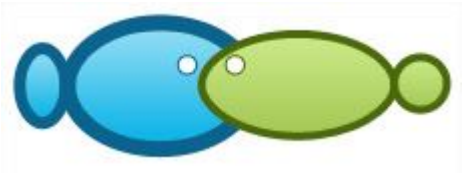

Biomolecular identification and optimization of growth performance and egg production in *Oithona* sp. under different salinity culture conditions

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Abstract. *Oithona* sp. is one of the copepod with the potential to serve as a live food organism for fish larvae and shrimps in estuary areas and seas due to its relatively small size, high nutritional content, and ability to adapt to changes in the temperature and salinity of its environment. Optimum salinity in the media could minimize the osmotic work load so as to increase growth and egg production in *Oithona* sp. This research was conducted to determine the optimum salinity for growth performance and egg production as well as to confirm the species of *Oithona* sp. that was used in this study through DNA barcoding. A completely randomized design was employed in this research with 6 treatments and 5 trials per treatment. The treatments consisted of culture media with salinities of 14, 18, 22, 26, 30, and 36 ppt. According to the results of species identification using DNA barcoding, this species belongs to Oithona and is 98% identical to *Oithona similis*. The salinity of the culture medium had a significant effect ($p < 0.05$) on growth performance and egg production in *O. similis*. A salinity level of 18 ppt in the medium gave the best growth performance (total density 16.54±0.24 ind mL^{-1}, population-specific growth rate of *Oithona* sp. 0.140±0.001 ind day^{-1}) and egg production (24.40±0.55 eggs ind^{-1}) for *O. similis* in culture. Based on the total density and egg production of *O. similis*, the optimum salinity for *O. similis* in the culture medium is 19.4 ppt.

Key Words: optimization, *Oithona* sp., osmolarity, growth, reproduction.

Introduction. *Oithona* spp. are important copepods in the marine trophic food web, due to their role as the main prey for the majority of fish and shrimp larvae (Sampey et al 2007; Drillet et al 2011). The population of this copepod genus is affected by the availability of its live food sources (Noyon & Froneman 2013), predators, and competitors (other copepods), as well as several abiotic conditions, such as temperature and salinity (Beyrend-Dur et al 2011). In a natural ecosystem, fish and shrimp larvae consume various species of copepod nauplii (Ma et al 2013). On the other hand, the aquaculture industry utilizes *Oithona* sp. nauplii as live feed for the rearing of fish and shrimp larvae (Santhanam et al 2011; Broach et al 2017). In addition, *Oithona* sp. is the preferred live food source for aquaculture, due to its short life cycle for biomass production (14-16 days), the suitability of its body size for the mouths of larvae (60-220 µm), and its unique swimming pattern, which increases consumption by larvae compared with *Artemia* and other rotifers (Lavens & Sorgeloos 1996). Milione & Zeng (2007) stated that *Oithona* sp. can adapt to sudden changes in salinity and temperature, and this capability increases its survival rate under fluctuating environmental stress. Moreover, the high unsaturated fatty acid and amino acid contents of this copepod make them nutritionally essential for the early stage of larval growth (Santhanam & Perumal 2012). All these

superior characteristics have led to mass production of *Oithona* sp. for aquaculture purposes.

In the culture of *Oithona* sp., Santhanam & Perumal (2012) stated that the initial density, use of microalgae as the feed, temperature, and salinity had a direct influence on the biomass production, growth performance, and survival rate of this copepod. Furthermore, water salinity was recognized as an important factor that impacted on the physiological conditions of feed consumption and growth in *Oithona* sp. (Kinne 1963). In addition, Beyrend-Dur et al (2011) explained that salinity also impacted on the osmoregulatory behavior, fecundity, and lifespan of this copepod. Previous studies found that every species of copepod has its own favorable salinity for growth and egg production (Cutts 2003; Molejón & Lajonchère 2003; Santhanam & Perumal 2012; Barroso et al 2015; Jose et al 2016; Broach et al 2017).

Unfortunately, *Oithona* sp. is rarely utilized as a live food organism in Indonesian aquaculture practices due to the lack of information about the optimum culture conditions for this copepod. Moreover, most of the *Oithona* used in Indonesia is of an unidentified species, for which suitable culture conditions based on the published literature are unclear, given that every species of this copepod has its own set of favorable environmental conditions. To address these problems, this research was conducted to determine the optimum salinity for growth performance and egg production, as well as to confirm the species of *Oithona* that was used in this study through DNA barcoding.

