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The Potential of Indonesian Microalgal Strains to Support Eastern White Shrimp (*Litopenaeus vannamei*) Aquaculture

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**THE POTENTIAL OF INDONESIAN MICROALGAL STRAINS TO
SUPPORT EASTERN WHITE SHRIMP (*Litopenaeus vannamei*)
AQUACULTURE**

BY

WA IBA

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOLOGY AND ENVIRONMENTAL SCIENCES**

UNIVERSITY OF RHODE ISLAND

2016

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

Indonesia is well known for abundant aquatic resources, both marine and freshwater, including fishes, zooplankton and phytoplanktonic microalgae. However, relatively little information is available about microalgal resources despite their potential to be used as live feed in the hatchery phase of aquaculture of a number of marine species. The use of local microalgae is desirable in local hatcheries because they tend to grow better with high yield under local conditions, thus reducing the risk of culture crash and production cost while preventing disease vectors that may be introduced by foreign microalgae strains. In this dissertation, the potential of Indonesian microalgae to be used in shrimp hatcheries is investigated by isolating and growing the microalgae from Kendari Bay and Wanggu River estuary in South East (SE)-Sulawesi, Indonesia, under controlled conditions, assessing the growth of shrimp larvae using the local isolates as food and measuring the nutritional content of these local microalgal isolates.

Four strains of microalgae were successfully isolated using flow cytometry and the micro-pipet isolation technique. Those strains are denoted as Kb1-2 identified as *Chaetoceros* sp, Kb1-3, Kb1-5 and Kb2-6 identified as *Melosira* cf *moniliformis*. *Melosira* cf *moniliformis* was excluded from the microalgal growth experiment due to its larger cell size which were $>10\text{ }\mu\text{m}$. The growth for 15 days and cell size of those strains was investigated under four different salinities, 20, 25, 30 and 35 psu and growth rates were compared to the growth of T-ISO (*Tisochrysis lutea*), *Chaetoceros neogracile* and *Tetraselmis chui*. Salinity did not affect the growth of Indonesian microalgae, similar to *T. chui*, *Ti. lutea* and *C. neogracile*, but did affect microalgal final

density. Two Indonesian microalgal strains, Kb1-3 and Kb1-5, had the highest yield, among all microalgae tested, whereas, yield of Kb1-2 was similar to *Ti. lutea* and *C. neogracile*. Cell sizes of three of the four Indonesian microalgae ranged from 1.2-11.8 μm , considered a suitable size for shrimp larvae which is $<20 \mu\text{m}$. The Indonesian strains may be potential for mass culture and to be used in shrimp hatcheries based upon the cell sizes, their ability to survive long culture periods and tolerate a wide salinity range.

The subsequent experiment to assess the suitability of the Indonesian microalgae strains as food for hatchery-reared white shrimp (*Litopenaeus vannamei*) larvae confirmed that Indonesian microalgae may be suitable as food for white shrimp larvae, supporting performance similar to that of well-established strains in terms of weight gain, ingestion rate and food conversion index. However, lower survival was observed in shrimp larvae fed on Indonesian strains compared to *T. lutea* and *C. neogracile*. Analyses of the nutritional content of Indonesian microalgae revealed that lipid, protein and carbohydrate contents of these strains in late log and stationary phase of growth and were comparable to *T. lutea* during the stationary phase of growth. Of Indonesian microalgae, Kb1-2 contained important ω -3, eicosapentaenoic acid and docosahexaenoic acid (EPA and DHA) and ω -6, arachidonic acid (ARA) when cultured at 25 and 35 psu. Kb1-3 strain contained ARA, EPA and DHA only when cultured at 35 psu whereas Kb1-5 only when cultured at 25 psu. Indonesian microalgae, Kb1-3, had a higher ARA concentration compared to other Indonesian strains and *T. lutea*. DHA in Indonesian microalgae was similar to *T. lutea* but *T. lutea* had a higher concentration of EPA. This suggests that the use of Kb1-2 and Kb1-5 strains in white

shrimp hatcheries may be best when cultured at 25 psu and Kb1-3 at 35 psu to supply necessary PUFA at optimal cells density. Other option is mixing these strains with other species, such as *T. lutea*, that contain higher levels of essential EPA.

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Life is a journey and everything that happens is for a reason as part of God's grand plan. The experiences that are a part of that journey serve as a path to a better understanding of life itself including the people we meet and in turn, serve us to help one another. My journey as a PhD scholar for almost four years at University of Rhode Island (URI) has been a journey full of people that offered me helping hands in one way or another and I am indebted to them for the rest of my life and this acknowledgment maybe is not enough to express my gratitude for their support and advice throughout that journey.

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PREFACE

This dissertation is presented in manuscript format, and is divided into five chapters. Chapter One is a literature review, serving as an introduction and providing context for this work and how it fits into our broader understanding of the importance of microalgae in aquaculture hatchery practices and the possibility to explore other geographical locations for potential microalgae strains that may be used for aquaculture. This manuscript has been published in Asian Fisheries Science. Chapter Two focuses on the growth characteristics of newly isolated microalgae from South East Sulawesi, Indonesia and compares them to other strains used extensively in aquaculture. This manuscript is in preparation for submission to Journal of Applied Phycology. Chapter Three addresses the nutritional composition of the newly isolated microalgae strains from SE- Sulawesi in comparison with *Tisochrysis lutea*, a widely studied and used strain for aquaculture. This manuscript is in preparation for submission to Aquaculture Research. Chapter Four explores the growth and survival of Eastern Pacific white shrimp (*Litopenaeus vannamei*) fed on the newly isolated microalgae strains from SE- Sulawesi. This manuscript is in preparation for submission to Journal of The World Aquaculture Society. Chapter Five is a speculative discussion on the dissertation as a whole, reflecting on the outcome of all four chapters. The appendix following these chapters includes additional data upon which figures and tables throughout the dissertation are based.

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CHAPTER 1

Microalgae in Eastern Pacific White Shrimp, *Litopenaeus vannamei* (Boone 1931)

hatcheries: A Review on Roles and Culture Environments

By

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Abstract

Demand for shrimp, particularly the eastern Pacific white shrimp *Litopenaeus vannamei* (Boone 1931), will continue to increase in Asian and worldwide seafood markets. Providing shrimp farms with a robust, healthy, and continuous supply of shrimp seed is a challenge that must be addressed to meet the demand. Shrimp feed during hatchery production still relies on live microalgae, despite many years of effort to find suitable full- or partial-replacement diet alternatives. Successful mass production of microalgae for hatchery feed to obtain good quality shrimp seedstock depends on a number of environmental factors that determine the growth and nutritional values of various microalgal species. These factors include nutrients in the culture medium, light intensity, temperature, salinity, and pH. An overview of the use and the culture of microalgae in shrimp hatcheries is also presented and outlines the need for research for optimisation of algal diets for the rearing of *L. vannamei* seedstock in Asian hatcheries. Finally, the possibilities of using local isolates for hatchery operation are also highlighted.

Introduction

Despite their critical functions in the aquaculture production chain, microalgae are not documented exhaustively compared to many other cultured organisms. FAO (2012) reported that only small quantities of microalgae are cultured for direct human consumption; this production consists of two species, *Spirulina* sp. and *Haematococcus pluvalis* J. Von Flotow 1844. These two species are cultured mainly for extraction of C-phycocyanin and astaxanthin, respectively. Borowitzka (2013) reviewed various high-value products derived from microalgae, from β -carotene and fatty acids to phycobilins and other unrevealed bioactive compounds, highlighting the potential importance of microalgal research in the future. In aquaculture, microalgal production is mainly conducted to provide live food for mollusc, shrimp, and fish culture. As the production of microalgae still is considered expensive (e.g. Walsh et al. 1987), several approaches have been attempted to replace microalgae in the hatchery with artificial diets. To date, however, microalgae still cannot be replaced fully with other types of feed, such as microbound diets and other enrichment diets such as Nutrokol, S. presso and Algamac (Gallardo et al. 2002; Sanchez et al. 2012; Ma and Qin 2012).

Research on microalgal culture since the early 1940's has led to wide use for feeding early life stages of cultured fish, shrimp, sea urchins (Conceicao et al. 2010; Carboni et al. 2012), and during the entire life cycle of cultured bivalves (Wikfors and Ohno 2001; Gonzalez-Araya et al. 2011). Microalgae are also used for growing and enriching zooplankton live feeds, such as *Artemia*, copepods, and rotifers (Chakraborty et al. 2010) that are fed subsequently to larval fish and shrimp. Microalgal use in the fish hatchery is known as the “green water technique” wherein microalgae are added

directly to larval rearing tanks along with the zooplankton that serve as food for the larvae. Green-water larviculture has been shown to increase larval quality of cultured fish species (Conceicao et al. 2010; Hemaiswarya 2011; Sanchez et al. 2012). Moreover, research on nutritional requirements of molluscs has led to improved algal culture procedures, particularly with the finding that f/2 culture medium is suitable for various species of microalgae, both in temperate and tropical regions (Wikfors and Ohno 2001). Work showing that cost reductions can be made in mass microalgal cultures by replacing f/2 medium with less expensive agricultural fertilisers (e.g. Valenzuela-Espinoza et al. 1999; Simental-Trinidad et al. 2001) has expanded nutrient options for microalgal mass culture.

Studies on the biochemical composition of microalgae have enabled aquaculturists to select superior species of microalgae, such as *Isochrysis* spp., (some now assigned to the new genus *Tisochrysis* (Bendif et al. 2013), *Pavlova lutheri* (assigned to new name *Diacronema lutheri* (Droop) Bendif and Véron 2011), *Navicula* spp., and *Chaetoceros calcitrans* (Paulsen) Takano, 1968, as food for various cultured species, including oysters, scallops, fish, and shrimp larvae (Blackburn et al. 2000; Burke 2000). Research has shown that microalgae with high contents of metabolisable sterols and essential fatty acids are desirable for aquaculture live feeds, particularly for rapid growth of oysters (Wikfors et al. 1984, 1991, 1996). Furthermore, dietary cholesterol is an essential nutrient for crustaceans as they cannot synthesise this sterol *de novo*, thus it or a precursor phytosterol must be supplied in the diet (Teshima 1982). Required sterols may be available naturally in microalgae, as shrimp juveniles cultured

with phytoplankton showed better growth and survival compared to shrimp grown in a non-phytoplankton culture method (Sanchez et al. 2012).

Shrimp aquaculture has increased rapidly within the last decades, and cultured shrimp continue to dominate international seafood markets. Aquaculture contributed 52% of the shrimp supply in the world market in 2009, reaching 3.8 million tonnes by 2010 (FAO 2012). During 2005-2009, world shrimp aquaculture production increased up to 7% annually, but decreased 3% from 2010 to 2011, and then increased again in 2012 (FAO, 2012). It was projected that shrimp aquaculture will have increased up to 10.3% in 2013 (Valderrama and Anderson 2012).

The eastern Pacific white shrimp, *Litopenaeus vannamei* (Boone 1931) is an increasingly popular and important cultured shrimp species after the decline in giant black tiger prawn (*Penaeus monodon* Fabricius 1798) production caused by white spot syndrome disease outbreaks world-wide, most devastatingly in Southeast Asia. As a result, the white shrimp has been introduced to most Southeast Asian countries in the past few years. *Litopenaeus vannamei* has been cultivated widely in Asia, North America, South America and the Pacific Islands because of high survival, rapid growth in intensive culture systems, and disease tolerance (Briggs et al. 2004). The proportional share of *L. vannamei* in global shrimp production continues to increase. *Litopenaeus vannamei* contributed 7% to global production in 2001, with a net volume of 0.2 million tonnes, but in 2009, this contribution had increased to 58%, with a volume of 1.6 million tonnes. The peak production of white shrimp was in 2007 when this species accounted for 68% of global production. It was predicted that in 2013, this

species will have contributed up to 69% of global shrimp aquaculture production (Valderrama and Anderson 2012).

As the demand for shrimp products from both local and international markets continues to increase, supply of shrimp larvae and post larvae to farmers is necessary to meet the growing demand. Hatchery seed stock, therefore, is a critical step in the production chain that needs to be addressed, in terms of technology and husbandry protocols. A survey conducted by the Global Aquaculture Alliance in 2011 revealed that seed stock quality and availability was still the main issue limiting production of high-quality shrimp in Asia (Valderrama and Anderson 2012). Accordingly, a research programme to explore the possibility of using local microalgae isolates that meet nutritional demands in shrimp hatcheries can contribute to ensuring that hatchery protocols are conducted appropriately to rear shrimp larvae and post larvae locally as robust, healthy, and cost-effective seed stock for commercial use. Local isolates of microalgae will reduce transportation cost and may help small-scale farmers to increase productivity and thus incomes. Local culture of microalgae will also ensure a continuous supply of live feeds to aquaculture hatcheries, particularly shrimp hatcheries. Finally, a small-scale and local installation of microalgae culture is a promising solution not only to overcome hatchery feed challenges, but will also provide employment that can drive economic growth.

In terms of sustainability, producing microalgae for aquaculture is considered to be more favourable compared to other fish feed production (Taelman et al. 2013). This study found that, based on life-cycle analysis of microalgae production for aquaculture, recycling of nutrients and energy savings were identified as important ways to increase

the sustainability of algae production. Up-scaling, reactor design improvements, enhancement of photosynthetic yield, and a good choice of location also contribute to a low resource and carbon footprint. Considering that microalgae still are an important part of shrimp hatcheries, this present review discusses the use of microalgae to feed early stages of white shrimp, as well as some environmental factors affecting the growth and nutritional content of microalgae necessary for shrimp growth and development. Therefore, the intent of this review is to provide an overview on the use of microalgae in shrimp hatcheries, particularly white shrimp and the possibilities of using local isolates for hatchery operations.

Shrimp nutrition: use of microalgae in early life stages of white shrimp

In natural environments, white shrimp larvae feed initially on phytoplankton and detritus, then on macrophytes, small molluscs, crustaceans, and zooplankton as they grow larger (Senanan et al. 2009). Microalgae are recognised widely to serve as nutritionally important live feeds for rearing shrimp in the hatchery. All nutritional components required for larval shrimp growth may be fulfilled with microalgae in the rearing tanks. Several research projects have shown that, in the culture environment, white shrimp larvae grew faster when co-cultured with phytoplankton (Gallardo et al. 2002; Sanchez et al. 2012; Ju et al. 2009; Nunes et al. 2011), underscoring the importance of microalgae in shrimp nutrition during hatchery rearing.

Approximately 16 genera of microalgae are used in aquaculture; strains used generally have a size range of 2-20 μm with most of the genera being planktonic (Gopakumar and Ignatius 2006). Several genera that are used commonly in shrimp aquaculture are summarised in Table 1. The listed genera of microalgae have been

widely used because of good nutritional profiles and ease of culture (Hemaiswarya 2011). Diatoms, particularly *Thalassiosira* spp. and *Amphipora* spp., were found to be ingested and digested readily by white shrimp juveniles (Kent et al. 2011). A cyanobacterial species, *Spirulina platensis* (Gomont) Geitler 1925: 344 (currently accepted name is *Arthrospira platensis* (Nordstedt) Gomont 1892), also was shown to play an important role in reducing nitrogen levels in black tiger shrimp rearing tanks (Chuntapa et al. 2003).

Research on other potential microalgal isolates for use in white shrimp aquaculture hatcheries will continue into the future as we explore local natural resources. For instance, local microalgae isolates from southeast (SE)-Sulawesi, Indonesia are not utilised yet, despite their potential to support aquaculture industries in the region. These local microalgae may have comparable growth rates and nutritional values to the imported counterparts isolated elsewhere in the world. Currently, shrimp aquaculture in SE-Sulawesi relies upon microalgae starters supplied from the south Sulawesi and Java regions (Fig.1) that impose high operational costs to aquaculture farms and hatcheries in the region. The price of microalgae starter culture obtained from Java and south Sulawesi such as *Spirulina* sp., *Chorella*, and *Dunaliella*, is in the range of US\$10-30·L⁻¹. That price can be reduced up to 50% if the starter culture were to be provided within SE-Sulawesi region. Importing microalgae from other regions also increases greenhouse gas (GHG) emissions associated with air and ground transportation (Edwards-Jones 2010) and exposes receiving areas to risks from non-native species invasions, including both the algae themselves and any contaminating microorganisms. To reduce both cost and non-native introduction issues, the use of

locally-isolated, cultured microalgae in local hatcheries may be considered. Locally-isolated microalgae with good growth and complete nutritional profiles will help small farmers to increase the productivity of their farms and thus their incomes, which is beneficial to drive economic growth.

We have isolated four strains of local microalgae from Kendari Bay, SE-Sulawesi. Those strains are noted as Kb1-2, Kb1-3, Kb1-5 and Kb2-6 identified as *Melosira moniliformis* (O.F.Müller) C.Agardh 1824. Growth experiments on these strains have been conducted in the Coastal Institute, University of Rhode Island. We found that the growth and biomass of these isolates, particularly the Kb1-3 strain, is comparable to *Chaetoceros neogracile* S.L. Vanlandingham 1968 and *Thalassiosira weissflogii* (Grunow) G. Fryxell & Hasle 1977 at high salinity (35 psu). We are now examining the nutritional content of these strains and identifying the strain using scanning electron microscopy before conducting feeding trials with white shrimp post larvae. These preliminary results suggested that local isolates may have potential for use in shrimp hatcheries, assuming the nutritional compositions of the algae are appropriate. Several studies have been conducted to analyse the nutritional composition of locally isolated microalgae that may be used for tropical aquaculture, e.g., Renaud et al. (1999), Nunez et al. (2002) and Martinez-Fernandez et al. (2006). Renaud et al. (1999) screened 18 strains of local microalgae from tropical waters of Australia and suggested that they may be used for mariculture. Similarly, a study by Nunez et al. (2002) tested the efficacy of employing local isolates of microalgae from north-eastern Venezuela as feed for white shrimp post larvae. They found that local isolates can be utilised by shrimp post larvae. Moreover, shrimp grew better and produced higher

biomass under local conditions at large scale production, thereby reducing the production cost.

As is true for other cultured animals, shrimp require sufficient levels of proteins, lipids, carbohydrates, minerals, and vitamins for normal growth and development. Nutritional requirements of shrimp larvae and juveniles were reviewed by Kanazawa (1982). Diatoms and brine shrimp have been used commonly for shrimp larval rearing in the hatchery with good results. Shrimp such as *Penaeus japonicus* Spence Bate 1888 need proteins and essential amino acids, with an optimum dietary protein level of 52-57% (Kanazawa 1982). Protein in microalgae is considered to be of good quality, with amino acid profiles comparable to those of other reference food proteins (Gallardo et al. 2002). This may be the reason why shrimp larvae exhibited poor growth when cultured using only a micro-bound diet without microalgae, although Pedroza-Islas et al. (2004) found that a microencapsulated diet with a wall composition of a polysaccharide blend could be used to rear white shrimp mysids. Protein contents of microalgae vary between species and range from 12% to 35%, and this value is influenced by culture medium (Becker 2004). In general, microalgae can be considered to be a good candidate for shrimp aquaculture if they contain a protein higher than 25% of dry weight, 8-30% carbohydrate, and approximately 10% lipid, specially including certain types of lipids such as the fatty acids C20:5 ω 3 and C22:6 ω 3 (Nunez et al. 2002). We summarised the nutritional requirement of shrimp and microalgal strains/species that produce these nutrients in Table 2 and 3.

Carbohydrates are utilised by shrimp larvae and juveniles in the form of disaccharides such as sucrose, maltose, and trehalose, and polysaccharides, such as

dextrin and starch (Kanazawa 1981). A study with *P. japonicus* juveniles by Kanazawa (1981) showed that maltose was much more readily utilised as an energy source than other sugars. Unlike dietary glucose, that is not converted to trehalose but quickly absorbed from the stomach and released to the blood, maltose is not absorbed from the stomach. Instead, this disaccharide is converted to glucose in the mid gut, then to trehalose in the hepatopancreas, and is finally released gradually into the blood. High variation is found in carbohydrate contents of microalgae, which ranged from 4.6% to 23% (Wikfors and Ohno 2001). This variation is determined by culture procedures employed (Ferreira et al. 2009). Carbohydrate content in *Nannochloropsis gaditana* L.M. Lubián 1982 decreased from 28.47% to 21.01% when this species was grown in semi-continuous culture with daily renewal rates of 10% or 50%, respectively.

Shrimp have been shown to have special needs in lipid metabolism. Fatty acids (FAs) are organic molecules typically found bound to other compounds such as glycerol, sugars, or phosphate head groups to form lipids. Lipids are integral components of cell structures, e.g. membranes, which are made up of phospholipids, and energy stores that often are composed of triglycerides. Through enzyme reactions, FAs can be released from lipids to become free fatty acids (FFAs). The biological activities of FFAs have roles in host defence against potential pathogenic or opportunistic microorganisms, e.g., growth inhibition or the direct killing of bacteria (Desbois and Smith 2010). Shrimp fed on mixtures of formulated and natural feed may have better immunological and nutritional condition when compared to those fed on formulated feed alone because of the greater amount of essential amino acids, fatty acids

(Poly Unsaturated Fatty Acids/PUFA and Highly Unsaturated Fatty Acids/HUFA), and other nutritional factors supplied by the live feed component.

Penaeid larvae have an absolute requirement for long-chain, polyunsaturated fatty acids, especially for C20 and C22: ω -3 and ω -6 compounds. In particular, shrimp growth seems to be promoted by docosahexaenoic acid (DHA) 22:6 ω -3 and enhanced further by eicosapentaenoic acid (EPA) 20:5 ω -3 (Castell 1982). Growth rates of protozoal stages of *Penaeus semisulcatus* De Haan 1844, *P. monodon*, and *P. japonicus* can be enhanced by dietary supplementation with 20:4 ω -6 arachidonic acid (ARA) (D'Souza and Loneragan, 1999). Deficiencies in the ω -3 HUFA contents of microalgae may cause mortalities and/or quality problems in shrimp. Measurable concentrations of EPA generally are present in diatom species (*C. calcitrans*, *C. neogracile*, *Skeletonema costatum* (Greville) Cleve 1873 and *Thalassiosira pseudonana* Hasle & Heimdal 1970), as well as in *Nannochloropsis* sp., *Tetraselmis suecica* (Kyllin) Butcher 1959, *Tetraselmis chui* Butcher 1959, and “*Chlorella minutissima* Fott & Nováková 1969” (in reality, a eustigmatophyte) (Brown 1997; Becker 2004).

Cholesterol is an essential nutrient that must be supplied in the diet of shrimp, directly or as a precursor, because crustaceans cannot synthesise this compound *de novo* (Teshima 1981). Cholesterol is the main sterol found in crustaceans and is required for normal development, survival, and growth. Cholesterol also is a precursor for sex hormones, molting hormones, and epidermis constituents in crustaceans (Lim 1998). Sterol content has been investigated by Gladu et al. (1991) for diatom species. These researchers found that five of seven diatom species (*T. pseudonana*, *T. weissflogii*, *Detonula confervacea* (Cleve) Gran 1900, *Cyclotella cryptica* Reimann, Lewin &

Guillard and *Nitzschia brevirostris* Hustedt examined in the study contained 24-methylenecholesterol and 24-methylcholesterol, which together made up 63-97% of the total sterols of these species. Moreover, stigmasterol was the principal sterol in *Amphora coffeaeformis* (C. Agardh) Kützinger 1844 (currently accepted name is *Halamphora coffeaeformis* (Agardh) Levkov 2009), and 24-ethylcholesterol was the major sterol in *Navicula pelliculosa* Hilse 1863: 68 (currently accepted name is *Fistulifera pelliculosa* (Brébisson) Lange-Bertalot 1997). Although cholesterol is not the predominant phytosterol, some phytosterols can partially substitute for cholesterol in the shrimp diet (Teshima 1981; Lim 1998).

Crustaceans, such as shrimp, absorb minerals from water but also need minerals and vitamins included in their diets. Kanazawa (1981) stated that mineral dietary supplement in shrimp is required as these compounds are lost in molted exoskeletons. Vitamin content is a major factor determining the nutritional value of microalgae. The microalgae showing relatively high concentrations of different vitamins were: *T. suecica* for thiamin, pyridoxin, nicotinic acid, pantothenic acid and ascorbic acid and *Chlorella stigmatophora* Butcher for vitamin A, tocopherol (vitamin E) and biotin (Fabregas and Herrero 1990). Some strains of *Dunaliella* such as *Dunaliella tertiolecta* Butcher, 1959 and *Dunaliella salina* (Dunal) Teodoresco, 1905 have been known to be a good source of β -carotene, riboflavin, cobalamin and folic acid and thus been exploited since 1980s for human consumption and aquaculture (Fabregas and Herrero 1990; Raja et al. 2007; Abu Reza et al. 2010; Borowitzka 2013). *Tisochrysis iso* (revised to *Tisochrysis lutea* El M. Bendif and I. Probert as in Bendif et al. 2013) and *Isochrysis* spp., also is a good source of fat-soluble vitamins (vitamin A and vitamin E,

biotin, ascorbic acid, and B-group vitamins (folic acid, nicotinic acid, pantothenic acid, biotin, thiamin, riboflavin, pyridoxine and cobalamin) (Coutinho et al. 2006).

Microalgal isolation and production techniques

Isolation and culture systems

Although several isolated microalgae species are readily available for aquaculture from research centres or large hatcheries, locally-isolated species may be preferred for local use to reduce transportation cost and assure constant availability. Successful isolation of specific microalgae from the local environment is determined by some important factors, including: environmental conditions (water quality of natural samples are thought to be controlled by habitat conditions and sampling time), taxonomic knowledge (to determine nutrients for culture of isolated species) and elimination of contaminants. Andersen and Kawachi (2011) suggested using caution and multiple methods to isolate unknown species. Microalgal isolation methods commonly used are: enrichment culture, single cell isolation by micropipette, agar isolation, dilution, gravity separation, and phototaxis techniques.

