2009).

2.2.1 Zooplankton live feed species and its limitations

The most common zooplankton live feed species used in commercial hatcheries are rotifers (*Brachionus plicatilis* and *B. rotundiformis*) and brine shrimp (*Artemia* spp.). Unlike artificial feeds, live food organisms are better in terms of acceptance, digestibility and nutrition (Dhont *et al*., 2013). Rotifers (*Brachionus* spp.) have been an essential part of the initial feeding of the larval marine fish and crustaceans since the 1970s (Conceicao *et al*., 2010). Rotifers are

used as first feed during the initial stages of larval rearing for a couple of days to weeks prior to replacing it with a larger prey species, usually *Artemia* nauplii as the fish grow larger (Conceicao *et al*., 2010). Some of the advantages of using rotifers as live food organisms are that they are easy to culture and can reach densities as high as over 1000 rotifers/mL (Dhont *et al*., 2013), have high reproductive rate which contributes to the high population growth rate, can tolerate a wide range culture conditions and handling. The main disadvantages of rotifers are that their nutritional composition lacks the appropriate HUFAs to satisfy larval nutrition which requires enrichment and mass cultures are prone to sudden collapses (Dhont *et al*., 2013).

Brine shrimp (*Artemia* sp.*)* is the most widely known and used live feed to culture larvae globally. Dormant *Artemia* cysts collected from the wild can be stored dry for years and easily hatched with predictable success, making it an indispensable aquaculture product. Nevertheless, the body size of *Artemia* nauplii is 450 µm, which is too large as initial live prey to most marine fish larvae with small mouth gape. The main disadvantage of using *Artemia* as live feed is the insufficient levels of DHA, EPA and ARA for fish nutrition (Rasdi & Qin, 2014). Furthermore, *Artemia* may metabolize the nutritional supplement ingested before being captured and they are also able to catabolize DHA back to EPA which limits the effectiveness of fatty acid enrichment to obtain a desired DHA: EPA content (Ohs *et al*., 2009). The main issue for commercial hatcheries is the fluctuating prices for *Artemia* cysts compared to the cheaper alternative of formulated food. Therefore, the price of *Artemia* cysts has been a major factor for each hatchery's cost-benefit structure and may not be the preferred feed in the long run (Dhont *et al.*, 2013).

2.2.2 Copepods as an alternative live feed

Copepods have been used as a live food for various marine fish larvae but their use in aquaculture remains sporadic due to challenges in upscaling copepod cultures in commercial setting (Ajiboye *et al*., 2011). The three main orders of copepods used in larviculture are Calanoida, Harpacticoida and Cyclopoida. Cyclopoids have an advantage over calanoids as they are easier to culture and maintain at high densities. The disadvantages of both cyclopoids and harpacticoids is the inability to harvest eggs as they bear their eggs until hatching and the lack of possibilities for egg storage. The process of tank cleaning and egg harvest cannot be combined which may result in more labour-intensive systems (Stottrup, 2006). Drillet *et al*. (2011) reviewed that the most important traits for live feed include a fast reproductive cycle, low mortality, biochemical composition and/or swimming behaviour. Copepods with fast reproduction and low mortality are advantageous in terms of viability of commercial cultures while the nutritional qualities and swimming behaviour are more specific to the needs of each fish larvae species (Drillet *et al*., 2011). Calanoid copepods are the most studied due to their pelagic nature, are natural prey to a wide range of fish larvae and the capacity of storing eggs but they cannot be cultured in high densities without negative side effects such as low hatching success and increased mortality.

The size range of copepod nauplius is ideal for the small mouth-gapes of many newly hatched fish larvae. They are smaller in size (38-220 μm) than the smallest strains of rotifers or *Artemia* (Ohs *et al*., 2009). Another advantage of copepods is their swimming behaviour which is a jerking (or zigzag) motion which provides a visual stimulus to pelagic predators and is important during first feeding as it can improve ingestion rates (Ajiboye *et al*., 2010; Rasdi & Qin, 2014). The

mechanisms differ between the naupliar (N1-N6) stages and the early C1 copepodite stage. Nauplii lack pereiopods which are used by copepodites for faster escape. However, different fish predators have different capture strategies which need to be considered. The broad spectrum of body size shows that copepods are suitable live prey candidates for early feeding due to having multiple stages of nauplii and copepodite before reaching mature adult size (Gemmell & Buskey, 2011).

Digestive enzymes in live prey are released in the gut of the larvae to assist in the digestive process. The retention time of copepods in digestive system of fish is much higher than that of *Artemia* and rotifers, validating that the former was better digested and offer better nutrient uptake for the fish compared to the latter (Olivotto *et al*., 2010; Camus, 2012).

Table 2.1: Summary of main nutritional characteristics of copepods compared to *Artemia* and rotifers as live feed (Cited from Camus, 2012).

Prey categories	Size range	Motion Pattern	Main nutritional characteristics				
			Digestive enzymes	Micronutrients and vitamins	HUFA	DHA/EPA ratio	Phospholipids
Artemia	400-500µm (Instar I)	continuous (weak feeding responses)	Low	deficient, enrichment needed	very low enrichment needed	generally <1, even when previously enriched	mostly triacyglycerols enrichment needed
Rotifer	~100-150µm (s type)	continuous (weak feeding responses)	Low	deficient, enrichment needed	low, enrichment needed	generally <1, even when previously enriched	mostly triacyglycerols enrichment needed
Copepod	<80µm (early nauplii)	"stop and go" (strong feeding responses)	higher level compared to Artemia and rotifer	Copepods generally contain more vitamins, pigments and trace minerals than rotifers and Artemia	naturally high level	substantially >1 significantly higher levels of DHA and EPA when compared to enriched rotifers or Artemia	more phospholipids (>50%) than triacylglycerols in proportion when compared to Artemia and rotifers

2.2.3 Nutritional value of copepods

Copepods are one type of zooplankton consumed by fish larvae in the wild. They are superior live prey due to containing high levels of HUFAs with

DHA/EPA ratios >1 which are significantly higher than enriched *Artemia* or rotifers (Rasdi & Qin, 2014). The biochemical composition (lipids, amino acids and micronutrients) of copepods display high stability over years and seasons regardless of environmental variations (van der Meeran *et al*., 2008). PUFAs dominated the total lipid content in three copepods (*Acartia grani*, *Centropages hamatus*, and *Eurytemora affinis*). Copepods contained moderate levels of lipids (6.9–22.5%), high amounts of DHA (8.3-24.6%) and EPA (13.9-42.3%) and also low levels of ARA (0-2.6%). Despite having low amounts of ARA, the ratio of DHA/EPA in copepods is better in terms of fulfilling the HUFA requirements of fish larvae for optimal development and quality (van der Meeren *et al*., 2008). The other advantage of copepods is the bioavailability of HUFA in their phospholipids compared to HUFA supplied in neutral lipids (namely in triaglycerols) in enriched *Artemia* (Conceicao *et al*., 2010). Phospholipids are an important dietary requirement for marine fish larvae for tissue growth and development (Sargent *et al*., 1999).

Copepods have better levels of free amino acids and an abundance of astaxanthin and iodine, and comparable amounts of vitamins C, E, B1 and B2 compared to *Artemia* and rotifers (van der Meeran *et al*., 2008). The amounts of proteins and free amino acids in wild zooplankton (mainly copepods) was higher than in enriched *Artemia* but free amino acids follow a species-specific pattern (Helland *et al*., 2003). Variations in both fatty acids and protein content in copepods also depended on their origin, the culture methods used and their life stage (egg versus nauplii) (Drillet *et al*., 2006). High levels of dietary proteins and amino acids are essential requirements for marine fish larvae as they have high growth potential and amino acids are used for energy (van der Meeran *et al*., 2008; Ajiboye *et al*., 2010). Several amino acids have been described as feeding

stimulants for fish larvae (Drillet *et al*., 2006) but some essential amino acids can also limit larval growth (Aragão *et al*., 2004). However, Ajiboye *et al.* (2010) emphasized that little attention has been paid to compositions of protein, amino acids, pigments and vitamins which are also important for copepod performance and survival compared to lipids and fatty acids.

2.2.4 Cyclopoid copepod: *Apocyclops dengizicus*

There are over 13,000 named species of copepods and approximately 700 known species of freshwater cyclopoid copepods in the world. All cyclopoids use grasping mouthparts to eat; smaller species are plankton feeders and larger species are predators that can consume large prey. The name "cyclops" describes their single eye spot which senses luminous intensity, a mechanoreceptor to detect prey (Marten & Reid, 2007). A species which is indigenous in the coastal waters of Malaysia is a planktonic cyclopoid copepod, *Apocyclops dengizicus* (Lepeshkin, 1900) (Figure 2.1). This species has six naupliar stages (85-240 μm) and five copepodite stages (320-680 μm), moulting each time before maturing to adult male or female (Farhadian *et al*., 2008b; Marten & Reid, 2007). Sexes are separate and easily distinguished. Copepods reproduce sexually and their eggs are contained within one or two egg sacs attached to the female genital segment until they hatch (Stottrup & McEvoy, 2008). *Apocyclops dengizicus* has a shield-like cephalothorax with a typical cyclopoid body form (see Figure 2.1). The first thoracic segment which is fused with the head is leg-bearing, followed by three increasingly narrower thoracic segments which give it a club-shaped appearance. An adult female has a pair of antennules with 11 segments, all bearing setae. The urosome consists of five segements, including a swollen second genital segment and three posterior segments. The fifth pair of legs occurs on the first urosome. Females carry paired egg sacs. In adult male *A. dengizicus*, they have symmetrical

antennules with 14 segments, with two parts strongly hinged. The urosome consists of six segments. Adult males are generally smaller in size than adult females (Fofonoff *et al*., 2018; Marten & Reid, 2007; Valderhaug & Kewalramani, 1979).

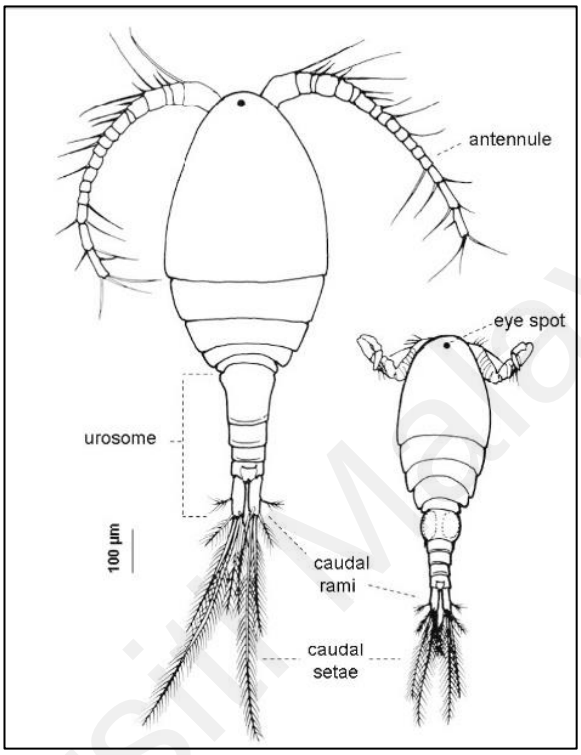

Figure 2.1: Key body parts of *Apocyclops dengizicus* (Adapted from Martens & Reid, 2007).

Only a few species of cyclopoids such as *Oithona* sp. and *Apocyclops* sp. have been reared in the laboratory compared to the widely studied calanoids *Acartia* and *Calanus* genera. These cyclopoids have the ability to survive in multi-generation cultures suitable as live feed for marine fish larvae (Drillet *et al*., 2011; Stottrup, 2006). The Taiwanese aquaculture industry has extensively studied the use of copepods, mainly *Apocyclops royi* and *Pseudodiaptomus annandalei*, for rearing finfish fry (Su *et al*., 2005). The advantages of cyclopoids are: 1) easier to culture than calanoids, 2) some species only take 4-5 days to reach sexual maturity, 3) omnivorous and fed on various microorganisms such as

phytoplankton and yeast, and 4) cyclopoid nauplii are easier to harvest than harpacticoids because they are planktonic (Stottrup, 2006).

2.3 Effects of abiotic factors on copepods

2.3.1 Salinity

Apocyclops dengizicus is able to tolerate a wide range of salinities from 0.5 to 68 ppt (Dexter, 1993). In a more recent study, the optimum salinity for maximum population growth rate (K=0.314) and fecundity (26.8±1.5 individuals/day) was observed at 20 ppt as compared to those cultured in 10 ppt and 30 ppt (Farhadian *et al*., 2014). Similarly, the optimal salinity for attaining highest productivity of *Apocyclops royi* was also reported at 20 ppt (Pan *et al*., 2016). This study also observed lower population growth rates at extreme salinities (0 and 5 ppt, 30 and 35 ppt). Lower clutch size was observed at salinities 0 and 5, whereas significantly lower nauplii and clutch production at salinities of 0 and 30. Salinity fluctuations are normal occurrence in marine ecosystems but suboptimal salinity may induce osmotic stress in copepods. Euryhaline copepods can survive through salinity fluctuations (where hypersalinity can be caused by evaporation and hyposalinity caused by rainfall) but only a narrow range of salinities are optimal for their physiological processes and fitness (Pan *et al*., 2016). In unsuitable salinity conditions, excessive energy is spent on adjusting their metabolism to regular their intercellular solute concentrations instead of their physiological performance (Kimmel & Bradley, 2001). Understanding the optimal salinity is essential in enhancing copepod production in manipulated aquaculture environments (Pan *et al*., 2016).

2.3.2 Temperature

Although *A. dengizicus* was able to reproduce and survive over a temperature range from 25°C to 35°C, its optimal growth, survival and high fecundity was

recorded at 35°C (Farhadian *et al*., 2014). Previous study on *A. royi* stated that increasing temperature range of 15°C to 35°C did not affect survival rate and production but mainly affected the growth rate and fecundity (Su *et al*., 2005). This is also evident in another study by Altaff and Janakiram (2015) which showed that a higher temperature range (31±1°C) resulted in a faster attainment of peak density compared to the lower temperature (26±1°C) culture. The egg production and hatching rate of *A. dengizicus* increased as temperature increased until it reached a temperature threshold (above optimal range), after which a decline begins. The authors recorded a statistically significant higher body length and width of adults of *A. dengizicus* in low temperature culture (Altaff & Janakiraman, 2015).

2.3.3 pH

Zooplankton thrive well in the pH range of 7.5 to 8.4 in oceanic waters. A review by Hansen *et al.* (2017) concluded that egg hatching by pH up to 9.0–9.5 was unaffected by a selection of euryhaline copepods, *Acartia* spp., *C. typicus* and *E. affinis*. Nauplii mortality was higher at pH 9.5 for both *Acartia* spp. and *C. typicus* compared to other pH regimes while *E. affinis* nauplii were unaffected by pH. It was found that pH tolerance is linked to their natural distribution. Oceanic-neritic copepods like *Oithona similis* showed less tolerance to elevated pH because oceanic-neritic habitats are quite stable in pH (range 7.5-8.5). In contrast, euryhaline species e.g. *T. longicornis*, *Acartia* spp., *C. typicus*, *P. elongatus* and *E. affinis* were more adapted to fluctuating pH environments because it is a common stressor in estuaries (Hansen *et al*., 2017). Jayalakshmi *et al.* (2016) found that acidification negatively affected fatty acid profile of harpacticoid copepod *Parastenhelia* sp. in vitro as HUFAs such as EPA, DHA and ARA were low at pH 4.0 ±0.3 compared to other pH tested.

2.3.4 Photoperiod

Photoperiod is a major abiotic condition which can be easily manipulated in an aquaculture setting with low costs (Nogueira *et al*., 2018). Copepods, like other organisms, are exposed to a daily cycle of daylight (day) and darkness (night). The daily cycle is usually divided into active and quiet periods where organisms are able to synchronize their behaviour to times of food availability and minimal predator activity to enhance their survival and reproduction (Marcus & Scheef, 2010). Organisms may also undergo biochemical and physiological adjustments so that their rhythmic functions and activities adjust to seasonal change (Marcus & Scheef, 2010). Growth, maturation and reproduction of aquatic invertebrates are generally inhibited by constant light (Miliou, 1992) where changes in illumination was found to affect endocrine activities such as reproduction behaviour, egg production, moulting and death in copepods (Omori & Ikeda, 1984).

Photoperiod had been shown to affect the reproductive performance, time required to maturation and total life span of cyclopoid *Mesocyclops* sp. as continuous light and total darkness significantly reduced total offspring production. However, several photoperiods tested did not significantly affect adult sex ratio and survival rate (Fereidouni *et al*., 2013). In contrast, continuous light 24:0 h LD was found to have positive effects on *A. dengizicus*. Total production, growth and survival were the highest at light regimes of 24:0 h LD and 12:12 h LD (Farhadian *et al*., 2014).

2.3.5 Light Intensity

Light intensity has not been a major abiotic factor studied in culturing copepods compared to light regimes. For *A. dengizicus* culture, the highest production and shortest development rate were achieved under a low light

intensity of 33.3 µmol photons m^{-2} s^{-1} (Farhadian *et al*., 2014). Light intensity is usually related to feeding behaviour but more research is needed to understand the relationship between feeding rates, light intensity, light cycles, oxygen production and the physiological status of copepods (Li *et al*., 2008). In the wild, positive light responses of predominantly herbivorous and omnivorous copepods in vertical migration of zooplankton is related to the plankton-rich upper water layer. Light is not the only determining factor in copepod behaviour which is also dependent on their ecological characteristics, feeding strategy and conditions (Martynova & Godeeva, 2010).

2.4 Conventional and non-conventional diets in zooplankton culture

2.4.1 Microalgae diet

Most studies on the dietary requirements of copepods are based on microalgae diets. Zooplankton production has been positively linked to certain amino acids and fatty acids in dietary algae. For instance, population growth of copepods and egg production were found to be positively correlated with algae consumption and its PUFA (EPA and DHA) content (Rasdi & Qin, 2014). A mixed diet of *Chaetoceros calcitrans* and *Tetraselmis tetrathele* was found to be the optimum diet for population growth in *A. dengizicus* culture (Farhadian *et al*., 2008a). It was reported that this combination had high DHA:EPA:ARA ratio of 0.66:1.21:1 (*C. calcitrans*) and 0.65:2.31:1 (*T. tetrathele*). ARA has an important role as a precursor to some prostaglandins and other biologically active compounds which regulates growth and reproduction of copepods (Sargent *et al*., 1997). In a study on *Paracyclopina nana*, the combination of *Tetraselmis suecica* (TET) and *Isochrysis galbana* (ISO) was found to be the optimum diet for mass culture of this species. In contrast, other microalgae diets containing high EPA and DHA showed lower fecundity and lower population growth of copepods. TET had a low

amount of EPA and no DHA but *P. nana* was able to biosynthesize DHA from linolenic acid, 18:3n-3 in TET (Lee *et al*., 2006). Both studies also emphasized that microalgae such as *Chlorella* sp. and *Nannochloropsis* sp. have hard cell walls which are indigestible for copepod nauplii (Cano *et al*., 2004).

