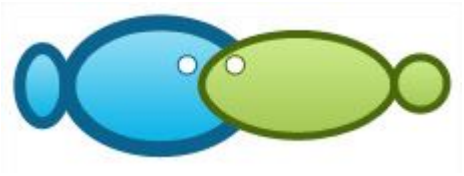

Some eco-physiological responses of *Chlorella vulgaris* culture in different environmental conditions

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Abstract. Growth responses of *Chlorella vulgaris* cultured at different environment such at laboratory, semi massal scale and massal scale seem dissimilar. This research evaluated *C. vulgaris*'s growth in respond to light intensity, temperature, ammonia, nitrate, orthophosphate, total bacteria and *Vibrio* bacteria at different culture environment. This result indicated that *C. vulgaris* grew higher at temperature and irradiation were about 28±0.7°C and 247-205 μE.m^{-2}s^{-1} respectively and preferring to use ammonia more than at temperature and irradiation were approximately 25±1.5°C and 88-3 μE.m^{-2}s^{-1} respectively and supplied by nitrate. Moreover as nitrogen source, ammonia is taken less in amount than nitrate by *Chlorella* to grow. Additionally at high growth of *C. vulgaris*, the number of total bacteria and *Vibrio* bacteria in culture environment were more stable. However as orthophosphate linearly declined at culture environment, *C. vulgaris* and total bacteria decreased whereas *Vibrio* bacteria increased.
Key Words: growth, ammonia, nitrate, orthophosphate, total bacteria, *Vibrio* bacteria.

Introduction. *Chlorella* is a green microalgae that is widely used and cultivated due to its capability to produce a range of substances. It contains proteins 51-57%, lipids 25-75% and carbohydrates 12-26% dwt^{-1} and it is also rich in fatty acids, antioxidants (carotenoids), vitamins and minerals (Harun et al 2010; Sharma et al 2011; Priyadarshani & Rath 2012). Recently *Chlorella vulgaris* is used for several purposes such as feeding in aquaculture, human nutrition and it is potential to be used as a biodiesel source and in environmental applications such as removal of excess organic/inorganic nutrients and heavy metals currently (waste-water treatment) (Harun et al 2010; Sharma et al 2011; Priyadarshani & Rath 2012; Zhang et al 2014). Since microalgae production as hatchery feeds increase, technical innovation is necessary to develop intensity and capacity of production systems (Brown & Blackburn 2013).

Production of *Chlorella* depends on many factors, the most important of which are nutrients availability, temperature, light, and bacteria density. These factors influence the growth of *Chlorella* and biomass composition due to changes in metabolism (Sharma et al 2011). The major nutrients for growth of common algae are nitrogen and phosphorus. Thus nitrate and ammonium are the most important nitrogen source for microalgae growth, and microalgae able to take up both ions energetically (Domingues et al 2011).

On the other hand bacteria should also be considered in control efficiency of mass algal culture systems and for the broader purposes since it is an inherent part of the biotic environment of microalgae and culture medium (Goecke et al 2013; Krohn-Molt et al 2013; Green et al 2015). Bacteria and microalgae interaction can be positive, in which microalgae growth promoted by bacterial products such as inorganic substance, carbon dioxide and other growth factors. However, bacteria could be negative on algae, leading to death of algae, while algae can suppress growth of bacteria by secreting antibiotic compounds, synthesis of ecto-enzymes, and competition for inorganic matters (Qu et al 2014). The mainly bacteria associated with *C. vulgaris* is Alphaproteobacteria (i.e., the genus *Sphingomonas*) (Krohn-Molt et al 2013). Therefore this present study evaluated

the growth response of *C. vulgaris* in respond to light intensity, temperature, ammonia, nitrate, orthophosphate, total bacteria and *Vibrio* bacteria cultivated at laboratory, semi massal and massal scales.

Material and Method. *C. vulgaris* was obtained from Laboratory of Live Feed, Center of Fishery and Mari-culture Ambon. Seawater used for microalgae culture had salinity 33 ppt and was previously filtered and sterilized. *C. vulgaris* culture used medium Guillard F/2 for laboratory (indoor culture) and semi massal scale (semi indoor culture), except for massal scale (outdoor culture) used commercial fertilizer (Table 1). *C. vulgaris* sp. was cultivated within six days. Each culture scale has three replications. Volume, initial density, temperature, irradiance of the three culture scales can be seen at Table 1.

