FRESHWATER ZOOPLANKTON DIVERSITY AND CULTURE OF SELECTED SPECIES WITH EMPHASIS ON REPRODUCTIVE CAPACITIES AND NUTRITIONAL CONTENTS

By

UMI WAHIDAH BINTI AHMAD DINI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chair : Professor Fatimah binti Md. Yusoff, PhD
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Zooplankton forms a critical link between the base of the aquatic food web and the top-level consumers; therefore, they are important live-feed organisms in the aquaculture industry. However, the unavailability and unstable production of highly nutritious live feeds are major bottlenecks for the aquaculture industry. Thus, understanding water quality and food requirements for zooplankton is pivotal to the success of sustainable production of zooplankton as live feed in hatcheries. Therefore, the current study aimed to evaluate the distribution and diversity of freshwater zooplankton and identify prospective candidates as live feeds. Subsequently, this study was conducted to ascertain the ideal conditions for zooplankton culture and to assess the effectiveness of microalgae-bacteria encapsulation in enhancing zooplankton quality. A study on zooplankton distribution was carried out at 20 different freshwater water bodies in Malaysia. Physicochemical parameters were measured in situ, while water and zooplankton samples were collected for nutrient analyses and for zooplankton identification and enumeration. Selected zooplankton species were molecularly characterised using the 18S rDNA approach. Each of the selected zooplankton species was exposed to several parameters, including temperature (25 °C, 27 °C and 30 °C), pH (pH 5, pH 7, and pH 9), unionized-ammonia, NH_3-N (0.1 mg L^{-1}, 0.3 mg L^{-1}, and 0.5 mg L^{-1}), and diets (13 diets, including mono and mixed diets). Each treatment was conducted in triplicate. Daily observations were performed for the life cycle, population density, and population growth rate. Proximate analysis was conducted according to standard methods, and fatty acids were analysed using a gas chromatograph–mass spectrometer (GC-MS). A total of 58 zooplankton species, consisting of 12 species of cladocerans, 10 species of copepods, and 36 species of rotifers, were recorded. Environmental parameters associated with eutrophication (high nutrients, chlorophyll *a*, turbidity, and low water transparency with a Global Test, ρ = 0.508) affect the diversity and composition of zooplankton. Several tolerant species such as *Ceriodaphnia cornuta*, *Brachionus calyciflorus*, *Keratella cochlearis*, *Trichocerca* *similis*, *Thermocyclops crassus*, and *Mesocyclops thermocyclopoides* can thrive in high eutrophic conditions, indicating that these species could serve as indicators. Among the three freshwater zooplankton groups, Cladocera was selected due to its reproduction mode and nutritional quality. Three species (*C. cornuta*, *Moina micrura* and *Diaphanosoma excisum*) of cladocerans were selected for cultivation based on their abundance and differences in size.The present results pointed out that the optimum water quality requirements for all three cladocerans were 27 ℃, pH 7, and 0.1 mg L^{-1} NH_3-N, which supported the fastest maturation, the highest reproductive capacities (production of eggs, offspring, and clutches), and maximized the nutritional contents of all cladocerans species. Furthermore, regarding diets, the combination of the bacterium, *Ochrobactrum haematophilum* with high-quality microalgae, especially the T10 diet (*Cyclotella meneghiniana* + *O. haematophilum*) found to be the ideal diet for all cladocerans, which enhanced the reproductive capacities and nutritional composition. Meanwhile, cyanobacteria diets (*Microcystis aeruginosa* and *Planktothrix agardhii*) were considered as poor diets which hindered the growth of cladocerans. However, the addition of *O. haematophilum* to *M. aeruginosa* and *P. agardhii* diets (T12 and T13) increased the population density and reproductive capacities of all cladocerans but not the nutritional contents. Overall, the selection of optimal culture conditions and microalgae-bacteria consortium have important implications for improving the production of high-quality cladocerans for sustainable cultivation, which can be utilized as high-quality live feeds in the aquaculture industry.