Enrichment culture is the most common culture and isolation method for microalgae; microalgae are induced to “bloom” by fertilisation (addition of nutrients to the water sample). Adding nutrients to the natural water samples is important to overcome possible nutrient deficiencies. Enrichment with nutrients is necessary, as the natural samples often are deficient in one or more nutrients. Microalgae survive in nature because of recycling processes making nutrients continually available in small quantities. Nutrients required for microalgal growth include the macronutrients nitrate or ammonia and phosphate (in an approximate ratio of 16:1), silicate (if growing

diatoms), and micronutrients, including various trace metals and the vitamins thiamin (B₁), cyanocobalamin (B₁₂) and sometimes biotin. These nutrients usually are mixed together in specific proportions to create microalgal culture medium (including formulations sold commercially). Walne medium and f/2 medium are the two most extensively used enrichment media and are suitable for the growth of most algae. There are also commercially-available nutrient solutions that are suitable for mass production of microalgae in large-scale, extensive systems. These solutions contain only the most important nutrients and are made of agriculture-grade fertilisers rather than laboratory-grade reagents. These culture media, sometimes together with aqueous soil extract, are the most common enrichment substances for growing microalgae (Andersen and Kawachi, 2011). Generally the amount of nutrients to be added to seawater must be moderate to avoid detrimental effects.

Hemaiswarya (2011) summarised some factors to be considered for production of microalgae, including: the biology of the alga; cost of land, labour, energy, water, and nutrients (climate if the culture is outdoors); and the type of final product needed. Microalgal production remains the main bottleneck to assuring the continuous supply of live feed to shrimp hatcheries. According to Enzing et al. (2014), several factors need to be addressed within 4 to 5 years to achieve the stability of microalgal feed production: 1) lowering the production costs of feed, 2) improvement of production technologies for the safety of food products, 3) stability/reliability of large cultures, 4) suitable strains, and 5) avoiding contamination and subsequent failure of the cultures. Using local isolates for shrimp aquaculture, as suggested earlier in this review, could become a solution to reduce the production cost and prevent contamination of foreign strains,

bacteria, and parasites. Furthermore, local microalgae tend to grow better under local environment conditions, thus reducing the risk of culture crash and would be easier to maintain in small scale hatcheries as suggested by Cotteau (1996) and Nuñez (2002).

Culture of microalgae for aquaculture purposes (rearing of molluscs, shrimp, and fish larvae) takes place mostly on-site, i.e., in the farms where they are utilised, although a new industry is emerging for the production of microalgae and delivery in lyophilised, frozen, or other forms to the farms (Navarro and Sarasquete 1998; Muller-Fuega 2004). Once in farms, stock cultures are kept under controlled conditions and protected from contamination by other microalgae, ciliates, and potentially harmful bacteria. Bags and cylindrical tanks are quite common approaches for the mass-production of microalgae on farms. In many parts of the world, however, particularly in Southeast Asia, facilities at the farm level are lacking and insufficient. Purchasing healthy seed from a local hatchery that utilises local microalgae would be one solution to reduce the production cost of farmers in developing countries.

Various techniques have been developed to grow microalgae on a large scale, ranging from less-controlled and extensive to mono-specific, intensive cultures. The controlled production of microalgae, however, still is a complex and expensive procedure. Semi-continuous and continuous cultures are commonly used in microalgal production in the hatchery. Semi-continuous culture, in which a percentage of the volume of the culture is harvested periodically and replaced with the same amount of fresh culture medium, is considerably easier and cheaper to establish. Semi-continuous culture management also produces microalgal biomass with high nutritional values (Ferreira et al. 2011).

Based on the intensity of the culture and average cell density from low to high, microalgae culture technology is divided into several types of containers: earth ponds, raceways, plastic polyethylene bags (100-500 L), open cylindrical tanks constructed of polymer fibre glass, and tubular and flat-plate photo-bioreactors. Earth ponds and raceways are more open systems, inexpensive, exposed to weather conditions, and more prone to contamination. In addition, the cell density remains at relatively low levels in comparison with the other culture systems. On the other hand, closed systems are much more expensive to operate, although they can produce high yields of microalgal biomass. Taelman et al. (2013) suggested that the closed photobioreactor for aquaculture purposes will be more profitable and produce higher biomass of microalgae if the design is improved, such as those of Provi APT (Proviron Advanced Photobioreactor Technology) (Fig. 2).

This system basically contains a plastic bag (12 m²) filled with water and placed on the ground. Each bag contains 35 embedded plastic panels in which the algae grow, yielding a reactive surface of 7 m². The Provi APT system is inexpensive to build as it consists entirely of plastic, and automated production can be employed. This design of photobioreactor has been used in trials to culture *Nannochloropsis* sp., which produced approximately 17 tonnes of dried algal biomass per hectare annually (Taelman et al. 2013). This kind of system can be developed on a smaller scale at hatchery level to protect the culture from contamination, to maintain the nutritional value, and also to ensure continuous supply.

Culture conditions and nutritional values

Culturing microalgae under optimal conditions is advantageous to achieve high nutritional values that are beneficial for shrimp larvae. Among other things, light intensity, day length, and nutrient concentrations are important factors regulating microalgae growth in culture and also affect the nutritional content (Renaud et al. 1991; Lewis et al. 2002; Meseck et al. 2005; Sheng et al. 2011; Huang et al. 2013). Several researchers also have shown that salinity, pH, and temperature are important factors determining the growth of microalgae and controlling the nutritional quality (Goldman et al. 1982; Schmidt and Hansen 2001; Lewis et al. 2002; Renaud et al. 2002; Khatoon et al. 2010; Gu et al. 2012). Table 4 summarises the optimal culture condition for microalgae species commonly used in shrimp aquaculture.

Microalgae most fundamentally need light (intensity and period) for photosynthesis. They need light for a photochemical phase of the photosynthetic process to produce Adenosine triphosphate (ATP), Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and use dark periods for biochemical phases synthesising essential molecules for growth (Cheirsilp and Torpee 2012). In the natural environment, microalgae utilise readily available sunlight easily. In controlled, indoor culture conditions, it is difficult to rely upon natural sunlight alone because of seasonal variability. The most common tool to provide illumination in indoor conditions is the fluorescent lamp. Optimal photoperiod to grow microalgae depends upon the place and season. The 12:12 h (light: dark) period may be appropriate to employ in the tropics, but in temperate regions, a 16:8 h photoperiod may result in better microalgal growth. A study by Harrison et al. (1990) found that light intensity at $300 \mu\text{mol photon m}^{-2}\text{s}^{-1}$

PAR was optimal for growth of *Isochrysis galbana* Parke 1949, *T. pseudonana* and *C. calcitrans*. Moreover, the study found that no consistent trend was observed in lipid content of those three microalgae under light limitation (less than 300 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR), and protein content was relatively constant for *I. galbana* and *T. pseudonana*, but decreased in *C. calcitrans* with lower illumination. However, a study by Renaud et al. (1991) found high light intensity in culturing *Isochrysis* sp and *Nannochloropsis oculata* (Droop) D.J.Hibberd 1981 decreased the ratio of total unsaturated fatty acids to total saturated fatty acids. A recent study by Khoeyi et al. (2012) noted that the maximum biomass of *Chlorella vulgaris* Beyerinck (Beijerinck) 1890 was recorded between 0.1 g and 2.05 g·L⁻¹ when the culture was exposed to 62.5 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR for a 16:8 h light/dark photoperiod; whereas, the maximum percentage of total saturated fatty acids (SFA) was 33.38% at 100 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR on a 16:8 h light/dark photoperiod. Other microalgae that are not popular for aquaculture, such as *Lobosphaera incisa* (Reisigl) Karsten, Friedl, Schumannn, Hoyer & Lembcke 2005, showed that at high light intensity (400 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR) and complete nutrient culture medium, the cells exhibited higher growth rates and high contents of total fatty acid and arachinoid acid compared to medium (200 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR) and low (35 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR) light intensity (Solovchenko et al. 2008). For long-term culturing of most microalgal species, light intensity at 10-30 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ PAR and low temperature (15°C) have proven to be reliable to keep cultures alive with minimal subculturing (Lorenz et al. 2011).

Nutrient enrichment also controls the growth, protein, carbohydrate, lipid, and fatty acid composition of microalgae. Sheng et al. (2011) showed that, in a P-depleted

treatment (f/2-P), the cell size of *T. weissflogii* was 1.48 times larger than that in the full-nutrient-limited (f/100) treatment and 2.67 times larger than that in P-saturated treatments (f/2 and f/10). Silica is necessary for optimal growth of diatoms and directly affects nutritional contents (Kröger et al. 1999; Ponomorenko et al. 2004; Sumper and Bunner 2006; Schwenk et al. 2013). Although Harrison et al. (1990) found that carbohydrate, lipid, and protein contents of *I. galbana*, *T. pseudonana* and *C. calcitrans* were not changed under 6 h silica (Si) starvation, providing silica in the culture medium is necessary for rapid growth of any diatom (Sumper and Bunner 2006; Schwenk et al. 2013). Moreover, the study by Harrison et al. (1990) also found that, under 2 days of nitrogen (N) starvation, does not change the cellular lipid percentage, but the protein percentage decreased, and the carbohydrate percentage increased. Solovchenko et al. (2008) showed that *L. insica* cultured in nitrogen- depleted medium contained increased arachidonic acid as a percentage of total fatty acids. Moreover, Huang et al. (2013) showed that the total lipid content, as well as the proportions of neutral lipid, in *Tetraselmis subcordiformis* (Wille) Butcher 1959 SHOU-S05, *Nannochloropsis oculata* (Droop) D.J.Hibberd 1981 SHOU-S14 and *Pavlova viridis* C.K. Tseng, J. Chen & X. Zhang 1991 (currently accepted name is *Diacronema viridis* (C.K.Tseng, Chen and Zhang) Bendif and Véron 2011) SHOU-S16, decreased significantly with increasing nitrogen supplementation. Eicosapentaenoic acid (EPA (20:5 ω 3)) and C16 fatty acids were significantly higher in *N. oculata* and *D. viridis*, respectively with increased nitrogen enrichment. This study also showed that *N.oculata* and *D. viridis* accumulated more 16-carbon fatty acids and fewer polyunsaturated fatty acids in nitrogen-limited media. Schwenk et al. (2013) revealed that lipid content of several

species of microalgae, including *I. galbana*, *Chaetoceros muelleri* Lemmermann 1898 and *T. pseudonana*, increased during early stationary phase as the nitrogen-limited environment shifted the energy supporting cell division to fatty acid biosynthesis and lipid accumulation. These findings imply that knowing growth phase (stationary or log phase) when harvesting microalgae is necessary to select the desired nutritional content of log-phase cells or stationary-phase cells that are either nutrient or light limited.

Salinity is another primary factor influencing the growth and biochemical composition of microalgae. For the purpose of this review, the standard unit for salinity is set to psu (Practical Salinity Unit), although literature has reported salinity in different units such as g.L^{-1} and mM. The gross chemical and fatty acid compositions of *Isochrysis* sp., *N. oculata*, and *Nitzschia* sp. were significantly different at different salinities (Renaud et al. 1992). Khatoon et al. (2010) reported experiments with marine diatoms (*Amphora* sp., *Navicula* sp. and *Cymbella* sp.) and a cyanobacterium (*Oscillatoria* sp.) at different salinities. Diatom growth was significantly higher at 35 psu than at lower salinity, but cyanobacterial growth was better at 25 psu. As for protein and lipid, significantly higher quantities were found in diatoms cultured at low salinities (15-25 psu), and an increase in carbohydrate was seen at high salinities (30-35 psu). Conversely, the cyanobacterium showed a significantly higher lipid content at 30-35 psu compared with other salinity levels, but no significant changes were observed in the protein and carbohydrate contents at different salinity levels. de Castro Araujo and Tavano Garcia (2005) showed that, at high salinity (35 psu), carbohydrates were enhanced, but lipids and protein decreased in *Chaetoceros* cf. *wighamii* Brightwell

1856 . Rao et al. (2007) found that growth of *Botryococcus braunii* Kützting 1849: 892 (race 'A') and its biochemical composition, i.e. hydrocarbon, carbohydrate, fatty acid, and carotenoids, were influenced by salinity. Under salinity of 34 mM (2 psu) and 85 mM (5 psu), a 1.7–2.25-fold increase in the relative proportion of palmitic acid, and a two-fold increase in oleic acid, were observed. A two-fold increase in carotenoid content was detected at 85 mM (5 psu) salinity with lutein (75% of total carotenoid) as the major carotenoid followed by β -carotene.

Another important factor affecting the growth and biochemical composition of microalgae is temperature. Temperature strongly influences cellular chemical composition, the uptake of nutrients, carbon dioxide fixation, and the growth rates for every species of algae. It is known that the growth rate will increase with temperature up to a maximal level. Above this temperature, growth rate decreases drastically (Raven and Geiden 1988). Temperature tolerance in microalgae is species-specific. Renaud et al. (2002) reported that the optimum temperature for growth of *Rhodomonas* sp. was 25–27 °C, but was 27–30 °C for a prymnesiophyte NT19. Furthermore, *Cryptomonas* sp., *Chaetoceros* sp. and *Isochrysis* sp. grew well at 33 and 35 °C. All species had significantly lower percentages of protein when grown at temperatures above 27 °C, but there was no consistent trend in the percentages of carbohydrate. *Chaetoceros* sp. had the highest percentage of lipid when cells were cultured at 25 °C, but *Rhodomonas* sp., *Cryptomonas* sp., NT19, and *Isochrysis* sp., had significantly higher amounts of lipid at temperatures within the range 27–30 °C. The highly unsaturated fatty acid (HUFA), eicosapentaenoic acid, 20:5 ω 3, was present in all species, with highest amounts in the prymnesiophyte NT19 (19.9% total fatty acids).

Percentages of 20:5 ω 3 were slightly lower at highest growth temperatures for all species. All species had lower percentages of 22:6 ω 3 at higher growth temperatures. *Chaetoceros* sp. and NT19 had moderate amounts of arachidonic acid, 20:4 ω 6 in the fatty acid profile (2.7-5.4% of total fatty acids). Highest arachidonic acid percentages were associated with growth temperatures within the range 27-30 °C. Only *Chaetoceros* sp. grew well at 35 °C, maintaining moderate percentages of protein, carbohydrate, lipid, PUFA, and HUFA (9.6% total fatty acids).

The optimum temperature for *C. vulgaris* ranges from 25 to 30 °C. (Sanchez et al. 2008). Several researchers have shown that biomass content, lipid, and fatty acid decreased when the culture conditions were changed from the optimal temperature range. Chinnasamy et al. (2009) reported an increase in biomass content and in chlorophyll content of *Chlorella* sp. at the optimum temperature (30 °C). Converti et al. (2009) reported that lipid content of this species increased from 5.9 to 14.7% when the temperature decreased from 30 °C to 25 °C; at temperatures above 38 °C, oleic acid, a monounsaturated omega-9 fatty acid, production increased.

The optimum temperature to grow *Scenedesmus* sp. is between 20-40 °C (Sanchez et al. 2008). Previously, Christov et al. (2001) studied *Scenedesmus* sp. at temperatures of 15-36 °C and found that, at lower temperatures the chlorophyll and protein levels were reduced, but levels of carotenoids, saccharides, and lipid were increased. These authors also observed an increase of 30% in sugars and lipids at extremely high temperature (36 °C).

Conclusions

As the demand for white shrimp products in world markets continues to increase, the continuous supply of healthy, inexpensive, and robust shrimp seed stocks to the farmers is important to maintain production of adult shrimp. Microalgae remain an important component of the aquaculture production chain, particularly for hatchery white shrimp feed, despite expensive culture installation and high production cost. Several studies and experiments have been conducted to replace the use of microalgae in white shrimp hatcheries, but to date research has showed that microalgae cannot be replaced completely. Partial substitution with other, low-price enrichment compounds and micro-bound diets is possible, but microalgae must be provided in the shrimp rearing tanks. Semi-continuous culture of microalgae, in which a portion of the biomass is harvested periodically, and nutrient medium is replaced at a constant level, is still the most-efficient procedure to be employed in shrimp hatcheries. Inexpensive outdoor culture installations may also be sufficient to supply microalgae to the hatcheries. In addition, smaller scale, inexpensive design photobioreactors may be installed at the hatchery level to minimize culture contamination, to maintain nutritional profiles, and to ensure continuous supply.

Adequate nutrients, light (intensity and period), salinity, and temperature are important factors in determining maximal growth and high nutritional value of microalgae. Readily available f/2 and Walne medium are the most common culture enrichments with sufficient nutrients to support growth of most algal species. The photoperiod of 12:12 h (light:dark) is suitable for culturing microalgae in the tropics, but in temperate areas a photoperiod of 16:8 generally is more favourable for growth

and nutritional content of microalgae. Salinity and temperature optimal levels for culturing microalgae are species specific. Knowledge about log and stationary phase during the culture period is important to ensure that microalgae are harvested when they have the desired nutritional contents.

Although there are approximately 5 genera of microalgae known to serve as good quality feed sources in white shrimp hatcheries, more research on other potential microalgal strains is recommended. This is mainly driven by the concern over sustainability and productivity of local farms. Using local microalgal isolates for white shrimp production will reduce the production cost to farmers, reduce the potential negative impact of shrimp farming on the environment, and increase the dependability and productivity of the farms to the benefit of local economies.

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TABLES

Table 1. Genera of microalgae commonly used in shrimp hatchery (modified from Hemaiswarya et al. 2011)

Genus/species	Class	Utilisation
<i>Isochrysis</i> (<i>Tisochrysis</i>)	Prymnesiophyceae	Feeding of zooplankton such as <i>Artemia</i> , and used in some shrimp hatcheries
<i>Tetraselmis</i>	Prasinophyceae	Best for larval shrimp and contains natural amino acids that stimulate feeding in marine animals, complement <i>Nannochloropsis</i> for producing rotifers as well as for feeding <i>Artemia</i>
<i>Thalassiosira weissflogii</i>	Bacillariophyceae	Applied in the shrimp and shellfish larviculture, considered by several hatcheries to be the single best alga for larval shrimp, also used for feeding copepods and <i>Artemia</i>
<i>Dunaliella</i>	Chlorophyceae	Source of vitamin A and B12 in some shrimp hatcheries and also for shrimp coloration
<i>Chaetoceros</i>	Bacillariophyceae	Source of vitamin A and B1 in some shrimp hatcheries
<i>Amphipora</i> spp	Bacillariophyceae	Feeding the white shrimp juveniles
<i>Spirulina platensis</i>	Cyanobacteria	Reducing nitrogen levels in black tiger shrimp rearing tanks

Table 2. Nutrition requirements of shrimp larvae (modified from Millamena 1996).

Nutrition	Requirement (%)	References
Protein	50-57	Kanazawa 1981
Essential Amino acids		
Arg	4.5%	
Lys	5.3%	
Met + Cys	3.3% (Cys, 0.4)	
Th	3.5%	
Val	3.7%	
Lipid	12-15	
Essential Fatty acids	20:4n-6, 20:5n-3 22:6n-3 2.6% n-3 PUFA < 0.5% 18:2n-6	
Cholesterol	0.05-0.5 (white shrimp) 1 (tiger shrimp)	
Carbohydrate	20	
Carotenoids	Desirable for coloration	
Vitamin C	50 mg as ascorbic acid	
		Castille et al. 2004

Table 3. Known nutrient profiles of some microalgae strain.

Microalgae strains	Nutrition supplied	Level (%)	References
<i>Chaetoceros muelleri</i>	Protein	45.56 ^a	Martinez-Fernandez et al. 2006
	Lipid	12.14	
	Carbohydrate	3.74	
	Vitamin C	16	
<i>Chaetoceros</i> sp.	Lipid	17	Renaud et al. 1999
	Protein	36.7	
<i>Skeletonema costatum</i>	Lipid	27.9	
	Protein	13.5	
<i>Tetraselmis suecica</i>	Vitamin A	27.07 ^b	Fabregas and Herrero 1990
	Vitamin C	17.45	
<i>Pavlova salina</i>	Carbohydrate	5.23 ^a	Martinez-Fernandez et al. 2006
	Lipid	30.55	
	Protein	52.91	
<i>Chlorella stigmatophora</i>	Vitamin A	5.13 ^b	Fabregas and Herrero 1990
	Vitamin E	69.44	
	Biotin	0.11	
<i>Thalassiosira pseudonana</i>	Phytosterol	63-97 ^c	Gladu et al. 1991
<i>T. weissflogii</i>	(24-methylenecholesterol and 24-methyl cholesterol)		
<i>Nitzschia breirostris</i>	Carbohydrate	21.01-28.47 ^d	Ferreira et al. 2009
<i>Nannochloropsis gaditana</i>	Lipid	18.60-35.34	
	Protein	36.19-60.39	
	PUFA	12.71-40 ^e	
	EPA	6.76-27	
<i>Dunaliella salina</i>	ARA	3.56-6.90	Abu-Rezq et al. 2010
	β- carotene	65.66-89.77 ^f	
<i>Isochrysis</i> sp. (T.Iso)	Lipid	29.99 ^a	Martinez-Fernandez et al. 2006
	Carbohydrate	3.33	
	Protein	47.77	
<i>Isochrysis galbana</i>	Vitamin A	19.57 ^b	Fabregas and Herrero 1990
	Vitamin C	30.44	

a= percentage of dry weight, b= percentage of total vitamin, c=percentage of total sterols, d=percentage of total organic content (sum of carbohydrate, lipid and protein), e=percentage of total fatty acids, f=percentage of total carotenoids

Table 4. Optimum culture environment of some microalgae species commonly used in shrimp hatchery.

Genus/Species	Optimum Culture Condition						Nutrient Yield (%)	References
	Photoperiod (Light:Dark)	Light	pH	T	Salinity (psu)	Medium		
<i>Chlorella vulgaris</i>	16:8	62.5		25-30		f/2	33.38 SFA	Khoeyi et al. 2012
<i>Thalassiosira weissflogii</i>	14:10	~ 100		18	31±1	f/2 and f/10	β- carotene	Sheng et al. 2011
<i>Isochrysis galbana</i>		300	8.0-8.3	21±1.5		Enriched	16 DHA	Harrison et al. 1990
<i>Thalassiosira pseudonana</i>		300		18	6	seawater	22.62 L	
<i>Isochrysis</i> sp.		300		18	6	f/2	27.2L	
<i>Isochrysis galbana</i>		300		18	35	f/2	12.07 L	Schwenk et al. 2013
						f/2		
<i>Chaetoceros</i> sp.	12:12	80±2	8.3±0.1	27-30	25±1	f/2	16.8 L	Renaud et al. 2002
Marine diatoms (<i>Amphora</i> sp. and <i>Navicula</i> sp.)	12:12	31.9	8.5	28	15-25	Conway	40 P 30 L 20 C	Khatoon et al. 2010
<i>Tetraselmis subcordiformis</i>		100		20±1	20	f/2	29.77 L	Huang et al. 2013*
<i>Nannochloropsis oculata</i>		150		25±1	20	f/2	35.85 L	
<i>Pavlova viridis</i>		100		20±1	20	f/2	32.10 L	

SFA = saturated fatty acids, P= Protein, L= lipid and C= Carbohydrate T=temperature in °C, Light=light intensity in $\mu\text{mol photon m}^{-2}\text{s}^{-1}$. * = nutrient yield is lipid, protein, carbohydrate, DHA, SFA or b-carotene content when nitrogen supplementation at 0.22 mmol N.L⁻¹

FIGURE LEGENDS

Figure 1. Source of microalgae starter culture for hatchery operation in SE Sulawesi.

Image from the map collections of the U.S Central Intelligence Agency

(<https://www.cia.gov/library/publications/cia-maps-publications/Indonesia.html>).

Figure 2. Typical Provi APT system for efficient microalgae culture (Michiels 2009).

FIGURES

Figure 1

Figure 2

CHAPTER 2

Growth characteristics of newly isolated Indonesian microalgae proposed as feed for shrimp hatchery

By

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Abstract

Microalgae were isolated from Kendari Bay and the Wanggu River estuary, Indonesia in summer 2013. Isolates denoted as Kb1-2, Kb1-3, Kb1-5, and Kb2-6 were established to evaluate possible use in Indonesian shrimp hatcheries. A batch culture experiment was conducted for 15 days under salinity levels of 20, 25, 30 and 35 practical salinity unit (psu) to investigate growth characteristics of the strains. *Tetraselmis chui*, *Tisochrysis lutea* and *Chaetoceros neogracile* were used to compare growth with Indonesian isolates. Cell numbers were measured every other day and cell size was measured from 50 live cells during the logarithmic phase. Cell sizes of three of the four Indonesian microalgae ranged from 1.2-11.8 μm , considered a suitable size for shrimp larvae. Indonesian strains started the logarithmic phase of growth at all salinities tested from 0 to 3 days after inoculation except Kb1-3 that started after a 3 day lag. Increasing cell density over culture period and division rate of Indonesian microalgae during logarithmic phase of growth were similar at all salinities tested, similar to *T. chui*, *Ti. lutea* and *C. neogracile*. However, final biomass after 15 days of culture of all microalgal strains was affected by culture salinities tested. Indonesian microalgal strains showed similar dry weight and ash free dry weight to smaller-cell strains, *Ti. lutea* and *C. neogracile*. Indonesian microalgal strains (other than Kb2-6) may be good candidates for mass culture in shrimp hatcheries based upon their cell size, ability to survive long culture periods, and wide salinity tolerance.