Table 1

Environmental condition of *Chlorella vulgaris* culture scales

No	Culture scale	Culture volume (L)	Initial culture density (cells/mL)	Temperature (^oC)	Irradiation ($\mu E.m^{-2}s^{-1}$)	Nutrients composition/L		
						F_2 (mL)	Trace elemen (mL)	Vitamin (mL)
1	Laboratory	6	2.4 x10^6	24±1.3	3-55	6	6	3
2	Semi Massal	100	4.3 x10^6	25±1.5	60-88	100	100	50
3	Massal	1000	6.6 x10^6	28±0.7	205-247	Urea 62 g, ZA 63 g, TSP 30 g, $FeCl_3$ 5 g, EDTA 5 g, Vit. B_{12} 6.5 g, $NHPO_4$ 7.5 g and KNO_3 50 g		

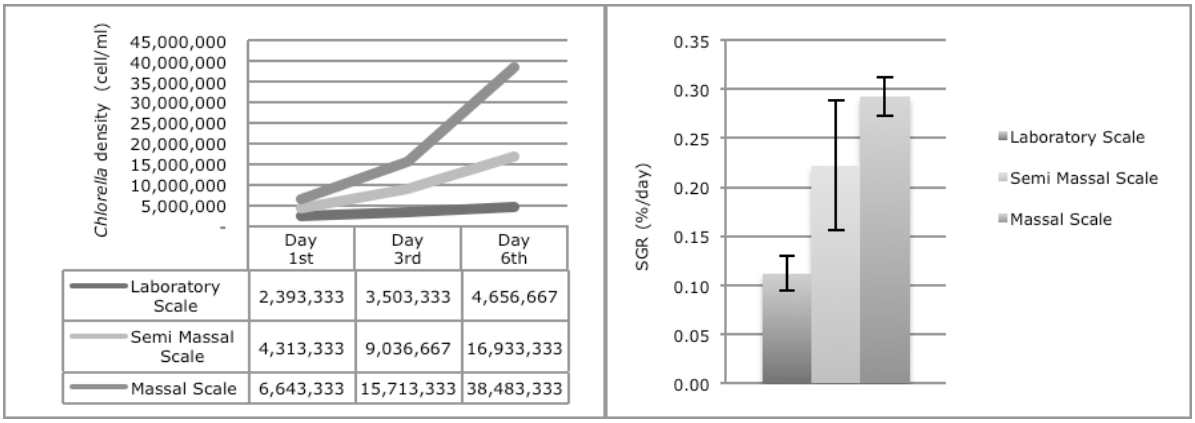
Data collection and analyzing. Sampling water quality was conducted within six days of culture period (until log phase) before *C. vulgaris* was taken for feeding rotifer and fish larvae. Sampling was performed once daily at 10.00 am while temperature and light intensity were measured three times a day at 07.00 am, 13.00 pm and 18.00 pm. *C. vulgaris* cells density was counted using the method described by Wagley (2012), while total bacteria cultured used TSA (Triptic soy agar) and *Vibrio* bacteria used TCBSA (Thiosulfate Citrate Bile salt Agar) subsequently both counting used colony counter. Ammonia, nitrate and orthophosphate concentration analyzed in spectrophotometer. *C. vulgaris* growth response indicates by specific growth rate (K) and doubling time (Dbt), was calculated used the formula described by Ashraf et al (2011).

$$K = Ln(D_t)-Ln(D_o)/ t_t- t_o$$

$$Dbt=1/K$$

Where: D_o is microalgae density (cell.mL^{-1}) at the beginning, D_t is microalgae density at t time, and t_t-t_o(day) was the period of time. Then all the data including growth rate, ammonia, nitrate and phosphate concentration and total bacteria and *Vibrio* bacteria density were analyzed descriptively using graphs.

Results and Discussion. Growth of *C. vulgaris* cultured at different culture environment is presented in Figure 1a. It seems that *C. vulgaris* culture at massal scale situated at outdoor had the best growth response compare to semi massal scale (semi indoor) and laboratory scales (indoor). *C. vulgaris* density multiplied greatly at massal scale, which achieved double density within 2.4 days, then followed by semi massal scale in 3.1 days and laboratory scale in 6.2 days. Growth rate of *C. vulgaris* is massal scale attained 0.29±0.02, and followed by semi massal scale (0.22±0.07) and laboratory scale (0.11±0.02) (Figure 1b).