Keywords: Cladocera; live food; microalgae; bacteria; nutritional quality
SDG: No poverty; zero hunger; life below water

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KEPELBAGAIAN ZOOPLANKTON AIR TAWAR DAN PENGKULTURAN SPESIES TERPILIH DENGAN PENEKANAN KE ATAS KAPASITI PEMBIAKAN DAN KANDUNGAN NUTRISI

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Zooplankton membentuk hubungan kritikal di antara asas siratan makanan dengan pengguna peringkat atasan; oleh itu, zooplankton adalah organisma makanan hidup yang penting dalam industri akuakultur. Walau bagaimanapun, ketidaksediaan dan ketidakstabilan pengeluaran makanan hidup yang sangat berkhasiat adalah halangan utama bagi industri akuakultur. Memahami kualiti air dan keperluan makanan untuk zooplankton adalah penting untuk kejayaan pengeluaran zooplankton sebagai makanan hidup secara mampan. Justeru, kajian ini bertujuan untuk menilai taburan dan diversiti zooplankton air tawar dan mengenal pasti spesies zooplankton yang sesuai sebagai makanan hidup. Seterusnya, kajian ini dijalankan untuk mengenal pasti kondisi yang ideal untuk pengkulturan zooplankton dan menilai keberkesanan gabungan mikroalga-bakteria dalam meningkatkan kualiti zooplankton. Kajian mengenai taburan zooplankton telah dijalankan di 20 lokasi air tawar yang berbeza di Malaysia. Parameter fizikokimia diukur secara in situ, manakala sampel air dan zooplankton dikumpul untuk analisis nutrien dan untuk pengenalpastian dan penghitungan zooplankton. Spesies zooplankton terpilih telah dicirikan secara molekul menggunakan kaedah 18S rDNA. Setiap spesies zooplankton yang dipilih telah didedahkan kepada beberapa parameter, termasuk suhu (25 °C, 27 °C dan 30 °C), pH (pH 5, pH 7, dan pH 9), ammonia, NH_3-N (0.1 mg L^{-1}, 0.3 mg L^{-1}, dan 0.5 mg L^{-1}), dan diet (13 diet, termasuk diet mono dan campuran). Setiap parameter diulang sebanyak tiga kali. Pemerhatian harian dilakukan untuk kitaran hayat, kepadatan populasi, dan kadar pertumbuhan populasi. Analisis proksimat telah dijalankan mengikut kaedah standard, dan asid lemak dianalisis menggunakan spektrometer jisim kromatografi gas (GC-MS). Sebanyak 58 zooplankton spesies, terdiri daripada 12 spesies Kladosera, 10 spesies Copepoda, and 36 spesies Rotifera telah direkodkan. Parameter alam sekitar

