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GROWTH CHARACTERISTICS OF NEWLY ISOLATED INDONESIAN MICROALGAE UNDER DIFFERENT SALINITY

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ABSTRACT

The aim of this study was to investigate the growth characteristics of microalgae strains isolated from Kendari Bay and the Wanggu River estuary, Indonesia. The growth of the isolates, denoted as Kb1-2, Kb1-3, Kb1-5, and Kb2-6, were evaluated under controlled conditions. A batch culture experiment of these strains except Kb2-6 was conducted for 15 days under salinity levels of 20, 25, 30, and 35 gL^{-1}. *Tetraselmis chui*, *Tisochrysis lutea,* and *Chaetocero sneogracile* were also culture and used as the growth references. Cell density was measured every day and cell size was measured from 50 live cells during the logarithmic phase. The cell sizes of three of the four Indonesian microalgae ranged from 1.2-11.8 μm, considered suitable for shrimp larvae. The Indonesian strains started the logarithmic phase of growth at all salinities tested from day 0 to day 3 after inoculation except for Kb1-3 that started the phase after a 3-day lag. Increased cell density over the culture period and division rate of Indonesian microalgae during the logarithmic phase of growth were similar at all salinities tested and similar to *T. chui*, *Ti. lutea,* and *C. neogracile.* However, the final biomasses after 15 days of culture of all microalgal strains were 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 strains (other than Kb2-6) are suggested as suitable live food candidates for mass culture in shrimp hatcheries based on their cell size, ability to survive long culture periods, and wide salinity tolerance.

KEYWORDS: microalgae; shrimp; aquaculture; Indonesia

INTRODUCTION

Microalgae have been mass cultured as live food for mollusk, shrimp, and finfish seeds and fingelings in hatcheries (Wikfors & Ohno, 2001; Martinez-Fernandez & Southgate, 2007; Kent *et al*., 2011; Iba *et al*., 2014). Replacing live microalgae with artificial diets or dried microalgae for several aquaculture species has so far been unsuccessful (Gallardo *et al*., 2002; Sanchez *et al*., 2014; Ma & Qin, 2012; Arney *et al*., 2015); however, partial substitution with artificial diets and frozen microalgal replacement diets are of promising alternatives (Gui *et al*., 2015; Southgate *et al*., 2016). Nevertheless, the availability of live microalgae in hatcheries for marine cultured species (mollusks, shrimps, and finfish) remains critical to ensure a continuous supply of healthy seed for grow-out aquaculture (Wells *et al*., 2017). A survey conducted by the Global Aquaculture Alliance in 2011 revealed that seedstock quality and availability were still the main issues in producing good quality shrimp in Asia (Valderrama & Anderson, 2012). With effective

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microalgal feeds, hatcheries are expected to produce robust, healthy and inexpensive seedstock for commercial application.

South-East Sulawesi (SE-Sulawesi, 02°45' to 06°15'S, 120°45' to 124°45'E), 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 of 153,000 km^2) of the SE-Sulawesi territory consists of marine areas (Sahrir *et al*., 2014). Local microalgae from SE-Sulawesi 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 cultures provided from the South Sulawesi and Java regions which incur high operational costs to aquaculture farms and hatcheries. The price of microalgae starter cultures obtained from Java and South Sulawesi such as *Spirulina*, *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 cultures were sourced from within the SE-Sulawesi region as a result of minimal transportation costs within the region. Importing microalgae from other regions also exposes receiving areas to risks from non-native species invasions, both the microalgae species and any contaminating microorganisms. To reduce transportation cost and non-native introduction issues, the use of locally-isolated cultured microalgae in local hatcheries should be encouraged.

Kendari Bay and the Wanggu River are economically important and ecologically critical for SE-Sulawesi, particularly for Kendari city, the province's capital city. Currently, Kendari Bay receives an excessive amount of anthropogenic run-offs and effluents, mostly in the form of sediment because of inappropriate land use in the surrounding areas. As most of the Kendari population lives in the Wanggu River watershed, this river also receives similar pressures in the form of organic and inorganic pollution. Habitats such as Wanggu River and Kendari Bay that receive high nutrient inputs from wastewater may have some species of microalgae that potentially can be grown easily in laboratory condition. 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. A previous study of phytoplankton identification in Kendari Bay had found four dominant strains of microalgae from three different classes (Iba *et al*., 2009). These strains could potentially be used in hatcheries once their growth characteristics under controlled conditions are known. This study, therefore, was aimed to characterize the growth of the newly isolated local strains of microalgae from Kendari Bay and the Wanggu River estuary of SE-Sulawesi, Indonesia.

