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Effect of Hydrocarbon-Polluted Seawater on the Cell Density of Microalgae *Scenedesmus vacuolatus* Shihira & Krauss

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Study the effect of hydrocarbon-polluted seawater on the cell density of microalgae *Scenedesmus vacuolatus* has been carried out in this study. Hydrocarbon pollution derived from the oil in the sea can inhibit the photosynthesis process of microalgae. This might impact the density of microalgae cells. The purposes of this study are to determine the effect of the concentration of hydrocarbon-polluted seawater on the density of *Scenedesmus vacuolatus* microalgae cells and to determine the optimum treatment to reduce total petroleum hydrocarbons (TPH) levels. A sampling of hydrocarbon-polluted seawater was taken at Kali Adem port, Jakarta. The treatment done in this research used a walne medium with the addition of 25% hydrocarbon-polluted seawater (A), 50% (B), 75% (C), and 100% (D). Control is Walne medium with sterile seawater that was not from the Kali Adem port. The results showed the highest average density of *Scenedesmus vacuolatus* cells was in the control sample. This can be seen from the results of the average cell density at the peak time of 29.48 x 105 cells / mL, as well as the log phase length of *Scenedesmus vacuolatus*. Measurement of TPH levels showed decreases of TPH in all treatments. The optimum treatment to reduce TPH levels is treatment B with a reduction percentage of 70.62%..

Key words: Kali Adem port, *Scenedesmus vacuolatus*, total petroleum hydrocarbon (TPH), Walne medium

Penelitian mengenai pengaruh air laut tercemar hidrokarbon terhadap kepadatan sel mikroalga *Scenedesmus vacuolatus* telah dilakukan. Pencemaran hidrokarbon yang berasal dari minyak di laut dapat menghambat proses fotosintesis mikroalga. Hal tersebut dapat berdampak pada kepadatan sel mikroalga. Penelitian ini bertujuan untuk mengetahui pengaruh konsentrasi air laut tercemar hidrokarbon terhadap kepadatan sel mikroalga *Scenedesmus vacuolatus*, serta mengetahui perlakuan yang optimum untuk menurunkan kadar total petroleum hidrokarbon (TPH). Pengambilan sampel air laut tercemar hidrokarbon dilakukan di pelabuhan Kali Adem, Jakarta. Perlakuan dalam penelitian adalah medium Walne dengan penambahan air laut tercemar hidrokarbon 25% (A), medium Walne dengan penambahan air laut tercemar hidrokarbon 50% (B), medium Walne dengan penambahan air laut tercemar hidrokarbon 75% (C), dan medium Walne dengan penambahan air laut tercemar hidrokarbon 100% (D). Kontrol yang digunakan adalah medium Walne dengan air laut steril yang bukan berasal dari pelabuhan Kali Adem. Hasil penelitian menunjukkan rata-rata kepadatan sel *Scenedesmus vacuolatus* tertinggi yaitu pada perlakuan kontrol. Hal tersebut dapat dilihat dari hasil rata-rata kepadatan sel pada masa puncak sebesar 29,48 x 105 sel/mL, serta panjang fase log dari *Scenedesmus vacuolatus*. Hasil pengukuran kadar TPH menunjukkan terdapat penurunan TPH pada seluruh perlakuan. Perlakuan optimum untuk menurunkan kadar TPH yaitu perlakuan B dengan persen penurunan sebesar 70,62%.

Kata kunci: Medium Walne, Pelabuhan Kali Adem, *Scenedesmus vacuolatus*, total petroleum hidrokarbon (TPH)

Port is one of the places with the most human activities, especially transportation. The number of transportation activities by ships causes common pollution in the port area is oil pollution. Oil pollution to the sea is the release of liquid petroleum hydrocarbon pollutants which mainly come from human activities (Priyadarshani *et al*. 2011). Petroleum hydrocarbon pollution in the sea has many negative impacts on the environment and living things (Wibowo 2018). Microalgae are photosynthetic organisms that require sunlight and CO_2 fixation to photosynthesis (Muchammad *et al*. 2013).