yang dikaitkan dengan eutrofikasi (nutrien, kekeruhan, dan klorofil *a* yang tinggi dengan Ujian global, ρ = 0.508) memberi kesan kepada diversiti dan komposisi zooplankton. Beberapa spesies toleran seperti *Ceriodaphnia cornuta*, *Brachionus calyciflorus*, *Keratella cochlearis*, *Trichocerca similis*, *Thermocyclops crassus*, dan *Mesocyclops thermocyclopoides* mampu bertahan dalam kondisi eutropik yang tinggi; menunjukkan bahawa spesies ini boleh berfungi sebagai indikator. Antara tiga kumpulan zooplankton air tawar, kladosera telah dipilih berdasarkan kaedah pembiakan dan kualiti nutrisi. Tiga spesies (*C. cornuta*, *Moina micrura* and *Diaphanosoma excisum*) kladosera telah dipilih untuk pengkulturan berdasarkan saiz dan kelimpahan yang berbeza. Keputusan menunjukkan bahawa kualiti air yang ideal untuk kesemua kladosera adalah pada suhu 27 ℃, pH 7, dan 0.1 mg L^{-1} NH_3-N yang didapati telah mempercepatkan kematangan, meningkatkan hasil pembiakan (pengeluaran telur, anak dan kantung telur), dan memaksimumkan kandungan nutrisi kesemua kladosera. Seterusnya, dari segi diet, campuran bakteria (*Ochrobactrum haematophilum*) dengan mikroalga yang mempunyai kualiti yang tinggi, terutamanya diet T10 (*Cyclotella meneghiniana* + *O. haematophilum*) didapati sebagai diet yang ideal untuk semua kladosera yang telah meningkatkan hasil pembiakan dan kandungan nutrisi. Sementara itu, diet cyanobacteria (*Microcystis aeruginosa* and *Planktothrix agardhii*) dianggap sebagai diet yang tidak elok kerana telah menyekat pertumbuhan kladosera. Walau bagaimanapun, penambahan campuran *O. haematophilum* ke dalam diet *M. aeruginosa* and *P. agardhii* (T12 and T13) telah meningkatkan ketumpatan populasi dan kapasiti pembiakan kladosera, tetapi bukan kandungan nutrisi. Secara keseluruhan, pemilihan keadaan pengkulturan yang optimum dan campuran mikroalga-bakteria sebagai makanan untuk kladosera mempunyai implikasi penting untuk meningkatkan pengeluaran kladosera berkualiti tinggi untuk pengkulturan kladosera secara mampan, yang boleh digunakan sebagai makanan hidup berkualiti tinggi dalam industri akuakultur.

Kata kunci: Kladosera; makanan hidup; microalga; bakteria; kualiti nutrisi
SDG: Tiada kemiskinan; sifar kelaparan; kehidupan bawah air

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%	Percentage
$(NH_4)_6Mo_7O_{24}$	Ammonium molybdate
<	Less than
>	More than
$\mu g\ L^{-1}$	Microgram per liter
µL	Microliter
µm	Micrometer
ALA	Linolenic acid
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
ARA	Arachidonic acid
BBM	Bold's Basal Medium
BIO-ENV	Biotic-environmental analysis
bp	Base pair
℃	Degree Celsius
$C_4H_4O_7SbK$	Antimonyl potassium tartarate
CCA	Canonical correspondence analysis
CFU	Colony forming unit
Chl *a*	Chlorophyll *a*
CTSI	Carlson Trophic Status Index
$CuSO_4\ 5H_2O$	Copper sulfate pentahydrate
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid

DO	Dissolved oxygen
EPA	Eicosapentaenoic acid
g	Gram
h	Hour
H’	Shannon–Wiener diversity index
H_2SO_4	Sulfuric acid
HABs	Harmful algal blooms
HCL	Hydrochloric acid
ind. L	Individual per liter
$K_2S_2O_8$	Potassium peroxobisulfate
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium nitrate
L	Liter
LA	Linoleic acid
m	Meter
mg	Milligram
mg L^{-1}	Milligram per liter
mL	Milliliter
mm	Millimeter
MUFA	Monounsaturated fatty acid
$N_2H_6SO_4$	Hydrazine sulfate
NaOH	Sodium hydroxide
NED	N-(1-naphthyl) ethylenediamine dihydrochloride
$NH_2C_6H_4SO_2$	Sulfanilamide
NH_3-N	Ammonia nitrogen

NH_4Cl	Ammonium chloride
nm	Nanometer
nMDS	Non-metric multidimensional scaling
NTU	Nephelometric turbidity unit
OD	Optical density
PCA	Principle component analysis
PCR	polymerase chain reaction
PRIMER	Plymouth Routines in Multivariate Ecological Research
PUFA	polyunsaturated fatty acid
r	Correlation
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SAFA	Saturated fatty acid
SD	Standard division
SGR	Specific growth rate
SIMPER	Similarity percentage analysis
Sp.	Species
TAN	Total ammonia nitrogen
TN	Total nitrogen
TP	Total phosphorus
TSB	Tryptic soy broth
UPM	Universiti Putra Malaysia
v/v	Volume/volume
$ZnSO_4$	Zinc sulfate heptahydrate