2.4.2 Marine microheterotrophs

Heterotrophs are organisms that obtain their energy (nutrition) from other sources of carbon compounds or materials. Examples of microheterotrophs include yeast, bacteria and some types of protist or microalga. These unicellular organisms have been used as food to enrich specific essential amino acids or fatty acids in live prey organisms (such as rotifers and brine shrimp) before feeding to fish larvae (Kang *et al*., 2006). Many planktonic copepods are filter-feeders and consume suspended particles such as phytoplankton, yeast and/or bacteria.

2.4.3 Marine yeast

Yeasts are found in almost every part of the aquatic environment. Marine yeasts are defined as being able to grow on or in a marine substrate (Kutty & Philip, 2008). Generally, marine yeasts inhabit near-shore environments in higher densities compared to deep-sea oceanic regions. The major genera isolated in a study by Kutty and Philip (2008) were *Candida*, *Crytococcus*, *Debaryomyces* and *Rhodotorula*. Marine yeasts are rich in proteins, lipids and vitamins. Yeast has a commercial importance due to its nutritional quality. It can biotransform raw material into yeast biomass (single-cell protein) and can be produced economically and efficiently due to its shorter generation time and use of inexpensive culture media (Kutty & Philip, 2008). Marine yeasts have been reported to be able to produce a variety of bioactive substances such as amino acids, glutathione, enzymes, vitamins and lipids with potential applications in a

wide range of industries including animal or aquaculture feed and waste treatment (Chi *et al*., 2009; Zaky *et al*., 2014).

Marine yeast had been investigated as an alternative dietary source in cultivating zooplankton such as rotifers and *Moina*. Marine yeast was compared to baker's yeast (*Saccharomyces cerevisiae*) as a diet for mass culture of rotifers and the use of *Candida* spp. mixed with algae *Chlorella* spp. was found to be successful for culturing rotifers (James *et al.,* 1987). The investigation revealed that marine yeast had better nutritional quality and higher amino acid content than baker's yeast which resulted in increased birth rate, increased overall production and better nutritional quality in rotifers. However, the yeasts were used as a supplement to microalgae and not as a single diet for rotifer culture (James *et al*., 1987). In another study, marine yeasts (*Debaryomyces hansenii* Yeast-14 and *Candida austromarina* Yeast-16) and a commercial diet was used to culture *Moina macrocopa*. They reported that *Candida austromarina* Yeast-16 provided better nutritional and dietary values than the commercial diet. The yeast diet increased the levels of DHA which resulted in a high DHA:EPA ratio in *M. macrocopa* (Kang *et al*., 2006).

2.4.4 Marine bacteria

Biotechnological processes have investigated the use of bacterial biomass as an alternative feed supplement and as probiotic resources in aquaculture and aquaculture systems (Banerjee *et al*., 2000). Bacteria are nutritious for fish and shrimps and their use as supplements or fish meal replacements have had positive effects (Azad *et al*., 2002; Aas *et al*., 2006; Shapawi *et al*., 2012). Probiotics in fish enhances immunity, larval survival and growth. Probiotic entry to fish larvae by the use of vectors such as rotifers and *Artemia* were reported to be a favourable route in increasing larval survival (Sayes *et al*., 2018). The use of bacteria also has

several advantages over conventional feed in aquaculture. Phototrophic bacteria, for example, can be cultured economically in limited space with cheap substrate, have a digestible cell wall, contains useful enzymes and their nutritional value can be manipulated genetically or by altering the carbon and nitrogen sources (Kobayashi & Kobayashi, 2001; Loo *et al*., 2013a).

Marine bacteria have been tested as diets for harpacticoid copepods, *Tisbe holothuriae* and *Paramphiascella vararensis*, and both species were able to grow and reproduce in long-term feeding experiments exclusively on a diet of dried bacteria (Rieper, 1978). In recent years, wastewater grown phototrophic bacteria, *Rhodovulum sulphidophilum*, was found to be able to support rotifer culture and produce rotifers containing biochemical content that was comparable to those fed with algae (Loo *et al*., 2015). This was an example of a cost-efficient rotifer production which utilized palm oil mill effluent (POME) as a substrate.

2.4.5 Marine protists

Thraustochytrids are heterotrophic marine protists that can be found in seawater, algae, estuaries, mangrove forest and sediment (Jaritkhuan & Suanjit, 2018). These marine protists produce high amounts of omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA), which is important for human health and aquaculture. They are also potential sources of other PUFAs such as EPA, DHA, asataxanthin, carotenoid pigments and other lipids (Raghukumar, 2008). For example, *Aurantiochytrium limacinum* isolated from fallen mangrove leaves contained high amounts of DHA compared to ten other isolated species and this strain was considered to have potential in aquaculture or commercial use (Jaritkhuan & Suanjit, 2018). Marine thraustochytrids are also able to be mass produced using low-cost substrates (commercial grade glucose and yeast extract from sodium chloride treated baker's yeast) at optimum culture conditions.

Biomass production was significantly affected by incubation temperature and salinity levels (Ludevese-Pascual *et al*., 2016). Thraustochytrids can also be cultivated using saline waste medium and glycerol, a by-product of the biodiesel industry, as a carbon source (Fortes, 2016). Recently, strains of *Aurantiochytrium* and *Parietichytrium* were fed to early stage Kuruma shrimp, *Marsupenaeus japonicus*, in a laboratory scale culture. It was reported that larvae fed on the KOU10 strain of *Parietichytrium* showed better survival, growth and development compared to other diets including microalgae *Chaetoceros calcitrans* (Sato *et al.*, 2018).

2.5 Palm oil industry in Malaysia

Malaysia is one of the world's leading producers and exporters of palm oil and palm oil products. Since its beginnings as a monoculture plantation crop in 1917, current oil palm cultivation has reached 5.81 million hectares in 2017 and has become one of the most significant contributors to Malaysia's Gross Domestic Product (GDP) (MPOB, 2018; Kushairi *et al*., 2017). Palm oil milling can be categorized into two general processes: dry or wet (standard) process and the latter is the most common way of extracting palm oil in Malaysia (Wu *et al*., 2009). For each tonne of crude palm oil to be produced from fresh fruit bunches, 5-7.5 tonnes of water are required and more than 50% of water will end up as POME (Wu *et al*., 2009). Fresh POME produced in crude oil extraction process are derived from three main sources, i.e. sterilizer condensate, separator sludge and hydrocyclone wastewater (Foo & Hameed, 2010).

2.5.1 Palm oil mill effluent (POME)

Fresh POME is described as thick, brownish, acidic colloidal mixture of water (95-96%), oil (0.6-0.7%) and suspended solids (2-4%), generated during the processing of fresh fruit bunch (FFB). POME contains amino acids (mostly

aspartic acid, glutamic acid, glycine, alanine and methionine), inorganic nutrients including sodium, potassium, calcium, magnesium, manganese, and iron, nitrogenous compounds, organelles, short palm fibres, oil residues and carbohydrates ranging from cellulose to simple sugars (Habib *et al*., 1997; Foo & Hameed, 2010). Other important characteristics are low pH value of about 4.5, high biological oxygen demand (BOD, 10,250 - 43,750 mg/L), chemical oxygen demand (COD, 16,000 – 100,000 mg/L), high amounts of suspended solids (5000–54,000 mg/L) and discharge temperature of 80-90°C. Compositions of POME vary depending on the quality of raw material and processing technique. It is also non-toxic as no chemicals were added during the extraction process (Foo & Hameed, 2010; Madaki & Seng, 2013). Raw POME also contains saturated and unsaturated fatty acids such as myristic acid, palmitic acid, stearic acid and oleic acid, and polyunsaturated fatty acids - eicosatrienoic acid (20:3n−6), eicosatetraenoic acid (20:4n−6) and eicosapentaenoic acid (20:5n−3) which are essential for the proper development of marine fish, shrimp larvae and fry (Wu *et al*., 2009).

The differences between undiluted treated POME and diluted treated POME includes lower ammonia-nitrogen levels, chemical oxygen demand (COD), suspended solids and lignin in the latter (Y Zahrim & Rajin, 2014). In a study of growing micro and macro algae in POME, different dilutions were used to enhance its biomass yield. The carbon source in a medium is used rapidly in heterotrophic cultures and large quantities of carbon source is needed to extend the growth period in a batch culture. Therefore, it is important to determine the optimal concentration of substrate without inhibiting growth (Kamyab *et al*., 2014).

POME is a lipid rich wastewater with very high BOD which is an environmental pollutant if left untreated. Management of POME is considered difficult and expensive to dispose properly (Madaki & Seng, 2013). Improper POME discharges into nearby lands and rivers has caused serious ecological impact on surrounding environments (Foo & Hameed, 2010). The palm oil milling industry has developed many POME treatment technologies including biological treatments as a sustainable strategy to eliminate palm oil residue wastes (Foo & Hameed, 2010).

2.5.2 Applications of POME

The possibilities of reusing POME as biotechnological products are vast due to its chemical composition. Applications of POME include production of biogas and other metabolites by fermentation, recovering bio resources or conversion into useful substitutes for animal feed and fertilizer (Wu *et al*., 2009). POME has been used as an alternative medium for algal biomass and lipid production (Shah *et al*., 2016).

In the aquaculture industry, POME has been used to produce high quality live food chironomid larvae (Habib *et al*., 1997) and used to culture marine microalgae (*Isochrysis* sp. and *Nannochloropsis* sp.) as supplementary diet to rotifer cultures (Vairappan & Yen, 2008). It has been successfully used to culture phototrophic bacteria (PB) for rotifer production. The POME enriched *Rhodovulum sulfidophilum* contained both EPA and DHA in its nutritional content (Loo *et al*., 2013a). It was then used to culture larval marble goby, (*Oxyeleotris marmorata)* which resulted in significantly higher levels of growth and survival compared to fish larvae given traditional diets of rotifers fed *Nannochloropsis* sp. (Loo *et al*., 2013b).

CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganism culture

The stock culture of marine microorganisms i.e. (1) bacteria, *Shewanella algae* (SA) and *Rhodovulum sulfidophilum* (RS); (2) yeasts – *Rhodotorula mucilaginosa* (RM) and *Meyerozyma guillermondii* (MG); and (3) protist - *Aurantiochytrium limacinum* (AL) were obtained from the Feed-to-Fish Laboratory, University of Malaya. These microheterotropic organisms were isolated from local coastal waters of Kuala Selangor, Selangor, Malaysia. These microbes were selected based on a previous study on POME-grown diets for *Moina* and rotifers (Loo *et al*., unpublished). The marine bacteria were maintained on 112 medium agar plates (refer to Appendix A), whereas the marine yeasts and protist were maintained on potato dextrose agar (PDA) plates (refer to Appendix A, Figure 3.1). All culture plates were stored in the chiller at 4-8°C.

Figure 3.1 *Meyerozyma guilliermondii* grown on PDA.

3.2 Substrate preparation

Palm oil mill effluent (POME) was collected from the separator sludge outlet at Sime Darby Plantation West Mill, Pulau Carey, Selangor, Malaysia. The fresh raw POME was kept in a cold room for 2 days to settle the solid particles. It was

then filtered through a 40µm filter and the filtrate was dispensed into plastic containers and kept in the freezer at -20°C.

POME medium was prepared by diluting 25% of POME into 75% distilled water (v/v). The salinity of each POME medium was specifically adjusted to each microorganism. The amount of sodium chloride added to each medium is the same as the salinity in the synthetic medium for each microheterotroph (Table 3.1). The medium was passed through a filter paper (Whatman 1001-090 Grade 1; Pore size: 11 µm) and 900 ml of filtered POME medium was then dispensed into a 1L Schott bottle prior to autoclaving the bottle containing POME medium at 121°C for 15 minutes.

3.3 Preparations of culture medium

3.3.1 Synthetic media

The synthetic medium for each type of microorganism is shown in Table 3.1 (refer to Appendix A for composition of synthetic medium). The type of synthetic medium, amount of sodium chloride and incubation period for each microorganism were predetermined by Loo *et al.* (unpublished). The pH medium was adjusted to 7.0 by adding hydrochloric acid (HCl) or sodium hydroxide (NaOH) before autoclaving the defined medium at 121°C for 15 minutes.

Table 3.1: Preparations of synthetic media, POME medium and incubation period for microorganism culture (Adapted from Loo *et al*., unpublished).

Name of Microorganism	Type of Synthetic Medium	Amount of NaCl (g/L)[1]	Incubation period (hours)
Shewanella algae (SA)	112 Medium	30	24
Rhodovulum sulfidophilum (RS)	112 Medium	30	24
Rhodotorula mucilaginosa (RM)	Potato dextrose broth	15	48
Meyerozyma guillermondii (MG)	Potato dextrose broth	30	24
Aurantiochytrium limacinum (AL)	Potato dextrose broth	15	48

[1] Amount of NaCl in synthetic medium and POME medium.

After inoculation with 10% inoculum (v/v) in sterile conditions, the bottles were incubated at continuous illumination under light intensity of 32.5 µmol photons m^{-2} s^{-1} at 30 ± 2°C for a specific duration (refer to Table 3.1).

3.3.2 Culture of microheterotrophs in POME

The inoculum of 100 ml containing microorganism culture in synthetic medium (Subsection 3.3.1) was inoculated into 900 ml of 25% POME medium in a sterile environment (Figure 3.2). The bottles were then incubated at continuous illumination under light intensity of 32.5 µmol photons m^{-2} s^{-1} and at a temperature of 30 ± 2°C for a specific duration for each microorganism (refer to Table 3.1). All microorganisms were cultured in autoclaved 25% POME media.

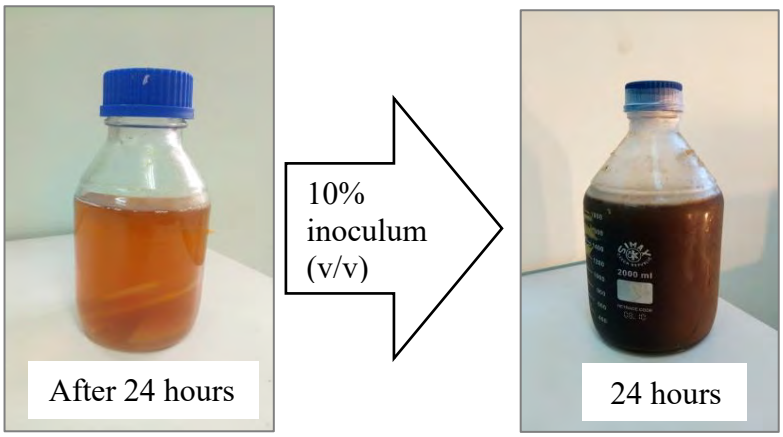

Figure 3.2: Inoculation of *Meyerozyma guilliermondii* cultured in PDB into POME medium at a volume ratio of 1:9.

3.3.3 Microalgae preparation

Instant microalgae Nanno 3600™ (*Nannochloropsis oculata*) was sourced overseas from Reed Mariculture, United States. The microalgal paste containing a concentration of 6.8 x 10^{13} cells/mL (as stated on the package) was diluted with sterile 10 ppt saline to obtain a final concentration of 2 x 10^5 cells/mL.

3.3.4 Feed concentration

The number of cells in the POME enriched microorganism culture was counted using a Neubauer counting chamber (0.0025mm^2 x 0.1mm) under a light microscope (Leica DM750) and then adjusted to a final concentration of 2 x 10^5 cells/mL by dilution with sterile 10 ppt saline water, unless otherwise stated.

3.4 Copepod stock culture

The stock culture of adult *Apocyclops dengizicus* was obtained from the Marine Culture Unit (hatchery), University of Malaya. The stock was maintained in 10L tanks and acclimatized at Feed to Fish Laboratory, University of Malaya under the laboratory conditions of 10 ppt salinity, pH 7-8 and temperature of 28°C to 30°C for at least one week prior to the experiments. Saline water for all

copepod cultures were prepared by mixing freshwater with synthetic sea salt (Instant Ocean© Sea Salt) and salinity was measured using a multi-parameter water quality meter (Eutech Instruments PCD650). Copepods were fed a mixed diet of POME-grown bacteria, yeasts and *Nannochloropsis* before selection of the best diet determined after Experiment 1 (refer to Subsection 3.5.2). During stock maintenance, each culture was transferred to a clean tank with total water change every five days.

3.5 Experimental designs

3.5.1 Experimental setup

Fifty *A. dengizicus* ovigerous females were separated from the stock culture and cultured in 500 mL beakers in 10 ppt saline at 30 ± 1°C in the water bath for 24 hours. After 24 hours, most eggs have hatched and ready for nauplii collection which was used for the experiment. The copepod culture in the 500 mL beakers were poured through a 150 µm nylon mesh to separate the adult female copepods in the population and then through a 40 µm nylon mesh to collect the nauplii (stage I) for the experiment. Saline water for all copepod cultures were prepared by mixing freshwater with synthetic sea salt (Instant Ocean© Sea Salt) until the required salinity concentration was achieved and then sterilised in 2L Schott bottles prior to each experiment. Experiments were conducted with an initial stocking density of fifty nauplii stage I per 250 mL of saline water (or 1 nauplius per 5 mL) and were cultured in 250 mL glass beakers. Each treatment was conducted in three replicates. The beakers were placed in an electric water bath (Memmert WB14) in completely randomized block and the opening of the water bath was covered with a transparent plastic sheet to maintain a constant temperature. Each beaker was aerated with the lowest setting of air bubbles through an air tube connected to a 10 mL glass pipette (see Figure 3.3). The

control conditions were: temperature of 30 ± 1°C, salinity of 10 ± 1 ppt, pH 7 ± 0.5, light intensity of 2.975 µmol photons/m^2/s (fluorescent ceiling light) and photoperiod of 12 h light: 12 h dark. Feeding frequency was two times per day (at 8:00 and 17:00). These culture conditions were maintained in all experiments, unless otherwise stated.

Figure 3.3: Experiment setting in a temperature-controlled water bath housing six replicates.

In situ water quality parameters (i.e. temperature (°C), pH, salinity (ppt) and dissolved oxygen (DO, mg/L)) were measured daily using a multi-parameter water quality meter (Eutech Instruments PCD650) and a portable dissolved oxygen meter (YSI 550A) to maintain the above mentioned culture conditions. The culture conditions were maintained with 50% sterile brackish water change every two days, addition of sterile freshwater if salinity was above the limit, addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH) to maintain the pH range and controlling the water bath temperature to maintain the temperature range.