Contact between the oil layer and microalgae on the surface of the water in addition to having an impact on the photosynthesis process will also have an impact on its density. Microalgae flexibility in morphology, physiology, and life cycle gives the ability to survive in polluted conditions (Amirlatifi *et al*. 2013). Response and tolerance of exposure to pollutant levels in each

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type of the microalgae are different. One of microalgae genus that is adaptive in oil-polluted waters is the *Scenedesmus*. The genus microalgae *Scenedesmus* can adapt to oil-contaminated environments through acclimatization of physiology and genetic mutations (Martinez *et al*. 2013). *Scenedesmus* is a green microalga that can be found in fresh to brackish water (Phinyo *et al.* 2017). Generally, the genus *Scenedesmus* has an ellipsoidal cell shape and forms a coenobitic with a multiple of 4 (Guiry and Guiry 2019). *Scenedesmus vacuolatus* is a species of the genus *Scenedesmus* which has a different shape. These microalgae are round in shape, do not have a prominent apex, and do not have cell-to-cell connections like the genus *Scenedesmus* in general. S. *vacuolatus* is very tolerant and adaptive to the environment. Lewis and Flechtner in 2014, found S. *vacuolatus* in macrobiotic crust in deserts (Lewis and Flechtner 2004). In addition, these microalgae are environmentally tolerant with a wide salinity range (Anand *et al*. 2019).

Hydrocarbon compounds in water can trigger eutrophication and cause an abundance of certain types of microalgae, resulting in changes in community structure. Besides affecting the structure of the microalgae community, hydrocarbons also affect the size and physiology of microalgae cells. In phytoplankton exposed to hydrocarbons, the cell size is reduced in line with the increase in petroleum hydrocarbon content (Nair *et al.* 2014). The response of each microalgae to pollutants, especially petroleum hydrocarbons is different. Al Obaidy and Lami (2014) conducted researched on chlorophyll A levels in *Microcystis flos-aquae* (Wittr.) Kircher and *Nostoc carneum* Agardh grown on a medium containing crude oil. In *Microcystis flos-aquae* (Wittr.) Kircher levels of chlorophyll A decreased, whereas in *Nostoc carneum* Agardh levels of chlorophyll A tended to increase (Al Obaidy and Lami 2014).

Microalgae are capable of contributing to the degradation of environmental pollutants either by directly altering pollutants, or by increasing the degradation potential of existing microbial (Semple *et al*. 1999). Several chemicals can be found in total petroleum hydrocarbons (TPH) hexane, jet fuel, mineral oil, benzene, toluene, xylene, naphthalene, fluorene, and other petroleum products. One of the benzene derivatives that can be degraded by microalgae is phenol. Phenol can be degraded via the meta-cleavage pathway. The meta-cleavage pathway in phenol degradation produces the final products, namely pyruvate and acetaldehyde. Pyruvate can be metabolized in the Krebs cycle, and acetaldehyde can be converted to acetyl-CoA with the help of acetaldehyde dehydrogenase (AcDH) (Patel *et al*. 2017).

The study aims to determine the effect of concentrations of hydrocarbon polluted seawater on the density of *Scenedesmus vacuolatus* microalgae cells, and determine the optimum treatment to reduce levels of total petroleum hydrocarbons (TPH).

MATERIALS AND METHODS

Design Experiment. This study used five variations of treatment with three repetitions; Control (0%), A (25%), B (50%), C (75%), and D (100%). This research used 2-litter gallons with the composition of *Scenedesmus vacuolatus* microalgae, Walne nutrient and vitamin solutions, hydrocarbon-polluted seawater samples, and also sterile seawater (not from the port) as a solvent.

