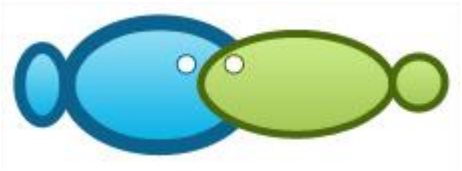

Transmission electron microscope analysis upon growth of lead acetate treated microalga, *Dunaliella* sp.

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Abstract. The objectives of the present research were to evaluate the effect of lead acetate on the growth of microalgae *Dunaliella* sp. and to its structure through Transmission Electron Microscope. *Dunaliella* sp. used in this study was taken from stock available at the Aquaculture Technology Laboratory of the Faculty of Fisheries and Marine Sciences, Sam Ratulangi University, Manado, Indonesia. The cell of *Dunaliella* sp. is motile and its shapes vary from ellipse to cylinder. Cell length varies depending on the shape of the cell. Radial symmetry shape has about 2.8-40 µm. Seawater used as the culture medium was taken from clean sea waters and far from settlements. Sea water was filtered using 0.45 µm filter paper with the help of an aspirator, then diluted with distilled water until it reached a salinity of 20 ppt. Microalgae *Dunaliela* sp. were cultured using walne medium in the culture chamber with a temperature of 25° C, salinity of 20 ppt with 20-watt tube light irradiation. Algae harvesting was done when the algal density had reached the exponential phase by calculating the density of microalgae every day using a haemocytometer assisted by a light microscope. After the algae reached exponential density, the samples were treated with lead acetate at 30, 50 and 80 ppm. After harvesting, the algae were centrifuged and subjected to the Transmission Electron Microscope (TEM) analysis. The specimens were prepared through a standard preparation of Plastic Block (Resin) consisted of fixation, dehydration, infiltration, embedding and curing. The density of microalgae was calculated using a haemocytometer under a light microscope. The research results showed that the density of *Dunaliella* sp. was not significantly different conidering different concentrations of lead acetate. The density of *Dunaliella* sp. had no relationship with the cell structure of each concentration of lead acetate used. Transmission Electron Microscope analysis showed the cell structure of *Dunaliella* sp. treated with lead acetate was damaged at the cell membrane and many cells were dead and damaged as compared to control.
Key Words: heavy metal, aquatic environment, pollutants, microalgae.

Introduction. Lead (Pb) is one of the pollutants included in the category of heavy metals. Lead can contaminate the aquatic environment and influence the life of organisms that live in it (Darmono 1995). Lead acetate [Pb $(CH_3COO)_2$] is a white crystalline compound and tastes sweet resulted from reaction of lead (II) oxide with acetic acid. Lead acetate is also called lead sugar because it tastes sweet. In aquatic organisms, lead can be accumulated in the body. The metal is absorbed through water and food. Lead content in waters that exceeds the threshold will be dangerous for the environment itself and especially for the existing organisms. One of the marine organisms that can accumulate lead is microalgae *Dunaliella* sp. Lead contents in aquatic environment are diverse (Connel & Miller 1995; Hutabarat & Evans 1995).

Balaira et al (2017) found that the density of microalgae *Dunaliella salina* at lead concentration of 1 ppm, 2 ppm, 3 ppm and control were very significantly different in which the concentration of 3 ppm could reduce more number of cells as compared with the concentration of 1 ppm and 2 ppm. Research results of Nasprianto et al (2019)

showed that the concentration of metals in macro-algae *Halimeda opuntia* in Totok Bay waters was 2.2 ppm, and in Blongko waters was 0.2 ppm. Simanjuntak et al (2016) reported that the growth of *Botryococcus braunii* decreased at the eighth days when treated with $HgCl_2$ compared to control and administration of this compound could reduce the concentration of chlorophyll-a pigment. Pigment concentration decreased following the toxicity of the mercury chloride compound ($HgCl_2$) with a concentration of 2 ppm which could give the highest toxic effect.

Microalgae are a group of organisms that are very diverse and have a variety of potential that can be developed as a source of food, and other chemicals. The content of compounds in microalgae varied depending on the type, environmental factors and nutrients. One species of microalgae that has the potential to be developed is *Dunaliella* sp. because this organism has been widely studied especially as a source of β-carotene and glycerol.

