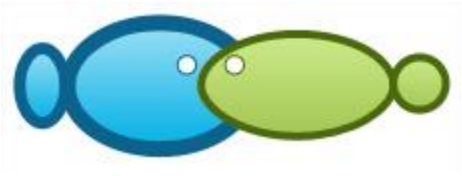

Growth of *Chlorella* sp. reared in a leachate enriched media

[1]Rosyadi, [2]Tengku Dahril, [2]Aras Mulyadi, [2]Sofyan H. Siregar, [2]Windarti

[1] Doctoral Program in Marine Science of Riau University, Pekanbaru, Indonesia; [2] Post Graduate Program of Riau University, Pekanbaru, Indonesia. Corresponding author: Rosyadi, rosyadi@agr.uir.ac.id

Abstract. During organic waste decomposition process, leachate water is produced and the leachate spill may pollute the environment. This liquid waste, however, is rich in organic compounds and it may be used as media for growing microalgae such as *Chlorella* sp. (Chlorophyta). To understand the best dosage of leachate water for growing the *Chlorella* sp, a study was conducted on the growth of *Chlorella* sp. reared in the leachate enriched media. There were 5 treatments, namely 5, 10, 15, 20 and 25% of leachate diluted in the water. These media were placed in plastic bottles (6 L) filled with 5 L of mixed leachate and freshwater. An the amount of 125 mL of *Chlorella* sp. culture ($13.5x10^6$ cells mL^{-1}) was put in each gallon and the gallons were placed indoor with natural sunlight and additional light sources of 40 Watts (2,000 lux LED). The *Chlorella* sp. was reared for 20 days and the media was sampled in the 5^{th} day. The measured *Chlorella* sp. parameters were the growth, abundance, proximate and nutrient content and the residual organic matter in the media. Results showed that the best growth and abundance of *Chlorella* sp. was obtained in the media with 10% leachate concentration ($5.116x10^3$ cells mL^{-1}, peaked in the 12^{th} day) and the proximate contents were: 30.27-32.82% protein, 7.84-8.46% fat and 27.65-30.02% carbohydrates. As the *Chlorella* sp. grew, the nitrate content in the media diminished to 0.804 mg L^{-1} on the 20^{th} day. Based on the data obtained, it can be concluded that the leachate water might be used to enrich the *Chlorella* sp. rearing media. In addition, this species was able to reduce the organic compounds in the media.
Key Words: domestic waste, liquid waste, microalgae culture, landfill.

Introduction. Indonesia, as a tropical region, has a great potential for the development of natural resources, especially aquatic organisms related to the utilization of sunlight. *Chlorella* sp. is a single-celled microorganism from the green algae group, including the Chlorophyta phylum, which has a high nutritional value. This organism requires sunlight for photosynthesis. *Chlorella* sp. contains protein, carbohydrates, fats, vitamins and minerals, which makes it an alternative source of food for humans and as a feed ingredient for livestock and fish or as a source of biofuel. Rachmaniah et al (2010) stated that *Chlorella* sp. has 51–58% protein, 12-17% carbohydrate, 14-22% fat and 4-5% nucleic acids. Furthermore, Sugiharto (2020) reports that *Chlorella vulgaris* has a crude protein content of 55.3, 10.3% crude fat and 5.80% crude fiber. *Chlorella* sp. are suitable for cultivation because they are easy and fast to breed. *Chlorella* sp. can be cultured easily, if a suitable culture medium for growth and reproduction is obtained. In the culture media, there must be a source of organic material as nutrition for *Chlorella* sp. Sources of organic compounds can be commercial fertilizers, solid or liquid waste containing organic substances.

Currently, there is a source of organic material that has not been widely used as a source of nutrition, namely the leachate of urban waste from landfills. Leachate is a by product of the waste decomposition process. In this case, if it is not collected or channeled, it may pollute the environment. Leachate water has the potential as a source of organic material that can be used by plants, including microalgae, namely *Chlorella* sp. The growth of *Chlorella* sp. is largely determined by nutrient availability and environmental condition. The growth of *Chlorella* sp. requires nutrients, consisting of

nutrients in the form of macronutrients such as N, K, Mg, S, P and Cl. Meanwhile, the micronutrient nutrients are Cu, Zn, Mn, B, Mo and Fe.