Key words: microalgae, shrimp, aquaculture, Indonesia

Introduction

Microalgae have been mass cultured for many years as live food for mollusk, shrimp, and fish culture during the hatchery stage (Wikfors and Ohno 2001; Martinez-Fernandez and Southgate 2007; Kent et al. 2011; Iba et al. 2014). Replacing live microalgae with artificial diets or dried microalgae has so far been unsuccessful (Gallardo et al. 2002; Sanchez et al. 2012; Ma and Qin 2012; Arney et al. 2015); however, partial substitution and frozen microalgal replacement diets may be promising (Gui et al. 2015; Southgate et al. 2016). Nevertheless, the availability of live microalgae in hatcheries for marine cultured species (fishes, shrimps and mollusks) remains critical to ensure a continuous supply of healthy seed for aquaculture.

The demand for shrimp products in both local and international markets continues to increase, partly because of increasing world population; therefore, a sufficient supply of shrimp larvae and post larvae to farmers is necessary to meet demand. Generating hatchery seed stock is one critical component in the production chain of shrimp for the aquaculture industry. A survey conducted by the Global Aquaculture Alliance in 2011 revealed that seed stock quality and availability still were the main issues in producing good quality shrimp in Asia (Valderrama and Anderson 2012). With effective microalgal feeds, hatcheries are expected to produce robust, healthy and inexpensive seed stock for commercial use.

South East Sulawesi (SE-Sulawesi), Indonesia, has a great potential for unexploited microalgae because of the region's abundant and diverse water resources as habitats for microalgae, both in fresh and seawater. Approximately 75 % (115,000 km² of 153,000 km²) of the SE-Sulawesi territory consists of marine areas (Ministry of Forestry of Government of Indonesia 2013). Local microalgae from SE-Sulawesi (02°45' to

06°15'S, 120°45' to 124°45'E) are not yet utilized, despite their potential to support biofuel production, food supplements, and aquaculture within the country. Most shrimp aquaculture in SE-Sulawesi currently relies upon microalgae starters supplied from the south Sulawesi and Java regions that impose high operational costs to aquaculture farms and hatcheries. The price of microalgae starter culture obtained from Java and south Sulawesi such as *Spirulina* sp., *Chorella*, and *Dunaliella*, is in the range of US\$10-30·L⁻¹ (Iba et al. 2014). That price can be reduced by up to 50% if the starter culture were to be provided within SE-Sulawesi region as the high transportation cost will be no longer part of the production equation. Importing microalgae from other regions also increases greenhouse gas (GHG) emissions associated with air and ground transportation (Edwards-Jones 2010) and exposes receiving areas to risks from non-native species invasions, including from the algae themselves and any contaminating microorganisms. To reduce cost and non-native introduction issues, the use of locally-isolated, cultured microalgae in local hatcheries may be considered. Microalgal research, therefore, is desirable, not only to conserve and make use of local resources, but to serve in supporting a number of economic activities including aquaculture in Indonesia.

Kendari Bay and the Wanggu River are important habitats and ecosystems that support SE-Sulawesi's economic growth and development. Currently, Kendari Bay receives many human inputs, particularly in the form of sedimentation because of inappropriate land use in surrounding areas. The Wanggu River drains into Kendari Bay and is one of the larger rivers in the Kendari municipal jurisdiction. As most of the Kendari population lives in the Wanggu River watershed, this habitat also receives pressures in the form of organic and inorganic pollution. Habitats such as the Wanggu

River and Kendari Bay that receive high nutrient input from wastewater may provide some species of microalgae that potentially can grow easily under cultivation in the laboratory. Cai et al. (2013) showed that several species of microalgae, such as *Chlorella* sp., *Scenedesmus* sp., *Oscillatoria* sp. and *Isochrysis* sp., some of which are commonly used in aquaculture hatcheries, tend to grow better in high nutrient effluent waters. In a preliminary study of phytoplankton resources in Kendari Bay, four dominant species of microalgae from three different classes were found (Iba et al. 2009). This study, therefore, was aimed to isolate and to characterize the growth of local strains of microalgae from Kendari Bay SE-Sulawesi that may be of potential use in shrimp hatcheries.

Materials and Methods

Sampling and Isolation of Microalgae

The samples for this experiment were collected from two locations: the marine Kendari Bay (3°58'55"S, 122°33'12"E) and the brackish water (estuary) of the Wanggu River (3°58'S, 122°35'E) at up to 2-m depth in each location. Fifty liters of seawater were filtered through a plankton net (25-µm mesh) to remove zooplankton. The filtrate was collected in the sample jar of the plankton net and then 30-mL samples from the filtrate were taken and distributed into two 15-mL Falcon tubes and placed in a cooler. Water samples containing natural microalgae were brought to the Laboratory of Fisheries (Haluoleo University) and kept overnight at room temperature before being brought to USA, specifically the National Marine Fisheries Service Milford Laboratory (Milford) and the University of Rhode Island (URI) for isolation. Fourteen days following collection, water samples were enriched in 10-mL culture tubes containing

Guillard's f/8 medium for marine species (Wikfors and Ohno 2001; Ponomorenko et al. 2009). Single-cell isolation at the URI was conducted using capillary pipettes according to procedures described by Andersen and Kawachi (2005)

At Milford, isolation was performed using a flow cytometer (JSAN Model DCS-260U Desktop Cell Sorter, Bay Bioscience Co., Ltd., Kobe, Japan). The samples were incubated under growth conditions (25°C, 12:12 light:dark, 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ PAR) for several days upon receiving to re-activate photosynthetic physiology. Microscope observation revealed 5-10 dominant microalgal taxa (organisms with chlorophyll fluorescence) in each field sample; several morphotypes were abundant. After concentration 10x by gentle centrifugation (500 x g), samples were run through the JSAN flow cytometer and visualized as plots of Forward Scatter (FSC, proportional to size) and FL3 (chlorophyll fluorescence) or Side Scatter (SSC, internal complexity) and FL3. Sort regions were drawn in the centers of aggregations of events with similar SSC/FL3 – analogous to targeting the bulls-eye – and the cytometer was programmed to deposit a single cell in each well of a 96-well plate using the plate-deposition unit on the instrument. Pre-sterilized well-plates were prepared to receive cells by aseptically pipetting 500 μL of sterilized f/2 medium into each well. As a precaution, the instrument also was programmed to deposit 50 cells into small test tubes containing 500 μL of sterilized culture medium, either f/2 or L1 (Guillard and Hargraves, 1993) enriched Milford Harbor seawater.

Micro-well plates containing sorted cells were covered to minimize evaporation and incubated under the growth conditions noted above. Every 2-3 days, wells were observed with an inverted light microscope for evidence of cell proliferation. Of 300+

wells receiving sorted cells, none yielded viable cultures; however, several test tubes into which 50 cells had been sorted showed growth. Growing isolates were transferred to 10-mL test tubes containing 5 mL f/2, f/2+Si, or L1 medium and incubated under growth conditions. Cultures that established robust growth were observed microscopically for provisional identification and determination of possible unialgal status. Cultures appearing to be unialgal were analyzed with an analytical flow cytometer (B-D BioSciences C6, San Jose, CA) to determine if more than one chlorophyll-containing particle was present. Following microscopic and flow-cytometric analysis, 3 unique, unialgal cultures were found: two appeared to be diatoms, which grew well in f/2+Si; and one was a brown flagellate that grew best in L1. These three isolates obtained from the flow-cytometric isolation effort were sent to the University of Rhode Island for perpetuation and experimentation.

Microalgal Culture

Microalgae were cultured based upon methods described by Martinez-Fernandez et al. (2006) and Rohani-Ghadikolaei (2012). Batch cultures of isolated microalgae were grown in 500-mL glass culture bottles containing 450 mL of sterilized seawater with f/2 medium, at 28-30°C, under a 12:12 h light: dark cycle with 160-170 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ PAR and gentle mixing once a day. Indonesian microalgal strains were inoculated at density ranging from $5.4\text{--}6.3 \times 10^4$ and $2.1\text{--}3.0 \times 10^4 \text{ cells mL}^{-1}$ for Kb1-2 and Kb1-3, respectively. A preliminary study with the newly-isolated strains, particularly Kb1-5, showed that they performed better with cell density above $5 \times 10^4 \text{ mL}^{-1}$ at inoculation; therefore, the inoculum at first day of the growth experiment was increased to facilitate good growth, therefore Kb1-5 was inoculated at density of $8.3\text{--}12.8 \times 10^4 \text{ cells mL}^{-1}$. Cell

density at inoculation for comparison species ranged from $0.2-0.6 \times 10^4$, $0.7-1.0 \times 10^4$, and 7.0×10^4 cells mL⁻¹ for *C. neogracile*, *T. chui* and *Ti. lutea*, respectively. The effect of salinity on microalgal growth was tested at 35, 30, 25 and 20 psu with four replicates for each salinity to accommodate a wide range of salinity possible for microalgae growth including their natural condition which is in the range of 22-25 psu. Seawater used in this study was artificial seawater (Instant Ocean Sea Salt mix) at initial salinity of 30 psu. To be practical, we added artificial salt (Morton Kosher Salt) to obtain 35 psu salinity and distilled water to decrease the salinity to 25 and 20 psu.

Three microalgae strains widely used in aquaculture were used as control species: *Ti lutea* (T-ISO; CCMP-1324), *T. chui* (CCMP-884) that were obtained from the shellfish laboratory at Roger Williams University, Bristol, RI, USA, and *C. neogracile* (strain Chaet B) from Milford Laboratory, Connecticut, USA. Cell density of cultured microalgae was recorded every two or three days by fluorometry (AquaFluor Handheld Fluorometer/Turbidimeter Turner Designs Sunnyvale California, USA) to measure increasing fluorescence intensity and wavelength distribution over culture period (Fig. 1). A measurement was conducted in each replicate for all salinities tested for 15 days. Furthermore, the cells during logarithmic phase were counted using a particle counter (Multisizer™ 4 COULTER COUNTER®, Beckman Coulter, Brea, California, USA). A regression relationship between Coulter counts and fluorometer readings was used to estimate cell counts from fluorometer readings. Finally, division rate (k) during logarithmic phase of the culture at each salinity for each species was calculated based upon cell counts (N) at times (t) (Guillard, 1973; Wood et al, 2005) as follows:

$$k = \frac{1}{\ln 2} [\ln (N_1/N_0)/(t_1 - t_0)] \quad (1)$$

Cell sizes of live cultured microalgae were obtained using image analysis software (Nikon NIS-Elements AR 3.0) to measure length, width and diameter of 50 non-dividing microalgal cells from each species, in logarithmic and stationary phases, and at each of the salinities.

Identification of the isolated strains was conducted using Scanning Electron Microscopy (SEM, JEOL 5900 LV system, SEM Tech Solutions, USA) on material preserved with 2% glutaraldehyde in seawater. The images of two strains of Indonesian microalgae, Kb1-3 and Kb1-5, were not successfully acquired using SEM; therefore, microphotographs of these strains were taken using a light microscope (Nikon Eclipse 80i equipped with a QImaging Retiga 2000R digital camera) at 40x magnification.

Yield of each cultured microalgal strain was calculated as an average of final cell density (cells·mL⁻¹) at stationary phase of 13 and 15 days of culture. In addition, dry weight (DW) and ash-free dry weight (AFDW) were measured following the method of Mohameini and Borowitzka (2013). At the end of experiment (Fig.1), four replicates of 100 mL of cultured microalgae at each salinity were filtered using pre-weighed, pre-combusted (1 h, 100 ± 5°C) GF/F filters (Ø = 47 mm), and rinsed with 10 mL 0.65-M ammonium formate to eliminate salts. Filters were oven dried at 100±5°C for 1 h, placed overnight over KMnO₄ salts in a vacuum desiccator, and weighed to obtain dry weight of the algae. The dried algae on filters were then combusted at 450°C for 5 h using a muffle furnace and weighed again to obtain AFDW. DW and AFDW were calculated using the following equations:

$$DW \left(\frac{\mu g}{mL} \right) = \frac{\text{Weight of filter with algae} - \text{weight of filter}}{\text{sample volume}} \quad (2)$$

$$AFDW \left(\frac{\mu g}{mL} \right) = \frac{\text{Weight of filter with dried algae} - \text{weight of filter after drying (DW)}}{\text{sample volume}} \quad (3)$$

Statistical Analysis

Data on cell density of all microalgal strains at each salinity tested were subjected to repeated measures ANOVA; whereas, the differences in cell size, yield, division rate, DW and AFDW across cultured microalgae were analyzed using two-way ANOVA. The differences in cell size, yield, division rate, DW and AFDW of individual microalgal strains at each salinity tested were analyzed using one-way ANOVA. All statistical analyses were performed using SAS Enterprise Guide 7.1.

Results

Indonesian Microalgal Isolates

We successfully isolated four strains of microalgae from Kendari Bay SE-Sulawesi, and these were denoted as Kb1-2, Kb1-3, Kb1-5 and Kb2-6 (Table 1). Kb1-2 is a *Chaetoceros* species (Class Bacillariophyceae) as shown by SEM and later confirmed by genomic sequences of 18S and 28S rDNA (Fig. 2, data on genomics are not shown here, paper is in preparation). The isolate labeled Kb1-3 has a coccoid morphology and sometimes cells clump together; Kb1-5 is irregular in shape and has relatively smaller sizes compared to other local isolates (Fig. 3). Neither Kb1-3 nor Kb1-5 were identified. Kb2-6 identified as *Melosira* cf. *moniliformis* forms colonies and has a larger cell size than 10 μm , suggesting that the strain is not suitable for aquaculture and therefore was excluded from subsequent culture experiments.

Cell Size and Growth of Indonesian Microalgal Isolates

Microalgal strains isolated from SE-Sulawesi, Indonesia ranged from 1.2 to 11.8 μm in size (Figs. 2 and 3, Table 1), and the differences in cell size within strains were significant. Each strain had a different mean cell size in response to salinity except for Kb1-5. Of Indonesian strains, Kb1-2 had the largest cell size when cultured at 30 psu; whereas, Kb1-3 had similar cell size at 35 psu to those cultured at 20, 25 and 30 psu. *C. neogracile* had a smaller cell size at 25 psu; whereas, *T. chui* was larger at 25 and 30 psu. *Ti. lutea* had larger cell sizes at low (20 psu) and high (35 psu) salinities tested, but cell size was smaller at 25 and 30 psu (Table 2). *T. chui* was the largest species across all salinities tested, and Kb1-5 was the smallest ($p < .0001$). Kb1-2 had a similar cell size to *C. neogracile*; whereas, cell size of Kb1-3 was similar to *Ti. lutea* (Table 5). Relatively higher percentages of smaller cell sizes were observed at higher salinities for most of the strains with the exception of *C. neogracile* and *T. chui* (data not shown).

Salinity or age of culture within salinity alone and the interaction of salinity and age of culture did not have a significant effect on the increase in cell density over time in all microalgal strains cultured. However, strain ($p = < .0001$), age of culture within strain ($p = 0.003$), and the interaction of culture age and strain ($p = < .0001$) did have a significant effect on cell density of algae. The highest cell densities were observed in Kb1-3 cultures at 25 psu on day 15 and Kb1-5 at 25 psu on day 8; 65.9 and 42.4 cells $\cdot \text{mL}^{-1} \cdot 10^5$, respectively. Most strains began to enter the stationary phase after day 10 except for Kb1-2 and Kb1-5 that reached stationary phase at day 8. Microalgal growth generally started to show a significant increase on day 3 after inoculation except for *T. chui* and *Ti. lutea* that experienced a lag phase on that day. Significant growth continued

up to day 8 for all Indonesian microalgal strains and *C. neogracile* except Kb1-5 at 20 and 30 psu that showed a decreasing growth after day 6. *T. chui* and *Ti. lutea* continued to have significant growth up to day 9 and 10, respectively (Fig. 4).

Division rates decreased as the culture reached a late logarithmic and onset of stationary phase (Fig. 5). There were no significant differences in division rate during logarithmic growth phase across all salinity levels tested as well as the interaction between salinity and strain among microalgal strains. The differences in division rate during log phase were between strains only ($p=0.002$). Within microalgal strains, salinity only affected the division rate of one Indonesian strain, Kb1-2 (*Chaetoceros* sp., $p<.0001$) but did not affect Kb1-5 and Kb1-3. Also, salinity did not affect the division rate during log phase of growth in *C. neogracile*, *T. chui*, or *Ti. lutea*. Among Indonesian microalgae tested, Kb1-5 showed the highest division rate during log phase, similar to *T. chui* and *C. neogracile*. Kb1-2 and Kb1-3 strains had lower division rates similar to *Ti. lutea* (Fig. 5).

Differences among microalgal strains ($p<.0001$), as well as salinity ($p<.0001$) and the interaction between strain and salinity ($p<.0001$), affected yields of cultured microalgae. Of Indonesian strains, higher final biomass was observed at 35 psu in Kb1-3; whereas, yields at other salinities were not significantly different from each other. Conversely, in Kb1-2 and Kb1-3, higher yield was obtained in both at lower 20 psu and at higher 30 and 35 psu salinity. Kb1-2 cultured at 25 psu produced the lowest yield; whereas, yield of Kb1-3 at the same salinity was similar to that at 20 and 30 psu. Final yields of *C. neogracile* and *T. chui* were not affected by salinity, unlike *Ti. lutea*. Final yield of *Ti. lutea* was significantly higher at 25, 30 and 35 psu than at 20 psu

(Table 3). Regardless of salinity, two Indonesian strains, Kb1-3 and Kb1-5, showed highest yields among all microalgae tested; whereas, yield of Kb1-2 was similar to *Ti. lutea* and *C. neogracile*. *T. chui* had the lowest yield but statistically similar to *C. neogracile* (Table 5).

Both dry weight (DW) and ash free dry weight (AFDW) across microalgae strains were not affected by salinity or the interaction between salinity and strains but by differences among strains themselves ($p < .0001$). DW and AFDW of Indonesian microalgal strains were similar at all culture salinities except for Kb1-2. Higher DW and AFDW of Kb1-2 were observed at 20, 30 and 35 psu than at 25 psu. Salinity affected DW and AFDW of *C. neogracile* and *Ti. lutea* but not *T. chui*. Higher DW and AFDW were obtained at higher salinity, 35 psu, in *C. neogracile* culture and at 30 and 35 psu in *Ti. lutea* culture, respectively (Table 4). Among microalgal strains, DW of all Indonesian microalgal strains was similar to *Ti. lutea* and *C. neogracile*. Moreover, DW of *T. chui* was the highest compared to the rest of the strains (Table 5).

Discussion

Of three distinct strains that were included in the growth experiment, one strain was identified as *Chaetoceros* sp., a diatom species. The size of microalgae in this growth experiment ranged from 1.2-11.8 μm , considered as either pico or nanoplankton, thus is suitable as feed for aquaculture hatcheries, particularly shrimp aquaculture as suggested by Brown (2002) and Becker (2004), and comparable to those extensively used in aquaculture, particularly *C. neogracile* and *Ti. lutea*.

Indonesian microalgae showed similar growth rates during logarithmic phase across all tested salinities, suggesting that they are euryhaline species, although they

produced different final biomass depending upon salinity. This is consistent with their natural habitat being an estuary that might be suitable for shrimp hatchery conditions. Diatom species, such as *C. neogracile* and Kb1-2 (*Chaetoceros* sp.) used in this study, tended to produce smaller cell sizes over time. Different classes of microalgae are known to undergo different cell division mechanisms, either reducing or increasing cell size during lifecycles. In diatoms, if rapid individual cell growth after asexual reproduction occurs, there will consequently be a reduction in average population cell size over time because of the reproductive mechanism in which one of the new daughter cells is always smaller than the parent cell (Round 1990; Lee 2008). As for flagellates without silica cell walls, such as *T. chui* and Prymnesiophyceae, such as *Ti. lutea*, used in this study, these taxa tend to produce similar or larger cell sizes under favorable environmental conditions (Danquah et al. 2009).

Salinity, in the range tested, was not a limiting factor for growth of any microalgal strains in this study. The growth is most likely limited by other environmental factors such as light intensity, temperature, or culture nutrients, which were not addressed in this study, and genetic traits of each strain as suggested by Hu et al. (1998), Lee and Kim (2002) and Wood (2005). Differences in cell density, thus dry weight and ash free dry weight, may be driven by cell size, cell density at inoculation, and harvest time, as well as other culture conditions such as light intensity and temperature (Hu et al. 1998; Richmond 2004). Richmond (2004) stated that output rate, i.e. yield of a microalgal culture, using *Spirulina* as a model, changed over time, becoming lower as the growth rate decreased during stationary phase of the culture cycle. In this study, Kb1-5 with the smallest cell size of all strains, yet with higher

inoculated cell numbers, showed the highest cell numbers over the culture period, but with considerably lower dry weight and ash-free dry weight at the end of experiment. Conversely, *T. chui*, with its larger cell sizes and lower inoculation numbers, exhibited the lowest cell numbers on all days of culture, but consistently exhibited higher dry weights across all salinities tested.

The growth of Indonesian microalgal strains, particularly Kb1-3 and Kb1-5, was higher than *T. lutea* although *T. lutea* showed a similar growth to Kb1-2, *Chaetoceros* sp. *Chaetoceros* sp. and Kb1-3 did not enter stationary phase at day 15 of culture, except for Kb1-3 at 25 psu. This growth pattern was different from Miller et al. (2014) who found that late stationary phase occurred at day 12 in cultured *Chaetoceros calcitrans*. Finally, maximal cell density of *T. lutea* during stationary phase reported here was almost three times lower than that reported by Huerlimann et al. (2010), but similar to that reported by Renaud et al. (1999) and almost six times lower than that reported by Fidalgo et al. (1998). The variation in final cell density may be attributable to different cell density at inoculation as well as culture conditions, such as light intensity, culture medium, temperature, and aeration (Fidalgo et al., 1998; Borowitzka, 2012; Gorgonio et al., 2013). Further studies would be needed to determine if the Indonesian strains stopped dividing because of nutrient or light limitation.

Although prolonged culture up to late stationary phase may compromise the harvested biomass as shown in this study with Kb1-2 and Kb1-5 strains, some research has shown that this practice will increase the nutritional value of microalgae particularly total lipid and fatty acid content (Patil et al. 2007; Schwenk et al. 2013; Nalder et al. 2015). If microalgae are to be used as food for aquaculture, however, it is important to

keep both cell density and nutrition variables at optimal levels; therefore, harvest for feeding purposes might best be conducted during late logarithmic phase or in this study at day 6 or 7 of culture. Our study on nutritional content of these newly isolated microalgal strains (Chapter 3) showed that their total lipid content after 15 days of culture or during stationary phase ranged from 11.8-74.0 % which was well above lipid requirement for shrimp hatchery feeding according to Nuñez et al. (2000). Furthermore, the abilities to survive under wide ranges of salinity and nutrient-deficient conditions are important traits of microalgae to be used in aquaculture. In the face of changing climate, increasing variability in weather conditions may alter the freshwater supply and thus salinity levels in estuaries (Taylor et al. 2012; Elliot et al. 2014, Schwe et al. 2014). Therefore, culturing euryhaline microalgae species for aquaculture that are accustomed to salinity fluctuations is one promising solution to continue producing high quality seafood products reliably over time. In summary, three of four local isolates from an Indonesian estuary were shown to have size, environmental tolerance, and growth characteristics consistent with needs as feeds in local shrimp hatcheries. This finding is consistent with an initiative to utilize local microalgae for aquaculture purposes to reduce production cost, promote sustainable and environmentally friendly aquaculture practices in the region and Indonesia in general. Finally, further identification of the strains using genetic tools such as DNA sequencing may be useful in revealing their taxonomic identity and may help to further describe the microalgal biodiversity in the region.

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TABLES

Table 1. List of strains that were isolated in this study, isolation location, date of isolation and size range. Tentative names are based on scanning electron microscopy (SEM) for *M. cf. moniliformis*. SEM and genome sequencing were used to identify *Chaetoceros* sp.

Strain Number	Tentative Species Name	Isolation Location	Date of Isolation	Size (µm)
Kb2-6	<i>Melosira cf. moniliformis</i>	Wanggu River Estuary	June 2013	10.6-26.9
Kb1-2	<i>Chaetoceros</i> sp.	Kendari Bay	August 2013	4.5-11.8
Kb1-3	Unidentified	Wanggu River Estuary	August 2013	1.2-10.0
Kb1-5	Unidentified	Wanggu River Estuary	August 2013	1.9-5.8

Table 2. Size ranges (mean $\pm$ SE) of cultured microalgae at each salinity tested. Numbers with common superscripts in a row are not significantly different from each other. SE=standard error of the mean from 50 sampled live cells.