The present study was conducted to evaluate the effect of lead acetate on the growth of microalgae *Dunaliella* sp. and its structure through Transmission Electron Microscope Analysis.

Material and Method

Test organism. *Dunaliella* sp. used in the present study was taken from stock available at the Aquaculture Technology Laboratory of the Faculty of Fisheries and Marine Sciences, Monado, Indonesia. The cells were motile and its shapes varied from ellipse to cylinder. Cell length varies depending on the shape of the cell. Radial symmetry shape has about 2.8-40 µm. The size of these cells was affected by the conditions of density and light intensity.

Seawater. Seawater used as the culture medium was taken from clean sea waters and far from settlements. Sea water was filtered using 0.45 µm filter paper with the help of an aspirator, then diluted with distilled water until it reached a salinity of 20 ppt. In 1,000 mL of seawater was added 1 mL of walne medium.

Research procedure. Microalgae *Dunaliella* sp. were cultured using walne medium in the culture chamber with a temperature of 25°C, salinity of 20 ppt with 20-watt tube light irradiation. Algae harvesting was done when the algal density had reached the exponential phase by calculating the density of microalgae every day using a haemocytometer assisted by a light microscope. After the algae reach exponential density, the samples were treated with lead acetate at 30, 50 and 80 ppm. The choice of this concentration was based on previous research in which at below 30 ppm, the alga is still alive in stressful conditions. After harvesting, the algae were centrifuged and continued with the Transmission Electron Microscope (TEM) analysis. The specimens were prepared through a standard preparation of Plastic Block (Resin) consisted of fixation, dehydration, infiltration, embedding and curing. Furthermore, the block was cut by providing a grid, a buffer film, and a trimming block. After the grid was stained, the specimens were observed using a transmission electron microscope at the Eijkman Institute for Molecular Biology, Jakarta, Indonesia.

Data collection. The data collected was the density of the alga after treated with lead acetate at different concentrations. The density was calculated using a haemocytometer under a light microscope.

Data analysis. Analysis of variance was applied to evaluate the effect different concentrations of lead acetate on the density of alga. The statistical analysis used SPSS 24 for windows.

Results and Discussion. The administration of lead acetate was done when the growth of microalgae was in the exponential phase, namely on the 9^{th} day. The density of algae before administration of lead acetate was 18×10^4 cells mL^{-1}. The first day after being

treated with 30 ppm of lead acetate, the density was 15.4 x 10^4 cells mL^{-1}, at concentration of 50 ppm was 14.2 x 10^4 cells mL^{-1}, at concentration of 80 ppm was 11.4 x 10^4 cells mL^{-1}; when compared with the control, which exhibited 16 x 10^4 cells mL^{-1}, the density of treated algae had decreased.

Table 1

Densities of *Dunaliella* sp. after lead acetate treatment

Day	*Density at …*			
	30 ppm	*50 ppm*	*80 ppm*	*Control*
1	15.4	14.2	11.4	16
2	14.8	12.6	16.2	13.6
3	16.6	17	14.8	18.6
4	15	11.2	11	16
5	13	11.6	11.2	15.8
6	13.4	11.4	11.4	16
7	13.4	10.8	11.8	13.4
8	11	9.2	8.4	12.2
9	9.2	9.4	7.6	11
10	8.8	9.6	10.6	10.8
11	9.4	9.6	9.4	10
12	10.8	7.8	10	9.8
13	10	9	11.2	9.4
14	11.8	8.4	13.2	9.2
15	10	8.8	9.6	9.4
16	9	8	9.2	13.4
17	8.4	9	8.8	11.4

Lead acetate is a heavy metal which is very toxic to *Dunaliella* sp. and can inhibit the growth of microalgae. In the research of Widiyani & Dewi (2014), the growth of *Chlorella vulgaris* was significantly influenced by the decreasing concentration of Cd. The density of *C. vulgaris* during culture were Cd 3 mg L^{-1} (1,015.97 cells mL^{-1}), Cd 1 mg L^{-1} (769.70 cells mL^{-1}), Cd 5 mg L^{-1} (719.55 cells mL^{-1}), and K (668.21 cells mL^{-1}). Statistical analysis showed that various concentrations of Cd had a significant effect on the growth of *C. vulgaris*.