Leachate contains a lot of nutrients needed by plants, including organic nitrogen (10-600 mg L^{-1}), ammonium nitrogen (10-800 mg L^{-1}), nitrate (5-40 mg L^{-1}), total phosphorus (1-70 mg L^{-1}) and total iron (50-600 mg L^{-1}). If the leachate was not treated, this liquid may pollutes the water around landfills, causing a decrease in environmental quality (Tchobanoglous et al 1993). *Chlorella* sp. microalgae have the ability to live in polluted waters and they are able to absorb high nutrient content in leachate water. The use of leachate water for culturing media of *Chlorella* sp. may beneficial as its growth may be improved and the organic materials content in the waste may be reduced. In addition, there has been no research that discusses the potential or benefit of leachate as a source of nutrients for the culture of *Chlorella* sp. Therefore, the study was conducted to find information about the growth of *Chlorella* sp. maintained on media enriched with leachate water from municipal waste.

Material and Method

Description of the study sites. The seeds of *Chlorella* sp. used in the study were obtained from stock at the Center Laboratory for Algae Research, Faculty of Fisheries and Marine Science, Universitas Riau, Pekanbaru. The organic material used as a nutrient was urban waste leachate, obtained from the landfills of Muara Fajar, Rumbai Pekanbaru, in which drilled well water wasused as a culture medium. The culture equipment used were: a gallon with a capacity of 6 L, 40 Watts fluorescent lamps, aerator pumps, binocular microscopes and computers, plankton net, micropipets, haemacytometers, analytical scales, centrifuges, microwave oven, pH meter, thermometer, DO meters and glass tools.

The research used an experimental method, with design usinga completely randomized design (CRD) comprising 5 treatments and 3 repetitions. The concentration of leachate in the treatments were P1=5%, P2=10%, P3=15%, P4=20% and P5=25%. The used water was collected from a drilled well. The volume of culture media was 5 L with an initial density of *Chlorella* sp. of $336x10^3$ cells mL^{-1}. *Chlorella* sp. abundance was calculated every two days, as well as their specific growth rate, biomass, proximate and amino acids content. Temperature data collection was carried out every two days and other water quality data such as pH, dissolved oxygen, ammonia, nitrate, phosphate, COD and BOD were measured every five days and heavy metals (Cu, Zn, Fe, Mn, Cd) measurements were made at the start and end of the study. A water quality analysis was conducted using a spectrophotometer (AAS), in the laboratory.

Statistical analysis. The abundance of *Chlorella* sp. during the study was determined by counting cells with a Neubauer haemacytometer (Mukhlis et al 2017) and by calculating the overall density with the formula $N=nx10^4$ (cells mL^{-1}), where n was the total number of computed cells (cells mL^{-1}) and N was the total number of cells mL^{-1} in each sample.The specific growth rate (μ) of *Chlorella* sp. was calculated according to Wood et al (2005): $\mu=Ln(N_2/N_1)/t_2-t_1$, in which N_2 was the cell population density at the observation time, N_1 was the cell population density at the initial time, t_1=initial time and t_2=observation time (days). The effect of different concentrations of leachate wastewater on the growth of *Chlorella* sp. was tested using the analysis of variance (ANOVA). Determinations of the *Chlorella* sp. abundance were carried out every two days.

Results

The abundance of Chlorella sp. population. The results of observations of the abundance of *Chlorella* sp. population during the study are presented in Figure 1. The growth of *Chlorella* sp. from the beginning of culture showed an exponential phase or accelerated growth. Therefore, from the measurement results, the number of cells hardly experienced a lag phase (adaptation). This was because at the time of cultivation the culture stock was already in an exponential phase, so that the inoculated cells could adapt to the new culture media. According to Istirokhatun et al (2017), the adaptation

phase will be shorter or even invisible if the cells inoculated come from an exponential phase culture.

