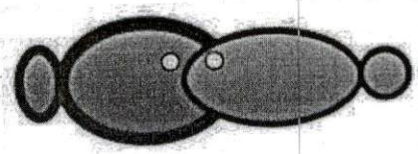

Evaluation of bleaching caused by different acidity degree (pH) levels in *Sargassum* sp.

[1]Arini Fadhla Wahyuningtyas, [1]Afif Mufidah, [1]Mochammad Amin Alamsjah [2]Agustono, [3]Pratiwi Pudjiastuti

[1] Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, Indonesia; [2] Department of Fish Health, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, Indonesia; [3] Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Indonesia. Corresponding author: M. A. Alamsjah, moch.amin.alamsjah16@gmail.com

Abstract. Indonesia is a country with high potential marine resources. *Sargassum* sp. is a marine seaweed that has high economic value. The unpredictable seasonal changes can cause fluctuation in the seawater pH (acidity degree). Fluctuation in seawater pH can interfere with the *Sargassum* sp. cell wall permeability. This can cause stress in *Sargassum* sp. and lead to bleaching condition. The aim of this study is observe the effect of pH difference in seawater towards the bleaching condition of *Sargassum* sp. This study uses different values of seawater pH, namely 5, 7 (control) and 9. The main parameters observed in this study were the thallus color gradation and the amount of chlorophyll-*a* of *Sargassum* sp. The supporting parameters observed were the structure and texture of the thallus and the characteristics of *Sargassum* sp. cells. The results from the low and high pH treatment groups show significantly different effects compared with the control group regarding thallus color gradation and the amount chlorophyll-*a* of *Sargassum* sp. ($p < 0.05$). The lowest color gradient in the thallus of *Sargassum* sp. was in the group with pH 5 (43.06%), then group with pH 9 (43.775%) and the highest was in the group with pH 7 (57.95%). The lowest amount chlorophyll-*a* of *Sargassum* sp. was found in the group with pH 5 (0.00522 µmol/cell), then in the group with pH 9 (0.00718 µmol/cell) and the highest amount chlorophyll-*a* of *Sargassum* sp. was in the group with pH 7 (0.00945 µmol/cell). This concludes that differences in acidity degree (pH) have different effects regarding the bleaching condition in *Sargassum* sp.

Key Words: bleaching, chlorophyll-*a*, pH, *Sargassum* sp.

Introduction. *Sargassum* sp. is class of Phaeophyceae or brown algae, which have high economic value. This species is widespread in Indonesia, but has not been widely cultivated (Tamayo & Del Rosario 2014). *Sargassum* sp. is commonly cultivated in salt waters, where many factors can affect its growth. The alga is one of the intertidal seaweeds that plays a distinguishing role in the region for marine animals and plants (Choi et al 2003; Nanba 1995).

Sargassum sp. contains alginic acid, which is widely used in food, cosmetics and pharmaceutical industries. The ability to stay stable at various pH levels can influence the total production of alginic acid (Bertagnolli et al 2014; Radulovich et al 2015). The water quality is important when considering the growth of the seaweed and it also affects the seaweed population. If nutrients are abundant, the algae can bloom, with negative effects on the ecosystem (De Ramon N'Yeurt & Iese 2015; Surbakti et al 2019).

Seawater generally has a pH value above 7, which means that it is mostly alkaline. However, in certain conditions, the pH value can be lower than 7, so it becomes acidic. Most of aquatic biota is sensitive to pH value changes, the ideal pH value for marine biota being between 7 and 8.5. At lower pH values (<4), most aquatic plants die, because they cannot tolerate low pH. Acidification is a condition which appears when CO_2 is continuously absorbed by the ocean (Sabine et al 2004). Water quality changes can cause the acidity of seawater to change, from alkaline (pH>7) to acidic (pH<7).

Sometimes, pH value fluctuations can interfere with the cell wall permeability of *Sargassum* sp. (Hurd et al 2014). However, the responses are species specific due to variations in carbon uptake from seawater (Mackey et al 2015; Wu et al 2008; Zou & Gao 2009).

Bleaching is the result of damage in seaweed chromatophores, the pigments being oxidized and degraded (Wisnuaji & Rochima 2015). Further pigment oxidation processes can cause a complete rupture of the isocyclic ring of the chlorophyll, resulting in color loss and compounds which have low molecular weight (Eskin & Sshahidi 2012).

The aim of this study is to observe the influence of pH difference towards bleaching condition in *Sargassum* sp. This study is expected to determine the pH value that can cause the greatest bleaching effect to *Sargassum* sp. The information presented can be used as a guideline regarding pH for the cultivation of *Sargassum* sp.