Halima et al (2019) reported that different concentrations of Pb significantly influenced the growth of *C. vulgaris* ($p<0.05$). Reduction in the concentration of Pb metal in culture media was Pb 10 ppm (96.8%), Pb 5 ppm (96.2%), Pb 1 ppm (90%) and there is no Pb found in control. Statistically it was shown that the density of *C. vulgaris* was significant effected by the decrease of Pb metal concentration ($p<0.05$). Sun et al (2020) found the contents of Hg, As, and Pb in microalgae cells cultivated with pure CO_2were 16.67%, 69.23%, and 70.33% that of CO_2 cultivated cells from flue gas. In fresh water cultivated cells, the Pb, As, and Hg contents were reduced by 38.46%, 15.38%, and 37.50%, respectively, compared with those cultivated with seawater.

The research of Kurniawan & Aunurohim (2014) found that Chlorella was able to survive at Pb and Zn concentration of 50 mg L^{-1} even though here was growth inhibition as compared to control. The absorption of metals in Chlorella increased with increasing metal concentration to 50 mg L^{-1} with P-values of 0.000 and 0.004. Similar result was also observed at mix metals treatment in which the adsorption increased with the increase of concentration.

The relationship between the maximum growth rate of *Spirulina plantesis* and different heavy metal (Cu) concentrations showed that the addition of 1 ppm heavy metal was needed for steady growth but the higher the concentration of heavy metal Cu, the lower the density of *S. plantesis*. *S. plantesis* can absorb heavy metal copper (Cu) in all treatments. The best absorption was observed at 3 ppm heavy metals reaching 97.886%. Heavy metal copper (Cu) affected the growth of *S. plantesis* at 5 ppm because this concentration was toxic to the growth of algae (Budi et al 2018).

Statistical analysis showed that there were no significant differences in the density of alga between different concentrations of lead acetate. The density of *Dunaliella* sp. had no relationship with the cell structure of each concentration.

TEM analysis. At the exponential phase of alga treated with 30 ppm lead acetate, there were still many algae that lived with normal cell structure, the damage was still not so visible but in the next phase the damage (not so severe) could be seen in the algae cell membrane. Figure 1 showed that the damaged cell membrane was not intact or not perfectly attach to cell organelles, probably due to a buildup of cytoplasmic fluid under the cell membrane because the circulation of heavy metals that penetrated into the cell through the cell membrane caused a swell.

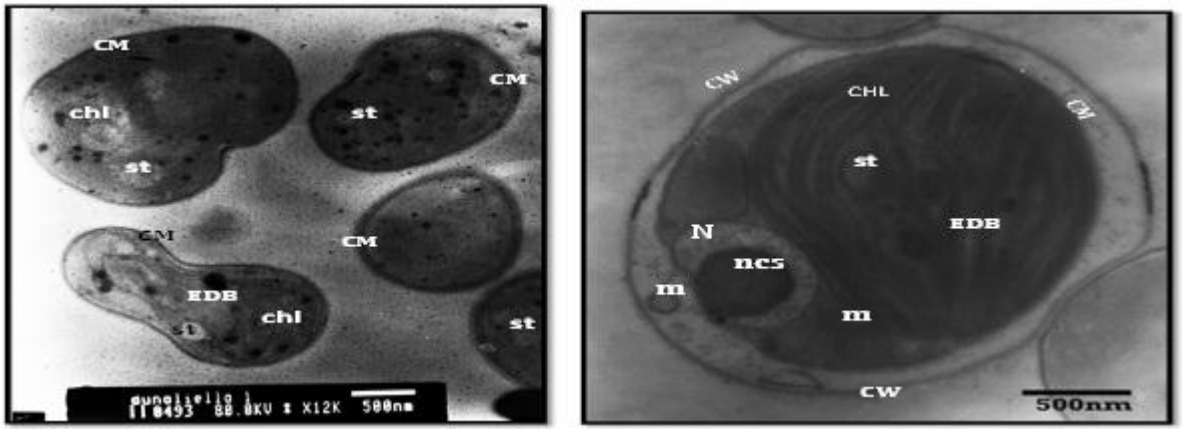

Figure 1. Cell structure of *Dunaliella* sp. at exponential phase after treated with 30 ppm lead acetate (CW: cell wall, CM: cell menbrane, EDB: electron dense body, M: mitochondria, Chl: chloroplast, St: starch) (original).