MATERIALS AND METHODS

Sampling and Isolation of Microalgae

The samples for this experiment were collected from two locations: the marine waters of 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 from which 30-mL of the filtrate sample was filled into two 15-mL Falcon tubes and placed in a cooler box. The water samples suspectedly containing natural microalgae were brought to the Laboratory of Fisheries (University of Halu Oleo), Indonesia and kept overnight at room temperature before being transported to the USA, at the National Marine Fisheries Service Milford Laboratory (Milford), Connecticut and the University of Rhode Island (URI), Kingston, Rhode Island for isolation procedures. Fourteen days following collection, water samples were enriched in 10-mL culture tubes containing Guillard's f/8 medium for marine species (Wikfors & Ohno, 2001; Ponomorenko *et al*., 2004). Single-cell isolation at URI was conducted using capillary pipettes according to the procedures described by Andersen & Kawachi (2005).

At Milford, the 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 μmol photons m^2 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 sample; several morphotypes were abundant. After concentration 10× by gentle centrifugation (500 × g), the 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 the 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 & Hargraves, 1993) -enriched Milford Harbor seawater.

Micro-well plates containing sorted cells were covered to minimize evaporation and incubated under the growth conditions stated above. Every 2-3 days, the 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 in which 50 cells had been sorted showed algae 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. The cultures that had established robust growth were observed microscopically for provisional identification and determination of possible unialgal status. The 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 the microscopic and flow-cytometric analyses, 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 and later denoted as Kb1-2, Kb1-3, and Kb-5 from the flow-cytometric isolation effort were sent to URI for perpetuation and experimentation.

Microalgal Culture

The microalgae were cultured based upon the methods described by Martinez-Fernandez *et al*. (2006) and Rohani-Ghadikolaei (2012). The 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 μmol photon m^{-2} s^{-1} PAR and gentle mixing once a day. These Indonesian microalgal strains were inoculated at densities ranging from 5.4-6.3 x 10^4 and 2.1-3.0 x 10^4 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 x 10^4 ml^{-1} at inoculation; therefore Kb1-5 was inoculated at density of 8.3-12.8 x 10^4 cells·mL^{-1}. The cell density at inoculation for comparison species ranged from 0.2-0.6 x 10^4, 0.7-1.0 x 10^4, and 7.0 x 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 gL^{-1} with four replicates for each salinity to accommodate a wide range of salinities possible for microalgal growth including their natural condition which is in the range of 22-25 gL^{-1}. The seawater used in this study was artificial seawater (Instant Ocean Sea Salt mix) at initial salinity of 30 gL^{-1}. To be practical, we added artificial salt (Morton Kosher Salt) to obtain 35 gL^{-1} salinity and distilled water to decrease the salinity to 25 and 20 gL^{-1}. Three microalgae strains widely cultured in aquaculture were used as reference species: *Ti. lutea* (T-ISO; CCMP-1324), *T. chui* (CCMP-884) obtained from the shellfish laboratory at Roger Williams University, Bristol, RI, USA, and *C. neogracile* (strain Chaet B) from Milford Laboratory, Connecticut, USA. The 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 the 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, the division rate (k) during the logarithmic phase of the culture at each salinity for each species was calculated based upon cell counts (N) at times (t) (Wood *et al*., 2005) as follows:

$$k = \frac{1}{\ln 2}\left[\frac{\ln\left(\frac{N_1}{N_o}\right)}{(t_1 - t_o)}\right] \qquad (1)$$

Cell sizes of the 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 40× magnification.

The 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 *et al*. (2013). At the end of the 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 (θ = 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 $KMnO_4$ salts in a vacuum desiccator, and weighed to obtain dry weight of the algae. The dried algae on the 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:

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

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

Statistical Analyses

Data on cell density of all microalgal strains at each salinity tested were subjected to repeated measures ANOVA; whereas, yield, division rate, DW and AFDW across cultured microalgae were analyzed using two-way ANOVA. The differences in yield, division rate, DW and AFDW of the 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 AND DISCUSSION

Microalgal Isolates

We successfully isolated four strains of microalgae from Kendari Bay SE-Sulawesi denoted as Kb1-2, Kb1-3, Kb1-5, and Kb2-6 (Table 1). Kb2-6 was isolated using single-cell pipette isolation whereas Kb1-2, Kb1-3, and Kb1-5 were from the 50 sorted cells of flow cytometer. 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). 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 could be identified suggesting they are new species needed to be further identified. Kb2-6, identified as *Melosir* a*cf.moniliformis*, forms colonies and has a larger cell size of more than 10 μm suggesting that the strain is not suitable for shrimp hatchery and therefore was excluded from the subsequent culture experiments.