Material and Method

Description of the study site and materials. The experiment was conducted in the Anatomy and Cultivation Laboratory and Chemical and Analyst Laboratory, from the Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, Indonesia. This experiment was conducted from November 2017 to January 2018. The materials used in this study were fresh *Sargassum* sp. from the waters of Saronggi Beach, Sumenep, Madura, 7°12'62.56"S 113°89'39.96"E. The algae were kept in seawater.

The materials used include 15x15x25 cm^3 aquariums, containers, jerry cans, aerators, aeration hoses, 1000 ml measuring cups, a 10 ml volume pipette, a drop pipette, chemical glassware, a digital scale, pinsets, pH paper, lux meters, thermometers, refractometers, aquarium shelves, cables, electrical terminals, test tubes, test tube racks and a round covet. A spectrophotometers (UV-Vis 722N model 325-1000nm), a charge Coupled Device microscope (CCD) and a Scanning Electron Microscope (JEOL JSM 7000F) were also used. The aquarium sterilization was done with chlorine and the seaweed extraction was performed using acetone 90%. The materials used to regulate the pH values were HCl and NaOH.

Study procedures setup. There were 3 treatment groups: the group with a low pH (pH 5), the control group (pH 7) and the group with high pH (pH 9). These groups were placed in the same environment, characterized by 30 ppt of salinity, 28°C temperature and light intensity around 3000 lux during the day, with aeration, simulating natural conditions. The environmental conditions for the normal growth of *Sargassum* sp. are water temperatures between 27–30°C, water depth between 0.5–10 m, salinity levels between 30–33.5 ppm, pH values between 6 and 9 and a current velocity of 0.2–0.4 m/s. Chlorophyll content in *Sargassum* sp. is very important for photosynthesis. Thus, they need high values for light intensity, near 3000 lux (Kadi 2005). Fresh *Sargassum* sp. was placed in each aquarium, weighing approximately 10 grams.

The monitoring of bleaching in *Sargassum* sp. was performed by determining the appearance of color gradations on the thallus, the amount of chlorophyll-*a*, the structure and texture of the thallus and its cell morphology. The observations were carried out by collecting 0.5 grams of thallus every day. Measuring the color gradation of the thallus of *Sargassum* sp. was conducted using the Color Analysis application. The body parts studied were the stipe, blades and air bladder. The color gradation data from the Color Analysis software was expressed as a percentage. The results were used to compare the bleaching rate from the first day to the fourteenth day of each group. At the end of the experiment, images of the *Sargassum* sp. from each treatment were collected with the Scanning Electron Microscope.

The chlorophyll-*a* amount of *Sargassum* sp. was determined by using a spectrophotometer. The calculations were carried out at the beginning and at the end of the experiment, by using a sample of grinded seaweed of about 10 mg, extracted in 5 ml of methanol 90%. The obtained solution was maintained for 24 hours in a refrigerator. The samples were centrifuged at 3000 rpm, for 5 minutes (Rohani-Ghadikolaei et al

2012), after which it was analyzed by spectrophotometry with wavelengths of 665 nm and 630 nm. The measurement unit used was µmol/cell (Lobban et al 1994).

The cell morphology was characterized by observing the tissue on a CCD microscope with 100x and 400x magnification, every 24 hours from the first day to the end of the experiment, totaling 14 days. The tissue was sampled by taking vertical and horizontal slices of the body parts of *Sargassum* sp., from the stipe, blades and air bladder. The cell morphology was studied for every sample and the cell integrity was monitored.

Statistical analysis. The color gradation and the amount of chlorophyll-*a* were analyzed using variance analysis (1-way ANOVA) with a confidence level of 95% with a Complete Random Design (CRD). Data on external body changes of *Sargassum* sp. (thallus structure and texture) and cell morphology (cell shape and size) obtained from the experiment were analyzed descriptively.

Results and Discussion. The results of thallus color gradation and the amount of chlorophyll-*a* of *Sargassum* sp. in each group can be seen in Table 1.

Table 1

The results of thallus color gradation and the amount of chlorophyll-*a* of *Sargassum* sp. in ANOVA analysis

Treatments	*The thallus color gradation of Sargassum sp. (%)*	*The amount of chlorophyll-a of Sargassum sp. (µmol)*
pH 5	43.06 ± 2.91^{b}	0.0052 ± 0.00047^{c}
pH 7	57.50 ± 4.15^{a}	0.0094 ± 0.00071^{a}
pH 9	43.61 ± 4.14^{b}	0.0072 ± 0.00062^{b}

Different superscript letter notations in one column show there are significant differences ($p < 0.05$).