The survival, F2 nauplii production and size (length and width) of *A. dengizicus* were studied for a period of 12 days. The number of copepods were estimated every 2-day intervals (refer to Subsection 3.5.2 for calculations). Total

count was performed on day 8 and 12 in Experiment 1 (Feed) and on days 6, 8 and 12 on all subsequent experiments. (Changes were made after Experiment 1 because majority of cultures reached maturity by day 6, contrary to our predictions.) Five subsamples of 10 mL from each beaker were taken out using a Henson Stempel pipette and transferred into a Sedgewick rafter cell. On total count days, the whole population of copepods in each culture beaker was passed through a 40 µm mesh net and then transferred into a Sedgewick rafter cell. Surviving copepods of various developmental stages (i.e. nauplius, copepodite, adult male, adult female and ovigerous female (refer to Figure 3.4)) were counted under a stereomicroscope (Leica EZ4) and returned immediately to the culture beaker after count. Methods of identification were based on descriptions by Anandan *et al.* (2013), Valderhaug and Kewalramani (1979) and cyclopoid life stage images by Park *et al.* (2005). The mean number of copepods was calculated from three replicates of each treatment.

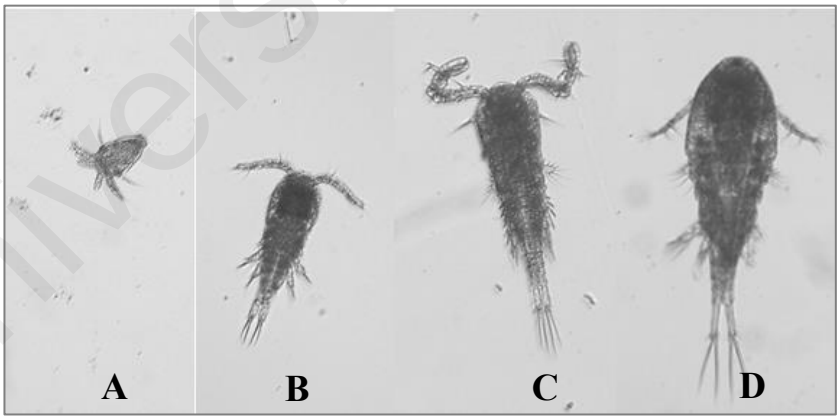

Figure 3.4: Life stages of *Apocyclops dengizicus* (image not to scale).
A = nauplius, B = copepodite, C = adult male, D = adult female.

3.5.2 Calculations for survival, nauplii production and sex ratio

Copepod survival was measured as the survival of the initial nauplii (stage I) population in the duration of 12 days. The initial number of nauplii was 50 individuals in Experiments 1 to 8 and 200 individuals in the scale-up experiment.

Calculations were as follows:

Day 2:

$$Survival\ of\ copepods\ (\%) = \frac{(N+C)}{N_i} \times 100 \quad (3.1)$$

Day 4:

$$Survival\ of\ copepods\ (\%) = \frac{(N+C+A)}{N_i} \times 100 \quad (3.2)$$

Day 6:

$$Survival\ of\ copepods\ (\%) = \frac{(C+A)}{N_i} \times 100 \quad (3.3)$$

Day 8, 10 and 12:

$$Survival\ of\ copepods\ (\%) = \frac{A}{N_i} \times 100 \quad (3.4)$$

Where N = number of nauplii, C = number of copepodites, A = total number of male and female adults and N_i = initial number of nauplii (stage I). The survival rates from a total of three replicates per treatment were calculated and averaged.

Reproduction was measured as the number of F2 nauplii production from day 6 onwards. It was calculated as the average number of nauplii (individuals) from three replicates per treatment.

The sex ratio was calculated using the population count from day 8 where the highest number of adults were observed across all experiments. It was calculated as the ratio of males to females in a population. The sex ratio on day 8 was presented as the mean value of three replicates per treatment.

3.5.3 Experiment 1 – Effects of POME-grown heterotrophic diets compared to a microalgae diet

The first experiment was conducted to evaluate the efficacy of different types of POME-grown microheterotrophs to support the survival, growth and reproduction of *A. dengizicus*. The feed concentration was 2.0 x 10^5 cells/mL (1 mL at 8:00 and 17:00) as suggested by Farhadian *et al.* (2008). The diets tested were: microalgae *Nannochloropsis oculata* (N), POME-grown: *Shewanella algae* (POME-SA), *Rhodovulum sulfidophilum* (POME-RS), *Rhodotorula mucilaginosa* (POME-RM), *Meyerozyma guillermondii* (POME-MG), *Aurantiochytrium limacinum* (POME-AL) and a mixed diet of POME-MG+AL (1:1 ratio) (see Table 3.2). This experiment determined which POME-grown microheterotroph diet was the optimal feed compared to microalgae for *A. dengizicus* in terms of highest survival (%) and number of F2 nauplii produced in the period of twelve days. The chosen optimal diet was used as the only diet in all subsequent experiments.

Table 3.2: Summary of the experimental variables and control conditions in biotic factor experiments (Experiment 1 to 3).

Experiment 1: Effects of POME-grown heterotrophic diets compared to a microalgae diet
Experimental variables: 1a. Nanno 3600 (*Nannochloropsis oculata*) 1b. POME-grown *Shewanella algae* (POME-SA) 1c. POME-grown *Rhodovulum sulphidophilum* (POME-RS) 1d. POME-grown *Rhodotorula mucilaginosa* (POME-RM) 1e. POME-grown *Meyerozyma guillermondii* (POME-MG) 1f. POME-grown *Aurantiochytrium limacinum* (POME-AL) 1g. Mixed diet of POME-MG and POME-AL (ratio 1:1) Control conditions (applicable to Experiment 1, 2 and 3): • Salinity: 10 ± 1 ppt • Temperature: 30 ± 1°C • pH: 7.0 ± 0.5 • Photoperiod: 12 hours light: 12 hours dark • Light intensity: 2.975 µmol photons m^{-2} s^{-1}
Experiment 2: Separate and combined effects of POME and MG diets
Experimental variables: 2a. POME-MG 2b. POME 2c. POME-MG biomass (POME-MGb) 2d. NO FEED
Experiment 3: Effects of POME-MG feed concentrations
Experimental variables: 3a. 1.0 x 10^5 cells ml^{-1} day^{-1} 3b. 2.0 x 10^5 cells ml^{-1} day^{-1} 3c. 4.0 x 10^5 cells ml^{-1} day^{-1} 3d. 8.0 x 10^5 cells ml^{-1} day^{-1}

3.5.4 Experiment 2 – Separate and combined effects of POME and MG diets on *A. dengizicus*

The optimal diet (POME-MG) determined in Experiment 1 (Subsection 3.5.3) was tested against positive and negative controls: only POME (positive control), POME-MG biomass (POME-MGb) and no feed (negative control) (see Table 3.2).

3.5.5 Experiment 3 – Effects of feed concentration

The third experiment studied the effects of different feed concentrations on *A. dengizicus* culture (Farhadian *et al*., 2008). The daily feed concentrations of POME-MG (determined as optimal diet in Experiment 1 – Subsection 3.5.3) tested were: 1.0 x 10^5 cells/mL (25%), 2.0 x 10^5 cells/mL (50%), 4.0 x 10^5 cells/mL (100%, control) and 8.0 x 10^5 cells/mL (200%) (see Table 3.2). Each feed concentration was made by measuring the original concentration of fresh POME-MG and diluting the feed with sterile 10 ppt saline to achieve the final concentration. If the original concentration was less than the desired concentration (e.g. 8.0 x 10^5 cells/mL), POME-MG biomass (prepared by centrifuging fresh POME-MG at the speed of 7000 rpm for 5 minutes at 4°C) was added until the required concentration was achieved. The best feed concentration overall was used for other remaining experiments.

3.5.6 Experiment 4 – Effects of salinity

The salinity experiment was tested to observe the effects of varying salinity levels on the survival, growth and reproduction of *A. dengizicus*. Prior to the experiment, the stock culture of *A. dengizicus* were separated into different 5L tanks and the salinity of the culture was gradually decreased or increased to obtain

the required salinity. Saline water for all copepod cultures were prepared by mixing freshwater with synthetic sea salt (Instant Ocean© Sea Salt) until the required salinity concentration was achieved and then sterilised in 2L Schott bottles prior to each experiment. The salinity levels tested were: 0 ppt (freshwater), 10 ppt, 20 ppt and 30 ppt (see Table 3.3). The salinities were maintained in the range ± 1 ppt by adding sterile saline solution (to increase salinity) or sterile freshwater (to decrease salinity) when needed. The type of feed used was determined in Subsection 3.5.3 and feed concentration in Subsection 3.5.5. The best salinity overall was used for other remaining experiments.

Table 3.3: Summary of salinity treatments and the control conditions in Experiment 4.

Experimental variables:	Control conditions:
4a. 0 ppt 4b. 10 ppt 4c. 20 ppt 4d. 30 ppt	• Feed type: POME-MG • Feed concentration: 4.0 x 10^5 cells ml^{-1} day^{-1} • Temperature: 30 ± 1°C • pH: 7.0 ± 0.5 • Photoperiod: 12 hours light: 12 hours dark • Light intensity: 2.975 µmol photons m^{-2} s^{-1}

3.5.7 Experiment 5 – Effects of temperature

The fifth experiment challenged *A. dengizicus* against 6 different temperatures ranging from 26°C to 36°C to determine the effects of temperature on the survival, growth and nauplii production of this species. Two thermal experiments were carried out due to insufficient equipment. Experiment 5A tested: 26°C, 28°C and 30°C, while experiment 5B tested: 32°C, 34°C and 36°C (see Table 3.4). The temperatures were maintained in the range ± 1°C. Other conditions in this experiment such as type of feed (Subsection 3.5.3), feed concentration

(Subsection 3.5.5) and salinity (Subsection 3.5.6) were previously determined. The best temperature overall was used for other remaining experiments.

Table 3.4: Summary of temperature treatments and the control conditions in Experiment 5.

Experimental variables:	Control conditions:
5a. 26°C 5b. 28°C 5c. 30°C 5d. 32°C 5e. 34°C 5f. 36°C	• Feed type: POME-MG • Feed concentration: 4.0 x 10^5 cells ml^{-1} day^{-1} • Salinity: 10 ±1 ppt • pH: 7.0 ± 0.5 • Photoperiod: 12 hours light and 12 hours dark • Light intensity: 2.975 µmol photons m^{-2} s^{-1}

3.5.8 Experiment 6 – Effects of pH

The pH experiment studied the effects of different pH levels on the survival, growth and reproduction of *A. dengizicus*. The pH levels studied ranged from pH 5 to 9, at intervals of 1 with an extra experimental condition of pH 7-8 (not adjusted) (see Table 3.5). pH values <4 and >9 causes acute toxicity to many copepod species and therefore was not included (Yamada & Ikeda, 1999; Hansen *et al*., 2017). The pH value of each treatment was maintained by adding hydrochloric acid (HCl) or sodium hydroxide (NaOH) and was kept within the range of pH value ± 0.5. The culture conditions used were previously determined in Subsections 3.5.3 – 3.5.7. The best pH overall was used for other remaining experiments.

Table 3.5: Summary of pH treatments and the control conditions in Experiment 6.

Experimental variables:	Control conditions:
6a. pH 5 6b. pH 6 6c. pH 7 6d. pH 7-8 6e. pH 8 6f. pH 9	• Feed type: POME-MG • Feed concentration: 4.0 x 10^5 cells ml^{-1} day^{-1} • Salinity: 10 ±1 ppt • Temperature: 30 ± 1°C • Photoperiod: 12 hours light and 12 hours dark • Light intensity: 2.975 µmol photons m^{-2} s^{-1}

3.5.9 Experiment 7 – Effects of photoperiod

This experiment evaluated whether exposure to light affected the survival, reproduction and body growth of *A. dengizicus*. The 250mL beakers containing fifty copepod nauplii were incubated under three photoperiod regimes of 24:0, 12:12 and 0:24 (Light: Dark in hours) (see Table 3.3) as described by Feredouni *et al.* (2013). The light intensity was kept constant and an opaque material was used to cover the opening of the water bath for the complete darkness treatment (0:24). Culture conditions were determined in previous experiments (Subsections 3.5.3 – 3.5.8). The best photoperiod overall was used for other remaining experiments.

Table 3.6: Summary of photoperiod treatments and the control conditions in Experiment 7.

Experimental variables:	Control conditions:
7a. 24 hr dark 7b. 12 hr dark: 12 hr light 7c. 24 hr light	• Feed type: POME-MG • Feed concentration: 4.0 x 10^5 cells ml^{-1} day^{-1} • Salinity: 10 ±1 ppt • Temperature: 30 ± 1°C • pH: 8.0 ± 0.5 • Light intensity: 2.975 µmol photons m^{-2} s^{-1}

3.5.10 Experiment 8 – Effects of light intensity

This experiment evaluated the effects of light intensity on the survival and reproduction of *A. dengizicus*. The three light intensities tested were: low (6.08 µmol photons m^{-2} s^{-1}), medium (14.85 µmol photons m^{-2} s^{-1}) and high (23.65 µmol photons m^{-2} s^{-1}) (see Table 4.3). The source of lighting for these treatments was from cool white fluorescent bulbs placed directly above the water bath to achieve the required level and was measured using a portable lux meter. Culture conditions were determined in previous experiments (Subsections 3.5.3 – 3.5.9). The best light intensity overall was used for other remaining experiments.

Table 3.7: Summary of light intensity treatments and the control conditions in Experiment 8.

Experimental variables:	Control conditions:
8a. 6.08 µmol photons m^{-2} s^{-1} 8b. 14.85 µmol photons m^{-2} s^{-1} 8c. 23.65 µmol photons m^{-2} s^{-1}	• Feed type: POME-MG • Feed concentration: 4.0 x 10^5 cells ml^{-1} day^{-1} • Salinity: 10 ±1 ppt • Temperature: 30 ± 1°C • pH: 8.0 ± 0.5 • Photoperiod: 12 hours light: 12 hours dark

3.5.11 Scale-up experiment of *A. dengizicus*

The final experiment of the study was to measure the survival (%) and F2 nauplii production of *A. dengizicus* in a scale-up capacity given the optimum diet and environmental parameters determined in previous Experiments 1 to 8. This scale-up experiment was to analyse if similar survival and reproductive rates were reproducible in a larger scale *A. dengizicus* culture. One litre of sterile saline water was placed into 1000 mL glass beakers and filled with 200 *A. dengizicus* nauplii (stage I naupliar). The initial stocking density was 1 nauplius per 5 mL.

The feeds tested were POME-MG and POME only (as determined in subsection 3.5.4 – Experiment 2) with the optimum feed concentration of 4.0 x 10^5 cells/ml daily per 250 mL of culture volume (Subsection 3.5.5 – Experiment 3) or 8 ml/daily of only POME. Each feed type had three replicates. The culture conditions were determined in prior experiments (Experiment 4 to 8) as optimal conditions for *A. dengizicus* culture and it was salinity of 10 ±1 ppt (Subsection 3.5.6), temperature of 30 ±1°C (Subsection 3.5.7), pH 8.0 ±0.5 (Subsection 3.5.8), photoperiod of 12 hours light and 12 hours dark (Subsection 3.5.9), and light intensity 6.08 µmol photons m^{-2} s^{-1} (Subsection 3.5.10).

3.6 Biochemical analysis

Fifty grams (wet weight) of each feed samples were prepared by using a refrigerated centrifuge machine at the speed of 7000 rpm for 5 minutes at 4°C, followed by rinsing the biomass twice with a 10% autoclaved saline solution and the samples were then kept in a -20°C freezer until fatty acid analysis. All analyses were outsourced to UKM Unipeq Sdn. Bhd., Selangor, Malaysia. The fatty acid methyl esters (FAMEs) were prepared in accordance with the standard IUPAC method 2.301 (IUPAC, 1987). Gas chromatography with flame ionization detector (GC-FID) was performed on a HP 5890 gas chromatograph equipped with a capillary column DB 225 (30m x 0.25mm x 0.25mm) using Supelco® 37 Component Fame Mix (Cat. No. 18919) as the standard. Additionally, samples of POME and POME-MG were also subjected to proximate analysis with methods based on AOAC 16th edition (1995): protein (No. 981.10), ash (No. 923.03), moisture (No. 950.46) and total fat using soxhlet extraction (No. 991.36). Carbohydrate and energy (by calculation) were analysed with in-house methods based on Pomeranz and Meloan (1987) and Pearson (1970) respectively. The

amino acid profiles were analysed using Waters Accq-Tag™ method (Waters, Milford, MA, USA) and run using high-performance liquid chromatography with fluorescence detection (HPLC-FLD) (refer to Appendix B for summarised list of methods).

The biomasses of *A. dengizicus* fed with POME-MG and only POME were prepared by harvesting the adult copepods after 8 days of culture with a 200 μm scoop net, followed by rinsing with sterile distilled water to discard unwanted debris and microbes and then immediately kept in a -20°C freezer prior to freeze drying using ALPHA 1-4 LSC basic. Lipid extraction on the freeze-dried copepod samples was conducted according to a modified method by Zhang *et al.* (2013) using hexane extraction solvent. A 15 mg copepod sample was weighed and then transferred into a test tube. Two millilitres of hexane were added into the sample test tube, vortexed to homogeneity and then left at room temperature for 2 hours prior to centrifuging at a speed of 3000 rpm for five minutes. After centrifugation, the supernatant was pipetted out into a pre-weighed glass beaker, sodium sulphate was then added to remove residual water and mixed well until it became a powdery liquid extract. The powdery liquid extract was filtered through the filter paper. The extraction procedure on the same sample was repeated three times. The sample solutions were collected and then left until dry in a laminar flow to concentrate the oil extract. The glass beaker containing the concentrated oil extract was weighed to determine the amount of lipid extracted from the copepod sample. The oil extract samples were kept in 2 mL amber glass vials in a -20°C freezer until fatty acid analysis as previously described.

3.7 Body growth (length and width) measurements of *A. dengizicus*

The body length of *A. dengizicus* was measured as the length of rostrum to caudal ramus (labelled 'A' in Figure 3.5). The width was measured as the width of the prosome or the widest part of the body (labelled 'B' in Figure 3.5) using an inverted microscope (Leica DMIL LED) with an attached camera. Images were measured in µm using the Leica Application Suite X (LAS X) software. Each treatment needed a minimum of 3 samples per life stage (nauplius, copepodite, adult male and adult female).

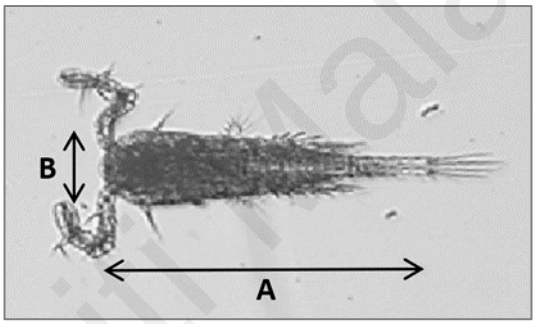

Figure 3.5: Body length (A) and width (B) measurements of a male copepod.