Strain	Size Measured	Size (µm) at different salinity (psu)				p-value
		20	25	30	35	
Kb1-2 (<i>Chaetoceros</i> sp.)	Length	4.9-9.5	4.5-9.4	5.5-11.8	5.0-10.6	0.0001
	Mean	6.6 $\pm$ 0.02 ^a	6.1 $\pm$ 0.02 ^a	7.3 $\pm$ 0.03 ^b	6.6 $\pm$ 0.03 ^a	
Kb1-3	Diameter	2.0-8.5	2.3-10.0	2.3-9.6	1.2-9.4	0.0067
	Mean	8.5 $\pm$ 0.03 ^b	5.2 $\pm$ 0.05 ^a	5.3 $\pm$ 0.05 ^a	4.3 $\pm$ 0.05 ^{ab}	
Kb1-5	Diameter	3.1-5.8	2.5-5.8	2.3-5.6	3.1-5.4	0.2278
	Mean	3.8 $\pm$ 0.01 ^a	4.0 $\pm$ 0.02 ^a	3.9 $\pm$ 0.01 ^a	3.7 $\pm$ 0.01 ^a	
<i>C. neogracile</i>	Length	6.7-11.0	4.0-8.7	5.6-8.0	5.8-10.7	<0.0001
	Mean	8.0 $\pm$ 0.02 ^a	5.4 $\pm$ 0.02 ^b	6.6 $\pm$ 0.02 ^c	7.5 $\pm$ 0.02 ^d	
<i>T. chui</i>	Diameter	7.3-12.5	10.0-15.6	10.8-16.6	7.8-13.4	<0.0001
	Mean	9.8 $\pm$ 0.03 ^b	12.6 $\pm$ 0.03 ^a	12.5 $\pm$ 0.03 ^a	10.2 $\pm$ 0.03 ^b	
<i>Ti. lutea</i>	Diameter	4.6-6.5	4.1-6.0	3.7-6.4	4.0-7.0	<0.0001
	Mean	5.2 $\pm$ 0.01 ^a	4.8 $\pm$ 0.01 ^b	4.6 $\pm$ 0.01 ^b	5.2 $\pm$ 0.01 ^a	

Table 3. Yield of cultured microalgae (mean $\pm$ SE) in 10^6 cells mL⁻¹ during stationary phase at different salinities. Numbers in a row with common superscripts are not significantly different from each other. SE=standard error of the mean from 8 points at

Strain	Yield (mean $\pm$ SE) at different salinity (psu)				p-value
	20	25	30	35	
Kb1-2 (<i>Chaetoceros</i> sp.)	2.3 $\pm$ 0.2 ^a	0.6 $\pm$ 0.03 ^c	1.8 $\pm$ 0.2 ^{ab}	1.7 $\pm$ 0.1 ^b	<.0001
Kb1-3	3.7 $\pm$ 0.1 ^b	4.4 $\pm$ 0.1 ^b	3.8 $\pm$ 0.1 ^b	6.5 $\pm$ 0.1 ^a	<.0001
Kb1-5	3.3 $\pm$ 0.3 ^a	1.6 $\pm$ 0.1 ^b	2.4 $\pm$ 0.4 ^{ab}	2.5 $\pm$ 0.1 ^{ab}	0.02
<i>C. neogracile</i>	1.0 $\pm$ 0.04 ^a	1.0 $\pm$ 0.05 ^a	1.0 $\pm$ 0.04 ^a	1.2 $\pm$ 0.05 ^a	0.27
<i>T. chui</i>	0.4 $\pm$ 0.01 ^a	0.3 $\pm$ 0.02 ^a	0.6 $\pm$ 0.01 ^a	0.7 $\pm$ 0.03 ^a	0.5
<i>Ti. lutea</i>	1.1 $\pm$ 0.01 ^b	1.5 $\pm$ 0.02 ^{ab}	1.3 $\pm$ 0.03 ^{ab}	1.6 $\pm$ 0.04 ^a	0.02

13 and 15 days of culture.

Table 4. Final weight (dry weight, DW $\pm$ SE, and ash free dry weight, AFDW $\pm$ SE) in μ g mL⁻¹ after 15 days of culture of Indonesian microalgal strains, *C. neogracile*, *T. chui* and *Ti. lutea* at different salinity. Numbers in a row with common superscripts are not significantly different from each other. SE=standard error of the mean from 4 replicates

Strain	Final Weight	Weight at different salinity (psu)				p-value
		20	25	30	35	
Kb1-2 (<i>Chaetoceros</i> sp.)	DW	175 $\pm$ 6 ^{ab}	107 $\pm$ 25 ^b	227 $\pm$ 36 ^{ab}	283 $\pm$ 51 ^a	0.02
	AFDW	135 $\pm$ 9 ^{ab}	85 $\pm$ 11 ^b	184 $\pm$ 23 ^a	204 $\pm$ 33 ^a	0.008
Kb1-3	DW	20 $\pm$ 6 ^a	46 $\pm$ 9 ^a	31 $\pm$ 10 ^a	29 $\pm$ 8 ^a	0.19
	AFDW	32 $\pm$ 5 ^a	48 $\pm$ 8 ^a	37 $\pm$ 7 ^a	40 $\pm$ 11 ^a	0.56
Kb1-5	DW	72 $\pm$ 16 ^a	35 $\pm$ 14 ^a	83 $\pm$ 24 ^a	27 $\pm$ 7 ^a	0.08
	AFDW	2 $\pm$ 8 ^a	32 $\pm$ 16 ^a	3 $\pm$ 6 ^a	18 $\pm$ 5 ^a	0.15
<i>C. neogracile</i>	DW	40 $\pm$ 5 ^b	21 $\pm$ 10 ^b	42 $\pm$ 10 ^b	151 $\pm$ 3 ^a	<0.0001
	AFDW	49 $\pm$ 5 ^{bc}	29 $\pm$ 7 ^c	56 $\pm$ 6 ^b	97 $\pm$ 5 ^a	<0.0001
<i>T. chui</i>	DW	640 $\pm$ 311 ^a	414 $\pm$ 264 ^a	757 $\pm$ 363 ^a	147 $\pm$ 32 ^a	0.44
	AFDW	521 $\pm$ 382 ^a	289 $\pm$ 118 ^a	179 $\pm$ 42 ^a	144 $\pm$ 20 ^a	0.56
<i>Ti. lutea</i>	DW	35 $\pm$ 15 ^c	21 $\pm$ 9 ^c	209 $\pm$ 7 ^a	131 $\pm$ 19 ^b	<0.0001
	AFDW	42 $\pm$ 8 ^b	29 $\pm$ 5 ^b	64 $\pm$ 6 ^b	141 $\pm$ 20 ^a	<0.0001

Table 5. Regardless salinity, size in μm , yield in $10^6 \text{ cells mL}^{-1}$, division rate (k), dry weight (DW) and ash free dry weight (AFDW) in $\mu\text{g mL}^{-1}$ of Indonesian microalgal strains, *C. neogracile*, *T. chui* and *Ti.lutea*. All values are mean $\pm$ SE from 50 replicates for size, 8 replicates for yield and 4 replicates for k, DW and AFDW

Strain/ Variables	Kb1-2 (<i>Chaetoceros</i> sp.)	Kb1-3	Kb1-5	<i>C. neogracile</i>	<i>T. chui</i>	<i>Ti.lutea</i>	p-value
Size	6.7 ± 0.1^b	4.7 ± 0.2^c	3.8 ± 0.05^d	6.9 ± 0.1^b	11.3 ± 0.1^a	5.0 ± 0.1^c	<.0001
Yield	1.6 ± 1.3^c	4.6 ± 2.6^a	2.5 ± 2.1^b	1.1 ± 0.4^{dc}	0.6 ± 0.7^d	1.4 ± 0.7^c	<.0001
k	0.9 ± 0.04^{dc}	1.0 ± 0.07^{bcd}	1.4 ± 0.04^a	1.1 ± 0.1^{abc}	1.3 ± 0.05^{ab}	0.8 ± 0.03^d	<.0001
AFDW	152 ± 15^{ab}	39 ± 4^b	14 ± 5^b	58 ± 7^b	283 ± 98^a	69 ± 13^b	<.0001

FIGURE LEGENDS

Figure 1. Diagram of microalgae culture sampling for one strain at four salinity levels (20, 25, 30 and 35 psu) tested in four replicates. One arrow describes volumes retrieved for growth analysis. The rest of the culture volume was harvested for subsequent biochemical composition (paper in preparation) of the algae.

Figure 2. Scanning electron micrographs of microalgal isolates in this study. Kb2-6 identified as the chain-forming *Melosira* cf. *moniliformis* (a), and Kb1-2 that belongs to *Chaetoceros* sp. (b).

Figure 3. Photomicrographs of Indonesian microalgae strains denoted as Kb1-3 (a) and Kb1-5 (b) at 40x magnification using light microscopy. Scale bar is 50 μm .

Figure 4. Increase in cell density of Indonesian microalgae *Chaetoceros* sp., Kb1-3, Kb1-5 (a, b, c, respectively) over culture period compared to *C. neogracile*, *T. chui* and *Ti. lutea* (d, e, f, respectively) at different salinities in psu. Standard error about the mean are represented by error bars from 4 replicates. Note that the y axes are not all on the same scale.

Figure 5. Division rate (k , doubling.day⁻¹) of cultured microalgae at different salinities, 20, 25, 30 and 35 psu, during logarithmic phase of growth. Log phase was at time intervals of 0 to 3 days for Kb1-2, Kb1-5 at all salinities tested, Kb1-3 at 25 psu and *C. neogracile* at 20, 25 and 30. Log phase of *C. neogracile* cultured at 35 psu was at time intervals of 6 to 8 days. Log phase was at time intervals of 3 to 6 days for *T. chui* at all salinity levels and Kb1-3 at 20, 30 and 35 psu, 6 to 8 days for *Ti. lutea* cultured at 25 and 30 psu and 8 to 10 days at 20 and 35 psu. Standard error about the mean are represented by error bars from 4 replicates.

Figure 1.

Figure 2.

Figure 3

a

b

Figure 4

c

d

e

f

Figure 5

CHAPTER 3

Effects of salinity on growth and nutritional content of newly isolated Indonesian microalgal strains with potential for use in shrimp hatcheries

By

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Abstract

A two-week batch experiment was conducted on three newly isolated Indonesian microalgal strains (Kb1-2 identified as *Chaetoceros* sp., Kb1-3 and Kb1-5) and *Tisochrysis lutea* to determine salinity effects upon the proximate composition and ω -3, eicosapentaenoic acid and docosahexaenoic acid, (EPA and DHA) and ω -6 (arachidonic acid/ARA). Salinity affected the total lipid content in Indonesian microalgal strains (Kb1-3, and Kb1-5, $p=0.03$) except *Chaetoceros* sp. Total lipid content in Kb1-3 was significantly higher at 25 and 35 psu (24.6 ± 1.4 and 12.9 ± 0.3 %, respectively) compared to 30 and 25 psu (4.4 ± 1.4 and 4.9 ± 0.5 %, respectively). Kb1-5 produced highest protein when cultured at 25 and 35 psu (47.7 ± 0.4 and 38.8 ± 0.8 %), decreasing at 20 and 30 psu to 26.2 ± 2.4 and 15.0 ± 1.1 %, respectively. Salinity levels affected carbohydrate content of only one Indonesian microalgal strain (*Chaetoceros* sp., $p=0.003$), but not Kb1-3 and Kb1-5. Carbohydrate content above 10% was observed in Kb1-5; whereas, in Kb1-3 the content ranged from 4-8%. ARA, EPA and DHA were not detected at 20 psu in *Chaetoceros* sp and at 35 psu in Kb1-5. EPA and DHA were not detected at all salinities tested in Kb1-3. ARA was absent in *T. lutea* at all salinities tested. DHA content of Indonesian strains was similar to *T. lutea*. Higher percentage of ARA to total fatty acids was found in Kb1-3, ranging from 2.42-7.90 % ($p=0.0008$). EPA was higher in *T. lutea*, ranging from 1.31-13.16 % of total fatty acids and it was similar to Indonesian strains, Kb1-5 ($p=0.03$). Lipid, protein and carbohydrate in Indonesian strain were within the range required for shrimp larvae suggesting that they may be potential to be used in shrimp hatcheries.

Keywords: Microalgae, nutrition, fatty acids, shrimp

1. Introduction

Local isolates of microalgae from different geographic locations have been investigated for use in various applications, including aquaculture, biofuel or bioproducts and bioremediation, with promising results (Brown et al., 1997; Renaud et al., 1999; Matsunaga et al., 1999; Nuñez et al., 2002; Yeesang and Cheirsilp, 2011; Talebi et al., 2011; Cai et al., 2013; Borowitzka, 2013; Ruangsomboon et al., 2013; Durvasula et al., 2015; Hende et al., 2016). In aquaculture, many cultured animals utilize live microalgae at certain life-history stages -- all growth stages of bivalve mollusks, and during larval stages of some fish and crustaceans -- underscoring the importance of microalgae in the aquaculture industry. In shrimp aquaculture in particular, the co-culture practice of microalgae and shrimp throughout or in some part of their growth cycle has resulted in significantly better growth and survival of the cultured species (Nuñez et al., 2011; Sanchez et al., 2012; Iba et al., 2014; ; Cao et al., 2014; Jamali et al., 2015).

The quality of microalgae as live food in aquaculture hatcheries is determined by several factors including: appropriate size, digestibility, robust growth under variable culture conditions, and above all, appropriate nutritional content (Brown, 2002). In general, microalgae can be considered good candidates for shrimp aquaculture if they contain protein higher than 25% of dry weight, 8-30% carbohydrate, and approximately 10% lipid (Tobias-Quinitio and Villegas, 1982). Usually, species with high fatty-acid content, particularly including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA), are the most desirable for shrimp aquaculture (Coutteau, 1996; Nuñez et al., 2002). Furthermore, cholesterol also is considered to be

an essential nutrient for shrimp; therefore, adequate cholesterol or metabolizable phytosterols is another desirable trait for microalgae to be used in shrimp aquaculture (Teshima et al., 1982; Lim, 1998; D'Souza and Loneragan, 1999).

Culture productivity and nutritional content of microalgae can vary based upon nutrients in culture medium and environmental conditions such as light intensity, temperature, and salinity (Renaud et al., 2002; de Castro Araujo et al., 2005; Schwenk et al., 2013). Salinity has been known to affect the lipid, protein and carbohydrate content of various species of microalgae, with different optimal ranges for different species (Renaud and Parry, 1994; de Castro Araujo et al., 2005; Khatoon et al., 2010; Zhila et al., 2011; Ruangsomboon et al., 2013). The present study was done to investigate the nutritional contents of newly isolated microalgae from Kendari Bay and the Wanggu River Estuary in South East Sulawesi, Indonesia under different salinity culture conditions. These two habitats are considered to be good potential sources of microalgae for use in regional white shrimp (*Litopenaeus vannamei*) hatcheries.

2. Material and Methods

2.1. Algal Culture

Three distinct microalgal isolates from Kendari Bay, denoted as Kb1-2 (*Chaetoceros* sp.), Kb1-3, and Kb1-5, were studied, and *Tisochrysis lutea* (T-ISO; CCMP-1324) was used as a comparison species. All microalgal strains have been cultured and maintained at the University of Rhode Island (URI) for more than a year. All four strains of microalgae were cultured in duplicate for 15 days in 1,000-mL Erlenmeyer culture flasks containing 600 mL of f/2 Guillard medium in artificial seawater at 28-29°C under 12:12 light: dark photoperiod at four different salinities: 20,

25, 30 and 35 practical salinity units (PSU) and under 160-170 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ PAR. Procedures of obtaining microalgal cell weight and measuring growth were described elsewhere (Chapter 2).

Microalgal cultures were harvested after two weeks of growth by filtering the algae onto pre-combusted (100°C, 1 h), pre-weighed, 47-mm GF/F Whatman filters prior to analysis. Pre-combusted filters were stored in a vacuum desiccator over a KMnO_4 dessiccant before being used for sample filtration. Respective volumes of cultured microalgae (Table 1) at each salinity were filtered onto pre-weighed, pre-combusted filters, and rinsed with 10 mL 0.65-M ammonium formate to eliminate salts. Filters were oven dried at $100 \pm 5^\circ\text{C}$ for 1 h, placed overnight over KMnO_4 salts in a vacuum desiccator, and weighed to obtain dry weight of the algae. Dry weight of microalgae was calculated using the following equations:

$$\text{DW (mg)} = \text{Weight of filter with algae} - \text{weight of filter} \quad (1)$$

All filters containing microalgal samples were folded and kept at -20°C if not dried immediately. If not analyzed immediately, the dried microalgal samples were further kept at -20°C up to 2 weeks before being subjected to biochemical analysis procedures (Gonzalez-Araya et al., 2011; Mohameini et al., 2012).

2.2. Nutritional Analyses

Total lipid, protein and carbohydrate contents of microalgae were analyzed based upon methods by Gonzalez-Araya et al (2012), Huang (2013) and Mohameini et al. (2012) from filtered and dried microalgal samples. Total lipid of the dried microalgae was extracted with chloroform–methanol–deionized water (2:1:0.8, v/v) according to the modified method of Bligh and Dyer (1959) as suggested by Mohameini

et al (2013). In brief, the microfiber filter holding cells from 200 mL of culture was crushed in a mortar and pestle after adding 4 mL of liquid nitrogen. The sample was extracted twice with fresh solvent mixture and centrifuged at 1,000-2,000 x g for 10 minutes or until a compact pellet was formed. The supernatant from samples was placed in a 20-mL tube with a screw cap and left for 24 h in the dark at -5° C for phase separation. The methanol/water layer on the top was removed with a very fine Pasteur pipette, then 6-8 drops of toluene were added to the chloroform layer to remove any small amount of remaining water. The chloroform layer was transferred to a dry and pre-weighed vial. After the toluene/water on the surface of the chloroform layer in the vial was carefully removed, the chloroform was evaporated using rotary evaporator. After evaporating, the lipid sample was kept in a vacuum dessicator over KOH pellets over night and then the vial was weighed using a 5 digit balance. The lipid content was measured gravimetrically and was calculated using the following equation:

$$\text{Lipid (\%)} = \frac{\text{WL}}{\text{WDA}} \times 100 \quad (2)$$

where, WL (mg) and WDA (mg) are the weights of the extracted lipid and the dry algae biomass, respectively (Huang et al, 2013; Mohameini et al, 2013).

The total protein contents of microalgae cultures were determined using the Lowry method as described by Mohameini et al (2013). After filtered samples were crushed with a mortar and pestle in liquid nitrogen, 10 mL of the Biuret reagent was added to the extracted sample, and 5 mL to tubes containing 5 mL protein standard, before they were placed in a heating block at 100±5°C for 60 min. After the tubes were removed from the heating block, 0.5 mL of Folin-Phenol reagent was added immediately while mixing with a Vortex stirrer. The tubes were placed in a 100±5°C

heating block for a further 20 min and finally allowed to equilibrate to room temperature for another 15 min before centrifuging at 1,000-2,000 x g for 5-10 min. Protein content of the supernatant was determined using spectrophotometry at 660 nm against bovine serum albumin (BSA) standard samples using the equation below:

$$\text{Protein content (\%)} = \frac{P_{\text{sample}}}{W_{\text{dw}}} \times 100 \quad (3)$$

when, P_{sample} = milligrams of protein in samples against protein standard and W_{dw} = total dry weight of the microalgae (mg).

The carbohydrate contents of microalgal samples were determined using a method described by Mohameini et al (2012), also known as the modified Phenol-Sulfuric Acid method. In brief, after adding 5 mL 1M H_2SO_4 , homogenized microalgal samples were incubated in a heating block at 100°C for 60 min. The samples were centrifuged at 1,000-2,000 x g for 5-10 minutes after having been incubated for ~30 min at room temperature. Two mL of supernatant were added to 1 mL of the phenol solution and mixed. After adding 5 mL concentrated H_2SO_4 and mixing again, the carbohydrate content was determined by spectrophotometry against a glucose standard curve at 485 nm. Carbohydrate content was calculated using the equation:

$$\text{Carbohydrate content (\%)} = \frac{C_{\text{sample}}}{W_{\text{dw}}} \times 100 \quad (4)$$

when C_{std} = milligrams carbohydrate in sample based upon standard curve comparison and W_{dw} = total dry weight of microalgae (mg).

Total lipid extracts were collected to determine fatty acid compositions of the microalgae. Fatty acids within the lipid extracts were saponified after dilution in 2 mL of chloroform and addition of 1 mL of 3N NaOH before heating to 90°C for 1 h. After cooling to room temperature, samples were neutralized using 1.8 mL of 3.6 N HCl and

reheated to 90°C for 10 minutes. Fatty acids from the membrane hydrolysate were extracted three times with 1 mL of a mixture of hexane:diethyl ether (1:1, v:v), and the organic layers were combined in an Erlenmeyer flask. The fatty-acid extract was dried overnight over anhydrous sodium sulfate with a cover before being filtered with regular filter paper (Whatman Grade 5, diameter 25 µm) into a round-bottom flask. The organic solvents were evaporated to dryness by rotary evaporator, and the fatty-acid hydrolysates were stored in a desiccator under argon at -80°C before further transesterification. The fatty acid extracts were derivatized to fatty acid methyl esters (FAME) using 5 mL boron tri-fluoride–methanol complex (BF₃-2CH₃OH) at 60°C for 5 min. C-12 (Lauric Acid) was used as an internal standard at 50 µg for *T. lutea* and 150 µg for Indonesian strains. After cooling at room temperature, 1 mL of water and 1 mL of hexane were added to the mixture. To transfer the esters into the non-polar solvent thoroughly, the vials were shaken vigorously. The upper organic/hexane layer was removed into an Erlenmeyer flask. Those fatty acids methyl esters (FAME) were dried over anhydrous sodium sulfate overnight, filtered with filter paper (Whatman Grade 5, diameter 25 µm), and the solvent was evaporated by rotary evaporator. FAME samples were diluted in chloroform for gas chromatography – mass spectrometry (GC-MS) analysis. If the samples were not analyzed immediately, they were stored in a dessicator under argon at -80°C. FAME mix (Supelco 37 Component FAME mix Sigma Aldrich USA) was used as a standard to identify ARA, EPA and DHA retention times by GC-MS.

Analysis of FAME prepared from lipid extracts was conducted using a GC-MS system with an Agilent gas chromatograph (6890 GC) in tandem with mass

spectrometry (5973 MSD, Agilent Technologies/19091J-413, USA) with one uL of diluted samples run at an injector temperature of 250 °C. An HP-5 capillary, fused silica, column (0.32 mm ID x 30 m x 0.25 µm (5%-Phenyl)-methylpolysiloxane film, equivalent to USP phase G27) was installed in the GC, with helium as the carrier gas. The GC outlet was connected to the mass spectrometer to ionize the samples, and the detector was scanned from 15-800 m/z at 1.91 scans/s. Extracted fatty acid methyl ester retention times in the total ion chromatograms were identified by comparison with peaks from the chromatograms of the Supelco FAME 37-fatty acid standard mix injected on the same day, and FAME identities were confirmed by matching relevant chromatographic peak fragmentation patterns with those of authentic standards in the NIST library database (NIST02.L). Quantification of fatty acids extracted from each microalgal strain was calculated based upon the relative ion abundance of the ¹³C-lauric acid methyl ester internal standard as follows:

$$\frac{\left(\frac{\text{Area of fatty acid}}{\text{Area of lauric acid}} \right) \times \text{weight of lauric acid in sample (}\mu\text{g)}}{\text{Lipid weight (mg)}}$$

2.3. Statistical Analysis

The effects of salinity on lipid, protein and carbohydrate content in each strain of microalgae were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA was also used to compare ARA, EPA and DHA across microalgal strains. Percentage data were transformed using square root transformation to meet ANOVA normality assumptions. Significant differences in the data (p<0.05) were analyzed with pairwise multiple comparison of means (Tukey test). All statistical analyses were done in SAS Enterprise Guide 7.1.

3. Results

3.1. Microalgal growth

Microalgal growth data and discussion was covered thoroughly in Chapter 2 of this dissertation. Most of strains cultured were harvested during onset of stationary or late stationary phase except Kb1-3 strain that was at late logarithmic phase of growth (Figure 4, Chapter 2).

3.2. Proximate composition

Proximate compositions of microalgal strains tested in this study varied with salinity. Lipid, protein, and carbohydrate contents of each strain are shown in Fig. 1. Salinity affected the total lipid content in Indonesian microalgal strains (Kb13, $p=0.0005$ and Kb1-5, $p=0.03$) except *Chaetoceros* sp. Total lipid content in Kb1-3 was significantly higher at 25 and 35 psu (24.6 ± 1.4 and 12.9 ± 0.3 %, respectively) compared to 30 and 25 psu (4.4 ± 1.4 and 4.9 ± 0.5 %, respectively). Kb1-5 showed a higher total lipid content at 20, 25 and 30 psu but not at 35 psu. Also, salinity affected the total lipid content of *T. lutea* which was approximately 20% at 30 and 35 psu compared to ca. 10% at 20 and 25 psu.

Protein content in all Indonesian strains was affected by culture salinity, similar to *T. lutea*. Similar protein contents were found in *Chaetoceros* sp. cultured at 20, 30 and 35 psu ($>30\%$) but not at 25 psu that was 19% ($p=0.0007$). Protein content was significantly higher when microalgae were cultured at low (20 and 25 psu) salinity in the Kb1-3 strain, and protein decreased at higher salinities (30 and 35 psu), similar to *T. lutea* ($p=0.03$ and 0.001 , respectively). Similar protein content was observed in Kb1-

5 cultured at both low (20 psu) and high (35 psu) salinity, with decreasing content found at 25 and 30 psu ($p=0.0003$)

Salinity levels affected carbohydrate content of only one Indonesian microalgal strain (*Chaetoceros* sp., $p=0.003$), but not Kb1-3 and Kb1-5. Carbohydrate content above 10% was observed in Kb1-5; whereas, in Kb1-3 the content ranged from 4-8%. *Chaetoceros* sp. showed a higher level of carbohydrate at 25 psu, significantly different from all other salinities. Higher carbohydrate content was found at high salinity (35 psu) in *T. lutea* whereas at other salinities tested, carbohydrate content was similar ($p=0.05$).

3.3. Fatty Acids

Of Indonesian microalgae, Kb1-2 contained essential ω -3 (EPA and DHA) and ω -6 (ARA) fatty acids at 25 and 35 psu but they were not detected at 20 and 30 psu salinity tested. ARA, EPA and DHA were all detected at 35 psu in Kb1-3 strains and at 25 psu in Kb1-5. EPA and DHA were not detected at 20, 25 and 30 psu in Kb1-3 (Table 1, Fig. 2). ARA was not detected in *T. lutea* in all salinities tested whereas EPA was absent at 25 psu. Regardless salinity, DHA content of Indonesian strains was similar to *T. lutea*. Higher percentage of ARA to total fatty acids was found in Kb1-3, ranging from 2.42-7.90 % ($p=0.0008$). ARA concentration in Indonesian strains, Kb1-2 and Kb1-5 was similar with *T. lutea*. EPA was higher in *T. lutea*, ranging from 1.31-13.16 % ($p=0.03$) of total fatty acids and it was similar to Indonesian strains, Kb1-5. DHA and EPA to total fatty acids in *T. lutea* was in the range of 1.2-13.2% and 1.9-4.8%, respectively (Fig. 2).