Material and Method. This study was conducted at the Coastal Area Development Laboratory (LPWP), Faculty of Fisheries and Marine Science, Diponegoro University, Jepara Campus, Central Java, Indonesia from April to July 2018. The *Oithona* sp. employed was from the culture collection of the Marine Culture Development Research Centre (BBPBL) in Lampung, Indonesia. The phytoplankton used in this study was *Chaetoceros calcitrans* from the Live Food Organisms Laboratory, Research Center of Brackish-Water Aquaculture (BBPBAP), Jepara, Indonesia.

Every liter of modified Walne media consisted of $NaH_2PO_4.2H_2O$ (20 g); $NaNO_3$ (100 g); Na_2.EDTA (45 g); $MnCl_2.4H_2O$ (0.36 g); $FeCl_3.6H_2O$ (1.3 g); and H_3BO_3 (33.6 g). Every 100 mL of the trace metal solution consisted of $ZnCl_2$ (2.1 g); $CoCl_2.6H_2O$ (2.0 g); $CuSO_4.4H_2O$ (0.9 g); and $(NH_4)_8.Mo_7O_{24}.4H_2O$ (2.0 g). Every 100 mL of vitamin solution consisted of vitamin B_{12} (0.01 g); thiamin (0.01 g); and biotin (200 µg) (Chilmawati & Suminto 2016).

The populations of *Oithona* sp. at the stadia, nauplii, copepodite, and mature stages were observed under a microscope (Olympus CYK41).

Preparation of culture media. Natural sea water was collected from Jepara beach then filtered using palm fiber and carbon charcoal to eliminate macro impurities. The filtered natural sea water was transferred to a fiberglass basin for sterilization using chlorine and sodium thiosulphate ($Na_2S_2O_3$). A total of 30 ppm of liquid chlorine was added to the filtered natural sea water and incubated for 24 h. Then, a total of 15 ppm of sodium thiosulphate ($Na_2S_2O_3$) was added and incubated for another 24 h or until the smell of chlorine had vanished. This water was used for mass culture of microalgae and *Oithona* sp. Water salinity treatments were established as 14, 18, 22, 26, 30, and 34 ppt. The conversion of salinity was calculated using an equation according to Anggoro (1992):

$$S2 = \frac{a \times S1}{n + a}$$

where: S2 = the targeted salinity (ppt);
a = the volume of initial natural sea water (L);
S1 = the salinity of initial natural sea water (ppt);
N = the volume of distilled water that was added (L).

Culture conditions of Chaetoceros calcitrans. The sterilized natural sea water was boiled then chilled until the water temperature reached 27-28°C and transferred to a transparent, 10-L plastic jar for cell culture. The *C. calcitrans* was cultured in 1 ppt of a

modified Walne media, at room temperature (26-28°C), with a salinity rage of 24-26 ppt, illumination by a lamp (4000 lux) for 24 h/day, and controlled aeration. The cell density (cells mL^{-1}) was calculated daily using a hemocytometer to determine the growth phase of phytoplankton. The biomass of *C. calcitrans* was harvested during the exponential phase (Creswell 2010).

***Culture conditions of Oithona* sp.** *Oithona* sp. was cultivated in 30 vials (30 mL in size), with a total culture volume of 10 mL/vial, without aeration, at a water temperature of 27-29°C, and at pH 8 for 20 days. The initial cell culture density of *Oithona* sp. was 1 cell mL^{-1} (Lee et al 2006). For a feeding step, the ad libitum method was applied. The *C. calcitrans* were counted using a hemocytometer, harvested by centrifugation, and then transferred to the *Oithona* sp. culture (Chilmawati & Suminto 2016). The amount of *C. calcitrans* given to *Oithona* sp. was 0.01 mg/ind or equal to 8.33×10^5 cell ind^{-1} (Lee et al 2006).