3.8 Statistical analysis

The mean and standard error for survival, nauplii production and male to female sex ratios of *A. dengizicus* were calculated from the three replicates of each treatment. The average and standard deviation of the copepod body length and width measurements among treatments were statistically analysed. Differences in treatment means were compared by one-way analysis of variance (ANOVA). The percentage of survival rates were arcsine-square root transformed to ensure a normal distribution. Parametric testings and appropriate data transformation were carried out to satisfy the requirements of homogeneity of variance and normal distribution. The statistical significance for all parameters were at 95% confidence interval ($p < 0.05$). If the ANOVA was significant,

differences between treatment means were compared using a post-hoc analysis, Tukey HSD test. All statistical analysis was carried out using IBM SPSS Statistics (Version 24).

CHAPTER 4

RESULTS

4.1 Effects of POME grown microheterotroph diets compared to a microalgae diet

All POME grown heterotrophic microorganism diets supported better survival of *A. dengizicus* as compared to copepods fed instant microalgae *Nannochloropsis oculata* (N), except POME-SA and POME-RS (see Figure 4.1). Overall, POME-MG gave the highest survival of 86.7±8.8% until the Day 10 of culture. On day 6 of culture, *A. dengizicus* fed with microalgae was the only diet with less than 70% survival. By day 8, the number of adult copepods fed bacterial diets (POME-SA and POME-RS) suffered massive mortality. POME-MG gave the highest adult survival on day 8 (92.0±7.0%), followed by POME-RM (88.0±4.2%), POME-AL (70.7±10.7%), POME-MG+AL (77.3±5.8), POME-SA (43.2±2.9%), POME-RS (40.7±1.8%) and lastly *N. oculata* (N) (39.3±4.7%) ($p < 0.05$) (Figure 4.1). However, the survival rates on day 12 between all diets were not significantly different ($p < 0.05$). POME-RM diet gave the highest survival rate (97.3±1.3%) followed by POME-MG+AL (69.3±6.4%) and POME-RS (69.3±24.7%) (see Figure 4.1).

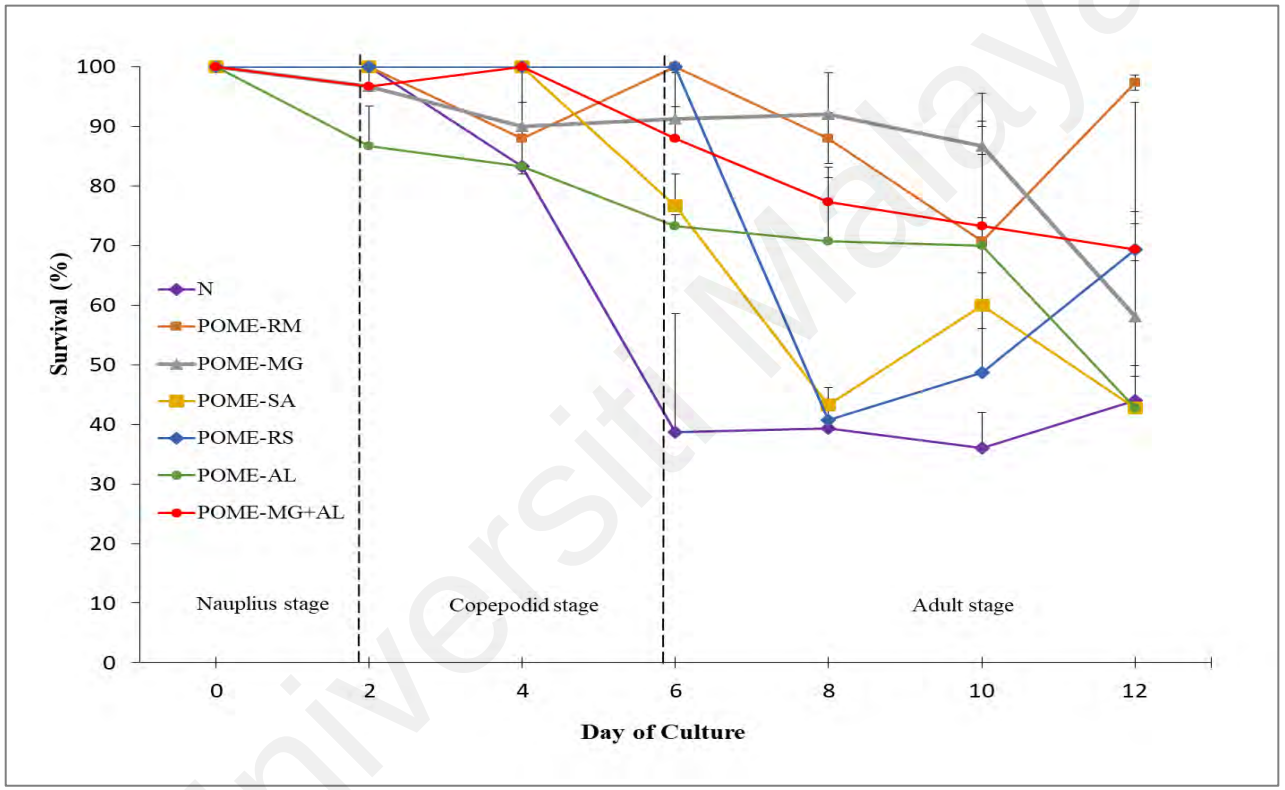

Figure 4.1: Survival (%) of *Apocyclops dengizicus* (mean±SE) given a variety of POME-grown heterotrophic microorganism diets and a microalgal diet over a period of 12 days.
N = *N. oculata* (or Nanno 3600), POME = POME-grown; RM = *R. mucilaginosa*, MG = *M. gulliermondii*, SA = *S. algae*, RS = *R. sulfidophilum*, AL = *A. limacinum*, MG+AL = mixed diet of *M. guilliermondii* and *A. limacinum* (ratio 1:1). Refer to Appendix C for summary of survival (%) in all experiments.

The highest number of F2 nauplii were observed in POME-MG treatments on day 6 (65.7±17.4), day 8 (142.0±34.3) and day 10 (186.7±37.1) (Table 4.1.1). Overall, the total number of F2 nauplii of copepods fed with POME-MG, POME-MG+AL, POME-AL and POME-RM were significantly higher ($p < 0.05$) compared to copepods fed with POME-SA, POME-RS and N which produced less than 10 nauplii (Table 4.1.1).

The sex ratios of *A. dengizicus* between all feed types were all female-biased populations (M/F <1). Copepods on a diet of POME-SA had the closest equal ratio of male to female (0.93±0.24) (Table 4.1.1). The water quality parameters of the treatments tested were measured every 2-day intervals and were within the stipulated range (refer to Appendix E for water quality data).

Table 4.1.1: F2 nauplii production and male to female sex ratio (mean±SE) of *Apocyclops dengizicus* fed on different types of POME-grown heterotrophic microorganism diets and a microalgal diet.

	Type of Feed						
	N	**POME-RM**	**POME-MG**	**POME-SA**	**POME-RS**	**POME-AL**	**POME-MG+AL**
F2 Nauplii Production							
Day 6	0.0 ± 0.0^{c}	44.4 ± 19.4^{b}	65.7 ± 17.4^{a}	8.3 ± 8.3^{c}	0.0 ± 0.0^{c}	13.7 ± 1.9^{b}	23.0 ± 13.0^{b}
Day 8	0.0 ± 0.0^{d}	2.7 ± 2.7^{d}	142.0 ± 34.3^{a}	0.3 ± 0.3^{d}	0.0 ± 0.0^{d}	54.7 ± 6.2^{c}	102.7 ± 25.4^{b}
Day 10	0.0 ± 0.0^{d}	97.2 ± 18.2^{c}	186.7 ± 37.1^{a}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	168.3 ± 14.2^{b}	141.7 ± 9.3^{b}
Day 12	0.3 ± 0.3^{c}	42.7 ± 17.7^{b}	51.3 ± 13.1^{b}	1.0 ± 1.0^{b}	1.3 ± 1.3^{b}	51.7 ± 13.0^{a}	35.7 ± 11.5^{b}
Sex Ratio (M/F)	0.80 ± 0.05^{a}	0.62 ± 0.13^{a}	0.73 ± 0.15^{a}	0.93 ± 0.24^{a}	0.59 ± 0.23^{a}	0.81 ± 0.17^{a}	0.66 ± 0.02^{a}

Mean of triplicate values; mean that does not share a common superscript letter in each row differ significantly ($p<0.05$; see Appendix D). Sex ratio values were calculated from data on Day 8. N = *N. oculata* (or Nanno 3600), POME = POME-grown; RM = *R. mucilaginosa*, MG = *M. gulliermondii*, SA = *S. algae*, RS = *R. sulfidophilum*, AL = *A. limacinum*, MG+AL = mixed diet of *M. guilliermondii* and *A. limacinum* (ratio 1:1).

4.1.2 Comparing survival and reproduction of *A. dengizicus* fed POME-MG with positive and negative controls

The survival (%) of copepods on diets of POME-MG and only POME decreased below 40% on day 6 and then increased on subsequent days (Figure 4.2). However, the survival of copepods between each diet on day 6 were not significantly different ($p < 0.05$). In contrast, the survival of copepods fed POME-MGb constantly decreased over the twelve-day period while copepods which were not fed (NO FEED) showed poorer survival compared to POME-MGb in the first eight days of culture but its survival was maintained until day 12 of culture. By day 12, the average survival for POME-MG (56.7±21.9%) and POME (52.7±5.2%) were higher (but not significantly different) than copepods fed on POME-MG biomass (12.7±5.9%) and no feed (27.3±15.4%) ($p > 0.05$) (Figure 4.2).

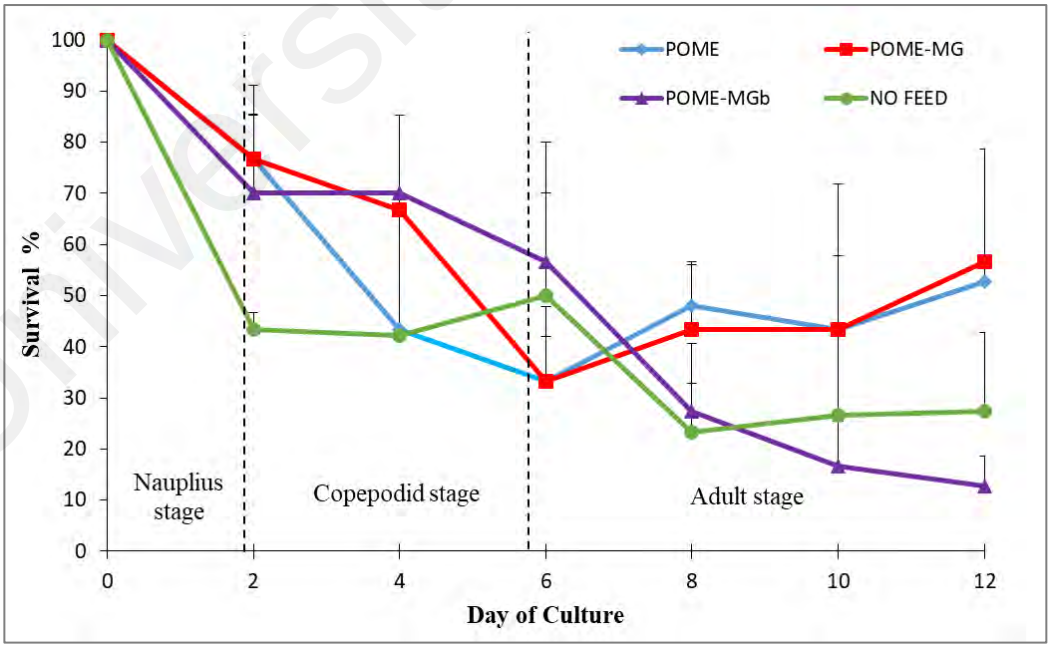

Figure 4.2: Survival (%) of *Apocyclops dengizicus* (mean±SE) given variations of POME-MG diets including only POME, POME-MG biomass and no feed over a period of 12 days.

The different feeds in Experiment 2 had varied effects on the nauplii production (Table 4.1.2). Both POME (42.0±33.0) and POME-MG (61.3±47.3) cultures produced large numbers of nauplii compared to those give POME-MGb (0.0±0.0) and NO FEED (9.7±9.7) on day 8. On day 10, the number of nauplii fed on POME-MG reached a peak of 211.7±64.1 individuals followed by POME with 120.0±52.5 individuals ($p < 0.05$). On day 12, POME feed gave the highest number of nauplii (49.7±11.1) followed by POME-MG feed (30.7±7.3) ($p < 0.05$). The cumulative number of nauplii from day 6 to 12 was highest in cultures fed on POME-MG (303.7±103.9) as compared to other feeds tested ($p < 0.05$) (Table 4.1.2). Therefore, POME-MG was the best diet for producing offspring compared to the other diets tested.

Table 4.1.2: F2 nauplii production and sex ratio (mean±SE) of *Apocyclops dengizicus* fed on variations of POME-MG diets including only POME, POME-MG biomass and no feed.

	Type of Feed			
	POME	**POME-MG**	**POME-MGb**	**NO FEED**
F2 Nauplii Production				
Day 6	3.3±3.3[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Day 8	42.0±33.0[a]	61.3±47.3[a]	0.0±0.0[a]	9.7±9.7[a]
Day 10	120.0±52.5[b]	211.7±64.1[a]	3.3±3.3[c]	16.7±14.2[c]
Day 12	49.7±11.1[a]	30.7±7.3[b]	1.0±1.0[c]	1.0±1.0[c]
Sex Ratio (M/F)	0.46±0.10[a]	0.98±0.12[a]	2.97±2.06[a]	0.52±0.21[a]

Mean of triplicate values; mean that does not share a common superscript letter in each row differ significantly ($p<0.05$; Appendix G). Sex ratio values were calculated from data on Day 8.

The sex ratios in POME only and NO FEED treatments were female-biased while the ratio of male to female was closer to equal in populations fed with POME-MG. Only POME-MGb treatments were highly male biased (M/F =

2.97±2.06) (Table 4.1.2). The water quality parameters recorded throughout the course of 12 days were recorded and were within the stipulated range (see Appendix E for water quality data).

4.1.3 Body length and width of *A. dengizicus* fed with a microalgae diet, POME-grown microheterotroph diets and its variations from Experiment 1 and 2

The body length and width measurements of *A. dengizicus* fed with different diets were compared in three main stages – naupliar, copepodid, adult male and female (Table 4.1.3). The average length and width of copepod nauplii were not significantly different ($p > 0.05$) across all treatments ranged from 163 µm to 212 µm in length and from 86 µm to 106 µm in width. The longest copepodids were on a diet of POME-SA (590±36 µm) and the widest copepodids fed on POME-RS (182±11 µm) which were both POME grown bacterial based diets (length and width: $p < 0.05$). In contrast, the largest adult males were on yeast-based diets of POME-RM, POME-MG and POME-MG+AL (length and width: $p < 0.05$). However, there was no significant variation ($p > 0.05$) in both length and width of adult females across all diets. The average length of female *A. dengizicus* ranged from 715 µm to 813 µm and the average width ranged from 213 µm to 246 µm (Table 4.1.3).

Table 4.1.3: Body length and width (mean µm±SD) of *Apocyclops dengizicus* fed with different diets in Experiment 1 and 2.

Feed	NAUPLIUS		COPEPODID		ADULT MALE		ADULT FEMALE	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)
N	174±124^{a}	86±35^{a}	536±43^{b}	166±19^{b}	646±60^{d}	174±11^{a}	720±81^{a}	213±16^{a}
POME-RM	212±83^{a}	102±25^{a}	400±49^{c}	141±9^{c}	700±46^{a}	170±8^{a}	810±55^{a}	236±31^{a}
POME-MG	193±60^{a}	106±24^{a}	429±62^{c}	146±21^{c}	710±29^{b}	174±8^{a}	805±110^{a}	246±30^{a}
POME-SA	-	-	590±36^{a}	173±9^{b}	660±38^{c}	170±13^{a}	813±120^{a}	222±38^{a}
POME-RS	-	-	507±17^{b}	182±11^{a}	673±42^{c}	168±8^{a}	779±82^{a}	246±28^{a}
POME-AL	158±52^{a}	88±16^{a}	430±71^{c}	154±21^{b}	672±28^{c}	173±13^{a}	752±71^{a}	224±36^{a}
POME-MG+AL	176±73^{a}	86±26^{a}	429±57^{c}	153±13^{b}	705±16^{b}	171±8^{a}	805±74^{a}	239±19^{a}
POME*	163±39^{a}	91±9^{a}	453±107^{b}	142±22^{c}	641±25^{d}	162±11^{b}	784±89^{a}	227±26^{a}
POME-MGb	-	-	495±46^{b}	155±10^{b}	635±60^{e}	168±13^{a}	715±60^{a}	225±15^{a}
NONE	176±0^{a}	92±0^{a}	425±119^{c}	141±16^{c}	572±27^{f}	148±8^{c}	722±55^{a}	224±16^{a}

Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly ($p<0.05$; Appendix H). N = *N. oculata* (or Nanno 3600), POME = POME-grown; RM = *R. mucilaginosa*, MG = *M. gulliermondii*, SA = *S. algae*, RS = *R. sulfidophilum*, AL = *A. limacinum*, MG+AL = mixed diet of *M. guilliermondii* and *A. limacinum* (ratio 1:1), POME* = POME medium only, POME-MGb = biomass of POME-grown *M. guilliermondii*, NONE = no feed.

4.2 Effects of feed concentration of POME-MG in *A. dengizicus* culture

The optimum feed concentration for both survival and nauplii production was 4.0 x 10^5 cells/ml daily of POME-MG. The survival (%) for all four feed concentration tested showed a decrease in survival over time (Figure 4.3). On day 6, the highest adult survival was 88.7±5.7% given with 4.0 x 10^5 cells/ml of POME-MG followed by copepods fed with feed concentrations of 2.0 x 10^5 cells/ml (74.0±2.3%) and 8.0 x 10^5 cells/ml (56.7 ±13.3%) while the survival of copepods fed with 1.0 x 10^5 cells/ml of POME-MG fell below 10% ($p < 0.05$). On the final day of culture (Day 12), all feed concentrations showed poor survival of below 35% ($p < 0.05$). The populations fed with 1.0 x 10^5 cells/ml of POME-MG stayed under 10% in survival from day 6 onwards.