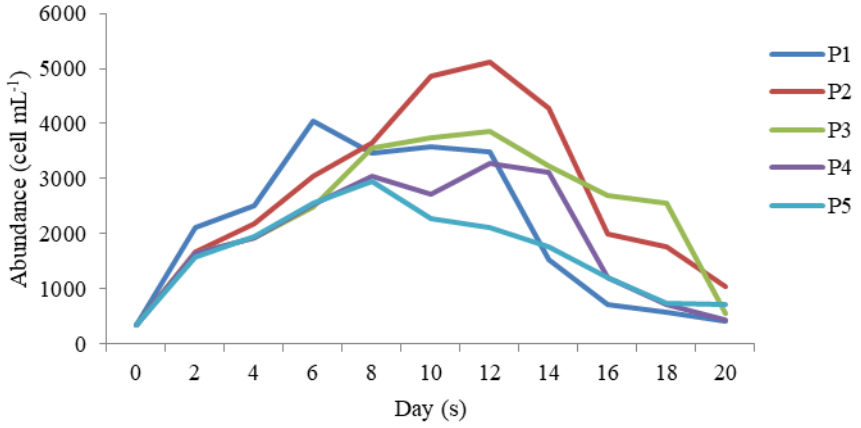

Figure 1. The abundance of *Chlorella* sp. (cells mL^{-1}) reared in leachate enriched media.

When culture was carried out, *Chlorella* sp. was able to optimally utilize the nutrients contained in the culture media for metabolism and reproduction. The growth of *Chlorella* sp. requires nutrients such as nitrogen, in which the leachate has enough nitrogen in the form of nitrate compounds for its growth and reproduction. The growth of *Chlorella* sp. given leachate with concentrations of 15, 20 and 25% grew slower than the concentrations of 5 and 10%, meaning that high concentrations *Chlorella* sp. took longer to adapt to the culture media. Chilmawati & Suminto (2010) reported that culture media that had a too high nutrient content would inhibit the growth of microalgae due to the need of a longer time for adapting.

In this study, the media enriched with 5% leachate water revealed that the growth increased exponentially and the algae abundance peaked on the 6^{th} day. Furthermore, the abundance of cells decreased on the 8^{th} day of observation and continued until the end of the study. In the treatment with 25% leachate water, *Chlorella* sp. experienced a population peak on the 8^{th} day and decreased on the 10^{th} day until the end of the study. In the media enriched with 10% leachate water, the abundance of cells increased exponentially until the 10^{th} day and peaked in the 12^{th} day. While in the leachate concentrations of 15 and 20%, the exponential phase occurred until the 8^{th} day and the population peak was on the 12^{th} day. Meanwhile, the stationary phase for concentrations of 10 and 15% was between day 10 to day 12.

Different concentrations of leachate resulted in different densities of *Chlorella* sp. Leachate with the concentrations of 5 and 10% resulted in higher cell density than with the other treatments. This can be caused by the concentration of nutrient. If the nutrient concentration was excessive, it would result in the inhibition of *Chlorella* sp. growth and even become a toxic material. Meritasari (2012) reported that excess nutrients could not be utilized effectively, resulting in a pile of toxic organic compound, so the effectiveness of cell metabolism would be directly disrupted and in the end could inhibit cell growth. Furthermore, on the 12^{th} day, all treatments had entered the death phase, namely the abundance of *Chlorella* sp. had decreased significantly until the end of the study. This was caused by the decreasing of nutrients availability in the culture media, so that there was no longer enough nutrients for cell growth and division. Then the cell density began to decrease because the nutrients in the leachate had begun to decrease by the culture time and the death rate was higher than the growth rate (death phase). According to Meritasari (2012), the death phase occurs when microalgae cells begin to die, marked by a decrease in cell abundance. Microalgae cell death occur due to: changes in the water quality towards a bad direction, worse environmental condition, too long cultivation age and a decrease in the nutrient content in the cultivation medium.