***Design of experiment*.** A completely randomized design with 6 treatments and 5 replications was carried out in this study. The salinity treatments consisted of 14 ppt (osmolarity: 405.48 mOsm L H_2O^{-1}); 18 ppt (osmolarity: 522.87 mOsm L H_2O^{-1}); 22 ppt (osmolarity: 640.27 mOsm L H_2O^{-1}); 26 ppt (osmolarity: 757.66 mOsm L H_2O^{-1}); 30 ppt (osmolarity: 875.06 mOsm L H_2O^{-1}); and 34 ppt (osmolarity: 992.45 mOsm L H_2O^{-1}). The cell density of *Oithona* sp. was calculated once every 4 days to obtain the total population size, growth performance (r), and egg production.

***Data collection and statistical analysis*.** The osmotic work level value was obtained by calculating the difference between the osmolarity of the culture media (*millieu exterieur*) and the osmolarity of the internal body fluid of *Oithona* sp. using a digital micro-osmometer (Roebling 4774, Germany) (Anggoro et al 2018). The osmolarity value of the internal body fluid of *Oithona* sp. was converted to units of ppt by using an equation suggested by Anggoro (1992):

$$\text{Salinity} = \frac{\text{Osmolarity}+5.4081}{29.3489}$$

***The population specific growth of Oithona* sp.** It was calculated using the initial and final population densities of each treatment. The population growth rate (r) was calculated using a formula proposed by Cheng et al (2011):

$$r = \frac{\ln Nt - \ln N_0}{t}$$

where: r = population growth rate (ind mL^{-1});
N_0 = initial density;
N_t = final density.

Egg abundance was calculated according to the following formula (Zamora-Terol et al 2014):

$$\text{Eggs abundance} = \frac{\Sigma\, s \times e}{\Sigma\, n}$$

where: s = amount of egg shack;
e = the average amount of eggs per egg shack;
n = number of the impregnated females.

Data were analyzed using the SPSS 16 software package with $p < 0.05$ (Ghozali 2006). Before further analyses, the data were analyzed for normality and homogeneity. ANOVA was applied to understand the influence of each treatment on the osmotic work level values, total population size, rate of population-specific growth, and egg production. A least significant difference (LSD) test was applied to explain significant differences

between median values in each treatment, while a polynomial orthogonal test was applied to determine the optimum salinity.

***Molecular identification of Oithona* sp.** Approximately 100 *Oithona* sp. were collected from the pure culture by using a micropipette, transferred to a centrifuge tube, and kept at -5°C to stun the sample. Then, the *Oithona* sp. was separated from the culture medium by orbital centrifugation (8500 r.p.m.), and the cells were reserved for DNA extraction, which was performed according to Cornils (2015) and Oliveira et al (2017) using the Chelex method. The sample was ground using a mortar and pestle and transferred to a PCR tube containing 100 µL of 10% Chelex. The mixture was centrifuged at 10,000 r.p.m., heated at 95°C for 25 min, and centrifugated again. The supernatant was collected and transferred to a new sterile microtube for further analysis. The COI region of the DNA was amplified by PCR using the universal marine animal primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Oliveira et al 2017). The following PCR cycling conditions were applied in this study: denaturation at 94°C (60 s), annealing at 39°C (60 s), extension at 72°C (90 s). The quality of the PCR product was analyzed by using gel electrophoresis and visualized using the GelDoc system (Sibero et al 2018). Furthermore, the PCR product was sent to 1st Base Malaysia for sequencing. A cladogram tree was reconstructed according to Sibero et al (2017) using the MEGA software package.

Results

***Species confirmation of Oithona* sp.** As mentioned, the *Oithona* sp. used in this study was a copepod collection from a research center, and there was no information on its exact species composition. According to the results of species identification using DNA barcoding, this species belonged to *Oithona* and was 98% identical to *Oithona similis*. The result of cladogram tree reconstruction of this species is displayed in Figure 1.