Growth Medium for Microalgae. The growth medium that will be used for the growth of *Scenedesmus vacuolatus* in the study is Walne medium. The first stage of making 1 liter Walne medium is to prepare distilled water as a solvent for trace metal solutions, vitamin solutions, and nutrient solutions. The next stage is the weighing of trace metal solution materials namely $ZnCl_2$ (2.1 g), $CoCl_2.6H_2O$ (2 g), $(NH_4)6Mo_7O_{24}.4H_2O$ (0.9 g), and $CuSO_4.5H_2O$ (2 g). The trace metal solution is dissolved into 100 mL distilled water and labeled. Weigh the ingredients of the vitamin solution, namely vitamin B12 (10 mg), vitamin B1 (10 mg), and biotin (200 µg). The vitamin solution ingredients are dissolved into 100 mL distilled water and labeled. The next stage is weighing the ingredients of nutrient solutions, namely $FeCl_3.6H_2O$ (1.3 g), $MnCl_2.4H_2O$ (0.36 g), H_3BO_3 (33.6 g), EDTA - 2Na (45 g), $NaH_2PO_4.2H_2O$ (20 g), $NaNO_3$ (100 g). The nutrient solution ingredients are dissolved into 1 L of distilled water and 1 ml of trace metal solution is added then labeled. One mL of nutrient solution has been made and then dissolved into 1 L of sea water that has been sterilized using a vacuum filter. The solution was sterilized using an autoclave at 121 °C for 15 minutes at a pressure of 0.2 MPa. After sterilization, add aseptic a 0.1 mL vitamin solution.

Observation of Microalgae Culture Colors. Microalgae culture colors were observed using Faber Castell's standard color table. Observations were made every day during the study.

Measurement of Microalgae Cell Density. Cell

size in starter culture, control culture, treatments A, B, C, and D were observed using a microscope at 1000x magnification. Microalgae cell density calculations are performed using an improved neubauer haemacytometer then observed under a microscope. The observations are then calculated using the following formula:

$$d = 10^4 \times Q \text{ cell/mL}$$

d: cell density
Q: average cell count per 1 mm^2

(Andersen 2005: 249)

Measurement of Total Petroleum Hydrocarbon (TPH). Measurement of total petroleum hydrocarbon content using gravimetric methods according to SNI 6989.10: 2011 standard. The measurement of TPH concentration is divided into four parts, namely oil and fat extraction, distillation, calculation of oil and fat content, and calculation of mineral oil content (total petroleum hydrocarbons).

Measurement of Environmental Parameters. Physical parameters observed were temperature (°C), salinity (‰), light intensity (lux), and culture color. Retrieval of temperature data is taken using a multiparameter water quality meter [Aquaread]. Measurement of light intensity is done with a lux meter [Uni-T]. Salinity levels were measured by using a refractometer [ATC Brix]. Chemical parameters observed were pH level, and dissolved oxygen (mg/L). Sample analysis was performed aiming to determine the level of chemical content in the water sample before and after the experiment. The pH data collection was carried out using pH paper, dissolved oxygen was measured using a multiparameter water quality meter device. All of these factors were observed every day during the study then recorded.

Statistical Methods. The data obtained were tested using the Saphiro Wilk normality test followed by the Kruskal Wallis non-parametric statistical test ($\alpha < 0.05$). These data were tested to determine whether or not there was a significant difference in the concentration of seawater contaminated with hydrocarbons on the density of microalgae cells *Scenedesmus vacuolatus*.

RESULTS

Observation of Microalgae Culture Colors. Figure 1 shows the change of microalgae cultures color during research. The color of the *Scenedesmus vacuolatus* culture in the control, A, B, C, and D medium on day 2 to day 9 experienced the same changes. On the 12th day of observation, the treatment medium C and D experienced a darker color change, namely moss green. The macroscopic appearance of the cultures on the control, A, and B treatment mediums were the same as the 9th day, namely grass green. On the 13th day of observation, all test cultures were the same color, namely grass green. The macroscopic color change of the culture on the 14th day only occurred in the control medium, A, and B treatment mediums, which were leaf green, while the C and D treatment mediums were still the same color as the previous day (T_{13}). On the 16th day of observation, the culture colors on control medium, A, and B were still the same color as the previous day (T_{14}), namely leaf green (leaf green), while the treatment medium C and D changed to sap green. The macroscopic appearance of the culture in the control medium, A, B, C, and D treatment mediums were the same color on the 19th to the 21st day, namely sap green. Overall, the color changes in the C and D cultures occurred more rapidly than in other cultures. This can be seen on the 12th and 16th days, the color of C and D cultures changed to a darker green.

Measurement of Microalgae Cell Size. Observation of *Scenedesmus vacuolatus* cell size was observed through the size of cells in starter cultures, control cultures, and each treatment. Microscopic observations of *Scenedesmus vacuolatus* cells cultured in starter cultures, control cultures, treatment A, and treatment B had a cell size range between 4—6 µm. The size of S. *vacuolatus* cells cultured in treatment C and D ranged from 4—5 µm. Overall, there is no significant difference in size for each treatment. In all treatments, the size of S. *vacuolatus* cells ranged from 4 - 6 µm. The difference in smaller cell size was only found in C and D medium.