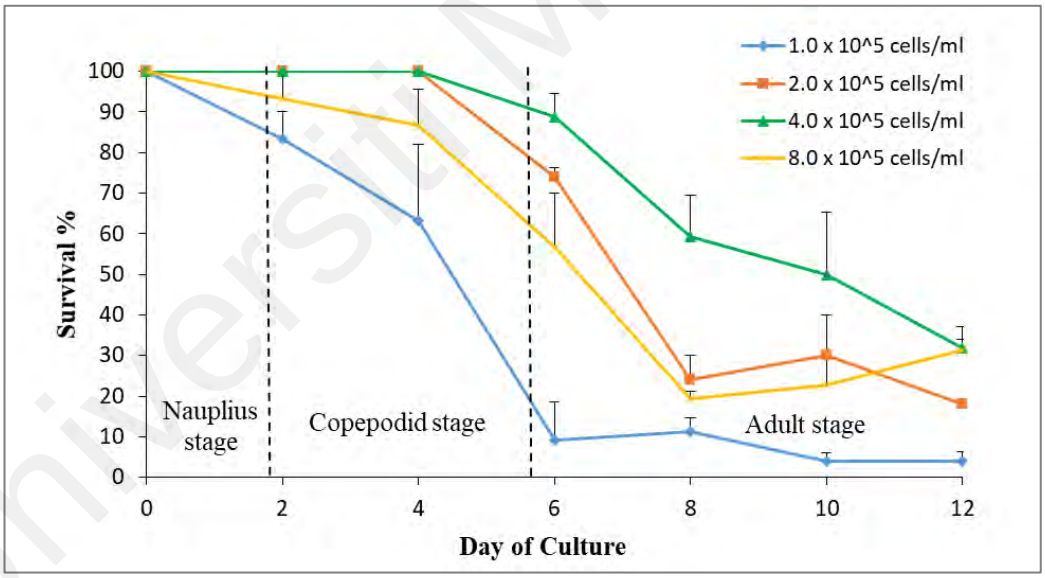

Figure 4.3: Survival (%) of *Apocyclops dengizicus* (mean±SE) given different concentrations of POME-MG feed over a period of 12 days.

Nauplii production reached a peak on day 10 in cultures given feed concentrations of 2.0 x 10^5 cells/ml (106.7±7.3) and 4.0 x 10^5 cells/ml (100.0±27.8) ($p < 0.05$). The F2 nauplii production between each feed concentrations were not significant on day 6 and day 12 (Table 4.2.1).

Table 4.2.1: F2 nauplii production and sex ratio (mean±SE) of *Apocyclops dengizicus* fed on different concentrations of POME-MG.

	Feed Concentration (cells/ml daily)			
	1.0 x 10^5	**2.0 x 10^5**	**4.0 x 10^5**	**8.0 x 10^5**
F2 Nauplii Production				
Day 6	0.7±0.7^a	48.0±20.0^a	42.0±12.6^a	4.0±0.6^a
Day 8	7.0±3.5^c	68.3±13.6^b	88.0±26.9^a	26.0±10.1^b
Day 10	6.0±1.0^c	106.7±7.3^a	100.0±27.8^a	43.3±11.0^b
Day 12	7.7±3.7^a	8.7±3.0^a	20.0±5.7^a	16.3±1.5^a
Sex Ratio (M/F)	x	0.94±0.18^a	0.74±0.15^a	0.81±0.13^a

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly (p<.050; Appendix H). Sex ratio values were calculated from data on Day 8. 'x' indicates sex ratio could not be determined due to very low survival.

The sex ratios in all feed concentration treatments (except 1.0 x 10^5 cells/ml) had female-biased populations (p > 0.05) (Table 4.2.1). The water quality parameters of the treatments were measured and was within the stipulated range (refer to Appendix E for water quality data).

4.2.1 Body length and width of *A. dengizicus* fed with different concentrations of POME-MG

The varying concentrations of POME-MG did not affect the body length and width in all developmental stages of *A. dengizicus* (p > 0.05) (Table 4.2.2). Copepods fed with feed concentrations between 1.0 x 10^5 cells/ml to 8.0 x 10^5 cells/ml of POME-MG had the average body lengths of 171-196 µm in the naupliar stage, 421-487 µm in the copepodid stage, 652-683 µm in adult males and 748-851 µm in adult females. The average body width ranged from 92-103 µm in the naupliar stage, 136-154 µm in the copepodid stage, 167-173 µm in adult males and 213-253 µm in adult females (Table 4.2.2).

Table 4.2.2: Body length and width (mean µm±SD) of *Apocyclops dengizicus* fed on different concentrations of POME-MG in Experiment 3.

Feed Concentration (cells/ml daily)	NAUPLIUS		COPEPODID	
	Length	**Width**	**Length**	**Width**
1.0 x 10^5	196±61[a]	103±18[a]	458±65[a]	154±13[a]
2.0 x 10^5	189±0[a]	92±0[a]	421±108[a]	136±25[a]
4.0 x 10^5	171±44[a]	94±23[a]	487±114[a]	152±19[a]
8.0 x 10^5	194±50[a]	97±18[a]	461±60[a]	148±18[a]
Feed Concentration (cells/ml daily)	**ADULT MALE**		**ADULT FEMALE**	
	Length	**Width**	**Length**	**Width**
1.0 x 10^5	683±18[a]	173±11[a]	851±54[a]	253±37[a]
2.0 x 10^5	652±14[a]	167±7[a]	748±94[a]	213±45[a]
4.0 x 10^5	676±43[a]	170±13[a]	845±70[a]	242±20[a]
8.0 x 10^5	678±19[a]	169±8[a]	780±66[a]	234±12[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly ($p<0.05$; Appendix F).

4.3 Effects of salinity on survival and nauplii production of *A. dengizicus*

A. dengizicus survived well in a wide range of water salinity except in freshwater (0 ppt) (Figure 4.4). Regardless of day of culture, salinity of 10 ppt produced the highest survival (%) for the duration of the experiment followed by 20 ppt and 30 ppt cultures. On total count day 6, the observed survival of *A. dengizicus* cultured in 10 ppt (58.0±5.8%) and 20 ppt (47.3±9.0%) were significantly higher ($p < 0.05$) compared to 30 ppt (28.0±18.1) and 0 ppt (4.7±1.8). However, by day 12 the survival in 10 ppt, 20 ppt and 30 ppt cultures ranged from 13.3-18.7% and were significantly different ($p < 0.05$) to 0 ppt which had no survival of copepods (Figure 4.4).

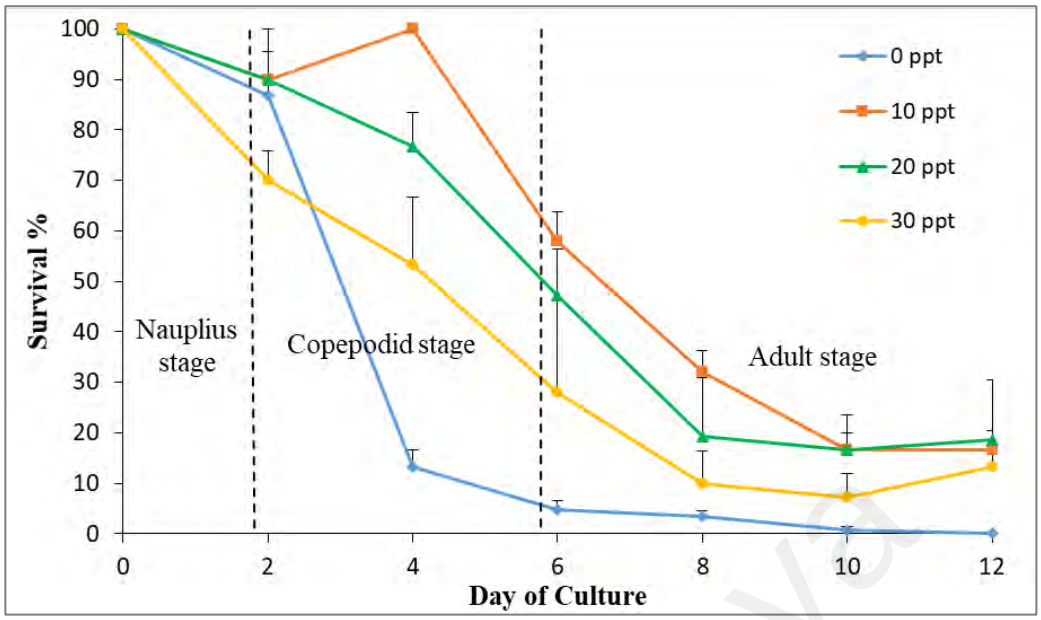

Figure 4.4: Survival (%) of *Apocyclops dengizicus* (mean±SE) in different salinities ranging from 0 to 30 ppt over a period of 12 days.

The highest cumulative total of F2 nauplii production was observed in salinity of 10 ppt (101.3±25.4) followed by 20 ppt (33.0±16.5), 30 ppt (29.0±12.6) and 0 ppt (4.3±2.0) ($p < 0.05$) (Table 4.3.1). The highest number of F2 nauplii was observed on day 8 in salinity of 10 ppt (61.3±21.1).

The sex ratios in 10 ppt and 30 ppt cultures were female-biased. Treatments with 20 ppt salinity had the closest equal ratio of male to female (M/F = 0.99±0.37) while the sex ratio in 0 ppt cultures was not obtained due to very low population survival (Table 4.3.1). The water quality parameters were observed every 2-day intervals throughout the duration of the study and were within the stipulated range (refer to Appendix E for water quality data).

Table 4.3.1: F2 nauplii production and sex ratio (mean±SE) of *Apocyclops dengizicus* in different salinities.

	Salinity (ppt)			
	0	10	20	30
F2 Nauplii Production				
Day 6	3.7±1.5[a]	8.3±4.9[a]	6.3±6.3[a]	3.3±3.3[a]
Day 8	0.7±0.7[b]	61.3±21.1[a]	2.0±1.2[b]	2.0±1.0[b]
Day 10	0.0±0.0[c]	13.0±4.6[a]	5.0±2.9[b]	2.0±2.9[b]
Day 12	0.0±0.0[a]	18.7±5.5[a]	19.7±14.7[a]	21.7±13.5[a]
Sex Ratio (M/F)	x	0.67±0.14[a]	0.99±0.37[a]	0.52±0.52[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly (p<.050; Appendix I). Sex ratio values were calculated from data on Day 8. 'x' indicates sex ratio could not be determined due to very low survival.

4.3.1 Body length and width of *A. dengizicus* cultured in different salinity levels

Salinity of 20 ppt had the largest nauplii individuals (length: 233±40; width: 122±13 µm), followed by in 30 ppt (209±19 µm; 98±18 µm) and in 10 ppt (155±57 µm; 91±19 µm) (length and width: $p < 0.05$). However, salinity did not affect body size of latter stages of copepods (copepodites and adults) as there were no significant differences ($p > 0.05$) found between the average length and width of copepods cultured between 10 ppt, 20 ppt and 30 ppt (Table 4.3.2). Results of 0 ppt cultures were unavailable due to poor survival by day 12 of experiment.

Table 4.3.2: Body length and width (mean µm±SD) of *Apocyclops dengizicus* cultured in different salinities in Experiment 4.

Salinity (ppt)	NAUPLIUS		COPEPODID	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
10	155±57[c]	91±19[c]	427±69[a]	144±20[a]
20	233±40[a]	122±13[a]	466±60[a]	151±18[a]
30	209±19[b]	98±18[b]	442±77[a]	147±18[a]
Salinity (ppt)	**ADULT MALE**		**ADULT FEMALE**	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
10	698±37[a]	177±10[a]	807±96[a]	245±31[a]
20	692±47[a]	179±11[a]	817±69[a]	224±27[a]
30	708±23[a]	184±6[a]	772±97[a]	229±25[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly (p<.050; Appendix F).

4.4 Effects of temperature on the survival and nauplii production of *A. dengizicus*

Temperatures ranging from 26°C to 36°C supported the survival and reproduction of *A. dengizicus* as shown in Figure 4.5 and Table 4.4.1. Higher temperatures (34°C and 36°C) had the lowest copepod survival from Day 0 until Day 6. On day 6, the highest survival were observed at 30°C (82.0±9.2%) and 28°C (79.3±15.9%) treatments ($p < 0.05$). However, the survival rates on day 8 of the lower temperatures tested (26-30°C) decreased significantly. Experiment 5A (26-30°C) was prematurely terminated on day 8. Therefore, the highest survival was found in 32°C (42.0±4.6%) on day 8 ($p < 0.05$). By day 12, the survival of *A. dengizicus* cultured in 32°C, 34°C and 36°C cultures were below 20% ($p < 0.05$).

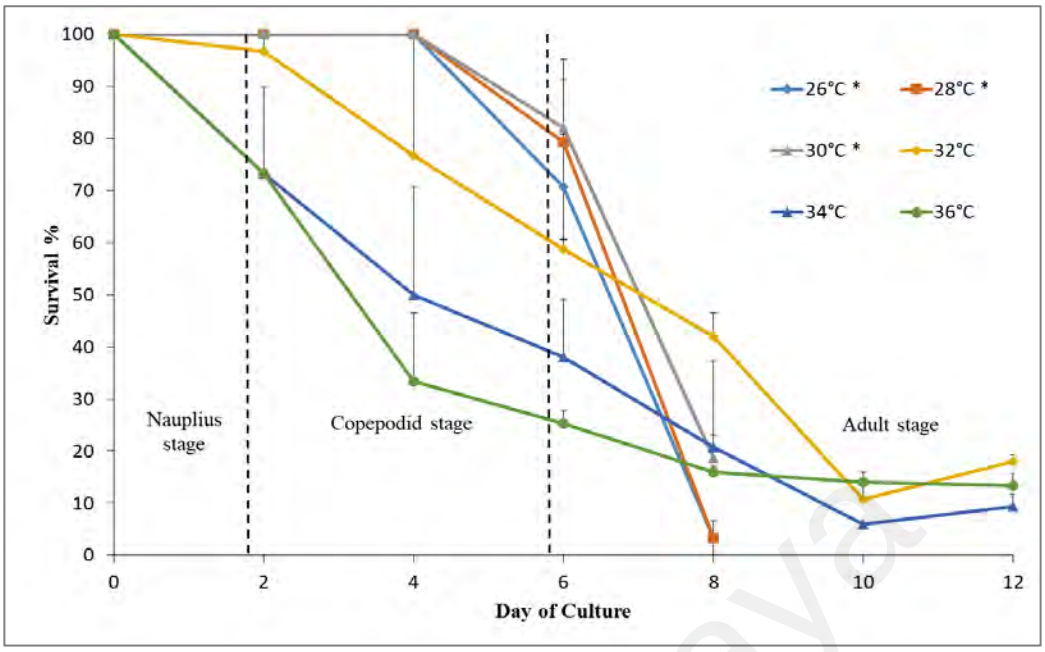

Figure 4.5: Survival (%) of *Apocyclops dengizicus* (mean±SE) in different temperatures ranging from 26°C to 36°C over a period of 12 days.

The overall nauplii production in Experiment 5 was low compared to previous experiments conducted in the control temperature of 30°C. The different temperatures tested (26°C to 36°C) did not significantly affect nauplii production ($p > 0.05$). Nonetheless, the total number of nauplii produced from day 6 to day 12 was highest in temperatures of 30°C (44.3±15.6) and 34°C (46.3±20.7) (Table 4.3.1).

Lower temperatures (26°C and 28°C) had female-biased populations (M/F <1) while temperatures above 30°C showed male-biased populations (M/F >1) ($p > 0.05$) (Table 4.4.1). The water quality parameters of the treatments tested were within the stipulated range (refer to Appendix E for water quality data).

Table 4.4.1: F2 nauplii production and sex ratio (mean±SE, n=3) of *Apocyclops dengizicus* in different temperatures.

	Temperature (°C)					
	26	**28**	**30**	**32**	**34**	**36**
Nauplii Production						
Day 6	1.0±0.6[a]	13.3±0.3[a]	27.7±8.7[a]	0.3±0.3[a]	27.3±16.4[a]	2.0±2.0[a]
Day 8	0.0±0.0[a]	0.0±0.0[a]	16.7±16.7[a]	16.3±10.7[a]	1.0±1.0[a]	5.0±4.0[a]
Day 10	x	x	x	0.3±0.3[a]	3.7±2.7[a]	1.0±1.0[a]
Day 12	x	x	x	0.3±0.3[a]	14.3±7.3[a]	12.0±7.4[a]
Total	1.0±0.6[a]	13.3±0.3[a]	44.3±15.6[a]	17.3±10.7[a]	46.3±20.7[a]	20.0±5.5[a]
Sex Ratio (M/F)	0.86±0.15[a]	0.82±0.17[a]	1.16±0.17[a]	1.94±0.96[a]	1.21±0.15[a]	1.51±0.58[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; Appendix J).
Sex ratio values were calculated from data on Day 8.

4.4.1 Body length and width of *A. dengizicus* cultured in different temperatures

Temperatures ranging from 26°C to 36°C did not affect the body length and width of adult males and females *A. dengizicus* ($p > 0.05$) (Table 4.4.2). Adult male *A. dengizicus* sizes ranged from 661-731 µm in length and 152-175 µm in width. Adult female sizes ranged from 808-846 µm in length and 218-253 µm in width. Data was inadequate for comparison in the naupliar and copepodid stage due to poor survival of the population by day 12.

Table 4.4.2: Body length and width (mean µm±SD) of *Apocylops dengizicus* cultured in different temperatures in Experiment 5.

Temperature (°C)	ADULT MALE		ADULT FEMALE	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
26	680±40[a]	175±11[a]	846±96[a]	233±41[a]
28	731±56[a]	164±11[a]	842±46[a]	253±16[a]
30	661±38[a]	173±8[a]	808±87[a]	240±19[a]
32	679±35[a]	155±17[a]	827±44[a]	237±6[a]
34	662±29[a]	184±26[a]	836±83[a]	222±27[a]
36	688±25[a]	152±19[a]	808±31[a]	218±27[a]

[1] Body dimensions of nauplius and copepodid not included due to inadequate data.
[2] Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly ($p<0.05$; see Appendix F).

4.5 Effects of pH on the survival and nauplii production of *A. dengizicus*

A. dengizicus were able to survive well and produce offspring in cultures with pH range of 5 to 9 as shown in Figure 4.6 and Table 4.5.1. In all pH treatments, survival decreased over time. Higher survival on day 6 were observed in neutral to slightly alkaline environments of pH 8 (61.3±24.5%), pH 7 (56.0±10.6%) and control pH 7-8 (52.0±5.8%) as compared to acidic (pH 5: 42.0±19.0% and pH 6: 38.0±13.1%) and alkaline (pH 9: 44.0±15.1%) environments. However, survival

was not significantly different between pH levels ($p > 0.05$). The same pattern continued until day 12 where survival fell below 20% (Figure 4.6).