Concentrations of fatty acids found in microalgal strains varied across salinity tested as shown in Table 1 and Appendix. The presence or absence of certain fatty acids in each microalgal strain varied with culture salinity; for instance, the short-chain, saturated fatty acid, pentanoic acid, (5:0) was present in Kb1-5 only when cultured at 20 and 30 psu and Kb1-3 at 30 and 35 psu; whereas, capric acid (10:0) was found only at 35 psu in Kb1-2 and at 25 and 35 psu in *T. lutea*. However, this fatty acid was found at all salinities tested in Kb1-5. The long-chain saturated fatty acid nervonic acid (23:0) was present only in *T. lutea* at 20 and 35 psu. Similarly, the long-chain, saturated fatty acid, lignoceric acid (24:0) was found only in Kb1-3 cultured at 20, 25 and 35 psu and in *T. lutea* cultured at 35 psu. In general, Indonesian microalgal strains contained the short- and long-carbon chain fatty acids, both saturated such as caproic acid (6:0), caprylic acid (8:0), and tridecanoic acid (13:0), and monounsaturated such as palmitoleic (16:1 ω 7) and oleic acid (18:1 ω 9).

All Indonesian microalgal strains distinctively contained the dicarboxylic acid (DCA) phthalic acid, which was not present in *T. lutea*. In *T. lutea*, some short-carbon-chain and saturated fatty acids such as pentanoic and capric acid were not present. *T. lutea* predominantly contained long-carbon-chain fatty acids, particularly at higher culture salinities tested (Table 1).

4. Discussion

Sufficient nutritional contents of microalgae determine efficacy as food for shrimp larvae in aquaculture hatcheries. Adequate levels of protein, lipid, and carbohydrate are particularly critical for normal growth and development of penaeid shrimp (Milamena, 1996; Nuñez, 2002; Gleccross, 2009). Our study showed that Indonesian microalgae,

as well as *T. lutea*, during stationary phase contained protein higher than required by shrimp larvae (ca. 25%), with comparable lipid and carbohydrate contents (around 10 and 5%, respectively), as suggested by Tobias-Quinito and Villegas (1982).

Total lipid contents of two Indonesian microalgae (except Kb1-2) and *T. lutea* were affected by salinity. Kb1-3 contained lower lipid at 20 and 30 psu, and higher lipid contents were found at other salinity levels tested. Higher lipid was obtained at 20 and 25 psu as well, as at 30 psu, in Kb1-5, with 35 psu giving the lowest lipid content. This finding is in agreement with study by Khatoon et al (2014) and Ruangsomboon et al (2013) who found that *Tetraselmis* sp., *Nannochloropsis* sp., and a newly-isolated species of *Scenedesmus dimorphus* from Thailand responded differently to salinity in terms of lipid. *Nannochloropsis* sp. and *Tetraselmis* sp. showed significantly higher lipid contents at 30 psu compared to 20 and 40 psu salinities; whereas, *S. dimorphus* accumulated higher lipid at 5 psu salinity. Also, lipid content in the *Chaetoceros* sp. analyzed in the present study was comparable to lipid in *Chaetoceros* sp. isolated from Innisfail, Queensland, Australia, reported by Renaud et al. (1999), that was 17% of dry weight.. Lipid content of *T. lutea* in the present study was higher compared to a study reported by Gorgonio et al. (2013), which was 10.54% during logarithmic phase of growth and similar with study by Huerlimann et al. (2010), which was 26.8% when cultured at seawater salinity and measured during the stationary phase of growth. Lipid content in microalgae was known to be affected by culture conditions, such as salinity, light intensity and temperature, as well as culture medium and growth phase (Renaud and Parry, 1994; Nalder et al, 2015). Nalder et al. (2015) found that total lipid increased almost two fold from 6.7 ± 0.8 pg/cell during log phase to 13.2 ± 1.2 pg/cell during late

stationary phase. Higher lipid production at certain salinities in microalgae is suggested as a coping mechanism under unfavorable conditions to reserve energy until favorable conditions resume (Kalita et al., 2011; Talebi et al., 2013).

Salinity affected protein content in Indonesian strains of microalgae and *T. lutea*. Generally, higher protein content was observed at lower salinity in each strain tested, although some variation occurred in *Chaetoceros* sp., and Kb1-5. Protein contents in Indonesian microalgal strains presented here were comparable to protein contents reported by Renaud and Parry (1994) for *Nannochloropsis oculata* and *Nitzschia frustulum* cultured at similar salinity levels as this study and harvested at late log phase. Furthermore, Renaud et al. (1999) reported similar protein content in *Chaetoceros* sp. from Queensland, also harvested at late log phase, to protein content of *Chaetoceros* sp reported here. Unlike the recent study by Khatoon et al. (2014), who reported that *Nannochloropsis* sp. and *Tetraselmis* sp contained higher protein content (ca. 45% of dry weight) at 30 psu when cultured under natural sunlight and harvested at logarithmic phase of growth, all Indonesian strains and *T. lutea* in this present study had lower protein contents at 30 psu. The protein content of *T. lutea* at 30 and 35 psu presented here at stationary phase is comparable to protein concentration of *Isochrysis galbana* reported by Fidalgo et al. (1998).

Carbohydrate contents in both Indonesian strains and *T. lutea* were also affected by the culture salinities. Among Indonesian strains, range of carbohydrate content was comparable to local strains from Australia as reported by Renaud et al (1999), but slightly lower than strains isolated from the South China Sea (Khatoon et al. 2014). Carbohydrate content of *T. lutea* presented here was comparable to *Isochrysis galbana*

reported by Fidalgo et al. (1998). Nevertheless, our study and other studies summarized by Mata et al. (2010), suggested that the differences in biochemical composition in microalgae were not only determined by culture medium and conditions such as salinity but also by the differences in strain that may be attributed to genetic differences. Therefore this finding is a promising result for exploring other local microalgal strains because there will be a chance to find several good strains for various purposes.

Our study showed that Indonesian microalgae contained similar DHA concentration with *T. lutea*. One strain of Indonesian microalgae, Kb1-3, contained ARA in higher concentration compared to other strains. EPA was higher in *T. lutea* compared to Indonesian microalgae but they contained almost complete profiles of fatty acids. This may be a factor that contributed to reasonable weight gain of shrimp larvae up to mysis stage when fed on Indonesian strains of microalgae (Chapter 4). These weight gains were comparable to a study by Piña et al (2006), although the survival of our shrimp was considerably lower. Survival rates of shrimp larvae fed Indonesian strains were similar to that of shrimp fed *C. neogracile* and *Te. chui*, with the exception of Kb1-2 (Chapter 4).

Shrimp larvae from the genus *Penaeus* and *Litopenaeus* require long-chain, polyunsaturated fatty acids, particularly those of C20 and C22: ω -3 and ω -6 compounds during metamorphosis to be able to survive this critical period and ω -3 continue to grow. Some species such as *P. japonicus* showed the ability to elongate and desaturate 18:3 ω -3 to produce long chain PUFA during larval stages (Castell, 1982; Teshima et al. 1992; Coutteau et al. 1996; D'Souza and Loneragan, 1999; Glencross, 2009). The level of these compounds in microalgae varies based upon culture conditions such as salinity,

although the results were contradictory as suggested by several studies. Several microalgal strains cultured at high salinity such as *Dunaliella* sp., *Nannochloropsis* sp., and *Nannochloropsis frustulum* showed low contents of polyunsaturated fatty acids (Xu and Beardall 1997; Hu and Gao 2006). Zhilla et al. (2011) suggested that salinity and growth phase may affect fatty-acid composition of microalgae in which polyunsaturated fatty acids were lower during the early phase of growth under high salinity but increased during late logarithmic and stationary phases. PUFA in *T. lutea* presented here was in agreement with findings of Ben-Amotz et al. (1985), who found that *Isochrysis* sp. cultured at high salinity contained high concentration polyunsaturated fatty-acid contents, but higher compared to Renaud and Parry (1994) who conversely found that these fatty acids in *Isochrysis* sp. decreased under high salinity culture conditions.

Another finding in the present study is the presence of Phthalic Acid in all Indonesian microalgal strains. Phthalic acid is a dicarboxylic acid that is usually found as an aerosol in the atmosphere (Mochida et al 2003) or as a product of diisobutyl phthalate (DiBP) hydrolysis catalised by the enzyme esterase (Ding et al, 2015). DiBP is one form of plasticizer phthalate esters (PAEs) that have become a public concern because of their effects on environmental contamination and toxicity in mammals (Ding et al, 2015). The C8 and C9 DCAs including phthalic acid have been proposed as oxidation products of unsaturated fatty acids such as oleic acid (Kawamura and Gagosian, 1987; Stephanou and Stratigakis, 1993). The presence of phthalic acid in all Indonesian microalgae may be an indicator that these strains were either able to hydrolyze DiBP by using specific enzymes or oxidation of unsaturated fatty acids. Conversely, presence of this compound could indicate sample contamination by plastic

components used in sample processing (e.g., pipet tips, tubes). Further study in this area will be an interesting topic to be explored in the future.

The present study showed that the proximate compositions of Indonesian microalgae were roughly comparable to *T. lutea* and that these isolates may have potential to be used as larval shrimp food. All essential fatty acids required by shrimp larvae, ARA, EPA and DHA, were present in Indonesian strains at 25 psu except for Kb1-3 which only ARA presented. Culturing Kb1-2 and Kb1-5 strains at 25 psu, therefore, may supply the necessary PUFA required for larval survival and growth at optimum cell density. Otherwise, the use of these Indonesian strains can be in conjunction with other species, such as *T. lutea*, that contain higher levels of essential PUFA especially EPA. Further study on the effect of other environmental factors, such as light intensity, temperature, and culture medium may reveal the best culture condition for Indonesian strains to yield optimal PUFA.

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TABLES

Table 1. Fatty acids concentration as percent of total fatty acids found in Indonesian microalgae strains and *T. lutea* at different salinity

Fatty Acids	Kb1-2				Kb1-3				Kb1-5				<i>T. lutea</i>			
	20	25	30	35	20	25	30	35	20	25	30	35	20	25	30	35
14:0	18.52	9.88	4.09	13.72		2.07	8.47	1.66	29.28	4.41	23.13	6.63	3.45	0.41	1.05	
15:0	0.15	1.08	0.16	1.47	0.61		0.80	2.67	4.02	0.97	4.16	0.43	1.12	0.99	1.00	
16:0	43.93	36.83	8.30	34.23	22.79	9.41	31.01	9.43	9.22	28.09	17.35	19.50	45.47	6.29	18.27	10.18
17:0		0.10		0.28	22.07	32.23	6.51	2.39	0.59	0.18	0.17	0.24			0.36	
18:0	14.67	12.16	12.22	10.42	11.01	4.02	17.20	5.48	4.26	12.26	7.43	8.88	15.27		5.53	3.65
20:0	0.27			0.73			0.20		0.59			0.39	12.40	16.09	20.69	23.53
21:0			14.58	4.73	11.90		2.68	7.13			0.33	0.21	1.42			0.59
22:0							0.75	4.25	0.18		2.77	3.23	1.05			
16:1 ω7	0.62		0.35	3.62				28.00	4.59	0.16	0.44					
16:1 ω9												0.12		0.40		0.70
18:1 ω9	0.38	0.44	0.18	3.08	0.75		0.35	1.45	2.25		0.45			0.41	0.88	0.57
20:1 ω9	0.88	2.09	0.39	0.45		1.35	2.52	1.41				3.11	3.66		2.01	0.56
18:2 ω6				0.57					0.27				2.89	28.58	21.96	26.72
18:3 ω3				1.12											0.44	0.49
20:2 ω6	0.21	0.16		0.09				1.50		1.75	0.27					
20:4 ω6		0.40		1.26	7.90	2.50	3.09	2.42	1.64	4.65	0.95	0.39				
20:5 ω3		1.23		0.09				1.10		11.51			1.31	6.14	10.88	13.16
22:6 ω3		0.85	0.09	3.73				3.50	1.48	2.20	1.46			1.85	4.79	4.27
Total Sufa	77.83	60.84	39.45	67.11	78.86	84.60	68.58	59.16	72.74	46.96	60.94	44.83	92.14	62.62	59.04	53.53
Tota Mufa	1.88	2.53	0.93	7.15	0.75	1.35	2.87	30.87	6.84	0.16	0.89	3.23	3.66	0.81	2.90	1.83
Total Pufa	0.21	2.64	0.09	6.85	7.90	2.50	3.09	8.51	3.39	20.11	2.68	0.39	4.20	36.57	38.06	44.64

Sufa= saturated fatty acids, Mufa= monounsaturated fatty acids, Pufa= polyunsaturated fatty acids

Table 2. Cell weight in pg.cell⁻¹, division rate (k) and final biomass in 10⁶ cells.mL⁻¹ of Indonesian microalgal strains and *T. lutea*. Values are mean $\pm$ SE from 4 replicates for k and 8 replicates for final biomass.

Strain/ Variables	Kb1-2 (<i>Chaetoceros sp.</i>)	Kb1-3	Kb1-5	<i>Ti.lutea</i>	p-value
Cell weight	22.1	20.7	15.6	21.8	<.0001
Biomass	1.6 $\pm$ 1.3 ^c	4.6 $\pm$ 2.6 ^a	2.5 $\pm$ 2.1 ^b	1.4 $\pm$ 0.7 ^c	<.0001
k	0.9 $\pm$ 0.04 ^{dc}	1.0 $\pm$ 0.07 ^{bcd}	1.4 $\pm$ 0.04 ^a	0.8 $\pm$ 0.03 ^d	<.0001

FIGURE LEGENDS

Figure 1. Proximate composition (% dry weight) of Indonesian microalgae (a=*Chateroceros* sp., b=Kb1-3, c=Kb1-5) and *T. lutea* (d) cultured at 20, 25, 30 and 35 psu. Bars indicate standard error (n=2). Bars with common letter for a particular component within a panel are not significantly different

Figure 2. Fatty acids distribution in Indonesian microlalgal strains in percent of total fatty acids (a=Kb1-2, b=Kb1-3, c=Kb1-5) and *T. lutea* (d) at different salinity (20, 25, 30 and 35 psu). Note that the y axes are not all on the same scale

Figure 1

a

b

c

d

Figure 2.

a

b

c

d

CHAPTER 4

Survival and growth of white shrimp (*Litopenaeus vannamei*) larvae fed newly isolated Indonesian strains of microalgae

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Abstract

Feeding experiments were conducted to investigate the ability of Indonesian strains of microalgae to support survival and growth of white shrimp (*L. vannamei*) larvae. Three Indonesian isolates (Kb1-2 identified as *Chaetoceros* sp., Kb1-3 and Kb1-5) and three standard aquaculture microalgal strains (*Chaetoceros neogracile*, *Tisochrysis lutea* and *Tetraselmis chuii*) were tested. Microalgae were cultured under natural, tropical conditions. Hatchery-reared shrimp larvae at the first protozoa stage (PZ_I) were distributed into 1-L Erlenmeyer flasks containing 250 mL of 0.2- μ m-filtered seawater in triplicate flasks and were fed single-species microalgal diets. After 8 days, more than 80 % of the shrimp larvae had metamorphosed to the mysis stage in all microalgal diets, except for Kb1-2 (*Chaetoceros* sp.) and the non-fed control which the larvae had all died on day 3 or 4 when most of them were still in protozoal stages. Survival of larval shrimp fed on Indonesian strains was similar to survival on *C. neogracile* and *Te. chuii*. Survival on *T. lutea* was highest among strains tested at the end of experiment. Higher final dry weight and weight gain were observed in larvae fed on Kb1-5, Kb1-3 and *C. neogracile* compared to the rest of the strains with similar ingestion rate and food conversion index (FCI) to *T. lutea*. Larvae fed on *Te.chuii* consistently had lower growth rate, final dry weight and weight gain. Indonesian microalgae may be suitable as food for white shrimp larvae, supporting performance similar to that of well-established strains; however, further studies with mixed algal diets may reveal greater performance than with any single strain alone.

Key words: white shrimp larvae, hatchery, microalgae, Indonesia

Introduction

Continuous and sustainable availability of microalgae is one critical factor in aquaculture hatcheries to ensure the production of robust and healthy larvae, including white shrimp. The demand for white shrimp larvae is increasing as the number of shrimp farms has grown, particularly in some countries such as India, Indonesia, and Ecuador (Nikolik and Heinhuis, 2015). These new farms are operated mainly to supply the high demand of shrimp products in local or international seafood markets. At the end of 2014, the US market alone imported 560,773 tons of shrimp, an increase of 12.6% from 2013 (Brown et al., 2015). Other large importers, such as Europe and Japan, are showing similar trends (Nikolik and Heinhuis, 2015), so there is an opportunity for shrimp hatcheries to produce shrimp larvae to support this increasing market demand.

Currently, hatchery practices worldwide still rely upon live microalgae cultured on site to feed early larval stages of white shrimp, despite some efforts to replace live microalgae with microencapsulated diets (Gallardo et al., 2002; Sanchez et al., 2012; Ma and Qin, 2012). Microalgae cultivated in shrimp hatcheries must maintain survival and growth, support metamorphosis, and provide natural color, as well as natural antibiotic activity for shrimp larvae to minimize the presence of pathogens that may affect the survival of larval shrimp (Iba et al., 2014). Sources of microalgal strains in shrimp hatcheries usually are well-established microalgal culture centers such as The *Commonwealth Scientific and Industrial Research Organization (CSIRO)* (<http://www.csiro.au>) in Australia (Martinez-Fernandez, 2007), Milford Laboratory (<http://www.nefsc.noaa.gov/nefsc/Milford/>) in United States of America (USA)

(Wikfors and Ohno, 2001), The Culture Collection for Algae and Protozoa (CCAP) (<http://www.ccap.ac.uk>) in Scotland, the Research Center for Oceanography, in Indonesia, UTEX Culture Collection of Algae (<https://www.utex.org>) in Texas, USA and National Center for Marine Algae and Microbiota (<https://ncma.bigelow.org/>), in Maine, USA. Alternatively, microalgae can be isolated from local, natural ecosystems. Established microalgal species from the culture centers have several advantages, such as known nutritional profiles and ease of culture. Known species of microalgae used in shellfish-hatchery aquaculture, including shrimp hatcheries, are *Isochrysis* (*Tisochrysis*) spp. *Thalassiosira weissflogii*, *Spirulina platensis*, *Tetraselmis* spp. and many *Chaetoceros* strains including *C. neogracile* and *C. calcitrans* (Hemaiswarya et al., 2011; Treece and Fox, 1993).

In some parts of the world, however, where shrimp hatcheries and farming exist, the supply of microalgal strains can be a problem. For example, in Southeast Sulawesi, Indonesia, hatcheries of white shrimp usually import microalgae from other regions of Indonesia. This practice poses several risks that include high cost, difficult availability, low productivity, and possible parasite contamination from handling and shipping. Importing microalgae from other regions also increases greenhouse gas (GHG) emissions during transportation (Edwards-Jones, 2010). To reduce both the cost and GHG emissions, the use of locally-isolated, cultured microalgae for local hatcheries may be considered. Locally-isolated microalgae with good growth and complete nutritional profiles will help small-scale hatcheries and farmers to increase the productivity of their hatcheries and farms and thus their incomes. Once the productivity increases, the farms will supply more shrimp to the markets that may decrease the

shrimp price and make them more affordable for local people. Finally, small-scale and local installations of microalgal culture will provide employment that can drive the economic growth of SE-Sulawesi and Indonesia in general. Therefore, in this study we investigated the possibility of using local isolates of microalgae from SE-Sulawesi in shrimp hatcheries by conducting a feeding study with white shrimp larvae fed newly isolated local microalgal strains.

Materials and Methods

Algal Culture

Three microalgal strains were isolated from Kendari Bay (3° 58' 56" S, 122° 35' 53.1" E) and Wanggu River Estuary (3°58'46.3"S, 122° 31' 54.4" E) South East Sulawesi, Indonesia. The microalgal strains are denoted as Kb1-2, Kb1-3 and Kb1-5. The strains have been maintained for more than one year in the laboratory at the University of Rhode Island, USA. The commonly used species *Tetraselmis chui*, *Tisochrysis lutea* and *Chaetoceros neogracile* were used for control comparison. The description of microalgal strains used in this feeding experiment is in Chapter 2. All microalgae were cultured semi-continuously in 1,000-mL Erlenmeyer flasks containing 600 mL artificial seawater f/2 medium at 29-30° C, at 30 and 35 psu salinity, with 30% daily harvests and replacement of the volume harvested with new medium. Higher salinity was chosen because previous growth trials with these strains showed better growth and yield at 30 and 35 psu (Chapter 2). Lighting was with natural, indirect sunlight in the range of 100–150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ PAR under natural photoperiod (approximately 12:12, light:dark cycle). Microalgal cultures were maintained at the

Laboratory of Fisheries University of Halu Oleo, Kendari, South East Sulawesi, Indonesia (4°00'28.10" S, 122° 31' 15.06" E).

Experimental Animals

A complete, randomized experimental design was applied in this research. The experimental design was based upon Nuñez et al (2002) and Piña et al (2006). All trial animals were hatchery-produced from the Situbondo Shrimp Development Center (SSDC), East Java, Indonesia. Larvae of *L. vannamei* were obtained during the naupliar stage (N_{IV} and N_V) and upon arrival were maintained at 29-30° C, salinity 30 psu, in 0.2-µm-filtered seawater in 10-L containers with abundant aeration at the Laboratory of Fisheries University of Halu Oleo, Kendari, South East Sulawesi, Indonesia. *Ti. lutea* was supplied twice a day to shrimp at a cell count of $1 \times 10^5 \text{ mL}^{-1}$ until the shrimp larvae reached the end of the naupliar stage (N_V) and underwent metamorphosis to protozoa I (PZ_I) stage (protocol based upon the Standard Operational Procedure/SOP at SSDC).

The experimental treatments were started when 50% of the larvae had reached PZ_I stage. Initial larval dry weights were measured by concentrating 3 replicates of approximately 25 larvae on tared GF/F microfiber filters, following the method used to obtain microalgal dry weight (Moheimani et al. 2012). On the same day, approximately 500 larvae were concentrated in 100 mL of seawater and distributed evenly into 20 sterile, 1,000-mL Erlenmeyer flasks containing 250 mL of sterilized seawater with gentle aeration, so that the larval density was at least 1 larva.10 mL⁻¹ (SSDC, 2015). Three replicates were employed for each microalgal strain tested. Two flasks were used as unfed controls without microalgae, and two flasks were used as microalgae-only

controls without shrimp. Temperature in all flasks was maintained by using continuous lighting with fluorescent lamps.

Larvae were fed twice daily at 7 am and 9 pm with increasing concentrations of algae at 1, 1.5, and 2 x 10⁵ cells mL⁻¹ for PZ_I, PZ_{II} and PZ_{III} stages, respectively. At feeding time, 4 mL of each algal strain at the appropriate density were centrifuged to remove culture medium and diluted with 4 mL 0.22-µm-filtered seawater before being given to the shrimp. Food ingestion was calculated based upon counts of microalgal cells in larval culture water 24 h after feeding. Specifically, 3 mL of water was sampled daily from each culture, and the number of cells was counted in duplicate by light microscopy using a Sedgwick-Rafter chamber. On this time, number of live shrimp larva was counted and, when possible, photographic images were taken. Observation of developmental stage of the shrimp was conducted every 24 h by examination of the larvae in culture flasks using a magnifying glass. Daily ration of feed was adjusted after 50% of larvae had reached the next developmental stage in at least three flasks of the feeding treatment. The experiment was terminated after more than 80% of the larvae had reached the first mysis stage (M_I) in one or more of the treatments. Final dry weight of shrimp was obtained from every replicate of each treatment after counting the larvae for final survival and subsequently filtering all of the surviving shrimps onto tared filter paper, dried at 100 °C for 1 h and weighing on an analytical balance.