Measurement of Microalgae Cell Density. The cell density of the starter culture of *Scenedesmus vacuolatus* entered into the test culture was 958,000 cells mL^{-1} in 1,800 mL of medium. The results of the mean cell density of *Scenedesmus vacuolatus* are listed in Table 2. These results are the average of three repetitions for each variation of the treatment medium. The growth curve of *Scenedesmus vacuolatus* is shown in Figure 2.

The average density of *Scenedesmus vacuolatus* cells on days 1 to 9 in the medium which was given hydrocarbon-polluted sea water (A, B, C, and D) was higher than in the control medium. The growth curve of *Scenedesmus vacuolatus* after day 9 showed that the cell density increased significantly. After the 9th to 21st day, the average density of *Scenedesmus vacuolatus*

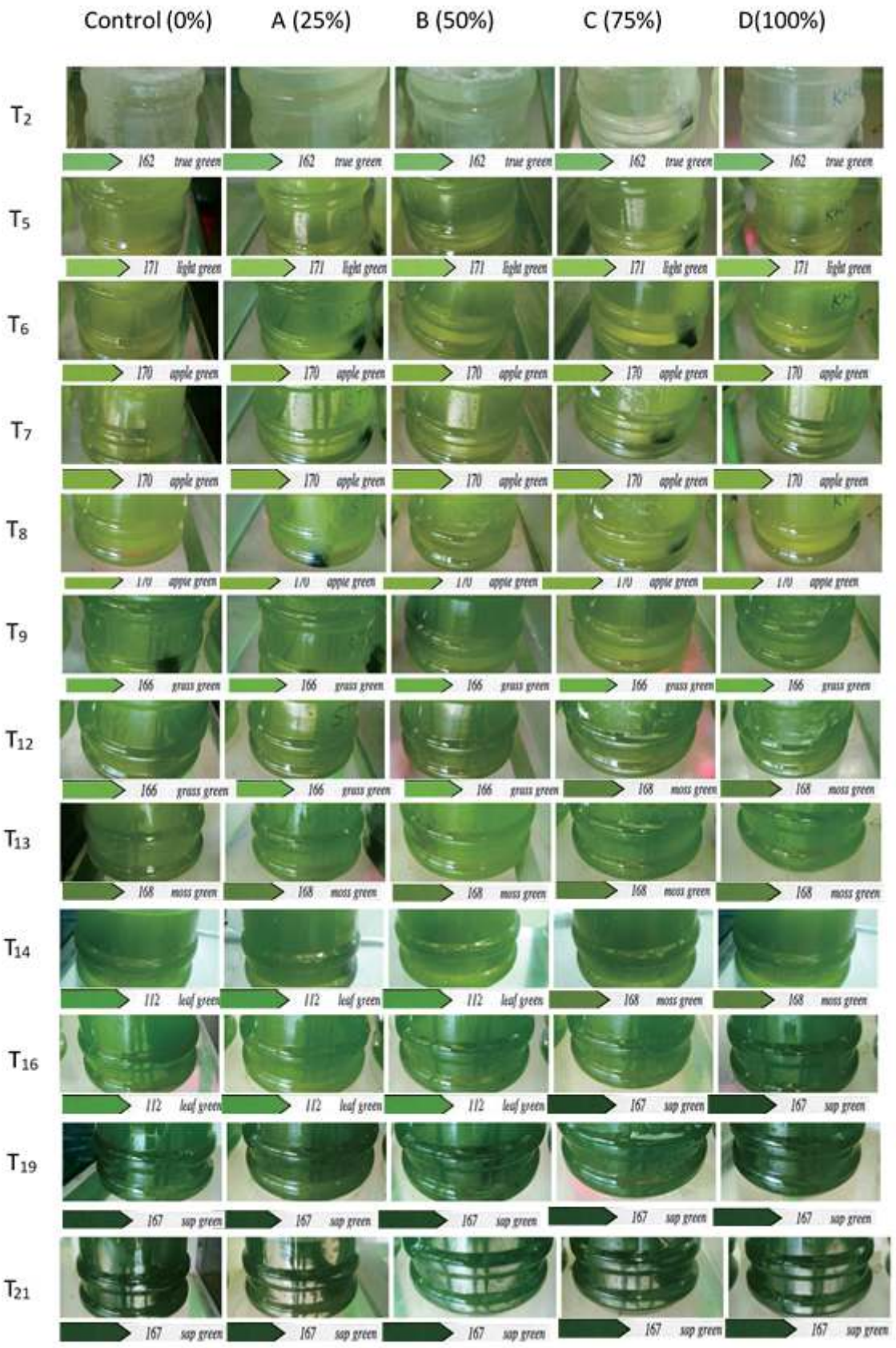

Fig 1 Macroscopic color appearance of the *Scenedesmus vacuolatus* culture from day 2 – 21.