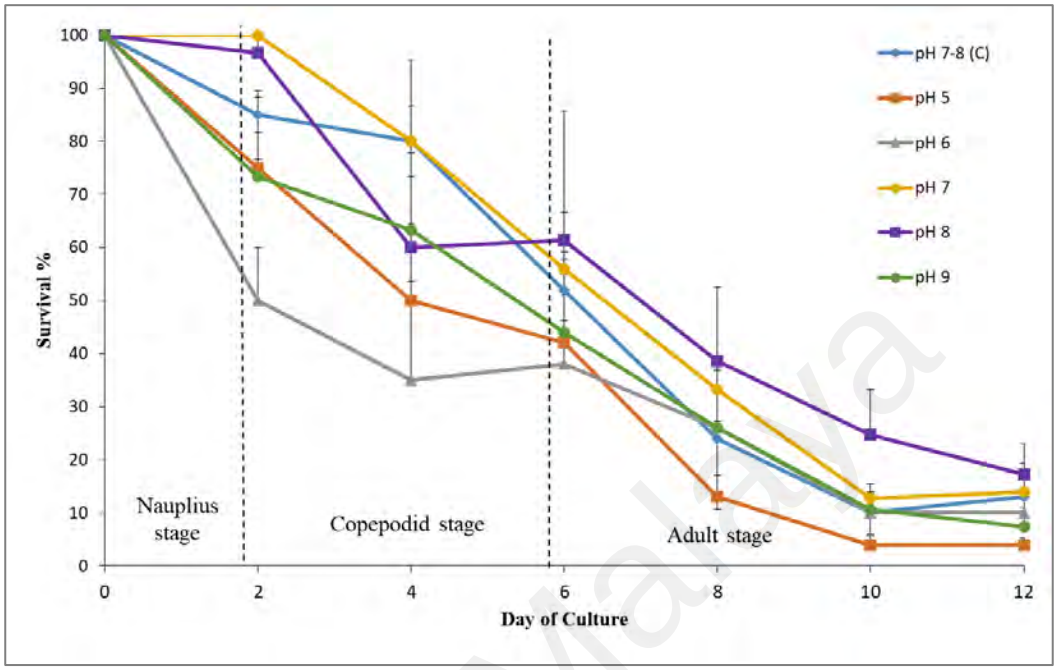

Figure 4.6 Survival (%) of *Apocyclops dengizicus* (mean %±SE) in different pH levels ranging from pH 5 to pH 9 over a period of 12 days.

The varying pH levels did not influence nauplii production ($p > 0.05$). The cumulative number of F2 nauplii observed between day 6 and 12 was highest in pH 8 treatments (98.7±25.8) followed by pH 7 treatments (70.3±10.4). All other pH treatments, including the control (pH 7-8), produced less than 30 nauplii in six days ($p < 0.05$) (Table 4.5.1).

The sex ratios in all pH treatments with the exception of pH 5 had male to female ratios close to M/F = 1 ($p > 0.05$). The populations in pH 5 treatments were highly male-biased (M/F = 2.46±0.74) (Table 4.5.1). Water quality parameters was observed every 2-day interval and were within the stipulated range (refer to Appendix E for water quality data).

Table 4.5.1: F2 nauplii production and sex ratio (mean±SE, n=3) of *Apocyclops dengizicus* in different pH levels.

	pH					
	Control	**5**	**6**	**7**	**8**	**9**
F2 Nauplii Production						
Day 6	3.0±2.5[a]	3.7±3.7[a]	2.7±2.7[a]	4.0±2.0[a]	14.7±14.7[a]	6.7±6.7[a]
Day 8	0.3±0.3[a]	12.7±8.6[a]	9.0±9.0[a]	29.0±5.6[a]	42.0±28.4[a]	7.7±2.3[a]
Day 10	7.0±7.0[a]	1.7±1.7[a]	3.7±2.0[a]	16.0±1.5[a]	19.7±7.8[a]	2.3±1.5[a]
Day 12	10.5±9.8[a]	0.0±0.0[a]	8.0±4.0[a]	21.3±6.9[a]	22.3±9.2[a]	9.7±8.2[a]
Sex Ratio (M/F)	1.02±0.33[a]	2.46±0.74[a]	1.18±0.82[a]	1.20±0.12[a]	0.92±0.55[a]	0.83±0.03[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; see Appendix K). Sex ratio values were calculated from data on Day 8.

4.5.1 Body length and width of *A. dengizicus* cultured in different pH levels

The different pH levels tested did not affect body size of *A. dengizicus* irrespective of their developmental stages ($p > 0.05$) except the length of adult males ($p < 0.05$) (Table 4.5.2). Across all pH treatments, the average length of a nauplius ranged from 136-171 µm and width ranged from 84-109 µm. Copepodids had an average length of 380-461 µm and width of 137-156 µm. Adult males were 652-705 µm in length and 163-178 µm in width. The average size of adult females were 816-901 µm in length and 222-265 µm in width (Table 4.5.2).

Table 4.5.2: Body length and width (mean µm±SD) of *Apocyclops dengizicus* cultured in different pH levels in Experiment 6.

pH	NAUPLIUS		COPEPODID	
	Length (µm)	**Width (µm)**	**Length (µm)**	**Width (µm)**
5	171±27[a]	96±16[a]	451±88[a]	147±18[a]
6	202±45[a]	106±16[a]	461±55[a]	156±22[a]
7	202±54[a]	109±15[a]	380±40[a]	141±6[a]
8	180±64[a]	96±21[a]	446±34[a]	156±13[a]
9	136±50[a]	84±18[a]	397±11[a]	137±6[a]
pH	**ADULT MALE**		**ADULT FEMALE**	
	Length (µm)	**Width (µm)**	**Length (µm)**	**Width (µm)**
5	705±34[a]	170±12[a]	807±102[a]	222±28[a]
6	683±27[a]	178±12[a]	816±64[a]	244±17[a]
7	652±26[a]	163±11[a]	852±29[a]	231±28[a]
8	674±34[a]	174±12[a]	901±65[a]	265±3[a]
9	705±28[a]	169±6[a]	870±117[a]	254±17[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly ($p<0.05$; Appendix F).

4.6 Effects of photoperiod on the survival and nauplii production of *A. dengizicus*

The three photoperiod regimes (24L:0D, 0L:24D and 12L:12D) did not significantly affect the survival (%) of *A. dengizicus* (Figure 4.7). Nonetheless, higher average survival was found in total darkness treatment (0L:24D). The survival on day 6 were highest in photoperiod regimes 0L:24D (70.0±11.0%) followed by 12L:12D (66.0±14.2%) and 24L:0D (65.0±9.4%) but they were not statistically different ($p > 0.05$). The survival continued to decrease until day 12 where it fell to 40±8.7% (24L:0D), 50.7±15.0 (0L:24D), 51.3±5.5% (12L:12D) and they were not significantly different ($p > 0.05$).

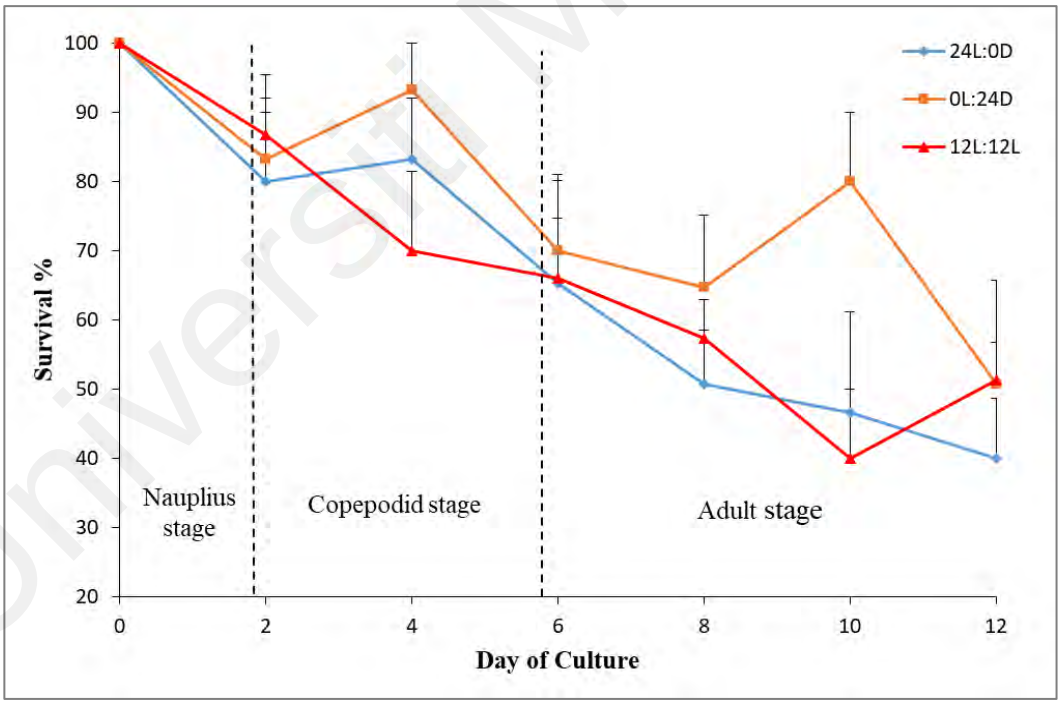

Figure 4.7: Survival (%) of *Apocyclops dengizicus* (mean±SE,) in different photoperiod regimes over a period of 12 days.

The numbers of nauplii produced between the three photoperiod regimes were not statistically significant from day 6 to day 12 ($p > 0.05$) (Figure 4.6.1). Nonetheless, nauplii production reached a peak on day 10 in all photoperiod

regimes – 48.3±26.8 (24L:0D), 38.3±8.3 (0L:24D) and 61.7±41.3 (12L:12D). The cumulative total of nauplii production in Experiment 7 ranged from 96.3±24.8 (24L:0D), 99.0±11.6 (0L:24D) and 128.0±60.9 (12L:12D) individuals (Table 4.6.1).

The sex ratio in continuous light treatment was equal male to female (1.00±0.09) and the populations in 12L:12D and 0L:24D were female-biased (0.79±0.14 and 0.42±0.05) ($p < 0.05$) (Table 4.6.1). The water quality parameters were within the stipulated range (refer to Appendix E for water quality data).

Table 4.6.1: F2 nauplii production and sex ratio (mean±SE) of *Apocyclops dengizicus* in different photoperiod regimes.

	Photoperiod (Light:Dark hours)		
	24:0	**0:24**	**12:12**
F2 Nauplii Production			
Day 6	0.0±0.0[a]	4.0±2.3[a]	6.7±3.5[a]
Day 8	24.7±9.2[a]	37.3±10.0[a]	37.3±14.3[a]
Day 10	48.3±26.8[a]	38.3±8.3[a]	61.7±41.3[a]
Day 12	23.3±3.2[a]	19.3±7.7[a]	22.3±10.8[a]
Sex Ratio (M/F)	1.00±0.09[a]	0.42±0.05[b]	0.79±0.14[ab]

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; Appendix L). Sex ratio values were calculated from data on Day 8.

4.6.1 Body length and width of *A. dengizicus* cultured in different photoperiod regimes

The different photoperiod regimes tested showed no significant differences ($p > 0.05$) on the body dimensions of copepods irrespective of their developmental stages except in nauplius length ($p < 0.05$) (Table 4.6.2). In all three photoperiod regimes, the body size of a nauplius ranged from 129-181 µm in length and 89-100 µm in width. Copepodids ranged between 399-476 µm in length and 139-156 µm in width. Adult males *A. dengizicus* had an average of 678-691 µm in length

and an average width of 170-180 µm. The average size of adult females were 856-870 µm in length and 250-253 µm in width (Table 4.6.2).

Table 4.6.2: Body length and width (mean µm±SD) of *Apocyclops dengizicus* cultured in different photoperiod regimes in Experiment 7.

Photoperiod (Light: Dark in hours)	NAUPLIUS		COPEPODID	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
0:24	129±29[a]	89±17[a]	399±63[a]	139±15[a]
24:0	179±58[a]	98±20[a]	476±183[a]	156±44[a]
12:12	181±43[a]	100±18[a]	449±77[a]	152±27[a]
Photoperiod (Light:Dark hours)	**ADULT MALE**		**ADULT FEMALE**	
	Length (µm)	**Width (µm)**	**Length (µm)**	**Width (µm)**
0:24	678±27[a]	170±11[a]	856±22[a]	250±7[a]
24:0	682±33[a]	175±8[a]	865±75[a]	254±29[a]
12:12	691±33[a]	180±11[a]	870±40[a]	263±13[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly ($p<0.05$; Appendix F).

4.7 Effects of light intensity on the survival and nauplii production of *A. dengizicus*

The *A. dengizicus* cultures were subjected to three different levels of light intensities (6.08, 14.85 and 23.65 µmol photons m^{-2} s^{-1}) and were able to survive and reproduce well in the tested conditions as shown in Figure 4.8 and Table 4.7.1. On day 6, survival was highest (87.3±7.0%) in low light (6.08 µmol photons m^{-2} s^{-1}), followed by 84.3±10.0% in high light levels (23.65 µmol photons m^{-2} s^{-1}) and 54.7±6.0% in medium light levels (14.85 µmol photons m^{-2} s^{-1}) (p <

0.05). By day 12, the survival in all three treatments decreased to less than 45% and was not statistically different ($p > 0.05$) (Figure 4.7).

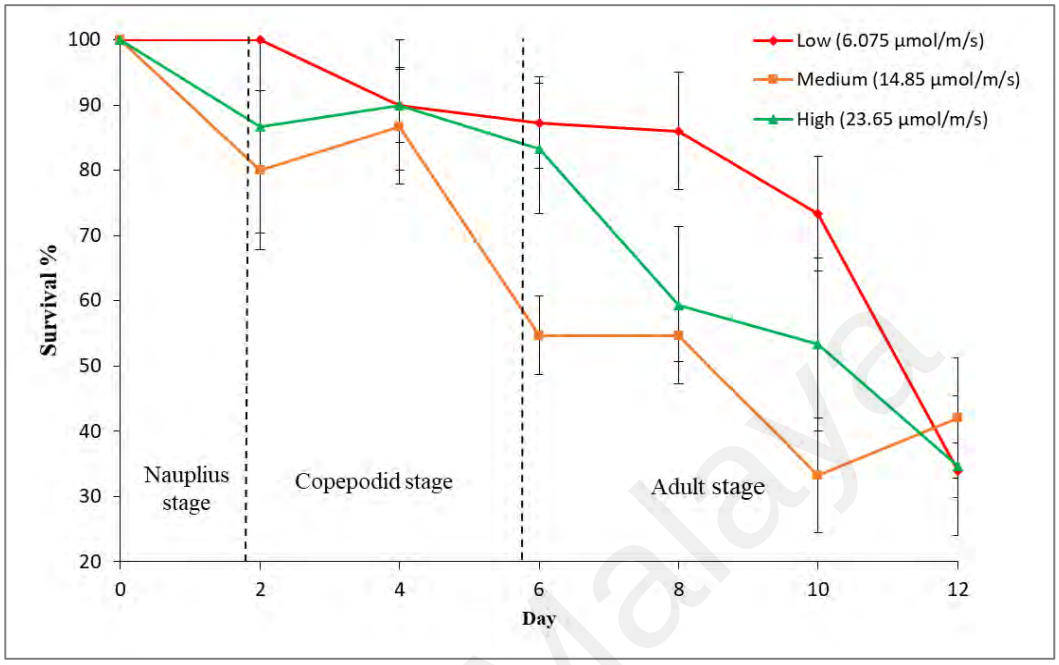

Figure 4.8: Survival (%) of *Apocyclops dengizicus* (mean±SE) in different light intensities over a period of 12 days.

The three levels of light intensity did not have a significant effect ($p > 0.05$) on the number of nauplii produced from day 6 to day 12. The cumulative total number of nauplii produced from day 6 to 12 was highest in cultures subjected to low light levels (90.7±23.2), followed by in medium light intensity (73.7±13.7) and in high light intensity (44.7±18.5) (Table 4.7.1).

Table 4.7.1: Nauplii production and sex ratio (mean±SE) of *Apocyclops dengizicus* in different levels of light intensity.

	Light Intensity (µmol photons m^{-2} s^{-1})		
	6.08 Low	**14.85 Medium**	**23.65 High**
Nauplii Production			
Day 6	36.3±14.9[a]	18.3±5.0[a]	14.7±4.4[a]
Day 8	30.3±6.4[a]	18.7±8.7[a]	20.0±10.4[a]
Day 10	15.0±7.6[a]	31.7±14.8[a]	6.7±4.4[a]
Day 12	9.0±4.0[a]	5.0±3.1[a]	3.3±1.3[a]
Sex Ratio (M/F)	1.00±0.10[a]	1.16±0.18[a]	1.09±0.17[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; Appendix M). Sex ratio values were calculated from data on Day 8.

The sex ratios in all light intensity treatments contained equal male and female populations ($p > 0.05$) (Table 4.7.1). The water quality parameters were observed every 2 days throughout the duration of the experiment and were within the stipulated range (refer to Appendix E for water quality data).

4.7.1 Body length and size of *A. dengizicus* cultured in different light intensities

Light intensities of 6.08, 14.85 and 23.65 µmol photons m^{-2} s^{-1} did not significantly affect ($p > 0.05$) body size of *A. dengizicus* except in adult females ($p < 0.05$) as shown in Table 4.7.2. The largest adult females were found in low light intensity treatment (length: 890±39 µm; width: 272±11 µm). The average size of adult females decreased as light intensity increased (Table 4.7.2).

Table 4.7.2: Body length and size (mean µm±SD) of *A. dengizicus* cultured in different light intensities in Experiment 8.

Light Intensity (µmol photons m^{-2} s^{-1})	NAUPLIUS		COPEPODID	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
6.08	217±37[a]	101±9[a]	435±110[a]	149±29[a]
14.85	202±33[a]	98±15[a]	461±85[a]	156±23[a]
23.65	183±105[a]	88±31[a]	429±104[a]	146±21[a]
Light Intensity (µmol photons m^{-2} s^{-1})	**ADULT MALE**		**ADULT FEMALE**	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
6.08	682±36[a]	186±7[a]	890±39[a]	272±11[a]
14.85	679±54[a]	173±7[a]	838±36[b]	250±22[b]
23.65	667±41[a]	172±17[a]	819±60[c]	251±17[b]

Mean of triplicate values; mean that does not share a common superscript letter in the same column differ significantly ($p<0.05$; Appendix F).

4.8 Comparison between POME grown *M. guilliermondii* (POME-MG) diet and POME only diet on *A. dengizicus* in a scale-up culture using optimal parameters

The average survivals of *A. dengizicus* fed with POME-MG and POME were similar throughout the 12-day period in 1L cultures (Figure 4.9). On day 6, the survival (%) between cultures fed with POME-MG and POME only were statistically different ($p < 0.05$). On day 6, the survival of copepods fed POME-MG and POME only were 88.5±3.9% and 72.7±2.9% respectively. On subsequent days, survival of *A. dengizicus* gradually decreased but the differences in survival between the two diets were not significant ($p > 0.05$). On the last day of experiment, survival of adult *A. dengizicus* decreased to 61.3±12.5% (POME-MG) and 41.8±1.8% (POME) (Figure 4.9).