Dry weight per cell of microalgae tested in this study was obtained from five replicate aliquots from microalgae-only control flasks during late logarithmic phase (8 days of culture) using methods described by Coutteau (1999). Microalgal density in 50 mL was determined using haemocytometer counts and weighing a known number of

cells on tared GF/F glass-fiber (diameter 47 μm) filters after washing with 0.65 M ammonium formate to remove salts. In addition, the same procedure was applied to triplicate control filters on which 50 mL of 0.22- μm filtered seawater was filtered. The filters were dried at 60 °C for 24 h to volatilize the ammonium formate and weighed on an analytical balance. The dry weight per algal cell was calculated using the following equation:

$$\text{DW (g. cell}^{-1}\text{)} = (\text{DW}_A - \text{DW}_C) \cdot (\text{N} \cdot \text{V})^{-1}, \text{ when:}$$

DW_A = average dry weight retained on algal filter (g), DW_C = average dry weight retained on control filter (g), N = algal density ($\text{cells} \cdot \text{mL}^{-1}$), V = volume of algal culture and filtered seawater filtered on algal and control filter, respectively (mL)

Shrimp growth was calculated using two methods: (1) as the percentage of increasing dry weight and (2) as specific growth rate (SGR) for eight days as follows:

$$\text{SGR} = \ln(W_t - W_0) \cdot t^{-1}, \text{ where:}$$

W_t = Shrimp dry weight at t day (8 days), W_0 = Initial dry weight of shrimp, t = days of experiment

The food conversion index (FCI) and gross growth efficiency (k_I) of shrimp larvae for each microalgae tested were calculated using equations from Piña et al (2006), based upon the respective mean values of dry weight of the individual food items, the mean values of ingestion, and the mean dry weight gain of the larvae, as follows:

$$\text{FCI} = \text{FS} \cdot \text{WG}^{-1} \text{ and } k_I = 100 \cdot \text{WG} \cdot \text{FI}^{-1}$$

where FS = food supplied (μg), FI = food ingested (μg) and WG = weight gain (μg) of dry weight.larva⁻¹

Statistical Analysis

The effects of different microalgae strains on shrimp growth, FCI and k_I were analyzed using one-way analysis of variance (ANOVA). Differences in shrimp survival were analyzed using mixed effect of repeated measure ANOVA. To meet ANOVA assumptions, percentage data were transformed using square root transformation. Significant differences in the data ($p < 0.05$) were analyzed with pair-wise multiple comparison of means (Tukey test). All statistical analyses were done in SAS Enterprise Guide 7.1

Results

Larvae of white shrimp survived and metamorphosed to the mysis stage on day 8 in all microalgae tested except for the Kb1-2 strain and the non-fed control (Figure 1). Microscope observation confirmed that Kb1-2 cultures were contaminated by *T. chui* and bacteria after four days of culture. On day 2 of the experiment, larvae fed the Kb1-2 were all dead in one replicate; all larvae in remaining replicates died on day 4 of the experiment when they were in PZ_{II}. Similarly, larvae in the non-fed control started to die on day 2 and subsequently were all dead on day 3 when still in PZ_I stage. Therefore, at those days, the larvae were filtered, dried and weighed to obtain the larval dry weight for these treatments. These two treatments were excluded from subsequent ANOVA analysis for survival and growth. Similar final survival was observed in white shrimp larvae fed on Indonesian microalgae strain (except Kb1-2) to *C. neogracile* and *Te. chui* that ranging from 7-20 %. *T. lutea* produced the highest larval survival among microalgae tested at 31 % (Fig. 1, Table 2).

Higher final dry weight was found in larvae fed on Indonesian strains Kb1-3 and Kb1-5 (45.1 and 70.8 $\mu\text{g.larvae}^{-1}$, respectively) that was similar to *C. neogracile* at 50.6 $\mu\text{g.larvae}^{-1}$. Shrimp larvae fed on Kb1-3 showed a similar final dry weight to *T. lutea* and *Te. chui* (21.7 and 28.4 $\mu\text{g.larvae}^{-1}$, respectively (Table 1). Weight gain and percentage of increase of weight of white shrimp larvae were significantly affected by microalgae that they had been fed. Shrimp larvae fed on one Indonesian strain, Kb1-5, had a similar percentage of total increase in weight and weight gain (108 % and 64 $\mu\text{g.larvae}^{-1}$, respectively) to those fed on *C. neogracile* (69 % and 43 $\mu\text{g.larvae}^{-1}$, respectively) but higher compared to *T. lutea* (39 % and 21 $\mu\text{g.larvae}^{-1}$) and *Te. Chui* (37 % and 21 $\mu\text{g.larvae}^{-1}$). Another Indonesian strain, Kb1-3, had a similar percentage of total increase in weight and weight gain to those fed on there other extensively use strains in aquaculture, *C. neogracile*, *T. lutea* and *Te. Chui*. Consequently, they had higher gross growth efficiency, growth rates and lower FCI ($p<.0001$, $p<.0001$ and $p=0.012$, respectively, Tables 1, 2 and 3).

Although shrimp larvae fed on Kb1-5 had a similar percentage of ingested food to *Te. chui* at 51 and 62 %, respectively, those larvae fed on *Te. chui* consistently had he highest FCI (53) and the lowest k_I (16) among larvae fed on other microalgae tested. Larval shrimp fed on *Te. chui* and *T. lutea* had a similar growth rate at 0.16 and 0.15 $\mu\text{g.larvae}^{-1}.\text{day}^{-1}$, respectively, being the lowest among larvae fed on other strains tested. Feeding on *Te. chui*, however, resulted in similar final dry weight and weight gain to those fed on *T. lutea* and Kb1-3 (Table 1 and 3).

Discussion

The performance of Indonesian microalgae in this study was similar to those of species that are used extensively for aquaculture hatcheries, except for the Kb1-2 strain. Bacterial and cross contamination may have interfered with feeding results with this strain, as massive bacterial contamination was observed under the microscope. The presence of bacteria in diatom feed cultures is common, and the relationship can be parasitic or symbiotic (Schaefer, 2001; Grossart et al, 2005). Mortality of larvae before reaching the end of the protozoa stage when fed the Kb1-2 strain suggested that bacteria present in the culture may have been harmful to the larvae, although several studies have found that diatom and bacterial interactions may support water quality beneficial for growth of shrimp in biofloc systems (Emerenciano et al, 2012; Hargreaves, 2013; Sakkaravarthi, 2015).

Survival of white shrimp larvae fed on Indonesian microalgal strains in this study was not significantly different *C. neogracile* and *Te.chui*. Survival of larvae was high (>90%) during early stages of protozoa and decreased as the larvae metamorphosed into later stages. This phenomenon is common in the shrimp larval life cycle (Rodriguez et al., 2012, Kiametha et al., 2011, Okauchi and Okuda, 2003). Metamorphosis is considered a critical time for larvae and therefore the feeding rate needs to be adjusted to meet larval nutrition requirements to support larval growth and survival. Also, maintaining water quality at optimal level during larval culture will aid the metamorphosis process and thus increase the chance of larval survival (Trece and Fox, 1993). Larvae fed Indonesian microalgal strains showed final survival ranging from 7-11 %, statistically similar to survival on extensively-used microalgal species

(13-20%) except for *T. lutea*. This larvae survival on Indonesian strains of microalgae can be considered relatively low compared to survival reported in the study by Piña et al. (2006) which was 50% on single algal species of Tahitian *Isochrysis* or TISO (which is *T. lutea* per Bendif, 2013), *Tetraselmis suecica*, or *Chaetoceros muelleri*. However, our result with a 32 % survival of larvae fed on *T. lutea* was comparable. Nuñez et al (2002) studied the protozoal stage of white shrimp with higher larval density and without water exchange, and reported PZ_{III} survival between 66-76%. By contrast, survival in "normal" hatchery operation has been reported to be 70-90% with 100-150 shrimp.L⁻¹ with water exchange (SSDC, 2015; Trece and Fox, 1993). Jamali et al (2015) showed that survival of PZ_{III} stage of white shrimp larvae fed a single diet of microalgae *I. galbana*, *Tetraselmis tetrahele*, or *C. muelleri* was 72-79%; whereas, the present study showed lower survival at that stage (4-60%).

Overall survival in the present study was comparable to results reported by Kiametha et al (2011), which was less than 30% when white shrimp larvae were fed *C. gracilis*. High variation in survival can be attributed to several factors, including sufficient quantity of microalgae supplied, water quality, suitable cell size, disease and appropriate nutrition in the algae to support growth and development of white shrimp larvae (Brown et al., 1997; Okauchi and Tokuda; 2003; Piña et al., 2006; Jamali et al., 2015). In this study we observed that water in most of shrimp larvae culture flasks was constantly in green and brown color suggesting that food availability might not be a limiting factor to survival. Low survival may be a result of interaction between poor digestion and bacterial infection due to non-water exchange system during the experiment although further clinical study is needed to confirm this. We observed that

larvae were able to ingest the microalgae supplied but they seemed to be unable to digest and thus convert these algae into metabolites substances that can be absorbed to support their survival.

Indonesian microalgal strains tested in this study were ingested at similar rates to those of well-known strains by white shrimp larvae. Larvae fed Indonesian strains, however, exhibited higher weight gains comparable to larvae fed *C. neogracile*, suggesting that the larvae were able to utilize the food for growth but not enough for survival. Although *T. lutea* is considered a good single-algal species to be used in shrimp hatcheries and is readily ingested by white shrimp juveniles due to its good nutritional profile and appropriate cell size (Iba et al., 2014; Hemaiswarya et al., 2011), this study showed that this species is inferior in terms of enhancing growth compared to *C. neogracile* and Indonesian strains Kb1-3 and Kb1-5. This finding confirmed the study by Pina et al (2006) who found that white shrimp larvae fed on *Isochrysis* sp. alone resulted in lower final weight of 27.11 $\mu\text{g.larvae}^{-1}$ compared to those fed on single diet of *C. muelleri* at 48.28 $\mu\text{g.larvae}^{-1}$. Therefore, considering no water exchange during larval culture, it is suggested that growth and survival of larvae in this study maybe correlated to the presence of bacteria in both algae and larvae culture. We observed during this experiment, the formation of aggregates at the bottom of the flask that may contained bacteria and remnant of microalgae. Hargreaves (2013) stated that aggregates (flocs) of algae, bacteria, protozoans, and other kinds of particulate organic matter such as feces and uneaten feed in green water microalgae system in shrimp culture may improve growth and survival of the species during grow out stages. However, the effect of bioflocs on larval stages during hatchery phase is not investigated

yet. Kb1-3, Kb1-5 and *C. neogracile* may have appropriate nutrition, cell size and possibly harbored a ‘good’ bacterial assemblages that supported the digestion of the cells, thus promoted the growth of shrimp larvae, but some such as Kb1-2, *T. lutea* and *Te.chui* may not have, resulting in poor growth of the larvae.

Dry weight gain of surviving larvae fed Indonesian strains showed comparable performance to weight gain on a well-known microalgal species, *C. neogracile*. Consequently, white shrimp larvae fed on Indonesian strains showed similar growth rates to those fed on *C. neogracile* which was not the case for *T. lutea* and *Te. chui*. This finding confirms similar studies by Nuñez et al (2002), who used a native microalgal strain (two strains of *Chaetoceros* sp.) from Venezuela and also supports numerous other studies that microalgae from the Class Bacillariophyceae are still preferred as food for shrimp larvae regardless of the source of the isolates (Nuñez et al, 2002; Okauchi and Okuda, 2003; Piña et al; 2006, Kiametha et al., 2011; Kent et al., 2011). Food conversion index of shrimp larvae was not significantly different among strains of microalgae tested, except for *Te. chui* which had the lowest FCI of other algal diets. This suggests that shrimp larvae may not be able to ingest the cells of *Te. chui* properly, resulting in lower weight gain per larva compared to other treatments. Other studies have shown that microalgae from the genus *Tetraselmis* may not be adequate for white shrimp larvae (Piña et al. 2006, Rodriguez et al. 2012, Jamali et al. 2015). Although *Chaetoceros* strains showed lower performance as feed of white shrimp compared to another diatom species, *T. weissflogii*, as reported by Kiametha et al. (2011), our study showed that Indonesian strains were comparable to *C. neogracile* as feed for white shrimp larvae and confirms previous studies finding that *Chaetoceros*

strains, such as *C. muelleri*, are suitable to feed early stages of shrimp larvae (Piña et al, 2006, Rodriguez et al, 2012, Jamali et al, 2015). Studies by Kiametha et al. (2011) and Kent et al (2011) showed that *T. weissflogii* was readily ingested and digested by shrimp larvae, resulting in higher growth and survival compared to *Chaetoceros* strains. Nevertheless, our study showed that Indonesian microalgae strains, *T. lutea*, and *C. neogracile* were able to support growth of shrimp larvae up to mysis stage in the absence of *T. weissflogii*.

This study showed that local Indonesian microalgal strains have potential to be used for larval rearing of white shrimp, at least up to the mysis stage. However, further study of the effects of mixed diets of Indonesian microalgal strains with well-known microalgal species upon larval survival, growth, and metamorphosis is recommended. We recommend experiments at commercial scale with water exchange to mimic the common practice in commercial hatcheries. Although longer term and larger commercial scale experiments may be needed to employ these newly isolated strains into aquaculture, this study is useful as an initial step to support aquaculture industries as well as to support algal research and its wider applications. Finally, the roles of water quality and bacterial assemblages in microalgal culture to support shrimp larval growth and survival in culture practice without water exchange are also interesting to be explored in the future.

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TABLES

Table 1. Initial and final dry weight (DW) of larvae *Litopenaeus vannamei* in $\mu\text{g.larvae}^{-1}$ fed with Indonesian microalgae strains, *C. neogracile*, *T. lutea* and *Te. chui* during protozoa stages. Standard errors (SE) about the mean are for 3 replicates for all strains. Numbers with same superscript letters indicate lack of significant differences ($p=0.05$, Tukey test)

Strain	Initial DW $\pm$ SE	Final DW $\pm$ SE
Kb1-3	6.8 ± 0.3	$45.1. \pm 1^{\text{ab}}$
Kb1-5	6.8 ± 0.3	$70.8 \pm 5^{\text{a}}$
<i>C. neogracile</i>	6.8 ± 0.3	$50.6 \pm 12^{\text{a}}$
<i>T. lutea</i>	6.8 ± 0.3	$21.7 \pm 8^{\text{b}}$
<i>Te. chui</i>	6.8 ± 0.3	$28.4 \pm 6^{\text{b}}$

Table 2. Microalgal cells dry weight in pg.cell^{-1} , final survival (mean $\pm$ SE), percentage of weight gain (PerWG.larvae $^{-1}$, mean $\pm$ SE) in % after 8 days of feeding experiment, and growth rate (SGR, mean $\pm$ SE) in $\mu\text{g.larvae}^{-1}.\text{day}^{-1}$ of white shrimp larvae fed on Indonesian microalgae strains, *C. neogracile*, *T. lutea* and *Te. chui* during protozoa stages. Numbers with same superscript letters in column indicate lack of significant differences (one way ANOVA, $p=0.05$ and Tukey test)

Strain	Dry weight	Final survival	Percent WG	SGR
Kb1-3	20.7	$11 \pm 1^{\text{ab}}$	$70 \pm 6^{\text{a}}$	$0.23 \pm 0.003^{\text{a}}$
Kb1-5	15.6	$7 \pm 2^{\text{b}}$	$108 \pm 5^{\text{a}}$	$0.29 \pm 0.002^{\text{a}}$
<i>C. neogracile</i>	30.8	$13 \pm 9^{\text{ab}}$	$69 \pm 20^{\text{a}}$	$0.23 \pm 0.003^{\text{a}}$
<i>T. lutea</i>	21.8	$31 \pm 12^{\text{a}}$	$39 \pm 12^{\text{b}}$	$0.15 \pm 0.003^{\text{b}}$
<i>Te. chui</i>	94.2	$20 \pm 9^{\text{ab}}$	$37 \pm 10^{\text{b}}$	$0.16 \pm 0.004^{\text{b}}$

Table 3. Food supplied (FS), food ingested (FI), weight gain (WG), food conversion index (FCI) and gross growth efficiency (k_I) of individual white shrimp larvae fed four Indonesian microalgae strains and three control species. Numbers with same superscript letters in column indicate lack of significant differences (one way ANOVA, $p=0.05$ and Tukey test)

Microalgae strains	FS		FI			WG $\pm$ SE	FCI $\pm$ SE	$k_I \pm$ SE
	N	DW	N	DW	% $\pm$ SE			
Kb1-3	8.8	182	4	93	51 $\pm$ 1 ^b	39 $\pm$ 5 ^{ab}	5 $\pm$ 0.1 ^a	43 $\pm$ 6 ^b
			5					
Kb1-5	8.8	137	5	84	61 $\pm$ 3 ^a	64 $\pm$ 4 ^a	2 $\pm$ 0.3 ^a	46 $\pm$ 6 ^a
			4					
<i>C. neogracile</i>	8.8	271	4	144	53 $\pm$ 0 ^b	43 $\pm$ 11 ^a	7 $\pm$ 31 ^a	30 $\pm$ 7 ^{bc}
			7					
<i>T. lutea</i>	8.8	192	4	98	51 $\pm$ 0 ^b	21 $\pm$ 7 ^b	18 $\pm$ 3 ^a	22 $\pm$ 8 ^c
			5					
<i>Te. chui</i>	8.8	829	5	514	62 $\pm$ 0 ^a	21 $\pm$ 5 ^b	53 $\pm$ 10 ^b	16 $\pm$ 1 ^d
			5					

N=total amount of microalgae cells supplied (FS) in 10⁶.larvae⁻¹ and in μ g.larvae⁻¹, ingested (FI) in 10⁶.larvae⁻¹ and in %, DW=Dry weight of algae from total cells supplied and ingested in μ g.larvae⁻¹, WG in μ g.larvae⁻¹ calculated as the mean of three replicates at the end of experiment.

FIGURE LEGENDS

Figure 1. Survival of the shrimp larvae over the eight-day experiment. Standard errors (SE) about the mean are for 3 replicates except for unfed-control that is for 2 replicates. SE are indicated by error bars symbol.

Figure 1

CHAPTER 5

DISCUSSION

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DISCUSSION

The use of live microalgae in aquaculture hatcheries, is very important for normal growth and development of the larvae, and so far has remained irreplaceable. Although some forms of preserved microalgae, i.e. frozen, dried, powder, have been explored for use for the rearing of larval stages of aquaculture species, they still cannot fully replace the living forms of microalgae (Gallardo et al. 2002; Sanchez et al. 2012; Ma and Qin, 2012; Arney et al. 2015; Gui et al. 2015; Southgate et al. 2016). We know that well-established microalgal strains that have been used in aquaculture belong to sixteen genera (Gopakumar and Ignatius, 2006) and out of them, only seven genera/strains have been used for shrimp aquaculture (Hemaiswarya et al. 2011). Isolation and use of local microalgal strains had been explored for their potential for use in local aquaculture operations (Renaud et al. 1991; Brown et al. 1997; Borowitzka, 1997; Nuñez et al. 2002). In shrimp hatcheries, live microalgae are used to feed larval stages from protozoa up to mysis stages and usually are cultured on site in the hatchery. Therefore, the availability of starter culture and subsequently the continuous or semi continuous microalgal culture in hatchery is very critical. Worldwide, widely known and used microalgae strains usually come from well-established hatchery, marine laboratory and algae centers, then shipped to the local hatchery to be scaled up and finally fed them to shrimp larvae.

Indonesia with its largest archipelagic seas of any country in the world with varies marine microhabitats provides a good opportunity for bioprospecting for new strains of microalgae that may be useful as live feeds for aquaculture. South East (SE) Sulawesi province on Sulawesi Island is one such location in Indonesia with the

potential to be explored as source of microalgal isolates for aquaculture because of its abundant marine resources in form of bays and coastal waters. Availability of local isolates that meet nutritional requirements of aquaculture species will reduce production cost and ensure that exotic strains that may harbor unknown bacteria and parasites will not enter local waters. This dissertation sought to explore these potential by: a) isolating, culturing, and partially characterizing algal strains from the Kendari region of Southeast Sulawesi; b) characterizing nutritional content of the new Indonesian microalgal isolates in varied culture environments and c) growing these newly isolated microalgae strains from SE-Sulawesi under controlled nutrient conditions and feeding those strains to white shrimp larvae (*Litopenaues vannamei*) as a model.

Previous studies have shown that environmental factors regulating the growth and production of microalgae under controlled conditions include light intensity, salinity, temperature and composition and concentration of nutrients in culture medium (Renaud and Parry, 1994; Borowitzka, 1997; Renaud et al. 1999; Hu, 2004). Salinity is considered to be a critical factor affecting microalgal growth and its effects are dependent upon the strain. Two groups of microalgal strains respond to high salinity are halophilic species require high salinity for optimum growth, and halotolerant strains have the ability to survive in saline medium but may have lower salinity optima (Rao et al. 2007). The ability of strains to thrive in highly variable salinity conditions while producing optimum nutrition is desirable for aquaculture and evidence has shown that some newly isolated microalgae possess those traits (Lee and Kim, 2002; Khatoon et al. 2014 Ruangsomboon et al. 2013; Borowitzka, 2013)

The results from microalgal growth experiments presented here show that Indonesian microalgae had similar growth across low and high salinity level suggesting that they are euryhaline or halotolerant species. Their growth is most likely limited by other environmental factors such as light intensity, temperature and culture nutrients, which were not addressed in this work (Hu et al. 1998; Lee and Kim, 2002; Wood, 2005; Rynearson et al. 2009; Whittaker et al. 2012). Differences in final cell density, thus dry weight and ash free dry weight, may be driven by cell size, cell density at inoculation and harvest time. Richmond (2004) stated that output rate, i.e. yield of a microalgal culture, using *Spirulina* as a model, changed over time, becoming lower as the growth rate decreased during stationary phase of the culture cycle. In this study, Kb1-5 with the smallest cell sizes and the lowest weight per cell among all strains, yet with higher inoculated cell numbers, showed the highest cell numbers over the culture period, but with considerably lower dry weight and ash free dry weight at the end of experiment. Conversely, *T. chui* with its larger cell sizes, higher cell weight and lower inoculation numbers exhibited the lowest cell numbers on all days of culture, but consistently exhibited higher dry weights across all salinities tested, except at high 35 psu.

Appropriate cell size and adequate nutrition are important microalgal traits that determine their use in aquaculture (Milamena, 1996; Brown, 2002; Becker, 2004; Nuñez et al. 2002). The results presented here showed that although the cell size of Indonesian microalgal strains was different depending on salinity, cell size variation is still within range desirable for larval shrimps (1.2-11.8 μm , considered as either pico or nanoplankton). Feeding experiments with white shrimp larvae fed on Indonesian microalgal strains showed that two Indonesian strains, Kb1-3 and Kb1-5, were ingested

at rates similar to *Tiostrea lutea*, a widely used species in aquaculture hatcheries. Indonesian strains also showed a comparable weight gain and survival to *Chaetoceros neogracile* and *Tetraselmis chui* suggested that these strains were digested and utilized by white shrimp larvae for growth.

Growth and survival of white shrimp were known to be affected by the nutrition content of microalgae as their food (Coutteau, 1996; Nuñez et al. 2002; Brown, 2002; Jamali et al. 2015). It is known that the nutrition of microalgae is varied depend on the strains as well as their culture condition such as temperature, light intensity, culture medium and salinity (Renaud and Parry, 1994; Renaud et al. 1999; Brown, 2002; de Castro Araujo et al. 2005; Khatoon et al. 2010; Zhila et al. 2011; Ruangsomboon et al. 2013; Khatoon et al. 2014). The results from the microalgal nutrition experiments presented here showed that proximate composition of Indonesian microalgae is affected by salinity with variation showed within the individual strains. The nutritional content of Indonesian microalgae, as well as *T. lutea*, during stationary phase contained protein higher than required by shrimp larvae (ca. 25%), with comparable lipid and carbohydrate contents (around 10 and 5%, respectively), as suggested by Tobias-Quinito and Villegas (1982).

The results presented here also showed that Indonesian microalgae contained polyunsaturated fatty acids (PUFA), ω -3, eicosapentaenoic acid and docosahexaenoic acid, (EPA and DHA) and ω -6, arachidonic acid (ARA), at 25 and 35 psu and were not all detected other culture salinities tested. Indonesian microalgae had a similar DHA concentration with *T. lutea* but higher ARA concentration. ARA was not detected at all salinities tested in *T. lutea* in this study but it had a higher EPA concentration compared

to Indonesian microalgal strains. This may be a factor that contributed to reasonable weight gain of shrimp larvae up to mysis stage when feeding on Indonesian strains of microalgae as showed in our feeding experiments. These weight gains in shrimp was comparable to shrimp in the study by Piña et al. (2006) although the survival was considerably lower. However, survival rate of shrimp larvae feed on two Indonesian strains was similar to the control cultures of *C. neogracile* and *Te. chui*. Nevertheless, these results and other results in studies summarized by Mata et al. (2010) suggested that the differences in biochemical composition in microalgae is not only determined by culture salinity, but also by the differences in strains that may be attributed to genetic differences. Therefore this is a promising result for exploring other local microalgal strains because there will be a chance to find several good strains for broad applications.

Another interesting results in microalgal nutrition experiment is the presence of phthalic acid in all Indonesian microalgae strains. Phthalic acid is a dicarboxylic acid that usually found as aerosol in the atmosphere (Mochida et al. 2003) or as a product of diisobutyl phthalate (DiBP) hydrolysis through enzyme esterase (Ding et al. 2015). DiBP is one form of plasticizer phthalate esters (PAEs) that have become a public concern because of their effects on environmental contamination and toxicity on mammals (Ding et al. 2015). The C8 and C9 DCAs include phthalic acid have been proposed as oxidation products of unsaturated fatty acids such as oleic acid (Kawamura and Gagosian, 1987; Stephanou and Stratigakis, 1993). The presence of phthalic acid in all Indonesian microalgae may be an indicator that these strains were either able to hydrolyze DiBP by using specific enzymes or they were formed as a result of routine housekeeping activity of oxidizing unsaturated fatty acids.

There has been a growing interest in Indonesia to find and develop local microalgal strains that maybe used for broad applications beyond aquaculture. The main bottleneck for this research in Indonesia is lack of expertise and available facilities. Training of individuals to be an experts in this area may help to better utilize local microalgal resources in support existing industries and at the same time open new opportunities to develop new industries to grow both the local and national economies. This work confirmed that local strains are potential to be utilized for white shrimp hatcheries based upon their appropriate cells size, robust growth under wide salinity regimes and considerably good nutrition values in terms of macro molecules, protein, lipid and carbohydrate. Furthermore, culturing the local microalgae strains at salinity 35 psu for Kb1-2 and Kb1-3 and at 25 psu for Kb1-5 will ensure that the yield will be optimal while maintaining their good nutritional values as feed for shrimp larvae. The use of these strains in white shrimp hatcheries can be in conjunction with other widely used species in aquaculture such as *T. lutea* that had higher concentration of EPA when cultured in the condition described in this study. Also, establishing repository for local microalgae in Indonesia, particularly in Kendari where the microalgal samples in this study was obtained, will be necessary to ensure continuous supply of high quality microalgal strains to the local hatcheries as well as to provide other researchers for broader application of microalgae.