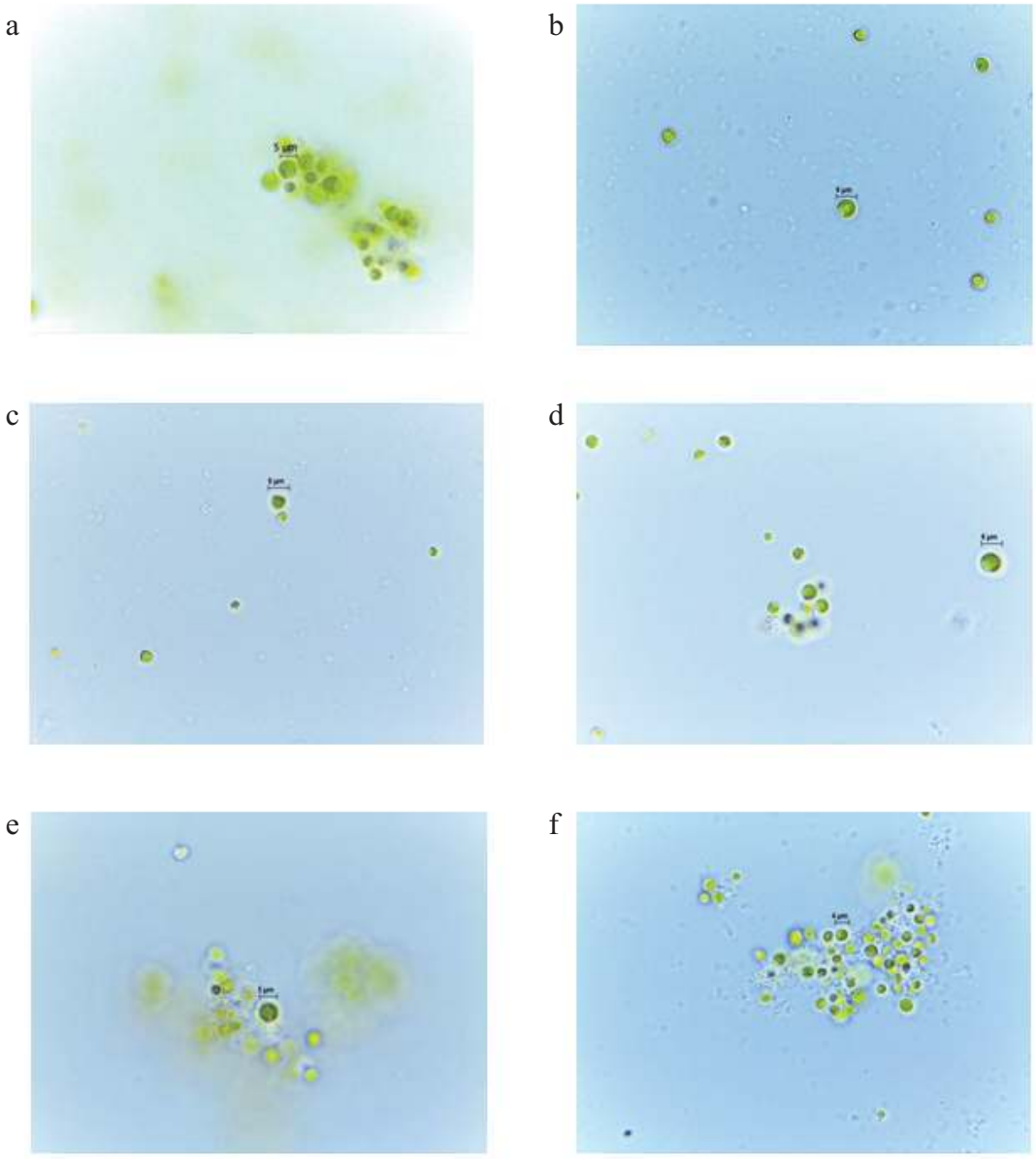

Fig 2 Microscopic observation of *Scenedesmus vacuolatus* on starter culture (a), control medium (b), treatment medium A (c), medium B (d), medium C (e), and medium D (f).

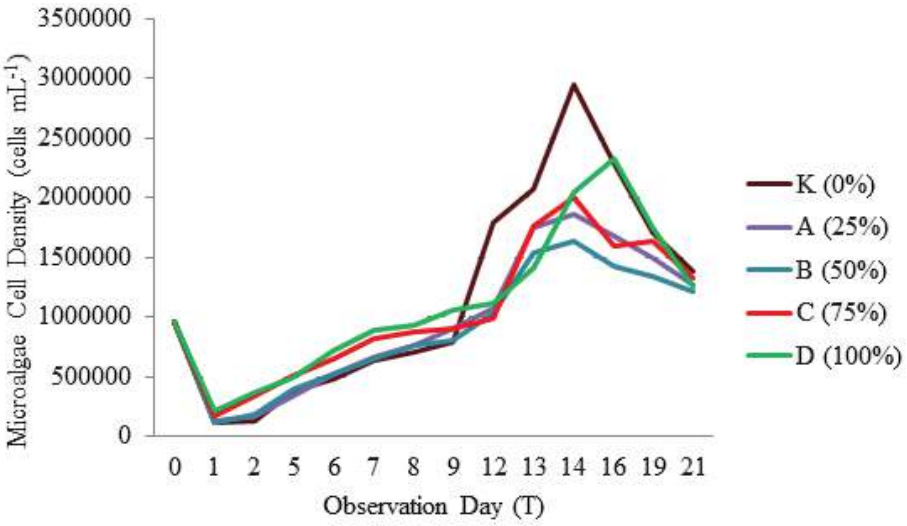

Fig 3 Growth curve of *Scenedesmus vacuolatus*.

Table 1 Average cell density of *Scenedesmus vacuolatus*

DAY	Cell Density (cell.mL^{-1}) (10^5)				
	Control (0%)	A (25%)	B (50%)	C (75%)	D (100%)
0	9,58	9,58	9,58	9,58	9,58
1	1,19	1,25	1,21	1,78	2,21
2	1,23	1,78	1,83	3,36	3,75
5	3,79	3,39	3,96	5,05	4,96
6	4,75	5,22	5,23	6,53	7,26
7	6,39	6,59	6,52	8,22	8,88
8	7,12	7,65	7,67	8,81	9,34
9	7,88	9,10	8,07	9,08	10,55
12	17,93	10,73	10,32	9,87	11,22
13	20,68	17,48	15,38	17,64	14,15
14	29,48	18,59	16,35	19,97	20,51
16	22,85	16,81	14,27	15,98	23,25
19	17,02	14,92	13,37	16,32	17,44
21	13,85	12,77	12,17	13,23	12,54

Table 2 Average of total petroleum hydrocarbon (TPH) level

Treatment	Total Petroleum Hydrocarbon (TPH) Level			
	Beginning (mg)	End (mg)	% Residue	% Decreasing
Control (0%)	0	0	0	0
A (25%)	719,38	266,65	37,07	62,93
B (50%)	1438,76	422,69	29,38	70,62
C (75%)	2158,14	840,71	38,96	61,04
D (100%)	2586,89	1597,91	61,77	38,23

cells in the control medium was higher than the other mediums.The growth continued until it reached the peak period on the 14th day for control, A, B, and C medium, while the culture in D medium reached its peak on the 16th day. The growth rate of *Scenedesmus vacuolatus* began to decline after day 14 in control, A, B, and C, whereas in culture D it occurred after day 16. This is indicated by a decrease in the cell density curve. Kruskal Wallis statistical test showed that there was no significant difference in the density of microalgae *Scenedesmus vacuolatus* cells to variations in the concentration of sea water contaminated with hydrocarbons. The increase or decrease in S. *vacuolatus* cell density was not directly affected by the differences in the treatment given.