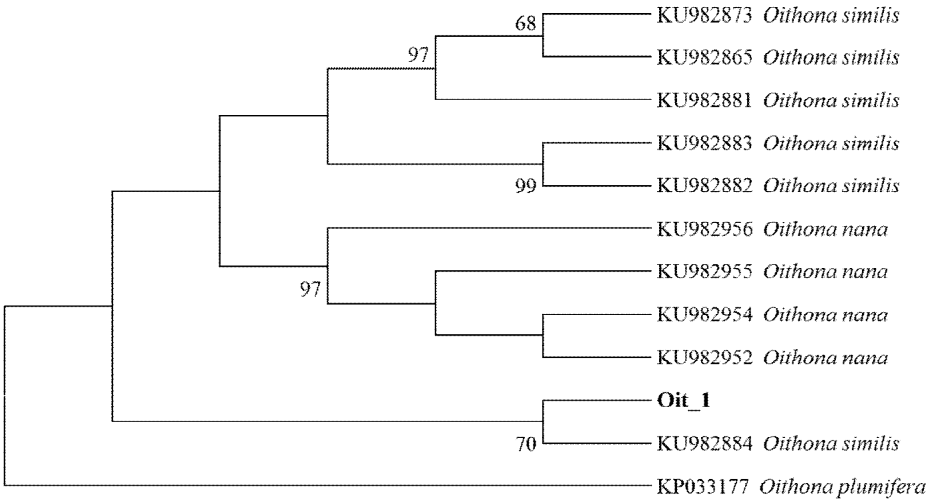

Figure 1. Cladogram reconstruction of *Oithona similis*.

Osmotic work level. The results of variance analysis indicated that the salinity level in the culture medium had a significant impact ($p < 0.05$) on the osmotic work level in *O. similis*. The LSD test result showed a significant difference between the median values in each treatment ($p < 0.05$). Table 1 shows the osmotic work levels of *O. similis* at different media salinity levels.

Table 1

The osmotic work levels of *O. similis* in different media salinity

Salinity (ppt)	*Osmolarity (mOsm L H_2O^{-1})*		*Osmotic work level (mOsm L H_2O^{-1})**
	Culture media	*O. similis internal body fluid*	
14	405.60±0.89	446±1.58	40.40±1.52[c]
18	522.8±1.30	553±1.48	30.20±1.48[a]
22	640.60±0.89	677±2.12	36.40±2.51[b]
26	757.20±0.84	683±2.74	74.20±2.68[d]
30	875.20±0.84	782.40±3.91	92.80±4.15[e]
34	992.80±1.10	890.40±6.47	102.40±6.31[f]

*Mean±SD. Different lowercase letters indicate a significant difference between treatments at $p < 0.05$.

O. similis population. According to the results of nauplii, copepodite, adult, and total *O. similis* enumeration on the last day of cultivation (Day 20), it was noted that most of the *O. similis* were in the nauplii stage at salinity levels of 14 ppt, 18 ppt, and 22 ppt. In contrast, at a salinity level of 26 ppt, 30 ppt, and 34 ppt, it was noted that *O. similis* was in the adult stage (Table 2).

Table 2

Numbers of nauplii, copepodite, adult and population of *O. similis* (ind 10 mL^{-1})

Salinity (ppt)	*Stadia*			
	Nauplii	*Copepodit*	*Adult*	*Population**
14	44.40±1.00	31.80±1.64	36.40±3.58	112.60±2.51[c]
18	68.80±0.89	37.40±0.89	59.20±3.03	165.40±2.41[a]
22	58.40±0.55	45.80±2.05	48.00±3.94	152.20±2.49[b]
26	22.80±1.30	25.60±0.89	57.80±1.64	106.20±1.79[d]
30	21.20±0.84	18.40±1.14	37.60±3.58	77.20±3.63[e]
34	20.20±0.84	12.80±0.84	34.40±2.30	67.40±1.82[f]

*Mean±SD. Different lowercase letters indicate a significant difference between treatments at $p < 0.05$.

The population growth curve of *O. similis* (Figure 2) indicated that the highest density on the last day of cultivation (Day 20) was attained at a salinity of 18 ppt (165.40±2.41 ind 10 mL^{-1}), while the lowest density was attained at a salinity value of 34 ppt (67.40±1.82 ind 10 mL^{-1}). Figure 2 shows that the density of *O. similis* increased significantly from Day 16 to Day 20 in each treatment.