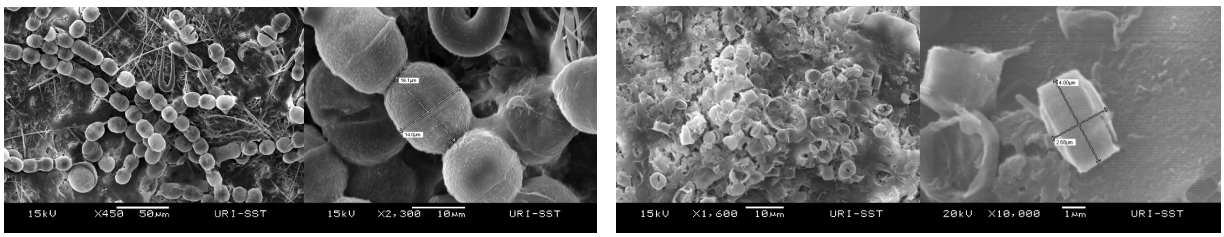
Of the three distinct strains that were included in the growth experiment, one strain was identified as *Chaetoceros* sp., a diatom species. The size of the microalgae in this growth experiment ranged from 1.2 to 11.8 μm, considered as either pico or nanoplankton, thus is suitable as feed for aquaculture 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*.

Microalgal Growth

The division rates decreased as the culture reached a late logarithmic and onset of stationary phase (Fig. 3). There were no significant differences in the division rate among microalgal strains during the logarithmic growth phase across all salinity levels tested as well as the interaction between salinity and strain. The differences in the division rate during log phase were among strains only ($p = 0.002$). Salinity only affected the division rate of Kb1-2 (*Chaetoceros* sp., $p = < 0.0001$) 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 the 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. 3).

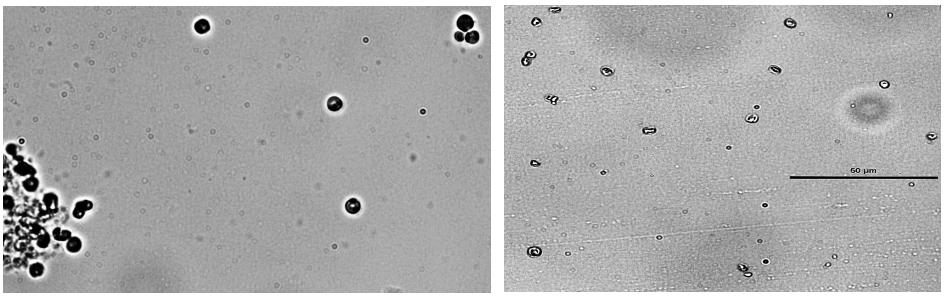
Table 1. List of strains isolated in 2013; location, date of isolation, and size range

Strain number	Tentative species name	Isolation location	Date of isolation	Size (μm)
Kb2-6	*Melosira* cf. *moniliformis*	Wanggu River Estuary	June	10.6-26.9
Kb1-2	*Chaetoceros* sp.	Kendari Bay	August	4.5-11.8
Kb1-3	Unidentified	Wanggu River Estuary	August	1.2-10.0
Kb1-5	Unidentified	Wanggu River Estuary	August	1.9-5.8



a b

Figure 1. Scanning electron micrographs of microalgal isolates in this study. Kb2-6 (a) identified as the chain-forming *Melosira* cf. *moniliformis*, and Kb1-2 (b) a *Chaetoceros* sp.



a b

Figure 2. Photomicrographs of Indonesian microalgae strains denoted as Kb1-3 (a) and Kb1-5 (b) at 40× magnification using a light microscopy. Scale bar is 50 μm.