Measurement of Total Petroleum Hydrocarbon (TPH). The total petroleum hydrocarbon (TPH) content shows the oil concentration in each treatment. The total petroleum hydrocarbon content can be determined from the residual weight of the oil after the extraction process. The TPH level in treatment medium A at the beginning of the study was 719.38 mg, and at the end of the study was 266.65 mg. The decrease in TPH levels in treatment medium A was 62.93%. The TPH level in the B treatment medium at the beginning of the study was 1438.76 mg, and at the end of the study was 422.69 mg. The decrease in TPH levels in the B treatment medium was 70.62%. The TPH level in the C treatment medium at the beginning of the study was 2158.14 mg, and at the end of the study was 840.71 mg. The decrease in TPH levels in the C treatment medium was 61.04%. The TPH level in the D treatment medium at the beginning of the study was 2586.89 mg, and at the end of the study was 1597.91 mg. The decrease in TPH levels in the D treatment medium was 38.23%. The highest decrease in total petroleum hydrocarbons was culture B, amounting to 70.62%. The results of measuring TPH levels in each treatment medium at the beginning and end of the study are listed in Table 3. The results of Kruskal Wallis's non-parametric statistical test (attachment 6) showed that there was no significant difference in the density of microalgae *Scenedesmus vacuolatus* cells to variations in the concentration of sea water contaminated with hydrocarbons. in

seawater does not directly affect the density of microalgae cells, but this study shows that S. *vacuolatus* can reduce the hydrocarbon levels contained in oil in sea water.

Measurement of Environmental Parameters. Observation of the environmental conditions of the test culture during the study was carried out by measuring temperature, pH, salinity, dissolved oxygen in the medium, and light intensity. Observation of the temperature of the test culture was carried out using a multiparameter tool. The results of the culture temperature observations ranged from 27.3 - 28.3°C. Observation of the salinity of the test cultures was carried out using a refractometer. The results of the salinity measurement during the study on treatment K and A were 28 ‰, while in treatment B, C, and D were 30 ‰. This difference occurs because the composition of solvent seawater is more in treatment K and A. The initial salinity measurement of solvent seawater is 28 ppt. Dissolved oxygen observation in the test culture was carried out using a multiparameter device. Dissolved oxygen data ranged between 7,1 - 9,5 mg L^{-1}. Observation of pH of the test culture was carried out using pH paper. The result of pH measurement in the test culture during the study was 7. Observation of light intensity at the place where the test culture was placed was carried out using a lux meter. The results of observations of light intensity in this study ranged from 7200 - 7900 lux.

DISCUSSION

The results of culture color observations in the study were compared to the Faber castell color table. Overall, the color changes in cultures C and D occur more quickly than in other cultures. This can be seen on the 12th and 16th day the colors of the C and D cultures change to a denser green. These changes can occur due to greater levels of oil pollutants. Exposure to oil pollutants in high concentrations has an impact on decreased levels of carbohydrates and microalgae protein (Lewis & flechtner 2004). Oil pollutants in seawaters contain many fractions of compounds which can be grouped into hydrocarbon and non-hydrocarbon fractions. At higher concentrations of oil pollutants, the more non-hydrocarbon fractions on the medium. The non-hydrocarbon fraction includes several inorganic compounds such as nitrogen, sulfur, phosphorus, iron and some trace elements (Anand *et al.* 2019). High nitrogen content can interfere with the process of formation of photosynthetic pigments in microalgae. That is because the deactivation process in photosynthetic pigment activity (Valotton *et al.* 2008).