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APPENDIX

A. Cells density of Indonesian microalgal strains *C. neogracile*, *T.chui* and *Ti.lutea* cultured at different salinity over 15 days culture period

Strain	Salinity (psu)	Cells density (cells·mL·10 ⁵) at days of culture						
		0	3	6	8	10	13	15
Kb1-2	20	0.6	2.7	9.7	10.9	15.1	18.0	27.7
Kb1-2	25	0.6	2.1	7.0	7.1	6.2	6.6	5.3
Kb1-2	30	0.6	2.2	7.2	8.2	8.2	14.9	20.9
Kb1-2	35	0.6	1.7	5.7	7.9	14.3	15.3	18.4
Kb1-3	20	0.2	0.5	5.0	12.5	25.8	34.6	39.4
Kb1-3	25	0.2	0.8	5.7	14.7	28.2	41.1	47.0
Kb1-3	30	0.3	0.7	5.4	14.1	28.1	34.7	40.6
Kb1-3	35	0.3	0.5	5.9	17.5	35.0	63.4	65.9
Kb1-5	20	1.3	8.2	21.5	11.9	15.7	39.4	27.5
Kb1-5	25	0.9	7.2	19.1	42.4	28.7	13.4	18.2
Kb1-5	30	1.2	6.7	39.7	16.9	7.5	15.7	9.4
Kb1-5	35	0.8	6.1	15.9	36.6	32.3	24.0	26.7
<i>C. neogracile</i>	20	0.0	0.1	0.7	2.4	5.5	9.4	11.1
<i>C. neogracile</i>	25	0.0	0.2	0.8	2.3	5.2	9.0	11.0
<i>C. neogracile</i>	30	0.0	0.1	0.8	2.1	5.6	9.5	11.3
<i>C. neogracile</i>	35	0.1	0.1	0.8	3.0	6.1	11.1	13.0
<i>T. chui</i>	20	0.1	0.1	0.5	1.0	2.4	4.3	4.5
<i>T. chui</i>	25	0.1	0.0	0.7	1.5	1.9	3.0	3.8
<i>T. chui</i>	30	0.1	0.0	0.6	3.5	3.5	5.7	6.1
<i>T. chui</i>	35	0.1	0.1	0.8	2.2	4.3	6.5	7.8
<i>Ti. lutea</i>	20	0.7	0.7	1.4	3.6	11.3	10.6	11.2
<i>Ti. lutea</i>	25	0.7	0.7	1.3	3.8	10.6	14.3	15.2
<i>Ti. lutea</i>	30	0.7	0.8	1.4	4.1	11.6	13.8	12.5
<i>Ti. lutea</i>	35	0.7	0.7	1.1	2.2	6.7	16.9	15.4

B. Fluorescence and cells density of Kb1-2 strain

Day	Salinity	Replicate	Fluorescence	Cells.mL $\cdot 10^{-5}$
1	20	1	8.665	0.6
		2	8.61	0.6
		3	8.036	0.6
		4	8.557	0.6
	25	1	9.058	0.6
		2	9.282	0.7
		3	9.529	0.7
		4	8.672	0.6
	30	1	7.894	0.6
		2	9.735	0.7
		3	9.068	0.6
		4	9.715	0.7
	35	1	7.755	0.5
		2	7.841	0.5
		3	9.399	0.7
		4	9.442	0.7
3	20	1	42.61	3.0
		2	35.33	2.5
		3	39.86	2.8
		4	35.61	2.5
	25	1	29.18	2.0
		2	31.15	2.2
		3	32.51	2.3
		4	28.09	2.0
	30	1	25.66	1.8
		2	34.28	2.4
		3	31.24	2.2
		4	36.27	2.5
	35	1	21.82	1.5
		2	20.97	1.5
		3	27.62	1.9
		4	24.77	1.7
6	20	1	136.5	9.6
		2	137.4	9.6
		3	170	11.9
		4	108	7.6
	25	1	111	7.8
		2	100.3	7.0

		3	74.2	5.2
		4	114.1	8.0
	30	1	100.1	7.0
		2	112.1	7.9
		3	88.76	6.2
		4	110.4	7.7
	35	1	67.9	4.8
		2	64.44	4.5
		3	91.4	6.4
		4	102.8	7.2
8	20	1	191.5	13.4
		2	136.8	9.6
		3	136	9.5
		4	160	11.2
	25	1	124.8	8.7
		2	99.58	7.0
		3	67.07	4.7
		4	113.4	7.9
	30	1	118.1	8.3
		2	110.3	7.7
		3	108.6	7.6
		4	129.9	9.1
	35	1	108.5	7.6
		2	95.54	6.7
		3	119.2	8.4
		4	128.1	9.0
10	20	1	157.1	11.0
		2	178.2	12.5
		3	291.1	20.4
		4	234.1	16.4
	25	1	94.41	6.6
		2	86	6.0
		3	90.16	6.3
		4	85.27	6.0
	30	1	164.2	11.5
		2	198.4	13.9
		3	203.8	14.3
		4	194.2	13.6
	35	1	188.3	13.2
		2	182.6	12.8
		3	230	16.1

		4	212.9	14.9
13	20	1	270.5	18.9
		2	277.1	19.4
		3	243.8	17.1
		4	235.6	16.5
	25	1	90.25	6.3
		2	96.58	6.8
		3	111.2	7.8
		4	77.23	5.4
	30	1	223.2	15.6
		2	202.3	14.2
		3	226.1	15.8
		4	199.8	14.0
	35	1	206.9	14.5
		2	195.8	13.7
		3	245.2	17.2
		4	223.9	15.7
15	20	1	385	27.0
		2	339	23.7
		3	412	28.9
		4	445.5	31.2
	25	1	64.16	4.5
		2	75.03	5.3
		3	101.5	7.1
		4	59.97	4.2
	30	1	315.9	22.1
		2	245.2	17.2
		3	294.9	20.7
		4	338.4	23.7
	35	1	241.4	16.9
		2	321.75	22.5
		3	227.9	16.0
		4	257.3	18.0

C. Fluorescence and cells density of Kb1-3 strain

Day	Salinity	Replicate	Fluorescence	Cells.mL $\cdot 10^{-5}$
1	20	1	0.707	0.2
		2	0.776	0.2
		3	0.742	0.2
		4	0.85	0.3
	25	1	0.726	0.2
		2	0.725	0.2
		3	0.793	0.2
		4	0.766	0.2
	30	1	0.709	0.2
		2	0.921	0.3
		3	0.872	0.3
		4	0.855	0.3
	35	1	0.916	0.3
		2	0.919	0.3
		3	0.931	0.3
		4	0.997	0.4
3	20	1	1.348	0.6
		2	1.291	0.6
		3	1.26	0.5
		4	1.092	0.4
	25	1	1.092	0.4
		2	1.131	0.5
		3	2.318	1.2
		4	2.089	1.1
	30	1	1.676	0.8
		2	1.251	0.5
		3	1.447	0.7
		4	1.421	0.6
	35	1	1.097	0.4
		2	1.39	0.6
		3	1.385	0.6
		4	1.127	0.4
6	20	1	3.033	1.7
		2	7.429	4.4
		3	15.6	9.6
		4	7.495	4.5
	25	1	2.047	1.0
		2	9.022	5.4
		3	12.96	7.9

		4	13.54	8.3
	30	1	10.43	6.3
		2	4.866	2.8
		3	12.91	7.9
		4	7.657	4.6
	35	1	10.62	6.5
		2	12.29	7.5
		3	8.462	5.1
		4	7.724	4.6
8	20	1	4.771	2.8
	20	2	17.5	10.8
	20	3	38.32	24.0
	20	4	19.85	12.3
	25	1	8.088	4.9
	25	2	21.57	13.4
	25	3	32.22	20.1
	25	4	32.93	20.6
	30	1	24.27	15.1
	30	2	13	8.0
	30	3	33.33	20.8
	30	4	19.96	12.4
	35	1	30.84	19.3
	35	2	33.73	21.1
	35	3	23.21	14.4
	35	4	24.24	15.1
10	20	1	10.32	6.3
	20	2	35.46	22.2
	20	3	77.22	48.6
	20	4	42.04	26.3
	25	1	18.56	11.5
	25	2	44.11	27.7
	25	3	56.12	35.3
	25	4	60.84	38.2
	30	1	49.22	30.9
	30	2	24.31	15.1
	30	3	67.87	42.7
	30	4	37.56	23.5
	35	1	62.59	39.3
	35	2	64.63	40.6
	35	3	45.95	28.8
	35	4	49.46	31.0

13	20	1	33.54	21.0
	20	2	63.45	39.9
	20	3	86.16	54.3
	20	4	37.26	23.3
	25	1	53.45	33.6
	25	2	85.5	53.8
	25	3	55.27	34.7
	25	4	67.22	42.3
	30	1	48.1	30.2
	30	2	55.34	34.8
	30	3	52.83	33.2
	30	4	64.4	40.5
	35	1	96.41	60.8
	35	2	116.6	73.5
	35	3	87.99	55.4
	35	4	101.6	64.0
15	20	1	68.96	43.4
	20	2	53.03	33.3
	20	3	84.19	53.0
	20	4	44.45	27.9
	25	1	76.16	47.9
	25	2	78.82	49.6
	25	3	67.58	42.5
	25	4	76.36	48.1
	30	1	49.01	30.8
	30	2	71.93	45.3
	30	3	65.2	41.0
	30	4	72.21	45.4
	35	1	76.28	48.0
	35	2	125.2	79.0
	35	3	100	63.0
	35	4	116.6	73.5

D. Fluorescence and cells density of Kb1-5 strain

Day	Salinity	Replicate	Fluorescence	Cells.mL $\cdot 10^{-5}$
1	20	1	6.398	1.0
		2	7.631	1.2
		3	9.314	1.5
		4	8.812	1.4
	25	1	5.313	0.8
		2	4.983	0.8
		3	8.336	1.3
		4	5.021	0.8
	30	1	7.46	1.2
		2	7.543	1.2
		3	7.528	1.2
		4	7.623	1.2
	35	1	4.918	0.8
		2	6.155	1.0
		3	5.401	0.8
		4	4.789	0.7
3	20	1	39.37	6.5
		2	56.93	9.4
		3	57.74	9.5
		4	46.07	7.6
	25	1	37.75	6.2
		2	26.51	4.3
		3	58.32	9.6
		4	52.12	8.6
	30	1	32.53	5.3
		2	33.65	5.5
		3	46.94	7.7
		4	49.92	8.2
	35	1	40.06	6.6
		2	37.35	6.1
		3	35.01	5.7
		4	35.67	5.9
6	20	1	148.5	24.5
		2	119.3	19.7
		3	110.5	18.2
		4	141.6	23.4
	25	1	85.99	14.2
		2	105	17.3
		3	166.7	27.5

		4	105.1	17.3
	30	1	243.9	40.3
		2	169	27.9
		3	270.2	44.7
		4	276.5	45.7
	35	1	86.37	14.2
		2	120.9	20.0
		3	99.91	16.5
		4	78.31	12.9
8	20	1	70.07	11.5
		2	94.58	15.6
		3	49.93	8.2
		4	75.05	12.4
	25	1	221.2	36.6
		2	269	44.5
		3	341.8	56.5
		4	194.6	32.2
	30	1	101.5	16.7
		2	75.28	12.4
		3	99.87	16.5
		4	133.8	22.1
	35	1	205.1	33.9
		2	240.9	39.8
		3	238.2	39.4
		4	201.8	33.3
10	20	1	67.09	11.1
		2	74.99	12.4
		3	87.59	14.4
		4	151.6	25.0
	25	1	221	36.5
		2	167.8	27.7
		3	215.8	35.7
		4	90.46	14.9
	30	1	60.29	9.9
		2	32.67	5.4
		3	39.21	6.4
		4	50.01	8.2
	35	1	124.6	20.6
		2	191.5	31.6
		3	188.4	31.1
		4	276.9	45.8

13	20	1	221.5	36.6
		2	227.2	37.6
		3	194.4	32.1
		4	310	51.3
	25	1	107.9	17.8
		2	66.64	11.0
		3	106.4	17.6
		4	44.58	7.3
	30	1	196.2	32.4
		2	28.4	4.6
		3	84.09	13.9
		4	70.78	11.7
	35	1	91.4	15.1
		2	129.8	21.4
		3	139.7	23.1
		4	219.7	36.3
15	20	1	136	22.5
		2	134	22.1
		3	150.1	24.8
		4	244.6	40.4
	25	1	125.4	20.7
		2	85.85	14.2
		3	151.7	25.1
		4	77.97	12.9
	30	1	297.9	49.3
		2	88.45	14.6
		3	198.8	32.9
		4	179.9	29.7
	35	1	217.7	36.0
		2	180.8	29.9
		3	103.4	17.1
		4	143.7	23.7

E. Fluorescence and cells density of *C. neogracile*

Day	Salinity	Fluorescence	Cells.mL $\cdot 10^{-5}$
1	20	0.495	0.02
1	20	0.53	0.02
1	20	0.748	0.05
1	20	0.779	0.05
1	25	0.73	0.04
1	25	0.411	0.01
1	25	0.419	0.01
1	25	0.539	0.02
1	30	0.485	0.02
1	30	0.946	0.07
1	30	0.817	0.05
1	30	0.576	0.03
1	35	0.919	0.06
1	35	0.903	0.06
1	35	0.73	0.04
1	35	0.944	0.07
3	20	1.158	0.09
3	20	1.495	0.13
3	20	1.659	0.14
3	20	1.87	0.17
3	25	1.732	0.15
3	25	1.176	0.09
3	25	3.245	0.31
3	25	1.296	0.10
3	30	1.367	0.11
3	30	1.141	0.09
3	30	1.717	0.15
3	30	1.347	0.11
3	35	1.642	0.14
3	35	1.713	0.15
3	35	1.115	0.08
3	35	2.26	0.21
6	20	5.713	0.58
6	20	11.4	1.18
6	20	4.698	0.47
6	20	6.634	0.67
6	25	5.764	0.58
6	25	6.469	0.66
6	25	14.86	1.55

6	25	5.786	0.58
6	30	10.26	1.06
6	30	7.797	0.80
6	30	6.311	0.64
6	30	5.399	0.54
6	35	6.681	0.68
6	35	4.928	0.49
6	35	4.336	0.43
6	35	16.23	1.70
8	20	14.64	1.53
8	20	43.94	4.66
8	20	11.01	1.14
8	20	22.3	2.35
8	25	18.22	1.91
8	25	35.97	3.81
8	25	13.62	1.42
8	25	18.56	1.95
8	30	30.06	3.18
8	30	25.51	2.69
8	30	14.77	1.54
8	30	11.12	1.15
8	35	24.4	2.57
8	35	17.51	1.84
8	35	14.77	1.54
8	35	55.5	5.89
10	20	42.42	4.50
10	20	86.72	9.23
10	20	27.64	2.92
10	20	50.14	5.32
10	25	40.46	4.29
10	25	55.65	5.91
10	25	69.1	7.35
10	25	29.1	3.07
10	30	91.84	9.78
10	30	66.03	7.02
10	30	32.93	3.48
10	30	21.74	2.29
10	35	61.05	6.49
10	35	40.33	4.27
10	35	32.5	3.44
10	35	94.6	10.07

13	20	72.78	7.74
13	20	127.5	13.59
13	20	62.04	6.59
13	20	90.03	9.58
13	25	88.16	9.38
13	25	81.02	8.62
13	25	102.1	10.87
13	25	65.22	6.93
13	30	112.8	12.02
13	30	94.76	10.09
13	30	75.14	7.99
13	30	73.79	7.85
13	35	93.71	9.98
13	35	97.03	10.33
13	35	92.83	9.88
13	35	131.7	14.04
15	20	81.89	8.71
15	20	148.2	15.80
15	20	87.79	9.34
15	20	98.99	10.54
15	25	100.7	10.72
15	25	106.7	11.36
15	25	103.4	11.01
15	25	103.5	11.02
15	30	117.7	12.54
15	30	98.56	10.49
15	30	105.6	11.25
15	30	101.4	10.80
15	35	111.5	11.88
15	35	116.9	12.45
15	35	103.4	11.01
15	35	158.1	16.86

F. Fluoresence and cells density of *T. chui*

Days	Salinity	Fluoresence	Cells.mL ^{-10⁻⁵}
1	20	2.006	0.11
1	20	1.868	0.10
1	20	1.296	0.07
1	20	1.603	0.09
1	25	1.523	0.08
1	25	2.005	0.11
1	25	1.199	0.07
1	25	1.568	0.09
1	30	1.256	0.07
1	30	1.24	0.07
1	30	1.571	0.09
1	30	1.226	0.07
1	35	2.339	0.13
1	35	1.652	0.09
1	35	1.276	0.07
1	35	1.42	0.08
3	20	1.02	0.06
3	20	1.384	0.08
3	20	0.802	0.04
3	20	0.59	0.03
3	25	1.29	0.07
3	25	1.197	0.07
3	25	0.742	0.04
3	25	1.307	0.07
3	30	0.359	0.02
3	30	1.506	0.08
3	30	0.859	0.05
3	30	0.783	0.04
3	35	0.806	0.05
3	35	1.06	0.06
3	35	1.031	0.06
3	35	0.86	0.05
6	20	7.9	0.43
6	20	10.55	0.57
6	20	11.8	0.63
6	20	9.629	0.52
6	25	37.67	2.02
6	25	7.521	0.40
6	25	13.6	0.73

6	25	20.11	1.08
6	30	9.45	0.51
6	30	10.06	0.54
6	30	13.22	0.71
6	30	14.06	0.76
6	35	8.842	0.48
6	35	14.16	0.76
6	35	16.77	0.90
6	35	19.82	1.06
8	20	18.91	1.02
8	20	13.03	0.70
8	20	35.03	1.88
8	20	10.42	0.56
8	25	64.39	3.45
8	25	13.42	0.72
8	25	40.05	2.15
8	25	46.44	2.49
8	30	20.53	1.10
8	30	35.77	1.92
8	30	36.05	1.93
8	30	27.11	1.45
8	35	13.19	0.71
8	35	51.42	2.76
8	35	44.02	2.36
8	35	55.09	2.95
10	20	9.325	0.50
10	20	22.54	1.21
10	20	90.58	4.86
10	20	53.58	2.87
10	25	87.8	4.71
10	25	14.84	0.80
10	25	58.79	3.15
10	25	41.03	2.20
10	30	34.22	1.84
10	30	119.4	6.40
10	30	44.94	2.41
10	30	60.91	3.27
10	35	18.35	0.99
10	35	106.8	5.73
10	35	76.02	4.08
10	35	117.7	6.31

13	20	29.67	1.59
13	20	28.36	1.52
13	20	151.7	8.13
13	20	108.2	5.80
13	25	104.9	5.62
13	25	29.93	1.61
13	25	128.4	6.88
13	25	55.96	3.00
13	30	40.95	2.20
13	30	208.7	11.19
13	30	43.12	2.31
13	30	134.6	7.22
13	35	49.87	2.67
13	35	126.6	6.79
13	35	88.1	4.72
13	35	217.5	11.66
15	20	13.7	0.74
15	20	16.18	0.87
15	20	168.3	9.02
15	20	140.8	7.55
15	25	157.4	8.44
15	25	30.4	1.63
15	25	162.1	8.69
15	25	61.66	3.31
15	30	35.77	1.92
15	30	213.4	11.44
15	30	29.33	1.57
15	30	178.2	9.55
15	35	27.54	1.48
15	35	123.1	6.60
15	35	149.2	8.00
15	35	281.4	15.08

G. Fluorescence and cells density of *Ti. lutea*

Day	Salinity	Fluorometer	Cells.mL $\cdot 10^{-5}$
1	20	0.144	0.7
1	20	0.128	0.7
1	20	0.127	0.7
1	20	0.106	0.7
1	25	0.133	0.7
1	25	0.151	0.7
1	25	0.167	0.7
1	25	0.171	0.7
1	30	0.182	0.7
1	30	0.177	0.7
1	30	0.156	0.7
1	30	0.21	0.7
1	35	0.026	0.7
1	35	0.13	0.7
1	35	0.163	0.7
1	35	0.145	0.7
3	20	0.33	0.8
3	20	0.043	0.7
3	20	0.326	0.8
3	20	0.224	0.7
3	25	0.01	0.7
3	25	0.401	0.8
3	25	0.52	0.8
3	25	0.323	0.8
3	30	0.349	0.8
3	30	0.328	0.8
3	30	0.372	0.8
3	30	0.383	0.8
3	35	0.317	0.8
3	35	0.22	0.7
3	35	0.176	0.7
3	35	0.292	0.7
6	20	2.121	1.3
6	20	1.736	1.2
6	20	3.761	1.8
6	20	2.065	1.3
6	25	1.628	1.1
6	25	1.954	1.2
6	25	3.054	1.6

6	25	2.022	1.3
6	30	2.77	1.5
6	30	1.972	1.2
6	30	2.638	1.4
6	30	2.693	1.5
6	35	1.487	1.1
6	35	0.887	0.9
6	35	1.357	1.1
6	35	2.29	1.3
8	20	9.981	3.6
8	20	4.295	1.9
8	20	16.17	5.5
8	20	8.339	3.1
8	25	9.619	3.5
8	25	7.883	3.0
8	25	16.59	5.6
8	25	8.486	3.2
8	30	13.29	4.6
8	30	6.936	2.7
8	30	10.85	3.9
8	30	15.03	5.1
8	35	4.377	2.0
8	35	3.352	1.7
8	35	5.094	2.2
8	35	8.279	3.1
10	20	38.2	12.1
10	20	15.29	5.2
10	20	50.74	15.8
10	20	38.96	12.3
10	25	26.78	8.7
10	25	32.18	10.3
10	25	54.53	16.9
10	25	19.42	6.5
10	30	39.7	12.5
10	30	25.26	8.2
10	30	30.41	9.7
10	30	51.07	15.9
10	35	16.54	5.6
10	35	14.95	5.1
10	35	20.48	6.8
10	35	29	9.3

13	20	31.24	10.0
13	20	46.63	14.6
13	20	27.5	8.9
13	20	28.14	9.1
13	25	55.38	17.2
13	25	30.48	9.8
13	25	39.31	12.4
13	25	58.19	18.0
13	30	47.2	14.8
13	30	46.23	14.5
13	30	47.88	15.0
13	30	34.53	11.0
13	35	47.16	14.7
13	35	70.92	21.8
13	35	60.59	18.7
13	35	39.37	12.4
15	20	31.75	10.1
15	20	55.61	17.3
15	20	30.43	9.7
15	20	23.65	7.7
15	25	51.29	16.0
15	25	29.72	9.5
15	25	45.71	14.3
15	25	67.65	20.9
15	30	49.55	15.5
15	30	36.36	11.5
15	30	48.21	15.1
15	30	24.58	8.0
15	35	46.49	14.5
15	35	56.43	17.5
15	35	51.67	16.1
15	35	43.07	13.5

H. Division rate (k) of cultured microalgae at different salinities, 20 25, 30 and 35 psu, during logarithmic phase of growth.