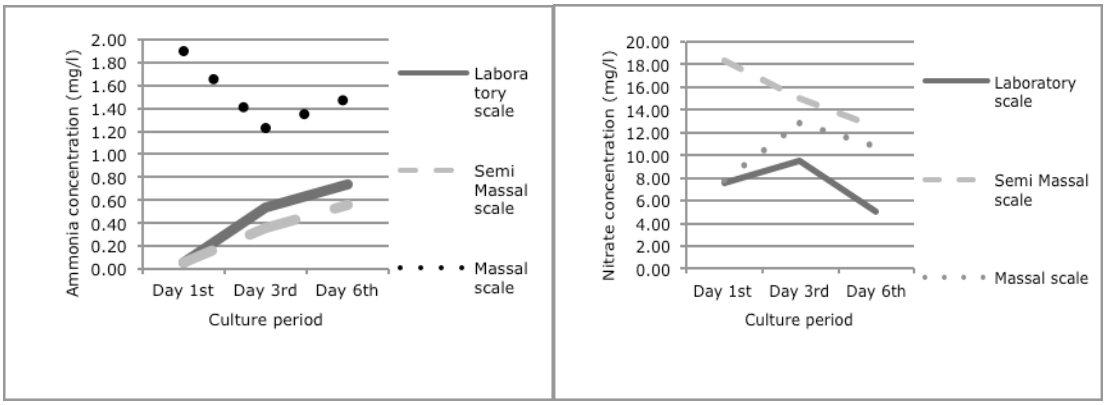


(a) (b)

Figure 1. *Chlorella vulgaris* density (a) and SGR (b) of *Chlorella vulgaris* at different culture scale.

This high growth response of *C. vulgaris* at massal scale is probably due to high temperature and irradiant which were about 28±0.7°C and 205-247 $\mu E.m^{-2}s^{-1}$ respectively, besides nutrients availability. This finding correspond to Sharma et al (2012) stated that temperature in range 25-30°C of natural light is best for growth and biochemical content of *C. vulgaris.* Moreover Munoz & Guieysse (2006) and Tang et al (2011) have been presented that light intensity in range of 200-400 $\mu E.m^{-2}s^{-1}$ and the optimal at 350 $\mu E.m^{-2}s^{-1}$ for algal growth generally.

This present study also demonstrated that *C. vulgaris* grew best at massal scale was supplied by urea, which can be assumed that ammonium from mineralized urea is preferred to take up by *C. vulgaris* than nitrate. It was indicated by decreasing of ammonia concentration within culture period that was about 0.41 mgL^{-1} in contrast to nitrate, which increased about 2.96 mgL^{-1} though nitrate concentration was more plentiful than ammonia concentration at culture media (Figure 2a and 2b).



(a) (b)

Figure 2. Concentration of ammonia (a) and nitrate (b) at different culture scales of *Chlorella vulgaris.*

On the other hand at semi massal and laboratory culture scales which nitrate from kalium nitrate (KNO_3) was nitrogen source, *C. vulgaris* preferred to take up nitrate than ammonia as showed in this study. Nitrate concentration at semi massal scale decreased approximately 5.93 mgL^{-1} and at laboratory scale was about 2.54 mgL^{-1} whereas ammonia increased about 0.51 mgL^{-1} and 0.69 mgL^{-1} respectively (Figure 2a & b). This finding clearly stated that *C. vulgaris* might utilize ammonia and nitrate as nitrogen source. However as green microalgae, *C. vulgaris* prefer to uptake ammonia since ammonia concentration at culture media was plentiful than nitrate. This result support Domingues et al (2011) statement that ammonium availability inhibited nitrate uptake of most microalgae and preference for ammonium was group specific and it was observed

mainly in green algae and cyanobacteria. Moreover in general cells take up nitrogen, Lomas & Glibert (1999) also stated that nitrate should be reduced intracellular into ammonium and then assimilated into amino acid so nitrate assimilation needs more energetically demanding than ammonium.

In relation to orthophosphate uptake by *C. vulgaris*, this research found that all culture scales definitely declined along culture period that were about 0.48 mgL^{-1} at massal scale, 0.39 mgL^{-1} at semi massal scale and 0.18 mgL^{-1} at laboratory scale (Figure 3). This finding of green microalgae *C. vulgaris* is similar to the most behave of green macro-algae due to have high internal nitrogen reserve in chlorophyll cells lead them require more external phosphate to grow (Jamal et al 2011).