The observation showed that the growth and abundance of *Chlorella* sp. for each treatment were different. The analysis of variance (ANOVA) revealed that the value of

calculated F was higher than in the F table (P<0.05), meaning that the treatment of leachate with different concentrations showed a significantly different response to the abundance of *Chlorella* sp. This indicates that leachate can be used as a source of organic compounds for the growth and reproduction of microalgae.

Table 1

Abundance data of *Chlorella* sp. (x10^3 cell mL^{-1}) during the research

Measuremen	Treatment				
day	P1	P2	P3	P4	P5
0	336.00±0.00	336.00±0.00	336.00±0.00	336.00±0.00	336.00±0.00
2	2100.00±86.6[b]	1666.67±236.3[a]	1633.33±275.4[a]	1633.33±104.08[a]	1566.67±189.3[a]
4	2500.00±492.4	2183.33±425.2	193.33±43.11	1916.67±288.7	1950.00±278.4
6	4050.00±2174.3[*]	3050.00±346.4	24833.33±682.5	2533.33±548.5	2550.00±624.5
8	3466.67±1504.4	3650.00±1243.9	3550.00±1353.7	3033.33±378.6	2950.00±1130.7[*]
10	3566.67±1088.9	4850.00±2357.4	3750.00±1868.1	2716.67±1681.0	2266.67±340.3
12	3483.33±1150.4	5516.67±1918.5	3866.67±2825.9[*]	3233.33±1270.2[*]	2100.00±377.5
14	1533.33±1172.9	4266.67±596.5	3216.67±2205.8	3116.67±2191.1	1766.67±125.8
16	700.00±50.00	2000.00±1743.6	2700.00±252.4	1200.00±1047.6	1200.00±1255.9
18	566.67±104.08	1750.00±1997.5	2550.00±1873.5	700.00±435.89	733.33±752.22
20	400.00±50.00	1033.33±812.9	550.00±396.86	433.33±189.30	716.67±728.58

Values are expressed as mean±STD. Superscript letters on the same line show significantly different results between treatments (P<0.05). *Population peak.

In the leachate treatment, the concentration of 5% was significantly different from the concentrations of 10, 15, 20 and 25%. This was because at a concentration of 5%, the growth had a peak abundance on the 6^{th} day of 4050.00x 10^3 cells mL^{-1} and the time was shorter when compared to other treatments. For a concentration of 10%, the abundance was 5516.67x10^3 cells mL^{-1}, with a population peak on the 12^{th} day, followed by leachate at 15% concentration of 3866.67x10^3 cells mL^{-1}, with a population peak on day 12 and the abundance for a 20% leachate concentration was of 3233,33x10^3 cells mL^{-1}, with the population peak on the 12^{th} day. The abundance for a 25% leachate concentration was of 2950.00x10^3 cells mL^{-1}, with the population peak on the 8^{th} day.

From the abundance perspective, the results for the leachate with a 10% concentration were larger than for the other treatments. However, from the growth rate perspective, the 5% concentration had a faster growth rate. This means that the use of nutrients from the culture media, by the microalgae, for growth and reproduction requires an optimum concentration of leachate, ranging from 5 to 10%. *Chlorella* sp. can grow quickly or become slower and even death can occur, depending on leachate concentration. Handajani (2006) reported that excessive nutrients could not be utilized effectively, so that it would produce a pile of toxic organic compounds, so the effectiveness of cell metabolism would be directly disrupted and at the end it could inhibit growth.

Discussion

Specific growth rate of Chlorella sp. (µm day^{-1}). The specific growth rate values during the study ranged from 0.097-0.198 µm day^{-1}, in which high values were obtained at leachate concentrations of 5% and 25%. It is presumed that this occurs because, with the provision of leachate concentration of 5%, *Chlorella* sp. could optimally absorb nutrients such as nitrates in the culture media for growth and reproduction. Widyantoro et al (2018) reported that nitrate was converted into nitrite by the nitrite reductase enzyme, then converted into ammonium ion, so that it could be utilized by *Spirulina platensis*. An optimal utilization of nitrate would increase the specific growth rate of *S. platensis*.