At the death phase of alga treated with 30 ppm lead acetate, damage occurred in the algal cell membrane area, apparently lysis was present and algal structure was abnormal (Figure 2). The structure of the chloroplast was not visible but starch grains still remained in damaged algal cells. Pb existed in the cell of most severe damage cells at 30 ppm. In the study of Shanab et al (2012), a dark dense electron was accumulated in the vacuole of *Pseudochlorococcum typicum* cells exposed to Pb.

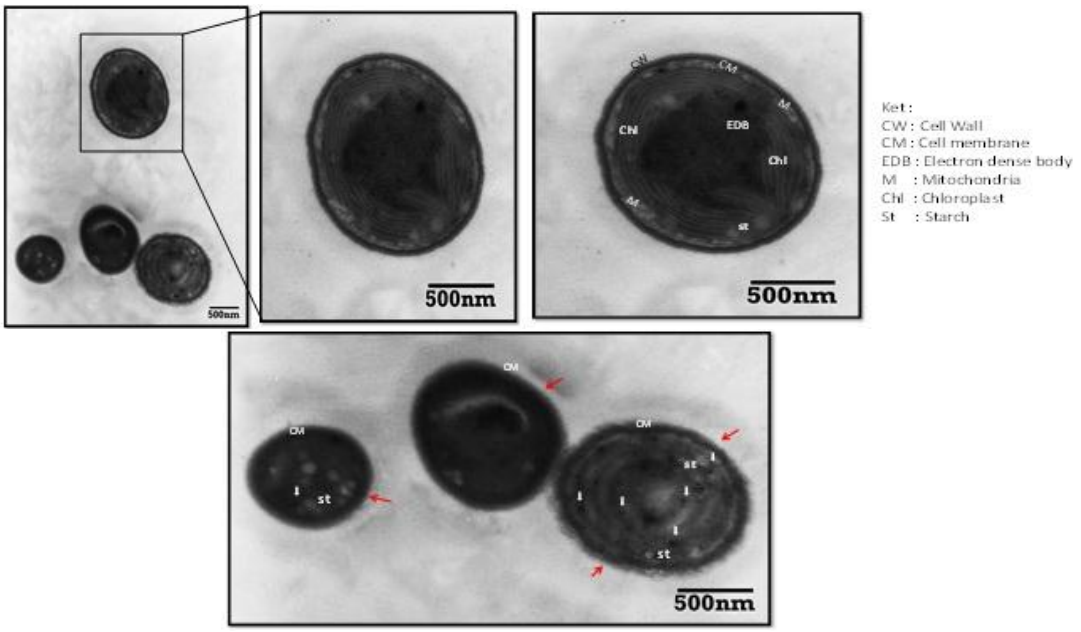

Figure 2. Cell structure of *Dunaliella* sp. at death phase after treated with 30 ppm lead acetate (original).

At the exponential phase of alga treated with 50 ppm lead acetate, the cell had started to damage more severely than in the 30 ppm exponential phase (Figure 3). At 50 ppm treatment, the damage was more severe, the algae structure became more abnormal, it was difficult to identify the chloroplast. Although the nucleus still appeared perfect but other cell organelles presented lysis and rupture. It was proven that at the death phase of 50 ppm, more cells were damaged and were dead, compared to the death phase at 30 ppm. It appeared that mitochondria were still present but the cristae were out of sight, this is likely the cell was at death condition so that the process of respiration by the mitochondria also did not worked (Figure 4). Mantiri et al (2018) also showed that the cells of *H. opuntia* from the waters of the Totok Bay analyzed through Transmission Electron Microscope (2500x magnification) experienced damage and lysis causing cell wall damage.