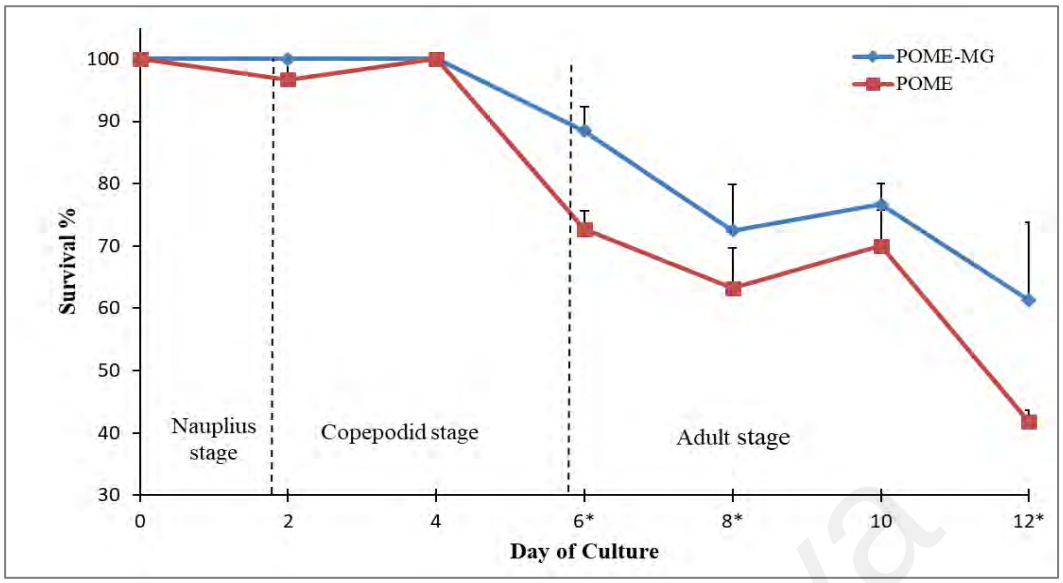

Figure 4.9: Survival (%) of *Apocyclops dengizicus* (mean±SE) given diets of POME-MG and POME in a scale-up experiment over a period of 12 days.

The average number of F2 nauplii present on day 6, 8, 10 and 12 in 1000 mL cultures fed with POME-MG and POME diets as shown in Table 4.8 were similar ($p > 0.05$; Appendix L). The cumulative total of nauplii produced in six days were 212±46.9 individuals in POME-MG treatments and 206±57.3 individuals in POME treatments ($p > 0.05$).

Table 4.8: F2 nauplii production and sex ratio (mean±SE, n=3) of *Apocyclops dengizicus* fed POME-MG and POME only in a scale-up experiment.

	FEED	
	POME-MG	**POME**
F2 Nauplii Production		
Day 6	22.3±3.9[a]	23.0±2.9[a]
Day 8	71.3±7.4[a]	69.3±6.5[a]
Day 10	50.0±16.1[a]	45.0±20.8[a]
Day 12	69.0±14.0[a]	69.3±22.6[a]

Mean of triplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; see Appendix N).

4.9 Nutritional profiles of POME based feed and *A. dengizicus*

The fatty acid compositions of POME based feeds (POME only, POME-MG, POME-RM, POME-SA, POME-RS and POME-AL) mainly consisted of palmitic acid, stearic acid, oleic acid and linoleic acid. Small amounts of α-linolenic acid (< 1.0%) were detected in POME, POME-MG and POME-RS. DHA was only detected in POME-AL (0.8%) (Table 4.9). Compared to their diets, *A. dengizicus* fed on POME only and POME-MG contained lower levels of palmitic acid (18.4% and 11.6%, respectively) but had very high levels of heptadecanoic acid (41.1% and 47.4%, respectively). The proportion of oleic acid was much lower in the copepods compared to their diets and neither ALA nor LNA was detected in the copepods (Table 4.9).

Table 4.9: Fatty acid compositions (% of total fatty acids) of the biomasses of POME, microheterotrophs cultured in POME and copepods.

Fatty acid		POME*	POME -RM	POME -MG	POME -SA	POME -RS	POME -AL	Copepods fed with POME	Copepods fed with POME-MG
Myristic acid	**C14:0**	3.6^{d}	3.4^{e}	4.6^{c}	5.1^{b}	2.8^{f}	11.3^{a}	-	3.6^{a}
Palmitic acid	**C16:0**	40.7^{b}	42.5^{a}	39.9^{c}	40.6^{b}	25.0^{e}	31.7^{d}	18.4^{a}	11.6^{b}
Heptadecanoic acid	**C17:0**	0.3^{d}	-	0.4^{c}	0.5^{b}	0.3^{d}	1.1^{a}	41.1^{b}	47.4^{a}
Stearic acid	**C18:0**	5.0^{d}	4.8^{e}	6.1^{c}	6.4^{b}	19.1^{a}	3.8^{f}	4.5^{a}	3.9^{b}
Oleic acid	**C18:1n-9**	29.5^{d}	33.6^{b}	30.0^{c}	28.1^{e}	37.8^{a}	12.3^{f}	13.8^{a}	5.8^{b}
Linoleic acid	**C18:2n-6**	8.3^{a}	8.3^{a}	7.1^{b}	6.7^{b}	6.6^{b}	2.3^{c}	-	-
α-Linolenic acid	**C18:3n-3**	0.9^{a}	-	0.5^{ab}	-	0.1^{b}	-	-	-
ARA	**C20:4n-6**	-	-	-	-	-	-	-	-
EPA	**C20:5n-3**	-	-	-	-	-	-	-	-
DHA	**C22:6n-3**	-	-	-	-	-	0.8^{a}	-	-
SFA		60.9^{c}	57.4^{d}	60.7^{c}	62.1^{b}	54.8^{e}	82.9^{a}	76.6^{b}	82.0^{a}
MUFA		29.9^{d}	34.4^{b}	36.7^{c}	31.1^{c}	38.5^{a}	13.5^{e}	23.4^{a}	18.0^{b}
PUFA		9.2^{a}	8.3^{a}	8.0^{ab}	6.7^{c}	6.7^{bc}	3.7^{d}	-	-
n-3 PUFA		0.9^{a}	-	0.5^{ab}	-	0.1^{b}	0.8^{a}	-	-
n-6 PUFA		8.3^{a}	8.3^{a}	7.5^{b}	6.7^{c}	6.6^{c}	2.9^{d}	-	-

See Appendix O for full list of fatty acids. Values are presented as mean ± standard deviation (n=2), means that do not share a common superscript letter in each row differs significantly (ANOVA, $p < 0.05$) between diets. Fatty acids in copepods (last two columns) were significantly different (T-test, $p < 0.05$). POME* = POME medium only, POME = POME-grown; RM = *Rhodotorula mucilaginosa*, MG = *Meyerozyma gulliermondii*, SA = *Shewanella algae*, RS = *Rhodovulum sulfidophilum*, AL = *Aurantiochytrium limacinum*.

When comparing POME and POME-MG, POME-MG had a higher percentage of protein (41.8%) and carbohydrate (24.8%), while POME had higher levels of lipid (27.1%), ash (28.0%) and energy (1772.9 kJ) (Table 4.10).

Table 4.10: Proximate composition and energy (dry basis) of POME and *Meyerozyma guilliermondii* cultured in POME (POME-MG).

Proximate composition (% in dry weight)	POME	POME-MG
Protein	38.80[a]	41.78[a]
Lipid	27.12[a]	11.0[b]
Carbohydrate	6.07[b]	24.81[a]
Ash	28.04[a]	22.39[a]
Energy (kcal/100g)	422.13[a]	364.80[b]
Energy (kJ)	1772.87[a]	1530.75[b]

Mean of duplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; T-test).

The amino acid composition between POME and POME-MG were similar (Table 4.11). However, the total percentages of non-essential amino acids in both feeds were higher (>52%) than the total amount of essential amino acids.

Table 4.11: Amino acid composition (% of total amino acids) of POME and *Meyerozyma guilliermondii* cultured in POME (POME-MG).

Essential	POME	POME-MG	Non-essential	POME	POME-MG
Histidine	1.7[a]	1.5[a]	Aspartic Acid	9.3[b]	8.9[a]
Threonine	5.4[a]	5.9[a]	Serine	5.7[a]	5.5[a]
Valine	6.9[b]	6.7[a]	Glutamic Acid	13.2[b]	12.7[a]
Methionine	5.4[b]	5.3[a]	Glycine	5.1[a]	5.2[a]
Lysine	5.9[a]	7.9[b]	Arginine	4.8[a]	4.9[a]
Isoleusine	6.0[b]	5.7[a]	Alanine	7.7[a]	7.4[a]
Leusine	9.4[b]	8.7[a]	Proline	3.6[a]	3.8[a]
Phenylalanine	4.2[b]	4.1[a]	Thyrosine	2.6[b]	2.5[a]
Tryptophan	1.8[a]	1.6[a]	Cysteine	1.4[a]	1.8[b]
TOTAL	46.6[a]	47.2[a]	**TOTAL**	53.4[a]	52.8[a]

Mean of duplicate values; mean that does not share a common superscript letter in the same row differ significantly ($p<0.05$; T-test).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Effects of POME based diets and its nutritional content on the survival and reproduction of *A. dengizicus*

The present study has shown that *M. guilliermondii* grown in POME (POME-MG) supported the development of *A. dengizicus* until maturity and produced a large number of offsprings compared to other POME grown microheterotroph diets and instant microalgae Nanno 3600 (*N. oculata*).

The food preferences of copepods are influenced by the morphological characteristics of the food item such as cell size, motility and probably taste as well (Rao *et al*., 2002). The marine yeast cells (*R. mucilaginosa* and *M. guilliermondii*) used in this study were approximately 2 to 6.5 µm (Mycology Online, 2016), the protist *A. limacinum* cells were 5 to 10 µm (Jaritkhuan & Suanjit, 2018) while marine bacterial cells (*S. algae* and *R. sulphidophilum*) were less than 2 µm (Munn, 2011). The actual cell sizes of microheterotrophs used in this study were not measured. The published cell sizes of the marine yeast cells in our study were comparable to algal cells *Isochrysis galbana* (3-6 µm) and *Nannochloropsis oculata* (2-4 µm) which were reported as the preferred diet of copepods compared to the much larger *Tetraselmis* species (*T. chuii*: 13-15 µm) (Farhadian *et al*., 2008; Wang *et al*., 2016; Pan *et al*., 2016). The survival of *A. dengizicus* fed bacterial diets (POME-SA and POME-RS) decreased significantly by day 8 which was when the population reached maturity. This showed that small cell sizes of *S. algae* and *R. sulphidophilum* were not preferred by adult *A. dengizicus*. Although the nutritional (fatty acid) values of POME-SA and POME-RS were comparable with POME-MG, their diets resulted in very low nauplii

production. The same concentration of feed was given regardless of cell size and perhaps it resulted in poor capture efficiencies (Kleppel *et al*., 1995) or it was insufficient for the development and/or maintenance of *A. dengizicus*.

The sex ratios in all feeding treatments were mostly female biased and ranged from 0.46 - 0.98 M/F (Table 4.1.1 and Table 4.1.2). *A. dengizicus* reared in the laboratory were reported to be female biased as female biased adult populations were suggested to be caused by natural mortality in males due to a shorter life span and predation of females on males especially when males were smaller than females (Mohamed *et al*., 2008).

A key factor in copepod reproduction is the chemical composition of diets, especially n-3 PUFAs for fecundity and egg production (biological parameters of reproduction) (Lacoste *et al.*, 2001). Previously, a few copepod studies reported that baker's yeast lacked EFA such as EPA, ARA and DHA as compared to microalgae such as *Isochrysis sp.*, *Nannochloropsis sp.* and *Tetraselmis sp.* (Farhadian *et al*., 2008; Pan *et al*., 2018). Similarly, the fatty acid compositions of POME and POME grown heterotrophic microorganisms also had low amounts of PUFAs (3.7-9.2%) and only POME-AL contained 0.8% of DHA. In contrast, our POME-based feeds contained large amounts of palmitic acid (25.0-42.5%), oleic acid (12.3-37.8%) and smaller percentages of stearic acid (3.8-19.1%) and linoleic acid (2.3-8.3%). Minimal amounts of α-linolenic acid were detected in POME, POME-MG and POME-RS (0.1-0.9%) (Table 4.8). The nutritional profile of POME, also the primary substrate for all the feeds, lacked ARA, EPA and DHA was similar to published data (Loo *et al*., 2013). Egg production in copepods has been largely associated with EPA (20:5n-3) due to the importance of long-chain PUFAs as a precursor for eicosanoids relating to hormone activities and in embryo development (Kleppel *et al*., 1998; Jonasdottir *et al*., 2009). The quality

of eggs produced, estimated by the hatching success, has been strongly correlated with DHA (22:6n-3) and to a lesser extent, α-linolenic acid (18:3n-3). DHA are metabolized to docosanoids which are hormones associated to neural functions (Jonasdottir *et al*., 2009). On the contrary, our POME based diets completely lacked EPA and DHA with small traces of α-linolenic acid detected in three diets (POME, POME-MG and POME-RS) but reproduction in *A. dengizicus* still occurred. Previous findings on *Acartia tonsa* also showed that a HUFA deficient diet of yeast-grown *Oxyrrhis marina* supported high rates of egg production (42.0±11.0 eggs female^{-1} day^{-1}) (Kleppel *et al*. 1998). Hence, more than one type of fatty acid is important for the total reproductive success in copepods because different stages in reproduction require specific nutritional needs. Egg production in *Temora longicornis* depended on ingested carbon and EPA from the diet and maternal fatty acid reserves while hatching success highly depended on DHA, ALA and fatty acid 18:5n-3 (Jonastoddir *et al*., 2009). Additionally, dietary fatty acid is not the only important component to copepod health and fecundity. Other factors include dietary macronutrients, micronutrients, protein, carbohydrates and amino acids have not been extensively discussed in copepod nutrition studies compared to fatty acids.

Based on our study, POME-MG contain high amounts of protein (41.78% dw) and carbohydrate (24.81% dw) but contained relatively low amounts of total lipid (11.0% dw). In contrast, instant microalgae *Nannochloropsis* (Nanno 3600™) contained significantly higher amounts of protein (62% dw) and lipids (18% dw) but low in carbohydrate content (8% dw) (Reed Mariculture, 2019). On the other hand, *Chaetocerous calcitrans* and *Tetraselmis tetrathele* which were the optimal microalgal diets for *A. dengizicus*, contained similar amounts of protein (38.1% and 41.9% dw) and carbohydrates (24.4% and 22.4% dw) as compared to POME-

MG and but the former diets had higher total lipid (19.2% and 16.1% dw) than the latter diet (Farhadian *et al*., 2008). Protein (as a source of amino acids) and lipids affects the consumers somatic growth and reproduction while carbohydrates are dietary energy sources for consumers (Taipale *et al*., 2016). Kleppel (1993) reviewed that nitrogen (as protein) limits copepod production in many areas and both ingestion and egg production rates of calanoid *A. tonsa* may be influenced by particulate organic nitrogen. When food quality was deficient in protein and lipid content, cladoceran *Daphnia magna* used a "sparing strategy" where carbohydrates were used for energy while protein and lipids were used for structural components to maximise somatic growth. Carbohydrates were also linked as an energy source during embryogenesis of copepod *Calanus helgolandicus* (Guisande *et al*., 1995). However, carbohydrates can only fulfil the short-term energy demands of zooplankton whereas lipids are important for long-term energy storage (Taipale *et al*., 2016). Therefore, *A. dengizicus* fed with POME-MG was still able to grow, develop and reproduce adequately due to the high nitrogen and carbohydrate content which may have substituted the role of dietary lipids.

Our marine yeast *M. guilliermondii* grown in POME (POME-MG) also has a higher PUFA content compared to baker's yeast, *Saccharomyces cerevisiae* (Payne & Ripangale, 2000). Previous findings have reported that calanoid copepod *Gladioferens imparipes* did not survive until maturity and poor population growth was observed in *A. dengizicus* on a diet of baker's yeast (Payne & Rippangale, 2000; Farhadian *et al*., 2008). Other factors that may affect survival rate and lack of population growth were possible hypoxia induced by high BOD of the residual POME (Loo *et al*., 2015), contamination and possible cannibalism (Guenther *et al*., 2015) which were not measured in this study.

Stottrup & McEvoy (2003) discussed possible copepod culture contamination by bacterial blooms, ciliates, other copepods and rotifers. Separate devices (such as sampling tools) should be exclusive for each tank to avoid contamination which was not practiced in this study due to equipment limitations. Uncontrolled proliferation of bacteria may degrade water quality resulting in copepod mortality. Microbial decomposition consequently leads to low dissolved oxygen concentration and ammonia accumulation in culture systems (Stottrup & McEvoy, 2003).

With regards to copepod size, the average lengths of nauplii was 163 to 212 µm while copepodids had average lengths of 425 to 590 µm (Table 4.1.3). The average size range of nauplii and copepodids were similar in all experiments. Karlsen *et al.* (2015) found that their copepod nauplii had overall lengths of 60 to 225 µm and copepodids had prosome lengths of 225 to 665 µm, similar to the sizes of *A. dengizicus* in this study. Their findings showed that copepod energy content increased relative to size which is an advantageous trait as a live feed product because fish larvae usually choose the largest prey item when offered prey of different sizes (van der Meeren *et al*., 1991). However, they also found that the growth rates of the Atlantic cod larvae were similar when fed with small and large zooplankton and larval dry weight was only slightly larger at the end of the experiment (47 days post hatch) (Karlsen *et al*., 2015). In a similar setup, Busch *et al.* (2011) also observed a similar pattern but far better growth was found in both zooplankton fed groups than the cod larvae fed rotifers. Therefore, major growth and development differences in fish larvae was most likely due to differences in nutritional compositions of copepods and rotifers/*Artemia*, particularly the low levels of taurine and/or protein in rotifers and *Artemia* were most likely to cause poor growth rates in cod larvae (Karlsen *et al*., 2015).

5.1.1 Feed concentration

The findings in this study indicated that 4.0 x 10^5 cells ml^{-1} day^{-1} of marine yeast *M. guilliermondii* cultured in POME (POME-MG) was the optimum feed concentration for *A. dengizicus* rearing (Table 4.2.1). Previous findings suggested algal densities between 2.5 x 10^4 to 5 x 10^5 cells ml^{-1} for *A. dengizicus* (Farhadian *et al*., 2008), 8 x 10^4 cells ml^{-1} for *A. royi* (Pan *et al*., 2016) and varied grazing rates of 40 to 12 x 10^4 cells ml^{-1} $hour^{-1}$ for *A. procerus* (Guenther *et al*., 2015). Biological parameters including survival, development and fecundity are significantly affected by feed concentrations (Wang *et al*., 2016). The survival from naupliar to adult of *Apocyclops borneoensis* fed with different algae treatments showed a general trend of increasing survival with increasing feed concentration but survival did not increase further beyond algae concentration of 8.50 µg C mL^{-1} (Wang *et al*., 2016). Female fecundity was also found to be significantly affected by feed concentration with a general trend of increasing food concentration leads to increasing fecundity rates. However, *A. borneoensis* fed *C. muelleri*, *S. costatum* and *N. Closterium* presented inhibiting effects when food concentrations were too high and differed for each diet (Wang *et al*., 2016). Excess feeding may result in feeding inefficiency and poor water quality (build up of tank detritus or nitrogenous waste products such as ammonia) due to degradation of uneaten food particles while insufficient feeding may result in nutrient deficiency and perhaps poor ingestion rates which was not measured in this study (Stottrup, 2006; Cowles *et al*., 1988).