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Zooplankton are microscopic aquatic organisms which respond quickly to many environmental stressors, such as hydrological changes and anthropogenic activity-induced water pollution. Zooplankton abundance and diversity are affected by abiotic and biotic factors. They represent the channel of transmission of energy flux and organic matter from the primary producers to the top consumers in aquatic ecosystems (Bakhtiyar et al., 2020). In addition, zooplankton are suitable for culturing as live food because they can reach a high population size rapidly which can assure a sustainable supply for aquaculture (Rasdi *et al*., 2020a; Chakraborty and Mallick, 2023). Zooplankton is also recognized as a superior live food based on its nutritional composition which may match the dietary requirements of aquaculture animals, particularly fish larvae, to foster their growth and reproduction (Samat et al., 2020b).

Zooplankton distribution, growth, reproductive capacities, and nutritional values are affected by environmental parameters and food sources. Temperature, pH, dissolved oxygen (DO), and ammonia are among the important parameters that have a substantial impact on zooplankton (Liang et al., 2020a; Qin et al., 2021; Xue et al., 2022). In general, warmer temperatures increase the metabolic rate of zooplankton, leading to higher growth, reproduction, and feeding rates (Garzke *et al*., 2020). However, decreased in monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) was observed when *Brachionus calyciflorus* exposed to increasing temperature (30 °C). Water pH affects the regular physiological processes of aquatic animals, such as the exchange of ions with water and respiration (Tresguerres et al., 2023). Acidic pH exposure, whether chronic or acute, can affect zooplankton metabolic activity, egg production, population growth, and survival (Lee et al., 2020; Gao et al., 2022). Ammonia is the primary nitrogenous product of the protein catabolism. Total ammonia-nitrogen consists of toxic un-ionized NH_3−N and nontoxic ionized NH_4^+-N, and their equilibrium is mainly dependent on temperature and pH. High levels of NH_3−N can disrupt the normal metabolism of aquatic organisms, resulting in growth retardation and inhibition of zooplankton metamorphosis (Han et al., 2022).

Of all the aforementioned factors, the food source is the most critical factor. Differences in fatty acid content among zooplankton taxa could affect their demands for essential fatty acids from their diet. Fatty acids especially polyunsaturated fatty acids (PUFAs) composition is important for the growth of zooplankton as they function as precursors for hormones involved in gonad development, reproduction, and immune responses (Ilić et al., 2019). Since Omega-3 and Omega-6 fatty acids cannot be synthesized de novo by zooplankton, they must be included in the diet, either as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) or as their precursors, such as α-linolenic acid (ALA: precursor of EPA and DHA) and linoleic acid (LIN: precursor of ARA). Therefore, these essential fatty acids must be supplied from diets to support zooplankton for the need for zooplankton growth. In nature, zooplankton are fed on a wide range of microalgal species and bacteria. Being the primary producers, microalgae are the zooplankton's main food source, which ultimately helps to enrich zooplanktonic organisms by giving protein, energy, vitamins, minerals, and amino acids (Altaff and Vijayaraj, 2021). At the same time, microalgae and bacteria also develop interactions over time. Several investigations have found that heterotrophic bacteria are important for microalgal growth and survival (Samo et al., 2018; Sandhya and Vijayan, 2019). Indirectly, these symbiotic microalgae–bacteria consortia with valuable chemical and energy compounds could be utilized to enrich the zooplankton or optimizing zooplankton growth and quality. In recent years, the enrichment of zooplankton as live feeds with high-quality microalgae and potential bacteria either by mono diets or mixed diets has become a popular approach to boost zooplankton production (Wenzel et al., 2021; Samat et al., 2020a).