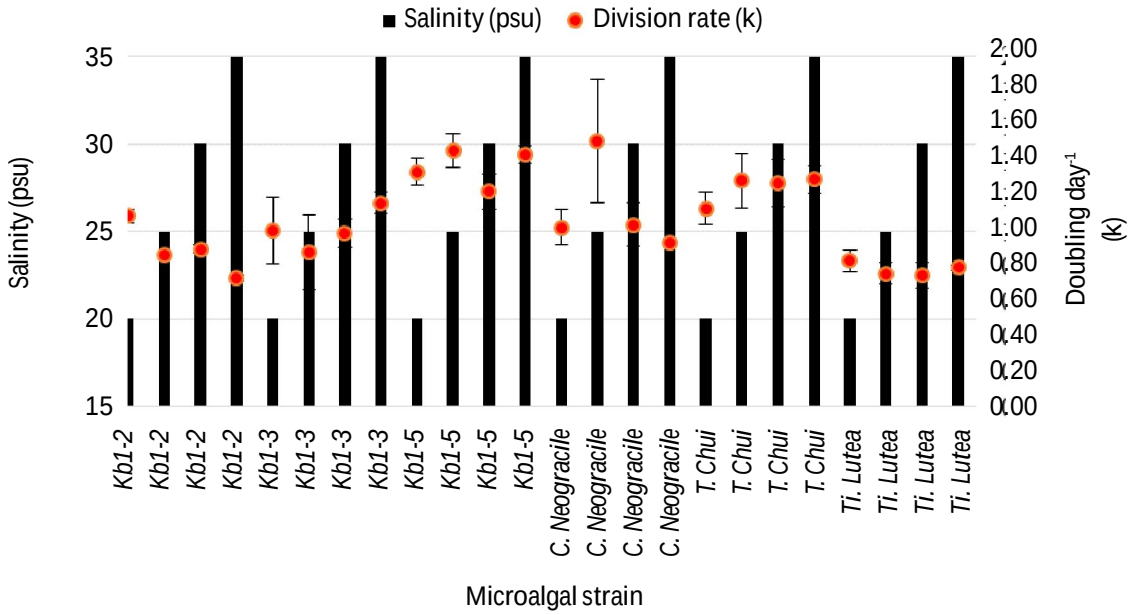


Figure 3. Division rate (k, doubling.day^{-1}) of cultured microalgae at different salinities, 20, 25, 30, and 35 gL^{-1}, during logarithmic phase of growth. Log phase was at time intervals of 0 to 3 days for Kb1-2, Kb1-5 at all salinities tested, Kb1-3 at 25 gL^{-1} and *C. neogracile* at 20, 25 and 30 gL^{-1}.

The differences among microalgal strains ($p = < .0001$) as well as salinity ($p = < .0001$) and the interaction between strain and salinity ($p = < .0001$) affected the yields of cultured microalgae. Of the Indonesian strains, higher final biomass was observed at 35 gL^{-1} in Kb1-3, whereas the 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 gL^{-1}) and at higher (30 and 35 gL^{-1}) salinities. Kb1-2 cultured at 25 gL^{-1} produced the lowest yield whereas yield of Kb1-3 at the same salinity was similar to that of 20 and 30 gL^{-1}. The final yields of *C. neogracile* and *T. chui* were not affected by salinity, unlike *Ti. lutea*. The final yield of *Ti. lutea* was significantly higher at 25, 30 and 35 gL^{-1} than at 20 gL^{-1}. Regardless of salinity, two strains, Kb1-3 and Kb1-5, showed the highest yields among all the microalgae tested, whereas the yield of Kb1-2 was similar to that of *Ti. lutea* and *C. neogracile*. *T. chui* had the lowest yield but statistically similar to *C. neogracile* (Table 2).

Both dry weight (DW) and ash free dry weight (AFDW) of all studied microalgae strains were not affected by the salinity levels or the interaction between salinity and strains but by differences among strains themselves ($p = < .0001$). DW and AFDW of the 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 gL^{-1} than at 25 gL^{-1}. Salinity affected DW and AFDW of *C. neogracile* and *Ti. lutea* but not *T. chui*. At higher salinity of 35 and 30 gL^{-1}, the DWs and AFDWs of *C. neogracile* and *Ti. Lutea* were higher (Table 3). Among the microalgal strains, the DWs of all three microalgal strains were similar to *Ti. lutea* and *C. neogracile.* Moreover, DW of *T. chui* was the highest compared to that of the rest of the strains (Table 3).

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. The highest cell densities were observed in Kb1-3 cultures on day 15 and Kb1-5 on day 8 at 25 gL^{-1}, with cell density of 65.9 and 42.4 cells·mL^{-1}·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 still experienced a lag phase. A significant growth continued up to day 8 for all microalgal strains and *C. neogracile* except Kb1-5 at 20 and 30 gL-1 that showed a decreasing growth after day 6. *T. chui* and *Ti. Lutea* continued to have a significant growth up to day 9 and 10, respectively (Fig. 4).