The results of measurements of the average density of *Scenedesmus vacuolatus* cells on days 1 to 9 in the medium given sea water contaminated with hydrocarbons (culture A, B, C, and D) are higher than in the control medium. This is caused by the ability of microalgae to accumulate and use oil (petroleum hydrocarbons) as a source of organic compounds (Al Obaidy & Lami 2014). Growth continues until it reaches its peak on the 14th day for cultures on medium K, A, B, and C, while culture on medium D reaches the peak period on the 16th day. This can occur because of the higher concentration of petroleum hydrocarbons in the medium D. Provision of crude oil to microalgae culture can prolong the growth phase and produce high biomass production (Semple *et al.* 1999). After the 9th day to the 21st day, the average density of *Scenedesmus vacuolatus* cells in the control medium was higher than that of the entire medium with the addition of hydrocarbon polluted sea water. This can occur due to the impact of residual petroleum hydrocarbons that are unable to accumulate or are degraded by microalgae. Petroleum hydrocarbons can inhibit the growth of microalgae by reducing the ability to absorb CO_2, photosynthesis, respiration, and cell division (Patel *et al.* 2017). The growth rate of *Scenedesmus vacuolatus* begins to decrease after the 14th day in cultures K, A, B, and C, whereas in culture D occurs after the 16th day. This is indicated by the decrease in the cell density curve. This occurs due to reduced nutrition in the medium and the effect of residual oil (petroleum hydrocarbons) which cannot be accumulated by microalgae. Petroleum hydrocarbons can be toxic to microalgae when forming thick oil layers around organisms, inhibiting gas diffusion, and destruction of cell membranes due to continuous hydrocarbon uptake (El-Dib *et al.* 2001).

The highest decrease in total petroleum hydrocarbons was in culture B, by 70.62%. This shows that *Scenedesmus vacuolatus* can reduce TPH levels optimally at 50% petroleum hydrocarbon concentration. Microalgae degrades hydrocarbons by accumulating and transforming the compound (Phinyo *et al.* 2017). Some chemicals that can be found in total petroleum hydrocarbons (TPH) are hexane, jet fuel, mineral oil, benzene, toluene, xylene, naphthalene, fluorene, and other petroleum products (EPA 2017). One of the hydrocarbons that can be degraded by microalgae is phenol which is a derivative of benzene compounds. Phenols can be degraded by microalgae through the

meta-cleavage pathway. The degradation process produces the final product, namely acetaldehyde, pyruvate and carbon dioxide. The three end products can be used in photosynthesis and microalgae cell respiration. Pyruvate can be metabolized in the Krebs cycle, and acetaldehyde can be converted to acetyl-CoA with the help of acetaldehyde dehydrogenase (AcDH) (Patel *et al.* 2017).

The temperature range in this study is included in the optimal temperature range for the growth of the genus *Scenedesmus* which is 25 - 30°C. The maximum growth rate of the genus *Scenedesmus* occurs at 30°C. Temperature fluctuations in culture are caused by changes in the temperature of the surrounding environment. Temperature affects the chemical composition of cells, nutrient uptake and CO_2, and microalgae growth rate.

Salinity measurement results in studies are higher than the optimum salinity range for *Scenedesmus* growth Salinity affects the ability of *Scenedesmus* cells to accumulate oil. In high salinity, the genus *Scenedesmus* is able to accumulate oil up to 36% dry weight (Kaewkannetra *et al.* 2012). According to Latala's (1991) study, the genus *Scenedesmus* can live up to 25 ‰ salinity. In this study, S. *vacuolatus* was grown in medium with sea water with salinity up to 30‰. This shows that S. *vacuolatus* is an adaptive microalgae in an environment with salinity stress.

Fluctuations in dissolved oxygen in the test culture are influenced by the density of *Scenedesmus vacuolatus* cells. It is also related to the oil layer in the medium and petroleum hydrocarbons that are accumulated by microalgae (Papa *et al.* 2009). The optimal pH range for growth of S. *vacuolatus* is 6.5 - 8.5. At pH more than 8.5 the growth rate will slow down (Neuwoener & Escher 2011). At pH 4.8 the growth rate of the genus *Scenedesmus* will stop (Nalewajko *et al.* 1997).

Light intensity in this study is included in the optimum range for *Scenedesmus* growth. The genus *Scenedesmus* is able to grow at the lowest light intensity of 2,500 lux (Latiffi *et al.* 2017). *Scenedesmus vacuolatus* species are able to grow optimally to a light intensity of 49,200 lux (Carbone *et al.* 2017).

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