Among freshwater zooplankton, cladocerans such as *Ceriodaphnia*, *Moina*, and *Diaphanosoma* have been explored as potential live feed candidates (Chilmawati et al., 2019; Neri et al., 2020; Suhaimi et al., 2022). Cladocerans reproduce by cyclic parthenogenesis, which involves alternation between the asexual and sexual phases as part of their reproduction. Females exhibit parthenogenesis during the asexual phase, in which they can self-replicate without fertilization, reproduce offspring that are all females, and reach a high population in a short period. In addition, cladoceran’s ability to produce ephippia (cysts/resting eggs) during the sexual phase is also one of the reasons to utilize them as live feed candidates. For culturing purposes, cladocerans display a high potential for large-scale production because of their high growth rate. Apart from elucidating the capabilities of cladocerans in high-density cultures, a better understanding of the factors influencing the production of high-quality zooplankton must be acquired improve the productivity of live feed in aquaculture.

1.2 Problem statements

Aquaculture production in Malaysia including freshwater, brackish, and marine aquaculture contributing significantly to the country's food security and economic growth. However, in the aquaculture industry, artificial feed is not always sufficient to fulfill the growth requirements of larvae compared to live food. Mass rearing of healthy fish larvae is a key step in the aquaculture production processes. The rearing of more sensitive freshwater and marine fish larvae depends heavily on live food, particularly zooplankton. During the hatchery and nursery phases, fish larvae undergo rapid growth and development in the early stages of their life cycle. During this time, their mouthparts (mouth sizes) undergo changes in size and structure. Larvae with smaller mouths may not be able to consume larger zooplankton, leading to poor nutrition and growth. Conversely, larvae with larger mouths may struggle to capture and ingest smaller zooplankton efficiently. They can only ingest specific live feed with a specific size. For example, the ideal size of live feed is 50–90 µm for silver carp larvae, 90–150 µm for grass carp larvae and 150–270 µm for bighead carp larvae (Dabrowski and Bardega, 1984). Matching the size of the live food to the developmental stage of the larvae helps ensure efficient feeding, optimal growth, and overall success in raising healthy fish fry for stocking or further grow-out (Lahnsteiner et al., 2023). At the moment, *Artemia* is the most preferred and reliable live food, however, the size of *Artemia* not suitable for all fish larvae developmental stages. Hence, different species of zooplankton with different size should be utilized. Nowadays, the mass production of high-quality zooplankton as live feed is a bottleneck for sustainable aquaculture production. A poor understanding of their optimum requirements renders large-scale zooplankton production. Previous studies have revealed that the successful mass culture production of zooplankton is immensely related to environmental conditions and food quality, which are prerequisites for growth and reproduction (Ogello et al., 2019; Hegab et al., 2020; Ramlee et al., 2021). In addition, to obtain high-quality live food, they should be fed with high nutritional food sources that influence the supply of various nutritional contents to zooplankton. The production of zooplankton depends on the biochemical composition of the microalgae and bacteria fed to them. However, mono-specific diets may cause nutritional deficiencies because of the inadequate content of one or more essential nutrients. To reduce this risk, several authors have suggested the use of mixed diets because their combined nutrient contents are more likely to meet the nutritional requirements of the target species (Rasdi et al., 2020c; Contreras-Tapia et al., 2020; Fouzi et al., 2021; Azani et al., 2022; Yuslan et al., 2022). From this study, potential zooplankton species were identified as candidates for live feed. Additionally, the optimal environmental conditions and diet necessary for their growth and reproduction were established. The findings from this study can be used to develop an economical and practical technique for high-quality live feed, which would be valuable to the aquaculture industry.

1.3 Objectives

The utilization of freshwater zooplankton as live feed is worth considering to meet the demand of the aquaculture sector. Hence, optimum conditions, especially environmental variables and food sources are crucial for high-quality zooplankton production. Therefore, the objectives of this study were:

1. To assess freshwater zooplankton community structure (species composition, distribution, and diversity) in natural and artificial lakes with different levels of eutrophication and to identify prospective candidates for live food production.

2. To investigate the effect of environmental factors (temperature, pH, and ammonia) on the reproductive performance, growth, and nutritional contents of the selected zooplankton species

3. To evaluate the dietary effect (mono-diets and microalgae-bacterial combined diets) on zooplankton growth performance and nutritional contents.

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