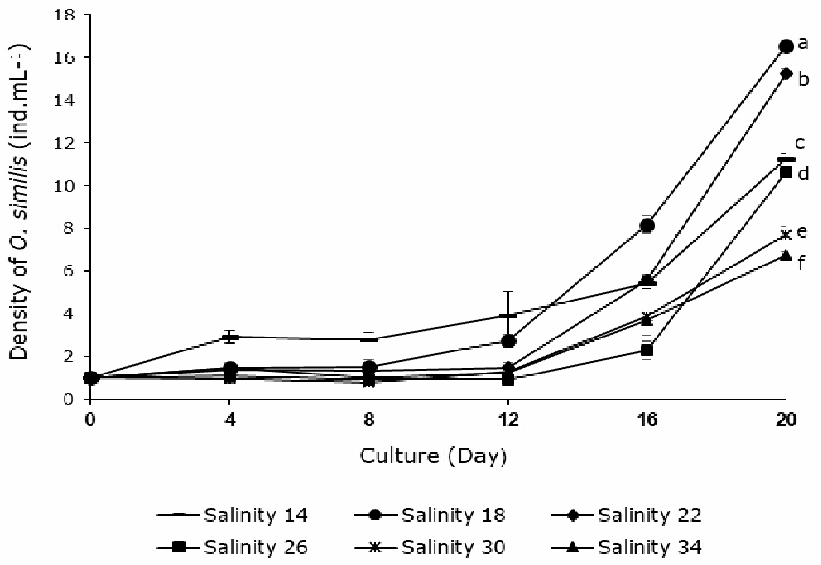

Figure 2. Growth performance of *Oithona* sp. at different salinities.

The correlation of *O. similis* density with media salinity is described by the curve shown in Figure 3. The result of a polynomial orthogonal test for the density of *O. similis* under different salinity conditions gave a quadratic function with $y = -0.042x^2 + 1.6321x - 1.6761$ and $R^2 = 0.7512$. In addition, this pattern also gave an optimum media salinity for *O. similis* cultivation at 19.4 ppt. R^2 values indicated that 75.12% of the *O. similis* density was affected by the salinity, while the other 24.88% was affected by other untested factors. Moreover, Figure 3 shows that the density of *O. similis* was inversely correlated with the response of osmotic work levels at different salinities.

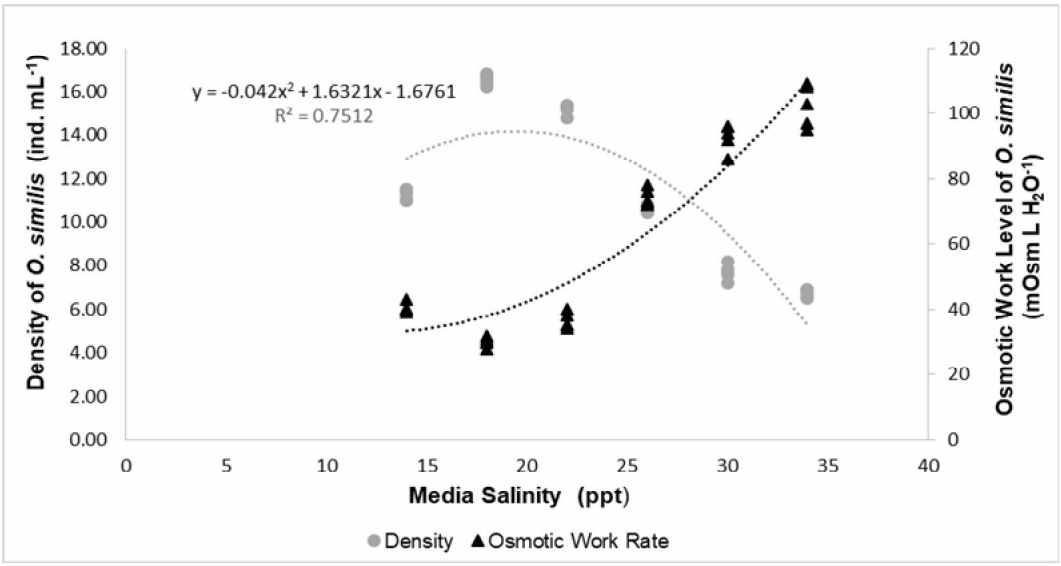

Figure 3. Correlation between the density of *Oithona* sp. and the osmotic work level at different salinities.

The population specific growth of O. similis. It was noted that salinity had a significant impact ($p < 0.05$) on the population-specific growth of *O. Similis*. The histogram in Figure 4 shows that the population-specific growth rate of *O. similis* reached its highest value at a salinity of 18 ppt with a cell number of 0.140±0.001 ind day^{-1}. The result of statistical analysis indicated that the growth rate in the 18 ppt salinity treatment was significantly different ($p < 0.05$) from those at salinity values of of 14 ppt (0.121±0.001 ind day^{-1}), 22 ppt (0.136±0.001 ind day^{-1}), 26 ppt (0.118±0.001 ind day^{-1}), 30 ppt (0.102±0.002 ind day^{-1}), and 34 ppt (0.095±0.001 ind day^{-1}).