Strain	Salinity (psu)	Replicate	k
Kb1-2	20	1	1.1
Kb1-2		2	1.0
Kb1-2		3	1.2
Kb1-2		4	1.0
Kb1-2	25	1	0.8
Kb1-2		2	0.9
Kb1-2		3	0.9
Kb1-2		4	0.8
Kb1-2	30	1	0.9
Kb1-2		2	0.9
Kb1-2		3	0.9
Kb1-2		4	1.0
Kb1-2	35	1	0.7
Kb1-2		2	0.7
Kb1-2		3	0.8
Kb1-2		4	0.7
Kb1-3	20	1	0.5
Kb1-3		2	1.0
Kb1-3		3	1.4
Kb1-3		4	1.1
Kb1-3	25	1	0.6
Kb1-3		2	0.5
Kb1-3		3	1.2
Kb1-3		4	1.3
Kb1-3	30	1	1.0
Kb1-3		2	0.8
Kb1-3		3	1.2
Kb1-3		4	1.0
Kb1-3	35	1	1.3
Kb1-3		2	1.2
Kb1-3		3	1.0
Kb1-3		4	1.1
Kb1-5	20	1	1.3
Kb1-5		2	1.5
Kb1-5		3	1.3
Kb1-5		4	1.2
Kb1-5	25	1	1.5
Kb1-5		2	1.2

Kb1-5		3	1.4
Kb1-5		4	1.7
Kb1-5	30	1	1.1
Kb1-5		2	1.1
Kb1-5		3	1.3
Kb1-5		4	1.4
Kb1-5	35	1	1.6
Kb1-5		2	1.3
Kb1-5		3	1.4
Kb1-5		4	1.5
<i>C. neogracile</i>	20	1	1.1
<i>C. neogracile</i>		2	1.2
<i>C. neogracile</i>		3	0.8
<i>C. neogracile</i>		4	0.9
<i>C. neogracile</i>	25	1	0.9
<i>C. neogracile</i>		2	1.6
<i>C. neogracile</i>		3	2.5
<i>C. neogracile</i>		4	1.1
<i>C. neogracile</i>	30	1	1.3
<i>C. neogracile</i>		2	1.1
<i>C. neogracile</i>		3	0.7
<i>C. neogracile</i>		4	1.0
<i>C. neogracile</i>	35	1	1.0
<i>C. neogracile</i>		2	1.0
<i>C. neogracile</i>		3	0.9
<i>C. neogracile</i>		4	0.9
<i>T. chui</i>	20	1	1.0
<i>T. chui</i>		2	1.0
<i>T. chui</i>		3	1.3
<i>T. chui</i>		4	1.3
<i>T. chui</i>	25	1	1.6
<i>T. chui</i>		2	0.9
<i>T. chui</i>		3	1.4
<i>T. chui</i>		4	1.3
<i>T. chui</i>	30	1	1.5
<i>T. chui</i>		2	0.9
<i>T. chui</i>		3	1.3
<i>T. chui</i>		4	1.4
<i>T. chui</i>	35	1	1.1
<i>T. chui</i>		2	1.2
<i>T. chui</i>		3	1.3

<i>T. chui</i>		4	1.5
<i>Ti. lutea</i>	20	1	0.9
<i>Ti. lutea</i>		2	0.7
<i>Ti. lutea</i>		3	0.8
<i>Ti. lutea</i>		4	1.0
<i>Ti. lutea</i>	25	1	0.8
<i>Ti. lutea</i>		2	0.6
<i>Ti. lutea</i>		3	0.9
<i>Ti. lutea</i>		4	0.7
<i>Ti. lutea</i>	30	1	0.8
<i>Ti. lutea</i>		2	0.6
<i>Ti. lutea</i>		3	0.7
<i>Ti. lutea</i>		4	0.9
<i>Ti. lutea</i>	35	1	0.8
<i>Ti. lutea</i>		2	0.8
<i>Ti. lutea</i>		3	0.8
<i>Ti. lutea</i>		4	0.8

- I. Dry weight (DW) and ash free dry weight (AFDW) in $\mu\text{g mL}^{-1}$ of Indonesian microalgal strains, *C. neogracile*, *T. Chui* and *Ti. lutea* at different culture salinity

Strain	Salinity	DW	AFDW
Kb1-2	20	156	124
Kb1-2	20	186	150
Kb1-2	20	212	152
Kb1-2	20	146	115
Kb1-2	25	179	116
Kb1-2	25	105	87
Kb1-2	25	67	69
Kb1-2	25	77	68
Kb1-2	30	159	123
Kb1-2	30	189	178
Kb1-2	30	235	208
Kb1-2	30	323	228
Kb1-2	35	207	149
Kb1-2	35	262	194
Kb1-2	35	433	298
Kb1-2	35	231	174
Kb1-3	20	11	23
Kb1-3	20	37	45
Kb1-3	20	16	28
Kb1-3	20	16	30
Kb1-3	25	24	25
Kb1-3	25	44	50
Kb1-3	25	65	62
Kb1-3	25	52	53
Kb1-3	30	26	36
Kb1-3	30	8	16
Kb1-3	30	54	51
Kb1-3	30	35	43
Kb1-3	35	9	13
Kb1-3	35	45	62
Kb1-3	35	26	34
Kb1-3	35	36	49
Kb1-5	20	45	9
Kb1-5	20	101	-23
Kb1-5	20	42	13
Kb1-5	20	100	8

Kb1-5	25	73	-6
Kb1-5	25	9	32
Kb1-5	25	18	30
Kb1-5	25	40	72
Kb1-5	30	153	-15
Kb1-5	30	47	14
Kb1-5	30	72	9
Kb1-5	30	59	3
Kb1-5	35	39	15
Kb1-5	35	16	29
Kb1-5	35	15	19
Kb1-5	35	37	7
<i>C.neogracile</i>	20	29	37
<i>C.neogracile</i>	20	52	59
<i>C.neogracile</i>	20	33	47
<i>C.neogracile</i>	20	46	52
<i>C.neogracile</i>	25	5	19
<i>C.neogracile</i>	25	3	17
<i>C.neogracile</i>	25	35	37
<i>C.neogracile</i>	25	42	45
<i>C.neogracile</i>	30	54	55
<i>C.neogracile</i>	30	24	47
<i>C.neogracile</i>	30	65	73
<i>C.neogracile</i>	30	24	47
<i>C.neogracile</i>	35	148	101
<i>C.neogracile</i>	35	144	88
<i>C.neogracile</i>	35	151	109
<i>C.neogracile</i>	35	160	91
<i>T. chuui</i>	20	137	105
<i>T. chuui</i>	20	67	79
<i>T. chuui</i>	20	1151	1663
<i>T. chuui</i>	20	1206	236
<i>T. chuui</i>	25	232	192
<i>T. chuui</i>	25	109	634
<i>T. chuui</i>	25	1200	226
<i>T. chuui</i>	25	114	104
<i>T. chuui</i>	30	51	60
<i>T. chuui</i>	30	1236	248
<i>T. chuui</i>	30	1513	182
<i>T. chuui</i>	30	227	226
<i>T. chuui</i>	35	67	97

<i>T. chuui</i>	35	177	181
<i>T. chuui</i>	35	127	126
<i>T. chuui</i>	35	217	170
<i>T. lutea</i>	20	46	43
<i>Ti. lutea</i>	20	45	54
<i>Ti. lutea</i>	20	16	20
<i>Ti. lutea</i>	20	32	49
<i>Ti. lutea</i>	25	7	33
<i>Ti. lutea</i>	25	27	32
<i>Ti. lutea</i>	25	43	37
<i>Ti. lutea</i>	25	7	15
<i>Ti. lutea</i>	30	207	61
<i>Ti. lutea</i>	30	189	49
<i>Ti. lutea</i>	30	217	75
<i>Ti. lutea</i>	30	222	69
<i>Ti. lutea</i>	35	114	114
<i>Ti. lutea</i>	35	164	181
<i>Ti. lutea</i>	35	84	98
<i>Ti. lutea</i>	35	161	170

J. Data of daily survival of shrimp larvae fed on Indonesian microalgal strains, *C. neogracile*, *T. lutea* and *Te. Chui*

Day	Strain	Replicate	Day/Time	Dead	Alive
1	Kb1-2	1	10:00 AM	0	25
Saturday	Kb1-2	2	Saturday, 8/29/15	0	25
8/29/2015	Kb1-2	3		0	25
	Kb1-3	1		0	25
	Kb1-3	2		0	25
	Kb1-3	3		0	25
	Kb1-5	1		0	25
	Kb1-5	2		0	25
	Kb1-5	3		0	25
	<i>C. neogracile</i>	1		0	25
	<i>C. neogracile</i>	2		0	25
	<i>C. neogracile</i>	3		0	25
	<i>T. lutea</i>	1		0	25
	<i>T. lutea</i>	2		0	25
	<i>T. lutea</i>	3		0	25
	<i>Te. chui</i>	1		2	25
	<i>Te. chui</i>	2		0	25
	<i>Te. chui</i>	3		0	25
	Control	1		0	25
	Control	2		0	25
1	Kb1-2	1	9:00 PM		
Saturday	Kb1-2	2	Saturday, 8/29/15		
8/29/2015	Kb1-2	3			
	Kb1-3	1			
	Kb1-3	2			
	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			
	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			

	Control	1			
	Control	2			
2	Kb1-2	1	7:00 AM	25	0
Sunday	Kb1-2	2	Sunday, 8/30/15	0	25
8/30/2015	Kb1-2	3		0	25
	Kb1-3	1		0	25
	Kb1-3	2		0	25
	Kb1-3	3		0	25
	Kb1-5	1		0	25
	Kb1-5	2		0	25
	Kb1-5	3		0	25
	<i>C. neogracile</i>	1		0	25
	<i>C. neogracile</i>	2		0	25
	<i>C. neogracile</i>	3		0	25
	<i>T. lutea</i>	1		0	25
	<i>T. lutea</i>	2		0	25
	<i>T. lutea</i>	3		0	25
	<i>Te. chui</i>	1		3	22
	<i>Te. chui</i>	2		0	25
	<i>Te. chui</i>	3		0	25
	Control	1		25	4
	Control	2		20	2
2	Kb1-2	1	9:00 PM		
Sunday	Kb1-2	2	Sunday, 8/30/15		
8/30/2015	Kb1-2	3			
	Kb1-3	1			
	Kb1-3	2			
	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			
	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			
	Control	1			

	Control	2			
3	Kb1-2	1	7:00 AM	25	0
Monday	Kb1-2	2	Monday, 8/31/15	22	3
8/31/2015	Kb1-2	3		23	2
	Kb1-3	1		18	7
	Kb1-3	2		13	12
	Kb1-3	3		19	6
	Kb1-5	1		19	6
	Kb1-5	2		20	5
	Kb1-5	3		19	6
	<i>C. neogracile</i>	1		17	8
	<i>C. neogracile</i>	2		11	14
	<i>C. neogracile</i>	3		19	6
	<i>T. lutea</i>	1		9	16
	<i>T. lutea</i>	2		17	8
	<i>T. lutea</i>	3		17	8
	<i>Te. chui</i>	1		17	8
	<i>Te. chui</i>	2		10	15
	<i>Te. chui</i>	3		22	3
	Control	1		25	0
	Control	2		25	0
3	Kb1-2	1	9:00 PM		
Monday	Kb1-2	2	Monday, 8/31/15		
8/31/2015	Kb1-2	3			
	Kb1-3	1			
	Kb1-3	2			
	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			
	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			
	Control	1			
	Control	2			

4	Kb1-2	1	7:00 AM	25	0
Tuesday	Kb1-2	2	Tuesday, 09/01/15	25	0
9/1/2015	Kb1-2	3		25	0
	Kb1-3	1		18	7
	Kb1-3	2		19	6
	Kb1-3	3		19	6
	Kb1-5	1		18	7
	Kb1-5	2		20	5
	Kb1-5	3		19	6
	<i>C. neogracile</i>	1		19	6
	<i>C. neogracile</i>	2		8	17
	<i>C. neogracile</i>	3		24	1
	<i>T. lutea</i>	1		10	15
	<i>T. lutea</i>	2		17	8
	<i>T. lutea</i>	3		17	8
	<i>Te. chui</i>	1		18	7
	<i>Te. chui</i>	2		13	12
	<i>Te. chui</i>	3		25	0
4	Kb1-3	1	9:00 PM		
Tuesday	Kb1-3	2	Tuesday, 09/01/15		
9/1/2015	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			
	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			
5	Kb1-3	1	7:00 AM	20	5
Wednesday	Kb1-3	2	Wednesday, 09/02/15	19	6
9/2/2015	Kb1-3	3		20	5
	Kb1-5	1		20	1
	Kb1-5	2		20	2
	Kb1-5	3		19	4
	<i>C. neogracile</i>	1		19	6

	<i>C. neogracile</i>	2		10	14
	<i>C. neogracile</i>	3		25	0
	<i>T. lutea</i>	1		10	15
	<i>T. lutea</i>	2		18	7
	<i>T. lutea</i>	3		18	7
	<i>Te. chui</i>	1		20	5
	<i>Te. chui</i>	2		13	12
	<i>Te. chui</i>	3		25	0
5	Kb1-3	1	9:00 PM		
Wednesday	Kb1-3	2	Wednesday, 09/02/15		
9/2/2015	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			
	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			
6	Kb1-3	1	7:00 AM	20	5
Thursday	Kb1-3	2	Thursday, 09/03/15	23	2
9/3/15	Kb1-3	3		20	5
	Kb1-5	1		23	2
	Kb1-5	2		21	4
	Kb1-5	3		22	3
	<i>C. neogracile</i>	1		23	2
	<i>C. neogracile</i>	2		16	9
	<i>C. neogracile</i>	3		25	0
	<i>T. lutea</i>	1		10	15
	<i>T. lutea</i>	2		18	7
	<i>T. lutea</i>	3		18	7
	<i>T. chui</i>	1		20	5
	<i>T. chui</i>	2		13	12
	<i>T. chui</i>	3		25	0
6	Kb1-3	1	9:00 PM		
Thursday	Kb1-3	2	Thursday, 09/03/15		

9/3/2015	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			
	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			
7	Kb1-3	1	7:00 AM	22	3
Friday	Kb1-3	2	Friday, 09/04/15	23	2
9/4/2015	Kb1-3	3		22	3
	Kb1-5	1		24	1
	Kb1-5	2		22	3
	Kb1-5	3		24	1
	<i>C. neogracile</i>	1		24	1
	<i>C. neogracile</i>	2		16	9
	<i>C. neogracile</i>	3		25	0
	<i>T. lutea</i>	1		10	15
	<i>T. lutea</i>	2		22	3
	<i>T. lutea</i>	3		20	5
	<i>Te. chui</i>	1		20	5
	<i>Te. chui</i>	2		15	10
	<i>Te. chui</i>	3		25	0
7	Kb1-3	1	9:00 PM		
Friday	Kb1-3	2	Friday, 09/04/15		
9/4/2015	Kb1-3	3			
	Kb1-5	1			
	Kb1-5	2			
	Kb1-5	3			
	<i>C. neogracile</i>	1			
	<i>C. neogracile</i>	2			
	<i>C. neogracile</i>	3			
	<i>T. lutea</i>	1			
	<i>T. lutea</i>	2			
	<i>T. lutea</i>	3			
	<i>Te. chui</i>	1			

	<i>Te. chui</i>	2			
	<i>Te. chui</i>	3			
8	Kb1-3	1	7:00 AM	22	3
Saturday	Kb1-3	2	Saturday, 09/05/15	23	2
9/5/2015	Kb1-3	3		22	3
	Kb1-5	1		24	1
	Kb1-5	2		22	3
	Kb1-5	3		24	1
	<i>C. neogracile</i>	1		24	1
	<i>C. neogracile</i>	2		16	9
	<i>C. neogracile</i>	3		25	0
	<i>T. lutea</i>	1		10	15
	<i>T. lutea</i>	2		22	3
	<i>T. lutea</i>	3		20	5
	<i>Te. chui</i>	1		20	5
	<i>Te. chui</i>	2		15	10
	<i>Te. chui</i>	3		25	0

E. Growth variables data of individual white shrimp larvae fed Indonesian microalgal strains, *C. neogracile*, *T. lutea* and *Te. Chui* after 8 days of culture except those fed on Kb1-2 and unfed control which was after 4 and 3 days, respectively

Strain	WG (ug)	FCI	DW FI (ug)	SGR	<i>kl</i> (%)	FI (%)	Increase WG (%)	Final DW (ug)
Kb1-2	5.7	10.8	10.4	0.15	55.1	16.74	21.4063	12.4
Kb1-2	3.6	17.4	9.0	0.09	39.5	14.55	11.1111	11.6
Kb1-2	3.6	17.4	9.0	0.09	39.5	14.55	11.1111	11.6
Kb1-2	3.6	17.4	9.0	0.09	39.5	14.55	11.1111	11.6
Kb1-2	8.6	7.2	30.9	0.23	27.9	50.00	36.7898	14.5
Kb1-2	8.6	7.2	30.9	0.23	27.9	50.00	36.7898	14.5
Kb1-3	41.5	4.4	100.9	0.25	41.1	55.41	77.7778	48.1
Kb1-3	41.5	4.4	100.9	0.25	41.1	55.41	77.7778	48.1
Kb1-3	41.5	4.4	100.9	0.25	41.1	55.41	77.7778	48.1
Kb1-3	57.0	3.2	79.6	0.26	71.6	43.69	89.0625	65.0
Kb1-3	57.0	3.2	79.6	0.26	71.6	43.69	89.0625	65.0
Kb1-3	22.9	7.9	92.1	0.20	24.9	50.57	48.8636	28.8
Kb1-3	22.9	7.9	92.1	0.20	24.9	50.57	48.8636	28.8
Kb1-3	22.9	7.9	92.1	0.20	24.9	50.57	48.8636	28.8
Kb1-5	68.1	1.3	123.3	0.30	55.3	89.81	127.7500	74.8
Kb1-5	68.9	2.0	73.3	0.28	94.0	53.36	107.6042	76.9
Kb1-5	68.9	2.0	73.3	0.28	94.0	53.36	107.6042	76.9
Kb1-5	68.9	2.0	73.3	0.28	94.0	53.36	107.6042	76.9
Kb1-5	42.7	3.2	75.2	0.26	56.9	54.76	91.0511	48.6
<i>C. neogracile</i>	78.6	3.4	144.1	0.32	54.6	53.15	147.4375	85.3
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>C. neogracile</i>	38.8	7.0	142.7	0.22	27.2	52.67	60.5903	46.8
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3

<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	7.6	25.1	98.0	0.10	7.8	51.08	14.3250	14.3
<i>T. lutea</i>	61.3	3.1	93.4	0.27	65.6	48.69	95.7292	69.3
<i>T. lutea</i>	61.3	3.1	93.4	0.27	65.6	48.69	95.7292	69.3
<i>T. lutea</i>	61.3	3.1	93.4	0.27	65.6	48.69	95.7292	69.3
<i>T. lutea</i>	37.7	5.1	99.7	0.25	37.9	51.96	80.3977	43.6
<i>T. lutea</i>	37.7	5.1	99.7	0.25	37.9	51.96	80.3977	43.6
<i>T. lutea</i>	37.7	5.1	99.7	0.25	37.9	51.96	80.3977	43.6
<i>T. lutea</i>	37.7	5.1	99.7	0.25	37.9	51.96	80.3977	43.6
<i>T. lutea</i>	37.7	5.1	99.7	0.25	37.9	51.96	80.3977	43.6
<i>T. chui</i>	38.5	21.5	531.6	0.24	7.2	64.13	72.2500	45.2
<i>T. chui</i>	38.5	21.5	531.6	0.24	7.2	64.13	72.2500	45.2
<i>T. chui</i>	38.5	21.5	531.6	0.24	7.2	64.13	72.2500	45.2
<i>T. chui</i>	38.5	21.5	531.6	0.24	7.2	64.13	72.2500	45.2
<i>T. chui</i>	38.5	21.5	531.6	0.24	7.2	64.13	72.2500	45.2
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
<i>T. chui</i>	12.0	69.0	504.7	0.11	2.4	60.88	18.7812	20.0
Unfed control	0.3	0.0	0.0	0.01	0.0	0.00	0.6250	7.0
Unfed control	0.3	0.0	0.0	0.01	0.0	0.00	0.6250	7.0
Unfed control	0.3	0.0	0.0	0.01	0.0	0.00	0.6250	7.0
Unfed control	0.3	0.0	0.0	0.01	0.0	0.00	0.6250	7.0
Unfed control	1.4	0.0	0	0.03	0.0	0.00	2.8883	7.2
Unfed control	1.4	0.0	0	0.03	0.0	0.00	2.8883	7.2

F. Chromatogram of Fatty Acids from FAME STD (Supelco 37 Component FAME mix Sigma Aldrich USA) by GC/MS Method Agilent 6890 GC with 5973 MSD

Abundance

G. Fatty Acids from FAME STD (Supelco 37 Component FAME mix Sigma Aldrich USA) identified by GC/MS Method Agilent 6890 GC with 5973 MSD

No	Systemic Name	Common Name	Formula	Length : °unsaturation	Molecular Weight (Da)	Retention Time (min)
1	Hexanoic acid	Caproic acid	C7H14O2	6;0	130	4.727
2	Octanoic acid	Caprylic acid	C9H18O2	8;0	158	7.869
3	Decanoic acid	Capric acid	C11H22O2	10;0	186	10.732
4	Undecanoic acid	Undecyclic acid	C12H24O2	11;0	200	12.041
5	Dodecanoic acid	Lauric Acid	C13H26O2	12;0	214	13.279
6	Tridecanoic acid	Tridecyclic acid	C14H28O2	13;0	228	14.450
7	Myristic Acid	Tetradecanoic acid	C15H30O2	14;0	242	15.609
8	Pentadecanoic acid	Pentadecyclic acid	C16H32O2	15;0	256	17.061
9	9-hexadecanoic acid	Palmitoleic acid	C17H32O2	16:1(n-7)	268	18.567
10	Hexadecanoic acid	Palmitic acid	C17H34O2	16;0	270	19.046
11	Heptadecanoic acid	Margaric acid	C18H36O2	17;0	284	21.601
12	9,12-octadecadienic acid	Linoleic acid	C19H34O2	18:2(n-6)	294	23.195
13	9-octadecanoic acid	Oleic acid	C19H36O2	18:1(n-9)	296	23.343
14	trans-9-Octadecenoic acid	Elaidic acid	C19H36O2	t18:1(n-9)	296	23.510
15	Octadecanoic acid	Stearic acid	C19H38O2	18;0	298	23.963
16	5, 8, 11,14 eicosatrienoic acid	Arachidonic acid (ARA)	C21H36O2	20:4(n-6)	320	27.032
17	11,14 eicosadienoic acid	Dihomolinoleic acid	C31H38O2	20:2(n-6)	322	27.419
18	11-Eicosanoic acid	Gondoic acid	C21H40O2	20:1(n-9)	324	27.532
19	9,12,15-Octadecatrienoic Acid	α-Linolenic Acid	C19H32O2	18:3(n-3)	292	27.550
20	Eicosanoic acid	Arachidic acid	C21H42O2	20;0	326	28.049
21	Heneicosanoic acid	Heneicosylic Acid	C22H42O2	21;0	340	29.856

22	4,7,10,13,16,19- Docosahexaenoic Acid	Cervonic acid (DHA)	C23H34O2	22:6(n-3)	342	30.168
23	Docosanoic acid	Behenic acid	C23H46O2	22;0	354	31.538
24	Tricosanoic acid	Tricosylic acid	C24H48O2	24:1(n-9)	368	33.243
25	15-Tetracosanoic acid	Nervonic acid	C25H48O2	24:1(n-9)	380	34.736
26	Tetracosanoic acid	Lignoceric Acid	C25H50O2	24;0	382	35.313

H. Chromatogram of Fatty Acids from *T. lutea* samples by GC/MS Method Agilent 6890 GC with 5973 MSD at 20 (a), 25 (b), 30 (c) and 35 (d) psu

Abundance

Time-->

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I. Chromatogram of Fatty Acids from *Chaetoceros* sp. samples by GC/MS Method
Agilent 6890 GC with 5973 MSD at 20 (a), 25 (b), 30 (c) and 35 (d) psu

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J. Chromatogram of Fatty Acids from Kb1-3 samples by GC/MS Method Agilent 6890 GC with 5973 MSD at 20 (a), 25 (b), 30 (c) and 35 (d) psu

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K. Chromatogram of Fatty Acids from Kb1-5 samples by GC/MS Method Agilent 6890 GC with 5973 MSD at 20 (a), 25 (b), 30 (c) and 35 (d) psu

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M. List of fatty acids found in Kb1-2, Kb1-3, Kb1-5 and *T. lutea* based on GC-MS at 20, 25 and 35 psu

Fatty Acids	Kb1-2				Kb1-3				Kb1-5				<i>T. lutea</i>			
	20	25	30	35	20	25	30	35	20	25	30	35	20	25	30	35
Sufa																
5:0							0.32	1.73	4.47		0.49					
6:0									1.06	0.24	1.55	0.30	8.79	3.06	4.58	4.35
8:0				0.08				2.06			0.28	0.58		8.30		
9:0				0.44					0.17	0.13	0.92	0.87				
10:0				0.26					0.43	0.18	0.85	1.11		0.41		0.42
11:0	0.18	0.27	0.10	0.29						0.14		1.48		0.57		
13:0	0.12	0.28		0.38	6.28	10.79	0.25	5.57	0.47	0.10		0.24	2.04	2.30		0.55
14:0	18.52	9.88	4.09	13.72		2.07	8.47	1.66	29.28	4.41	23.13	6.63	3.45	0.41	1.05	
15:0	0.15	1.08	0.16	1.47	0.61		0.80	2.67	4.02	0.97	4.16	0.43	1.12	0.99	1.00	
16:0	43.93	36.83	8.30	34.23	22.79	9.41	31.01	9.43	9.22	28.09	17.35	19.50	45.47	6.29	18.27	10.18
17:0		0.10		0.28	22.07	32.23	6.51	2.39	0.59	0.18	0.17	0.24			0.36	
18:0	14.67	12.16	12.22	10.42	11.01	4.02	17.20	5.48	4.26	12.26	7.43	8.88	15.27		5.53	3.65
19:0		0.25		0.08			0.23		3.45							
20:0	0.27			0.73			0.20		0.59			0.39	12.40	16.09	20.69	23.53
21:0			14.58	4.73	11.90		2.68	7.13			0.33	0.21	1.42			0.59
22:0							0.75	4.25	0.18		2.77	3.23	1.05			
23:0													1.12			0.57
24:0					1.07	2.64		2.79								0.73
26:0						16.38		4.29	3.81							
27:0					0.77	4.52	0.17	6.41	6.90		0.54	0.24		17.09	5.33	6.60
28:0					2.37	2.54		3.31	3.84			0.24		7.11	2.22	2.36
34:0										0.27	0.95	0.27				

Mufa																	
16:1 ω7	0.62		0.35	3.62				28.00	4.59	0.16	0.44						
16:1 ω9												0.12		0.40		0.70	
18:1 ω9	0.38	0.44	0.18	3.08	0.75		0.35	1.45	2.25		0.45			0.41	0.88	0.57	
20:1 ω9	0.88	2.09	0.39	0.45		1.35	2.52	1.41				3.11	3.66		2.01	0.56	
Pufa																	
18:2 ω6				0.57					0.27				2.89	28.58	21.96	26.72	
18:3 ω3				1.12											0.44	0.49	
20:2 ω6	0.21	0.16		0.09				1.50		1.75	0.27						
20:4 ω6		0.40		1.26	7.90	2.50	3.09	2.42	1.64	4.65	0.95	0.39					
20:5 ω3		1.23		0.09				1.10		11.51			1.31	6.14	10.88	13.16	
22:6 ω3		0.85	0.09	3.73				3.50	1.48	2.20	1.46			1.85	4.79	4.27	
ArC ₈	20.26	33.81	59.53	18.89	12.48	11.55	25.45	1.46	17.03	32.76	35.48	51.55					
DCA																	
Total Fa	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Total Sufa	77.83	60.84	39.45	67.11	78.86	84.60	68.58	59.16	72.74	46.96	60.94	44.83	92.14	62.62	59.04	53.53	
Tota Mufa	1.88	2.53	0.93	7.15	0.75	1.35	2.87	30.87	6.84	0.16	0.89	3.23	3.66	0.81	2.90	1.83	
Total Pufa	0.21	2.64	0.09	6.85	7.90	2.50	3.09	8.51	3.39	20.11	2.68	0.39	4.20	36.57	38.06	44.64	