5.2 Optimal abiotic factors for survival and reproduction of *A. dengizicus*

5.2.1 Salinity

The effects of environmental parameters on *A. dengizicus* life cycle were examined and the optimum salinity was found to be 10 ppt for both nauplii to

adult survival and nauplii production (Table 4.3.1). The findings in this study showed that *A. dengizicus* preferred brackish to marine waters compared to freshwater environment. In contrast to our results, previous studies found 20 ppt to be the optimal salinity for *A. dengizicus* (Farhadian *et al*., 2014) and *A. royi* (Pan *et al*., 2016). The differences in survival and nauplii production may be due to differences of experimental parameters and type of diet. Low salinity treatments (0 and 5 ppt) were reported to produce low clutch size while high salinity treatments (30 and 35 ppt) was hypothesized and assumed to reduce hatchability and naupliar survival due to suboptimal conditions which resulted in unsustainable population survival (Pan *et al*., 2016). Due to osmotic stress, the energy demand increases for required osmoregulation. In hypo and hypersalinity conditions, the fecundity rate of *P. nana* was significantly reduced because energy was expended for adaptation and/or homeostatic maintenance instead of biological processes (Lee *et al*., 2017). Ultimately, 10 ppt was chosen as the optimal salinity as it will be more cost-effective to culture in inland hatcheries using synthetic ocean salt compared to 20 ppt.

The body lengths of *A. dengizicus* in different salinities tested (10, 20 and 30 ppt) did not show variation in all developmental stages (Table 4.3.2). Salinity variations in copepod environments have been reported to be a crucial factor in determining the length of copepods, *Acartia clausi* (Gaudy *et al*., 1988). However, it was found that a combination of various factors such as temperature and salinity or chlorophyll and particulate carbon affected the body proportions (prosome:urosome) of *A. tonsa* studied during three different seasons but mainly temperature and food abundance were the determining factors in the varying morphological characteristics of a species (Gaudy & Verriopoulos, 2004). Riccardi and Mariotto (2000) also found that salinity variations in the Lagoon of

Venice did not affect copepod size (i.e. calanoids *A. clausi*, *A. tonsa*, *Paracalanus parvus*, *Centropages ponticus*; cyclopoids *Oithona nana*, *O. similis*; and the harpacticoid *Euterpina acutifrons*) and hypothesized that the species have built tolerance towards salinity changes or that food availability can counter the energy demands needed for osmoregulation and might not be expressed in body size.

5.2.2 Temperature

The present results showed that *A. dengizicus* was able to develop, survive and reproduce well over a range of temperatures between 26°C to 36°C. The optimal temperature was inconclusive in the present study due early termination of experiment because of poor water quality emerging on day 7 in all treatments. Nonetheless, treatments of 28°C and 30°C shared the highest survival (Figure 4.4) and 30°C produced the most F2 nauplii on day 6 (Table 4.4.1). Increased temperature can shorten maturation rate and decrease growth time (Altaff & Janakiranam, 2015). In their study, *A. dengizicus* cultured in 31±1°C reached peak density at the end of the second week compared to attainment of peak density at the end of the third week in 26±1°C treatment. Furthermore, Lee *et al.* (2017) discussed that copepods are adapted to survive within a certain range of temperature and change of ambient temperature will affect its life-cycle parameters. Below ambient temperatures (15°C and 20°C) resulted in reduced fecundity and decreased developmental time in *P. nana* in response to the energy trade-off for maintaining homeostasis (Lee *et al*., 2017).

The body length of adult *A. dengizicus* did not show significant variation across in the temperatures tested (Table 4.4.2). Cyclopoids *Oithona nana* and *O. similis* were also reported to be unaffected by temperature and did not show seasonal variations in size in comparison to several calanoid species (Ricciardi & Mariotto, 2000). In contrast, a laboratory study found that adult male and female

A. dengizicus was slightly larger in size in low temperature conditions (26°C) compared to those cultured in high temperature conditions (31°C). However, no variation in length or width was found in the nauplii and copepodids of *A. dengizicus* between the two temperatures (Altaff & Janakiram, 2015).

5.2.3 pH

This study found that there were no differences in survival (Figure 4.5) and nauplii production between pH ranges of 5 to 9 (Table 4.5.1). The highest average survival (%) were found in treatments of pH 8, 7 and control pH 7-8 (not adjusted). There was also no correlation between pH levels and *A. dengizicus* body lengths (Table 4.5.2). This showed that *A. dengizicus* was unaffected by pH levels 5 to 9 which indicated that they are able to survive, grow and reproduce in a wide range of pH levels. These findings are similar to Hansen *et al.* (2017) where copepods were also unaffected by pH up to 9.0-9.5. Euryhaline species were more adapted to fluctuating pH environments because it is a common stressor in estuaries. Both *Acartia* sp. caught from the wild and *Acartia tonsa* cultured in a laboratory showed significantly higher nauplii mortality in pH 9.5 compared to lower pH range which indicated that copepod species from both habitats had the same response to different pH levels (Hansen *et al*., 2017).

5.2.4 Photoperiod

The results in this study found that there were no differences in survival (but highest observed survival was in continuous darkness) and nauplii production of *A. dengizicus* between the photoperiod regimes 24 L: 0D, 12L: 12D and 0L: 24D (Figure 4.6 and Table 4.6.1). Our results are in agreement with a study by Nogueira *et al.* (2018) where population growth of *A. grani* was not significantly affected by photoperiod after a 12-day culture. In contrast, Farhadian *et al.* (2014) found that the survival percentage and total production was highest under

continuous illumination of 24L: 0D and 12L:12D. The discrepancy between our results and previous studies on *A. dengizicus* may be caused by differing culture conditions such level of light intensity (33.3 µmol photons m^{-2} s^{-1}) and salinity of 20 ppt in their study (Farhadian *et al*., 2014) compared to 2.975 µmol photons m^{-2} s^{-1} and salinity of 10 ppt used in our study (see Table 3.6). It is also possible that female copepods adapted their egg production during the acclimatization period to the different light conditions and therefore it is important to evaluate the effects of photoperiod and analyse reproductive parameters over a longer duration (Nogueira *et al*., 2018). In *Mesocyclops* species (incubated at 26±1°C in freshwater at 1100 lux ≈ 14.85 µmol photons m^{-2} s^{-1}), the survival percentage between five photoperiod regimes tested was not significantly different but photoperiod affected reproductive performance, maturation time and total life span. The highest mean offspring production for *Mesocyclops* sp. was at 08L:16D < 12L:12D < 16L:08D respectively. Photoperiod affects the synthesis of moulting hormones in crustaceans by regulating the moulting rhythm which strongly impacts development time (Fereidouni *et al*., 2013). Even though continuous light produced highest mean egg production, egg hatching success and fastest developmental rate in *Acartia sijiensis* (cultured at 30±1°C in 30 ppt at 1250 lux ≈ 16.88 µmol photons m^{-2} s^{-1}), it also resulted in shorter life expectancy and signs of decreasing egg production over time (Camus & Zeng, 2008).

5.2.5 Light intensity

A. dengizicus was able to survive and produce nauplii in three different light intensities of 6.08, 14.85 and 23.65 µmol photons m^{-2} s^{-1} (Figure 4.7 and Table 4.7.1). There were no significant variations overall in the biological parameters (survival, F2 nauplii production and body size) measured between treatments. Nonetheless, low light treatments exhibited slightly higher averages of naupliar I

to adult survival and nauplii production compared to higher light intensities. Farhadian *et al.* (2014) has reported that *A. dengizicus* can survive in light intensities of 33.3 to 162.1 µmol photons m^{-2} s^{-1} and maximum production occurred in low light levels compared to medium and high light levels. The light intensities tested in this study were not highly varied (differences of ~8.8 µmol photons m^{-2} s^{-1}) compared to differences of 50-80 µmol photons m^{-2} s^{-1} between light levels in the study conducted by Farhadian *et al.* (2014) which may be reflected in the results. Blue wavelength (425 nm) enhanced the survival, growth and reproduction of harpacticoid copepod *Tisbe holothuriae* compared to green and red wavelengths of higher fluence rate (UV irradiance on non-flat surfaces such as the ocean). This occurrence may be an adaptation since blue wavelengths are able to penetrate greater depths in water than other wavelengths and the possible significance of vertical migration for copepods is to avoid zones of high light intensity (Miliou, 1992). Unfortunately, most studies have discussed the effect of photoperiod on copepods but did not state the level of light intensity.

5.3 Evaluation of the fatty acid composition of *A. dengizicus* fed with POME-MG

In this study, essential fatty acids (EPA and DHA) were not detected in *A. dengizicus* fed with POME-MG and POME but were composed of high percentages of saturated fatty acids (SFA; 82.0%) and monounsaturated fatty acids (MUFA; 18.0%). In contrast, cyclopoid copepods fed on mono and mixed algal diets were reported to have PUFAs in their fatty acid compositions with a ratio of DHA/EPA >1 (Sargent *et al*., 1997) as shown in Table 5.1. Similarly, calanoid copepod *Pseudodiaptomus annandaleii* fed with diets of POME and POME-SA+AL also contained fatty acids ARA (1.3%), EPA (3.9%) and DHA (7.0%) (Table 5.1). The diet POME-SA+AL compared to POME-MG have similar fatty acid composition, levels of protein and carbohydrate with the

exception of POME-AL which contained 0.8% of DHA (Table 4.9). Unlike *P. annandaleii*, *A. dengizicus* in this study did not demonstrate the ability to biosynthesize EPA and DHA from dietary short chain fatty acids LNA and ALA, a result contrasting to previously reported studies (Monroig *et al*., 2013; Desvilettes *et al*., 1997; Rasdi *et al*., 2016). The majority of the fatty acid composition of *A. dengizicus* fed with POME-MG and POME was heptadecanoic acid (C17:0), an odd-chain saturated fatty acid that is not commonly reported in high amounts in copepods or other zooplankton species. Heptadecanoic acid is synthesized by α-oxidation of stearic acid (C18:0) which is synthesised from palmitic acid (C16:0) (Rezanka *et al*., 2015). The differences between the nutritional content of *A. dengizicus* fed with POME-MG compared to previously published studies may reflect the nutritional value of the diet and the lack of fatty acid conversion in the copepods. Pan *et al.* (2018) has reported that fatty acid conversion rate seems to be low in copepods after comparing the fatty acid composition between the algae diet and the copepods. For example, in their study, *T. chuii* contained 23.64% ALA but the copepod (*P. nana*) fed on this diet only had 4.05% of DHA which revealed a low self-conversion rate (Pan *et al*., 2018). The same pattern was also reported with the EPA-rich diet of *N. oculata*. Although *T. chuii* and *N. oculata* contained high proportions of precursor fatty acids for EPA and DHA synthesis, the low DHA production in their study showed that precursor fatty acids may not be efficiently taken by the copepods due to low digestibility (Pan *et al*., 2018). In this study, the proportions of LNA and ALA in the POME and POME-MG were low and the diets did not contain EPA or DHA which was consequently reflected in the lack of essential fatty acids in *A. dengizicus* fed with these diets. However, this study is the first to evaluate the

Table 5.1: Fatty acid compositions (% in total fatty acids) of copepods.

Fatty Acid	*A. dengizicus* fed POME-MG	*P. annandaleii* fed POME-SA+AL[1]	*A. dengizicus* fed *T. tetrathele*[2]
C14:0	3.6	3.1	3.2
C16:0	11.6	17.6	30.3
C17:0	47.4	17.1	-
C18:0	3.9	3.3	6.4
C18:1n9	5.8	6.8	7.2
C18:2n6	-	8.2	2.4
C18:3n3	-	10.5	3.1
C20:4n6 ARA	-	1.3	1.5
C20:5n3 EPA	-	3.9	8.4
C22:6n3 DHA	-	7.0	20.2
SFA	82.0	49.9	41.2
MUFA	18.0	18.1	13.0
PUFA	-	31.9	37.6
DHA/EPA	-	1.8	2.4

[1] Abd Aziz (2019).
[2] Farhadian *et al.* (2008b).

fatty acid composition of copepods on POME based diets and the discrepancy in the measured fatty acid content may also be due to improper conditions in freezing and storage of copepod samples. However, this study is the first to evaluate the fatty acid composition of copepods on POME based diets and the discrepancy in the measured fatty acid content may also be due to improper conditions in freezing and storage of copepod samples. Due to difficulties in obtaining the appropriate amount of copepods in dry weight, the storage of copepod samples in -15°C to -20°C for a few months before freeze drying and subsequent lipid extraction can result in loss of polar lipids and accumulation of free fatty acids compared with copepods stored at -80°C or immediate lipid

extraction of fresh animals (Ohman, 1996). This current study showed that the lack of EPA and DHA in the fatty acid composition of *A. dengizicus* is not suitable for larval diets which require an optimal ratio of 2:1 of DHA to EPA (Sargent *et al*., 1997). However, POME-MG was able to support the development, survival and reproduction of *A. dengizicus* as a non-algal diet. Further improvements can be made to its dietary fatty acid composition to improve the fatty acid content of the copepods. Suggestions include *A. dengizicus* to be fed on a better quality POME-MG diet (by modifying substrates or altering culture conditions) or enriching the copepods with some fatty acids before they are offered to the fish larvae which are also current practices on rotifers and *Artemia* in larviculture to compensate for missing HUFAs (de Lima *et al*., 2013).

5.4 Economic viability of producing copepods using POME-grown feed

The main benefit of POME-grown feeds compared to microalgae is its low production cost. The estimated cost to produce POME-MG is MYR 31.40/L, equivalent to USD 7.55/L (refer to Appendix P). The simplicity in MG culture is suitable for low-technology small-scale farms in the tropics where they lack technological knowledge in producing microalgae and production cost is high (Loo *et al*., 2013). A similar product POME-BAC used as feed for rotifers can be produced at an estimated cost of USD 23.29/kg of dry weight (Loo *et al*., 2013) in comparison to USD 75.00/L for microalgal paste Nanno 3600 (Reed Mariculture Inc., 2018) and predicted cost of USD 11.90-70.10/kg for algae in three different cultivation systems (Chauton *et al*., 2015). Acien *et al*. (2018) discussed that microalgae biomass production cost below USD 5.00/kg is demanded by the

aquafeed market. Though POME based feeds may not meet this price, it offers a cheaper alternative for aquaculturists.

The biological benefits of copepods as live feed for larval rearing have been discussed extensively in literature. A study by Abate *et al.* (2016) assessed the cost-effectiveness of copepods in commercial juvenile turbot farming compared to a common commercially used live feed item, *Artemia,* and overall results were relatively cost-effective. The calculated profit per fry (without fixed costs) when fed with semi-intensive cultured copepods was USD 0.54 compared to a profit of USD 0.12 when fed with *Artemia*. With further improvements, the application of copepods as a complement/substitute to *Artemia* in the future is inevitable due to uncertainties in *Artemia* availability and high cost which may limit the expansion of the aquaculture industry (Abate *et al*., 2016).

5.5 Limitations

a) Difficulty in differentiating copepods of various generations over the twelve-day culturing period. Survival from Day 6 onwards was calculated using the number of adult copepods in a population. However, it was unknown whether the adult copepods survived from the initial population (F1 nauplii) or were from the F2 generation. This may have caused the sudden increase in survival (%) on Day 8 or Day 10 in some experiments.

b) Equipment limitations include the sharing of sampling devices, water quality meters and water baths between two researchers may have caused cross-contamination. The sample size and number of replicates were also limited by time and equipment in the duration of the study.

c) Copepod samples were analysed for fatty acid composition but were insufficient for full nutritional profiling. Mass culturing of copepods and harvesting a total dry

weight of 50g was not possible due to time constraint and samples had to be stored in the freezer (at -20°C) for a few months.

5.6 Recommendations for future research

a) Optimisation of *M. guilliermondii* in POME or other wastewaters rich in EPA and/or DHA to further enhance its biomass production and nutritional content.

b) Study the combined effects of two abiotic factors such as salinity and temperature or photoperiod and light intensity. This current study looked at each abiotic factor singly and results might potentially change when different factors interact.

c) Investigate the effects of POME-MG as a supplement to algae diet as mixed feed for *A. dengizicus*.

d) Evaluate the feasibility of culturing fish/shrimp using POME-MG fed *A. dengizicus* compared to copepods fed with algae diets and other zooplankton.

e) Pilot-scale mass culture of *A. dengizicus* raised on a diet of POME-MG in captivity.

5.7 Conclusions

This study confirms the potential of utilizing waste-grown microheterotrophs as feed for culturing copepods cheaply and sustainably. The significant findings of this study were marine yeast *M. guilliermondii* cultured in POME (POME-MG) supported good survival, growth and reproduction of *A. dengizicus* compared to other POME-grown microheterotrophs. The mean overall survival of copepods fed POME-MG was 85.6±5.7% with a cumulative total of 445.7±55.4 F2 nauplii individuals produced throughout twelve days of culture (Objective 1). The highest survival and nauplii production of *A. dengizicus* was found in salinity of 10 ppt.

However, other abiotic factors such as temperature, pH, photoperiod and light intensity did not significantly affect the survival, F2 nauplii production and body size of *A. dengizicus* (Objective 2). All POME-grown microheterotroph diets and POME contained high levels of palmitic acid (C16:0) ranging from 25.0% to 42.5% and oleic acid (C18:1n-9) ranging from 12.3% to 37.8%. The diets also contained varied levels of PUFAs ranging from 3.7% to 9.2%. Only POME-grown thraustochytrid protist *Aurantiochytrium limacinum* (POME-AL) contained DHA (0.8%). POME-MG contained protein (41.8%), lipids (11.0%), carbohydrate (24.8%) and essential amino acids (47.2%). The fatty acid composition of POME-MG lacked EPA and DHA which was consequently reflected in the fatty acid profile of *A. dengizicus* (Objective 3). Optimum laboratory-scale culture conditions for *A. dengizicus* were identified to be POME-MG concentration of 4.0 x 10^5 cells/ml daily per 250 ml culture, salinity of 10 ± 1 ppt, temperature of 30 ± 1°C, photoperiod of 12 hours light: 12 hours dark under low light intensity of 6.08 µmol photons m^{-2} s^{-1} (Objective 4). It is worth noting that all experiments in this study were conducted in a laboratory and results may not be fully reproducible in a large-scale copepod production. This research has highlighted the responses of *A. dengizicus* to different abiotic factors and identified that a POME-grown microheterotroph diet could be used as an alternative feed for copepod culture which benefits both the aquaculture industry and the palm oil industry.

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