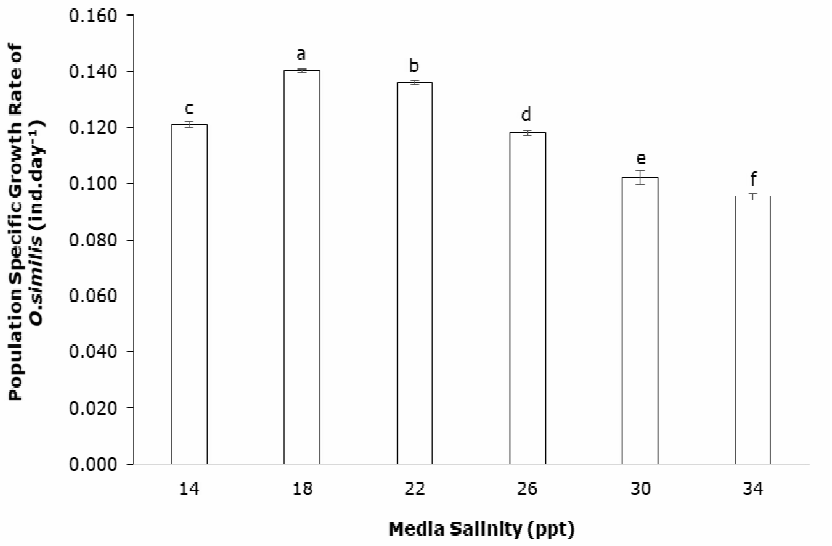

Figure 4. Histogram of the population-specific growth rate of *O. similis*.

The curve of population-specific growth of *O. similis* under different culture salinity conditions is shown in Figure 5. The polynomial orthogonal test resulted in a quadratic function given by the equation $y = -0.0002x^2 + 0.0076x + 0.0058$ and $R^2 = 0.8401$, and the optimum salinity for this parameter was 19.0 ppt. The R^2 value indicated that salinity influenced as much as 84.01% of the population-specific growth rate of *O. similis*, while 15.99% was affected by other untested factors.

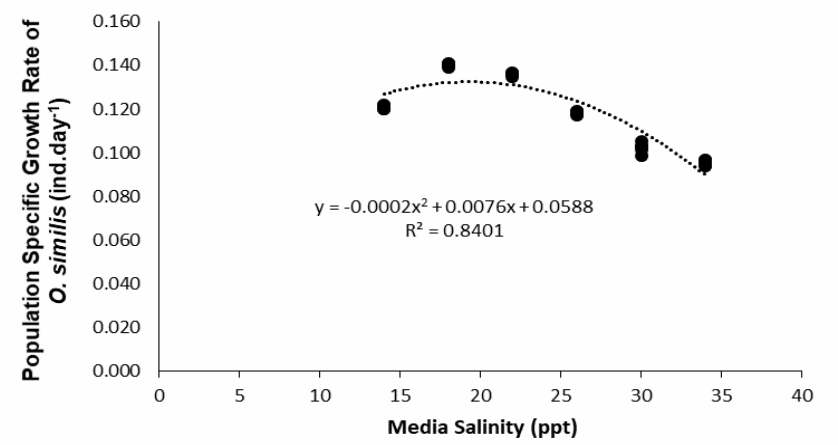

Figure 5. Correlation between media salinity and the population-specific growth of *O. similis*.

Egg production of O. similis. It was noted that the analysis of variance showed a significant effect ($p < 0.05$) of salinity on egg production. The histogram in Figure 6 shows that a salinity value of 18 ppt gave the highest egg production (24.40±0.05 egg ind^{-1}), and this treatment was statistically significantly different from the other salinities. The egg production at a salinity value of 14 ppt (19.80±0.45 egg ind^{-1}) was not significantly different from 26 ppt (19.60±0.89 egg ind^{-1}); on the other hand, it was significantly different from 22 ppt (23.00±0.89 egg ind^{-1}), 30 ppt (17.20±0.84 egg ind^{-1}), and 34 ppt (17.60±0.55 egg ind^{-1}).