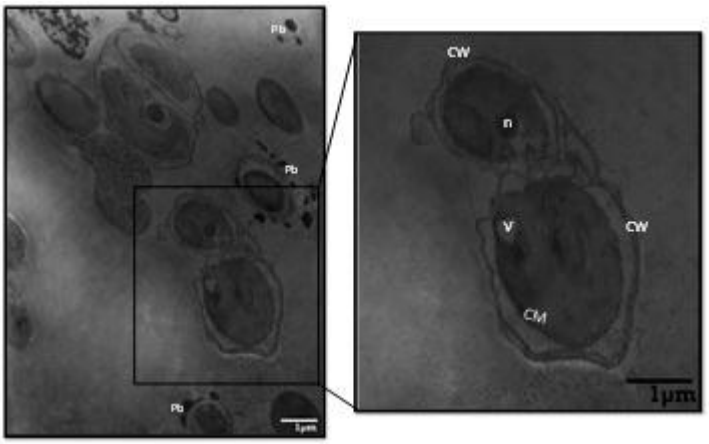

Figure 3. Cell structure of *Dunaliella* sp. at exponential phase after treated with 50 ppm lead acetate (original).

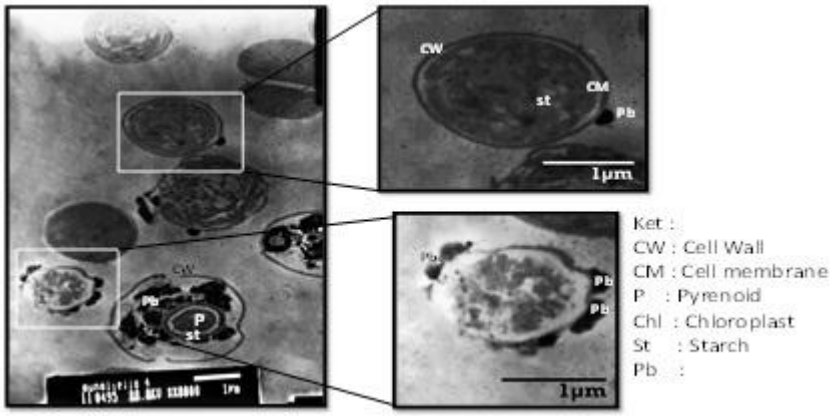

Figure 4. Cell structure of *Dunaliella* sp. at death phase after treated with 50 ppm lead acetate (original).

In the 80 ppm exponential phase, there were still cells alive but there were cells that had already experienced damage and death at higher level than at the 50 ppm exponential phase (Figure 5). At the death phase of 80 ppm the cell was dead and damaged creating a hole (Figure 6). Kepel et al (2018) reported that the cell wall around the chloroplast was damaged due to the arsenic (As) present at the environment which was bound to the cell wall.

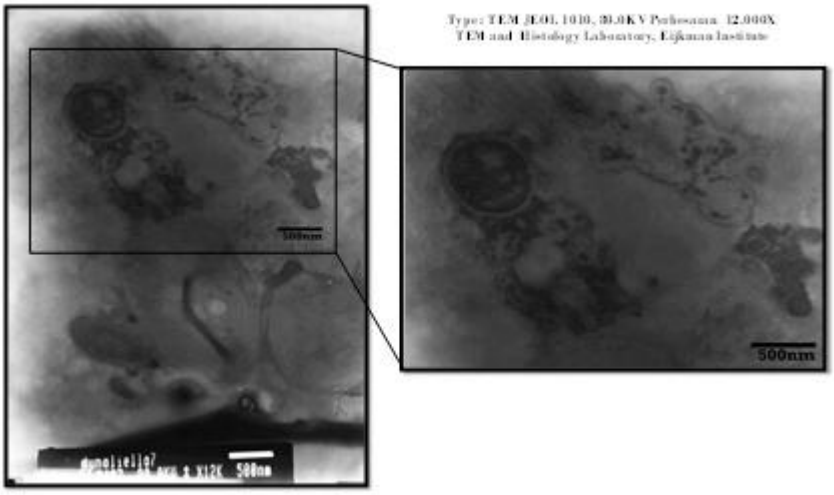

Figure 5. Cell structure of *Dunaliella* sp. at exponential phase after treated with 80 ppm lead acetate (original).