Then the high value of the specific growth rate at concentrations of 5 and 25% could be caused by the short peak day of the population, namely on the 6^{th} and the 8^{th} day. Apart from these factors, it could also be influenced by various environmental factors supporting the growth of *Chlorella* sp., such as the light intensity and aeration

system. Aulia et al (2017) reported that the difference of specific growth rate in each treatment was caused by the ability of cells to absorb nutrients contained in the culture media. Furthermore, it was mentioned that the specific growth rate value could be used as a measure to determine the carrying capacity of the media for the cell growth.

The specific growth rate for leachate administration with concentrations of 10%, 15% and 20% was lower than at the concentrations of 5% (0.198 μm day^{-1}) and 25% (0.146 μm day^{-1}). This can be due to the longer time of nutrients consumption for *Chlorella* sp. growth, so that the population peak day was also delayed to the 12^{th} day. For the provision of leachate with concentrations of 5 and 25%, the population peak time was shorter, namely on the 6^{th} and 8^{th} day, due to the exponential phase of *Chlorella* sp. Then its growth began to decline, due to a lower availability of nutrients in the culture media, except for the leachate at 10% concentration, where the peak day of the exponential phase occured on the 10^{th} day. According to Tetelepta (2011), the growth of *Chlorella* sp. has stopped because the availability of nutrients in the culture media is no longer sufficient, resulting in a nutritional competition and finally a decline in the cell number occurs due to the cells starvation.

Proximate and amino acid content in Chlorella sp. The protein content of *Chlorella* sp. among the treatments at the end of the study ranged from 30.27 to 32.82%. There were differences in the protein content among the treatments with different concentrations of leachate wastewater, but during the study it did not show a different effect.

Table 2

Data of proximate content of *Chlorella* sp. (%) due to the treatment

Parameter	*Action*				
	P1	*P2*	*P3*	*P4*	*P5*
Water content	27.28 ± 0.02^{c}	27.18 ± 0.02^{b}	26.18 ± 0.02^{a}	27.34 ± 0.01^{d}	27.36 ± 0.01^{d}
Ash content	3.79 ± 0.01^{a}	4.02 ± 0.02^{c}	4.47 ± 0.02^{e}	4.09 ± 0.02^{d}	3.87 ± 0.01^{b}
Protein	32.82 ± 0.03^{e}	31.13 ± 0.02^{b}	31.90 ± 0.02^{c}	30.27 ± 0.03^{a}	32.06 ± 0.02^{d}
Fat	8.46 ± 0.02^{d}	8.07 ± 0.02^{c}	8.06 ± 0.01^{c}	7.90 ± 0.02^{b}	7.84 ± 0.01^{a}
Carbohydrate	27.65 ± 0.01^{a}	29.60 ± 0.01^{d}	29.39 ± 0.06^{c}	30.02 ± 0.01^{e}	28.86 ± 0.02^{b}

Values are expressed as mean±STD. Superscript letters on the same line show significantly different results between treatments ($P<0.05$).

This can be due to the measurement of the proximate content for all treatments carried out, at the end of the study. In this case, at the time of measurement, the population growth of *Chlorella* sp. was already in the death phase, so that the number of cells also decreased. Likewise, the chlorophyll content had also decreased. Protein formation was determined by the availability of the elemental nitrogen present in the culture medium. Christiani et al (2017) state that nitrogen is a major component for protein formation and cell multiplication. Microalgae are able to carry out the photosynthesis process and produce energy which can be transformed into proteins. Furthermore, Oktaviani et al (2017) state that the supply of nitrate as a higher macronutrient can be used as a building block for chlorophyll structures and protein structures. Chrismadha et al (2006) say that nitrogen and phosphorus play a very important role as a constituent of protein compounds in cells, so that the deficiency of these two elements causes the cells to experience a decrease in protein content.