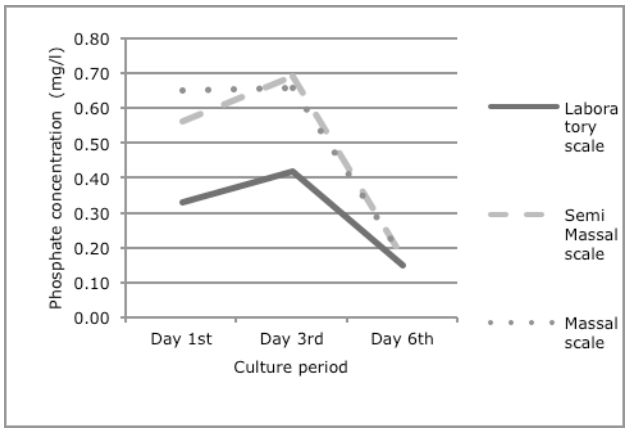
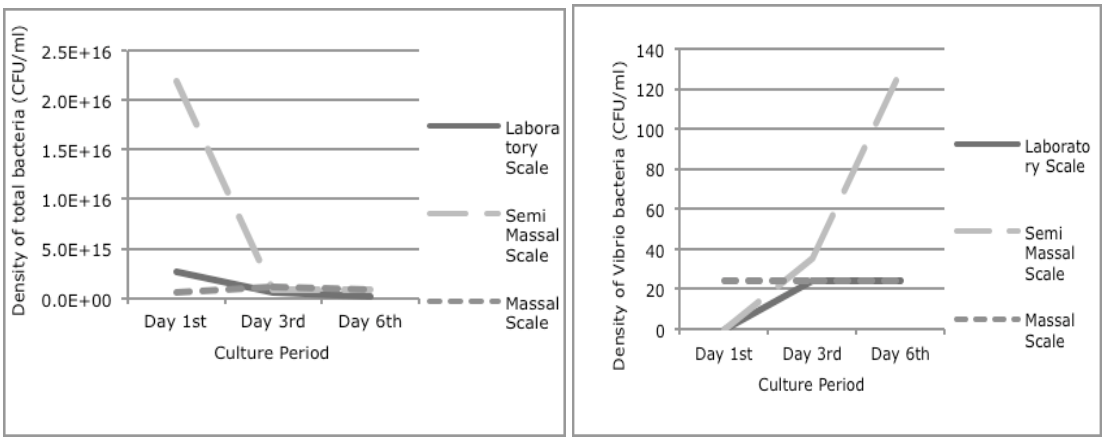


Figure 3. Concentration of orthophosphate at different culture scales of *Chlorella vulgaris.*

From microbiology point of view, this present study showed that total bacteria of all culture scales declined as *C. vulgaris* density multiply and orthophosphate decreased whereas *Vibrio* bacteria increased particularly at semi massal as shown (Figure 4a dan b).



(a) (b)

Figure 4. Density of total bacteria (a) and *Vibrio* bacteria (b) at different culture scales.

It was also describe that *C. vulgaris* grew better at massal scale which both total bacteria and Vibrio bacteria densities more stabled that were respectively in range 10^{14} $CFU.mL^{-1}$ and ≤25 $CFU.mL^{-1}$ along culture period. In contrast to the low growth of *C. vulgaris* cultured at laboratory and semi massal scales where total bacteria decreased approximately 10 $CFU.mL^{-1}$ and 10^2 $CFU.mL^{-1}$ as it followed by increasing of *Vibrio* bacteria on average 13 CFU/mL at laboratory scale and 130 $CFU.mL^{-1}$ at semi massal scale along culture period. This present result clearly illustrate that nutrients regulate the relationship between microalgae and bacteria (Fuentes et al 2016) and orthophosphate is the most competitive nutrient that can exist between bacteria and algae (Amin et al 2012) since bacteria can act as re-mineralizer in high nutrients level, but in low nutrients level it becomes as competitor (Guerrini et al 1998). Thus the fact of total bacteria

decreased in culture media as *C. vulgaris* density rise in line with Qu et al (2014), who stated that the growth of *C. vulgaris* is promoted at low concentrations of bacteria due to an inhibition effects on bacteria growth through such substance secreted by *C. vulgaris* at logarithmic phase. In addition decreasing of total bacteria and increasing of *Vibrio* bacteria could be related to increasing of N/P ratio in culture media since orthophosphate linearly declined. This finding support Obernosterer & Herndl (1995) statement, that limited availability of inorganic and organic phosphorus and N/P ratio also appears to have a severe impact on bacterial metabolism mainly total bacteria. Thus at low concentration of orthophosphate, opportunistic bacteria such as *Vibrio* is probably more efficient in utilizing orthophosphate than total bacteria and microalgae cells as well.

Conclusions. It can be concluded that beside of high light intensity and temperature, orthophosphate concentration is also definitely influence the growth of *C. vulgaris* since its sufficiency in culture medium might control *Vibrio* bacteria as well. Moreover as nitrogen source, ammonia is more efficient than nitrate for *C. vulgaris* culture.

Acknowledgements. This research is acknowledged to the Directorate of Research and Community Service, Ministry of Research, Technology and Higher Education that funded this research through Competitive Grants Programme. The same remarks are also submitted to the Head and staff of Center of Fisheries and Mari-culture Ambon for all the facilities provided during this research.

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Received: 17 August 2016. Accepted: 21 September 2016. Published online: 27 September 2016.
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How to cite this article:
Jamal E., Luturmas A., 2016 Some eco-physiological responses of *Chlorella vulgaris* culture in different environmental conditions. AACL Bioflux 9(5):1030-1035.