Indonesian microalgae showed similar growth rates during the logarithmic phase across all tested salinities, suggesting that they are euryhaline species, although they produced different final biomasses. 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 *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 their lifecycles. In diatoms, if rapid asexual reproduction occurs, there will consequently be a reduction in average population cell size over time because of the reproductive mechanism in which one

Table 2. Yield of cultured microalgae (mean ± SE) $\times 10^6$ cells.mL^{-1} during stationary phase at different salinities from 8 points at 13 and 15 days of culture. Values in a row with the same superscripts are not significantly different ($p > 0.05$)

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

Table 3. Dry weight (DW) and ash free dry weight (AFDW) in after 15 days of culture of Indonesian microalgal strains, *C. neogracile, T. chui,* and *Ti. lutea* at different salinities, expressed as $\mu g.mL^{-1}$. All values are mean ± SE from 4 replicates. Values in a row with the same superscripts are not significantly different ($p > 0.05$)

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

of the new daughter cells is always smaller than the parent cell (Round, 1990; Lee, 2008). Flagellates without silica cell walls such as *T. chui* and Prymnesiophyceae such as *Ti. lutea* used in this study tend to produce similar or larger cell sizes under favorable environmental conditions (Danquah *et al*., 2009).

The salinity level used in the study was not a limiting factor for the growth of the microalgal strains evaluated. 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 & Kim (2002), and Wood *et al*. (2005). The differences in cell density, DW, and AFDW may be caused 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 the output rate or yield of a microalgal culture, using *Spirulina* as a model, changed over time and became lower as the growth rate decreased during the 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 the 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 the Indonesian microalgal strains was higher than that of *T. lutea* although *T. lutea* showed a similar growth to *Chaetoceros* sp. *Chaetoceros* sp. and Kb1-3 did not enter stationary phase at day 15 of culture, except for Kb1-3 at 25 gL^{-1}. This growth pattern was different from that of found by Miller *et al*. (2014) where the late stationary phase occurred at day 12 in cultured *Chaetoceros calcitrans.* Finally, the maximum cell density of *T. lutea* during the stationary phase reported here was almost three times lower than that of reported by Huerlimann *et al*. (2010), but similar to that of reported by Renaud *et al*. (1999) and almost six times lower than that of reported by Fidalgo *et al*. (1998). The variation in the final cell density may be attributable to the different cell densities at inoculation as well as culture conditions, such as light intensity, culture medium, temperature, and aeration (Fidalgo *et al*., 1998; Borowitzka, 2013; Gorgonio *et al*., 2013). Further studies are needed to determine whether the cell of Indonesian microalgae strains stopped dividing because of nutrient or light limitation.