The results of the analysis of the fat content of *Chlorella* sp. during the observation ranged between 7.84 and 8.46% and the carbohydrate content was between 27.65 and 30.02%. There were differences in fat and carbohydrate content among the treatments during the study. The difference of nutritional values contained in *Chlorella* sp. can be caused by the difference of nutrient values of each treatment, such as nitrogen. In addition, it can also be caused by the intensity of light entering the culture media, although in the study the parameters were not measured. Sugiharto (2020) reported that several factors could cause variation in the nutritional value of *Chlorella*

vulgaris, namely light intensity, aeration, stress and production conditions. Then in the growing medium, nitrate and phosphate are two elements that absolutely must be available in microalgae culture media. Hu & Gao (2006) stated that nitrogen in nitrate is one of the macronutrients needed to form protein, fat and chlorophyll.

Glutamic acid and aspartic acid had the highest contribution to the amino acids content, compared to other types of amino acids. This can be due to the different nutrient elements concentration in each treatment. The types of amino acids that were high in nutrients content were glutamic acid and aspartic acid, compared to other amino acids. In the research results of Armaini et al (2016), the concentrations of glutamic acid and aspartic acid of the culture of *Chlorella pyrenoidosa* in the bean sprouts extract media were also the highest among all amino acids. Furthermore, Amanatin & Nurhidayati (2013) reported that nitrogen was an important building block for amino acids, amides, nucleotides and nuclei proteins.

Water quality temperature. The temperature of the culture media during the study ranged between 25 and 26^{0}C. The temperature of the culture media during the observation was relatively stable because *Chlorella* sp. was cultivated indoor, with sufficient and smooth air circulation. Prabowo (2009) reported that a temperature ranging from 25 to 30^{0}C was optimal for the growth of *Chlorella* sp. and could increase 2-3 times the biological activity of organisms. The temperature range was determined in accordance with *Chlorella* sp.'s metabolic activities. Chisti (2007) stated that the temperature for microalgae proliferation generally ranges from 20 to 30^{0}C.

pH. The results showed that the pH of the culture media during the study ranged between 4.8 and 8.1. The initial culture pH increased until the 5th and 10th day of observation, then decreased until the end of the study. The increase in the pH of the culture media occurred at the beginning of the observation, in the exponential phase. In this phase, there is a rapid growth and increase in the population of *Chlorella* sp, with the increase in the intensity of photosynthetic activity. Therefore, due to this activity, the oxygen content in the culture media also increased. In subsequent observations, the pH value of the culture media had decreased, due to a decline of the *Chlorella* sp. Population, which experienced death and decomposition. This condition caused the pH of the culture media to decrease until the end of the study, resulting in disrupted cell biochemical processes, thus affecting the growth of *Chlorella* sp. cells. Maharsyah (2013) reported that the optimum pH range for *Chlorella* sp. growth was between 4.5 and 9.3. The pH value of the culture media during the study was appropriate for the metabolism and growth of *Chlorella* sp.

Nitrate. The nitrate content in the culture media during the study ranged from 0.804 to 2.075 mg L^{-1}, at appropriate levels for the growth and reproduction of *Chlorella* sp. According to Aprilliyanti et al (2016), an optimal growth of phytoplankton requires a nitrate content ranging between 0.9 and 3.5 mg L^{-1}. The nitrate content in the culture media decreased from the beginning to the end of the observation, in all treatments. This is because *Chlorella* sp. required nitrogen nutrients derived from nitrate compounds for its metabolic processes and growth. Likewise, during the death phase, the death rate was faster than the reproductive rate, so that the decreased number of cells consumed less nitrate for metabolism and growth. According to Kawaroe et al (2009), the supply of nitrate in water decreases with an increasing microalgae growth. Amanatin & Nurhidayati (2013) stated that nitrogen is an important and essential ingredient for cell division, so nitrogen is essential for growth.

The effectiveness of nitrate in 5% of leachate was of 30.44%, whereas for a 10% concentration of leachate it was of 36.22%, in 15% leachate concentration it was of 50.22%, in 20% concentration of leachate it was of 46.63% and in 25% concentration of leachate it was of 42.29%. A higher value of nitrate effectiveness was observed at leachate concentrations of 15, 20 and 25% compared to concentrations of 5 and 10%, probably due to the increase of nitrate content with the leachate concentration in the culture media.