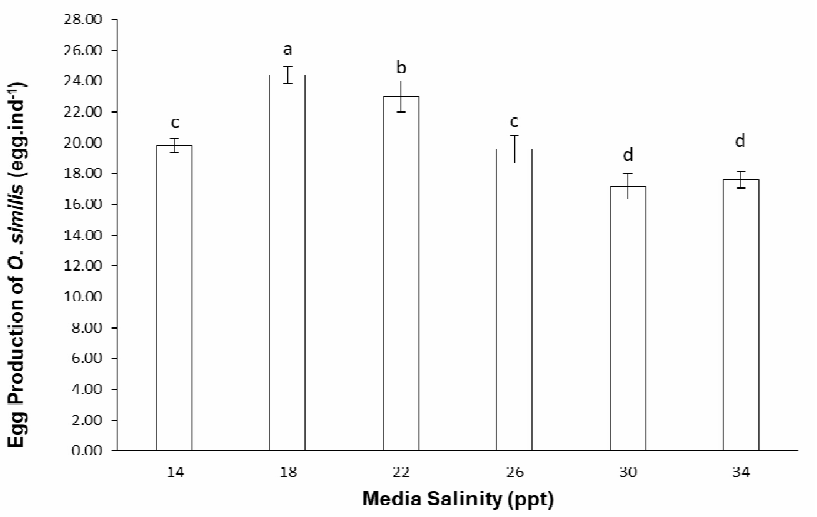

Figure 6. Histogram of egg production at different salinities on the last day of cultivation.

The curve of *O. similis* egg production under different culture salinity conditions is shown in Figure 7. The polynomial orthogonal test also resulted in a quadratic function given by the equation $y = -0.0279x2 + 1.0821x + 11.669$, $R^2 = 0.5849$, and the optimum salinity was determined to be 19.4 ppt. The R^2 value indicated that salinity influenced as much as 58.49% of egg production in *O. similis*, while 41.51% was affected by other untested factors.

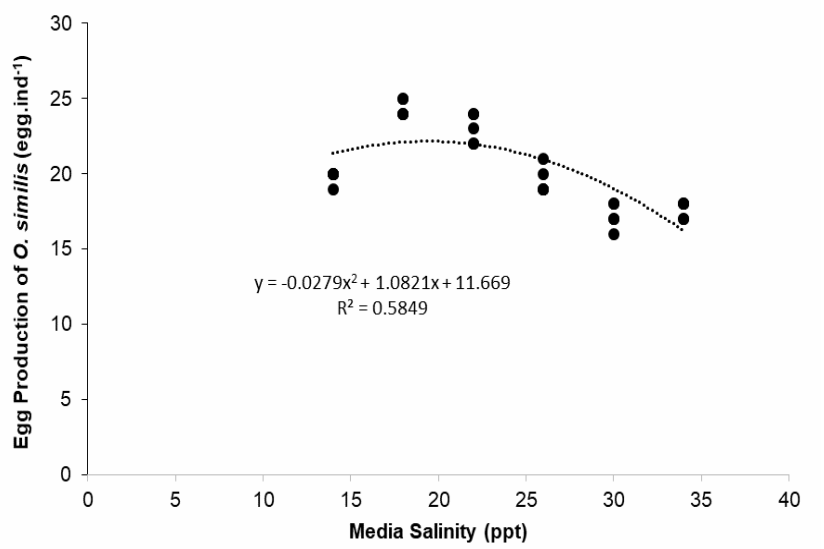

Figure 7. Correlation between salinity and egg production in *O. similis*.

Discussion. The results of our molecular approach confirmed that the copepod used in the study was *O. similis* (Figure 1). This species is known as a commensal marine copepod with a worldwide distribution (Jose et al 2017; Wang et al 2017). Regarding its physical characteristics, this species is commonly used as a live feed organism in aquaculture. In addition, several studies have employed other species of this copepod genus, including *O. colcarva*, *O. hebes*, *O. rigida*, and *O. oculata*. However, each species has its own set of culture conditions favorable for growth (Molejón & Lajonchère 2003; Santhanam & Perumal 2012; Barroso et al 2015; Jose et al 2016; Broach et al 2017).