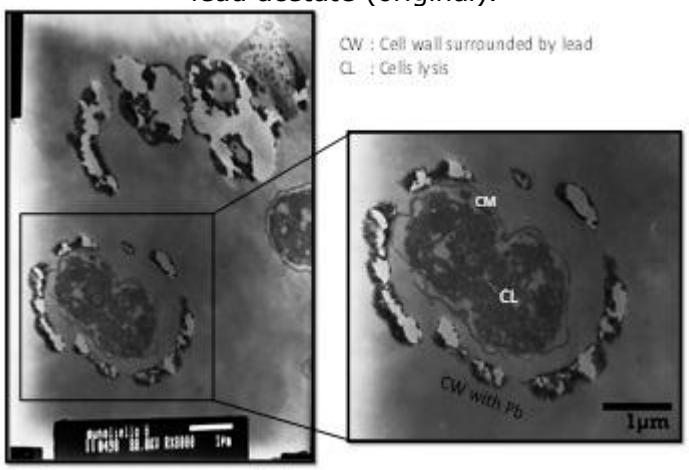

Figure 6. Cell structure of *Dunaliella* sp. at death phase after treated with 80 ppm lead acetate (original).

The cell structure of *Dunaliella* sp. without lead acetate treatment looked normal and there was no damage observed (Figure 7).

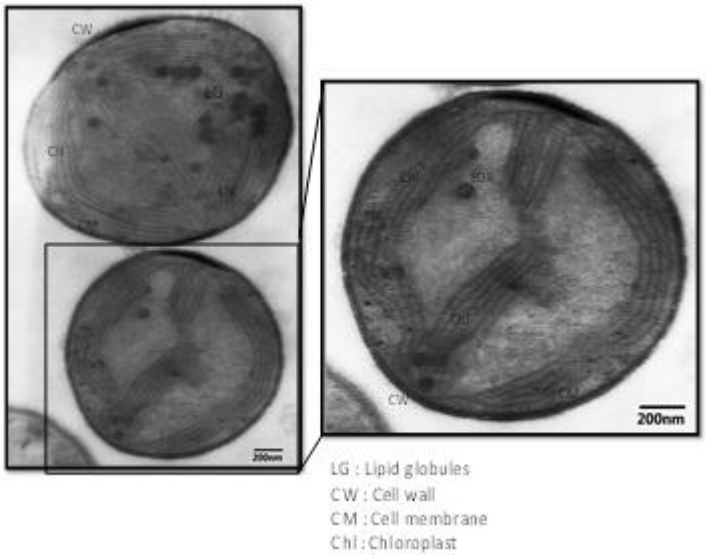

Figure 7. Cell structure of untreated *Dunaliella* sp. (original).

In the present study, lead metal acetate was found in *Dunaliella* sp. treated with 30 ppm, 50 ppm and 80 ppm concentrations. All concentrations could inhibit cell growth. Kepel et al (2018) reported that plasma cells and organelles of macro algae (*Ulva* sp.) collected from Totok Bay waters of Southeast Minahasa Regency were not affected by heavy metal and were in good condition. Hazeem et al (2019) reported that silver nanoparticles (Ag NPs) had toxic effects on the marine microalgae *C. vulgaris*. Transmission electron microscopy analysis (TEM) revealed that NP Ag was present in microalgae cells and formed large aggregates in culture media (Hazeem et al 2019). The Ag + ion, in the form of $AgNO_3$, and assessed at higher concentrations caused inhibitory effects.

Conclusions. The density of algae before administration of lead acetate was 18 x 10^4 cells mL^{-1}. The density at the first day after being treated with 30 ppm lead acetate was 15.4 x 10^4 cells mL^{-1}, with a concentration of 50 ppm which was 14.2 x 10^4 cells mL^{-1}, and with a concentration of 80 ppm was 11.4 x 10^4 cells mL^{-1}, against control of 16 x 10^4 cells mL^{-1}, the growth of the treated algae has decreased. The cell structure of *Dunaliella* sp. treated with lead acetate was damaged at the cell membrane and many cells were dead and damaged as compared to control where the cell structure looked normal and there was no damage. Statistically there were no significant differences between the treatments given with different concentrations. The density of *Dunaliella* sp. had no relationship with the cell structure regardless of treatments concentration.

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