Iron (Fe). The results of Fe measurement at the beginning of the study in each treatment ranged from 0.28 to 0.58 mg L^{-1}. At the final, the Fe content for all treatments was the same and the value was detected to be less than 0.2 mg L^{-1}. *Chlorella* sp. metabolism and growth, apart from requiring elements in the form of macronutrients, also requires micronutrients such as Fe. According to Effendi (2003), microalgae require relatively small amounts of Fe for cytochromes and chlorophylls. It also plays a role in the enzymatic system and in the electron transfer during the photosynthesis process, but excessive levels of Fe can inhibit the fixation of other elements. ANOVA showed that the value of calculated F was higher than in the F table ($P<0.05$). This means that the use of Fe by *Chlorella* sp. had a significant effect.

During the culture, *Chlorella* sp. could take advantage of Fe for reproduction and growth. This can be seen from the Fe content at the end of the observation, with a value of less than 0.2 mg L^{-1}. Elemental Fe presence in certain amounts is needed by living organisms, but in excessive amounts it could cause toxic effects. In microalgae, the metal ion Fe plays a very important role in the regulation of cell metabolism, as a cofactor for the photosynthesis process (Millaleo et al 2010; Allen et al 2011). The permissible Fe content in water, according to the water quality standards, is 0.3 mg L^{-1}. This means that the Fe content in the culture media at the end of the study was below the water quality standard based on Government Regulation No. 82 of 2001. From the measurement data, it was found that Fe absorption in *Chlorella* sp. reached an effectiveness of 66% for a concentration of 25% leachate, greater than for the concentrations of: 5% (29% effectiveness), 10 and 15% (43% effectiveness) and 20% (59% effectiveness). *Chlorella* sp. had a high tolerance to the absorption of heavy metal ions. Purnamawati (2015) stated that microalgae are able to reduce the metal concentration of the medium, usually through absorption and bioaccumulation.

Conclusions. The provision of leachate water with different concentrations affected the growth, abundance and proximate content of *Chlorella* sp. The best growth of *Chlorella* sp. was at a leachate concentration of 10%, with an abundance of 5116.67×10^3 cells mL^{-1} and the peak population on the 12^{th} day. In addition, the growth of *Chlorella* sp. also affected the organic materials concentration contained in the leachate, such as nitrate and iron. The effectiveness of Chlorella in reducing the inorganic materials in the media such as nitrate and iron ranged from 30.44 to 50.22% and 29 to 66%, respectively.

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Conflict of interest. The authors declare no conflict of interest.

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Authors:
Rosyadi, Islam Riau University, Faculty of Agriculture, Department of Aquaculture, Jl. Kaharuddin Nasution KM 13, Pekanbaru 28284, Indonesia, e-mail: rosyadi@agr.uir.ac.id
Tengku Dahril, Riau University, Faculty of Fisheries and Marine Science, Department of Aquatic Resources Management, Jl. H.R. Subantas KM 12.5, Pekanbaru 28293, Indonesia, e-mail: dahril2011@gmail.com
Aras Mulyadi, Riau University, Faculty of Fisheries and Marine Science, Department of Marine Science, Jl. H.R. Subantas KM 12.5, Pekanbaru 28293, Indonesia, e-mail: aras_mulyadi@yahoo.co.id
Sofyan Husein Siregar, Riau University, Faculty of Fisheries and Marine Science, Department of Marine Science, Jl. H.R. Subantas KM 12.5, Pekanbaru 28293, Indonesia, e-mail: sofyan.siregar@lecturer.unri.ac.id
Windarti, Riau University, Faculty of Fisheries and Marine Science, Department of Aquatic Resources Management, Jl. H.R. Subantas KM 12.5, Pekanbaru 28293, Indonesia, e-mail: windarti.unri@gmail.com
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