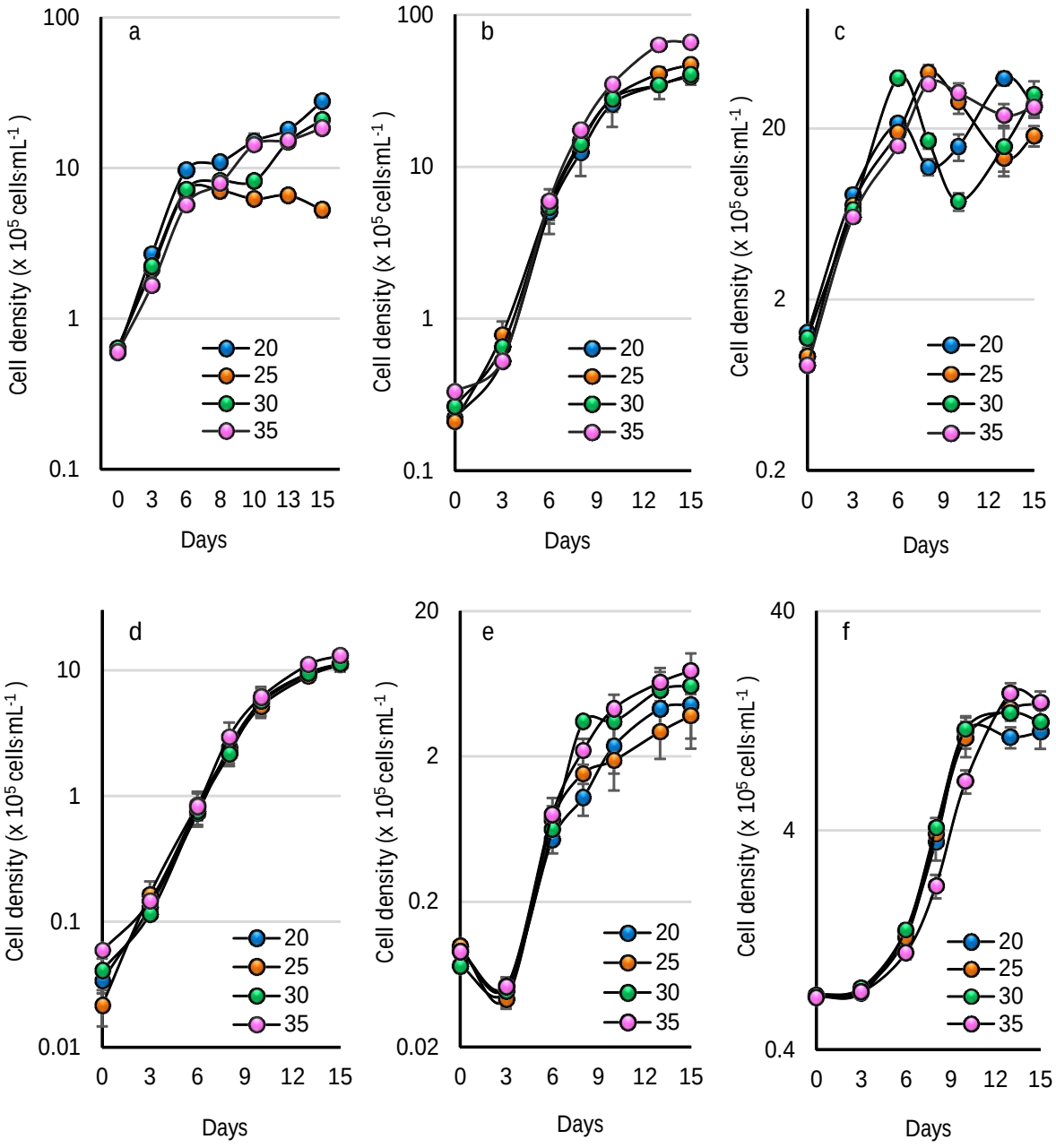


Figure 4. Increase in cell density of Indonesian microalgae *Chaetoceros* sp., Kb1-3, Kb1-5 (a, b, c, respectively) over culture period compared to *C. neogracile, T. chui,* and *Ti. lutea* (d, e, f, respectively) at different salinities in gL^{-1}. Standard error about the mean are represented by error bars from 4 replicates. Note that the y axes are not all on the same scale.

Although a prolonged culture up to late stationary phase may compromise the harvested biomass as shown with Kb1-2 and Kb1-5 strains, some research has shown that this practice increases the nutritional value of microalgae particularly total lipid and fatty acid content (Patil *et al*., 2007; Schwenk *et al*., 2014; Nalder *et al*., 2015). In order to be viably used as live food in aquaculture, it is imperative to keep both the microalgae cell density and nutrition variables at optimal levels; therefore, the harvest for feeding purposes might best be conducted during the late logarithmic phase (at day 6 or 7 of culture). Our study on nutritional content of these newly isolated microalgal strains (Iba, 2016) showed that the total lipid contents after 15 days of culture or during stationary phase ranged from 4.4-25 % DW which is well above the lipid requirement for shrimp in hatchery (Nuñez *et al*., 2002). Furthermore, the ability to sur-

vive under a wide range of salinity and nutrient-deficient condition is an important trait of microalgae in aquaculture. In the face of a 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; Schwenk *et al*., 2014). Therefore, culturing euryhaline microalgae species accustomed to salinity fluctuations is one of promising solutions to continue producing high quality seafood products reliably over time.

CONCLUSION

The growth and final yield of the Indonesian microalgal strains were not affected by the different salinity level and comparable to the reference strains *T. lutea*, *C. neogracile,* and *T. chui*. Three of the four microalgal strains have size, salinity tolerance, and growth characteristics suitable as live feed in marine fish hatcheries. These findings are in line with the initiative to utilize local microalgae for aquaculture purposes to reduce production cost, promote sustainable and environmentally friendly aquaculture practices in the region and in Indonesia in general.

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