Media salinity plays a vital role that has a direct effect not only on the osmolarity of the media but also on the osmoregulation of aquatic organisms (Anggoro & Subandiyono 2010). Salinity has been noted as one of the masking factors for copepod culture. Inappropriate media salinity causes physiological stress, resulting in additional osmoregulation and demands for respiration, leading to the suppression of growth rates (Kimoto et al 1986). The findings of previous studies were corroborated by our current result that salinity had a direct impact on the osmotic work levels of *O. similis.* This occurred because the salinity pressure impacted on energy use in this copepod. Increasing the salinity also increased the osmotic work level in *O. similis* because more ions (Cl^-, Na^+, Mg^{2+}, Ca^{2+}, and K^+) in the culture medium and inside the cell required osmotic control. In a hypotonic solution, the osmolarity of the intracellular fluid is higher than that of the culture media, which causes ions to exit the cell into the environment; therefore, *O. similis* must balance the osmolarity by increasing water intake and vice versa (Anggoro & Nakamura 2005).

The salinity level in the media determines the osmotic pressure of water organisms such as *O. similis*, so it must adapt to the media salinity through osmoregulation. When the media condition is hypotonic, the osmolarity of the intracellular fluid is higher than that of the media; this will cause water to move into the body (interieur millieu) and ions (inorganic osmoeffector) to exit into the environment (exterieur millieu) through diffusion (Anggoro & Subandiyono 2010). On the other hand, when the media is in the hypertonic state, the internal body fluid osmolarity is lower than that of the media; this will cause water to exit the body into the environment through osmosis, passing through the gills and skin. Under this condition, the culture will perform hypoosmotic regulation; one way is by increasing the amount of sea water ingested to maintain the equilibrium of body fluids (Anggoro & Nakamura 2005).

Growth will occur if the aquatic organism can maintain homeostasis, a stable set of internal conditions that allow it to carry out physiological activity in the body (Anggoro et al 2018). This notion was supported by Chen et al (2006), who stated that theoretically, copepods (*O. similis*) can easily decrease their energy expenditure to adjust the osmoregulation system when they are living in an optimal salinity environment; as a

result, excess energy can be used to maximize growth and reproduction. Energy expenditure for osmoregulation can be suppressed if the organism is well cultivated in isosmotic media so that the utilization of the feed is efficient and produces rapid growth (Jobling 1994).

Copepods (*O. similis*) can maintain osmoregulation under different environment salinity conditions (Chen et al 2006). The different species of *Oithona* also show that the level of physical adaptation and hydrology parameters, including the characteristics in which its cosmopolitan, also with the species with close distribution range (Dahms et al 2015). Chen et al (2006) stated in their research that egg production and the density of stadia *nauplii* increased in parallel with increasing salinity from 5 to 15 ppt but decreased gradually with increasing salinity from 15 to 35 ppt. Still, *O. similis* is a species of copepod that can preserve its life in salinities of 30 to 35 ppt, even though, based on the research, growth is not maximal compared with lower salinity conditions.

According to all parameters, the growth and breeding of *O. similis* is optimal at a salinity of 19.0-19.4 ppt. This was supported by the number of copepods that reached the adult stage, the total population size, and the high egg production. Under favorable salinity conditions, the energy that is ordinarily used for osmoregulation will be allocated to growth and other biological activities such as somatic activities and reproduction (Anggoro 2005; Chen et al 2006; Beyrend-Dur et al 2011). Furthermore, a suitable salinity level will increase the hatching rate of *O. similis* eggs. Chen et al (2006) stated that the eggs of *O. similis* were well hatched in a range of salinity between 15 and 20 ppt and had a lower probability of hatching at a salinity value of > 35 ppt. A similar result was also reported that explained the influence of salinity on the copepod's fecundity, with the highest fecundity occurring at a salinity of 15 ppt (Beyrend-Dur et al 2011). In the current study a similar result was obtained: *O. similis* had the highest number of eggs, number of adults, and population size at a salinity of 19.0-19.4.

Conclusions. According to the results of species identification using DNA barcoding, this species belonged to *Oithona* and was 98% identical to *Oithona similis*. The salinity of the culture media has a significant effect on the growth performance and egg production of *O. similis*. A media salinity of 18 ppt gives the best growth performance (total density 16.54±0.24 ind mL^{-1}, population-specific growth rate of *O. similis* 0.140±0.001 ind day^{-1}) and egg production (24.40±0.55 eggs ind^{-1}) in a culture of *O. similis*. Based on the total density and egg production, the optimum salinity of *O. similis* culture media is 19.4 ppt.

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