Thallus color gradations and the amount of chlorophyll-a of Sargassum sp. The results of variance analysis (ANOVA) showed that each pH treatment had a significantly different effect on the color gradation in the thallus of *Sargassum* sp. ($p<0.05$). The analysis was continued using Duncan's multiple distance test with a 0.05 in significance level. Duncan's multiple distance test results showed that the values of the control group (pH 7) were significantly different ($p<0.05$) from the values of group pH 5 and pH 9. The values from the high pH treatment group were not significantly different ($p>0.05$) from the ones in the low pH treatment group. This means that the pH had an effect on the color gradation of thallus (bleaching) of *Sargassum* sp., pH 5 and 9 being able to cause discoloration in *Sargassum* sp. A pH value different than 7 can produce lower color gradations. The highest color gradation of the thallus of *Sargassum* sp. was observed in the control group, with 57.95% (Figure 1). The lowest color gradation is found in the pH 5 group (43.06%).

The results also show that each pH treatment had a significantly different effect on the chlorophyll-*a* content of *Sargassum* sp. The analysis was continued by using the Duncan multiple distance test with a 0.05 degree of confidence. Duncan's multiple distance test results show that treatments pH 5, 7 and 9 were significantly different ($p<0.05$). Based on the test results from ANOVA, the highest chlorophyll-*a* content of *Sargassum* sp. was in the control group with pH 7 (0.0094 µmol/cell), then in the high pH group with pH 9 (0.0072 µmol/cell) and the lowest was in the group with pH 5 (0.0052 µmol/cell).

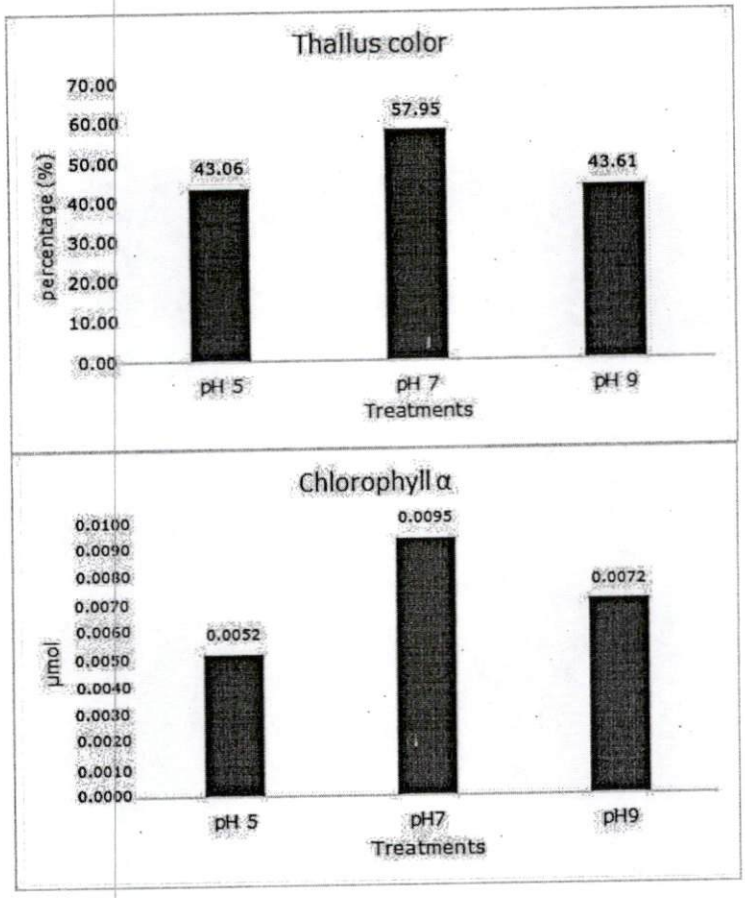

Figure 1. The thallus color gradation and chlorophyll-*a* amount of *Sargassum* sp.

Changes in the structure, texture, and cell morphology of the thallus of Sargassum sp. The information regarding the structure, texture and cell morphology of the thallus of *Sargassum* sp. is presented in Table 2.

Table 2

The structure, texture, and cell morphology of the thallus of *Sargassum* sp.

Treatments	*The structure and texture of the thallus of Sargassum sp.*	*The cell morphology (shape and size of cells) of the thallus of Sargassum sp.*
pH 5	The air bladder was separated from the thallus; the texture was soft and easily be broken.	Irregular, thin, and damaged cell walls. The cell size was various.
pH 7	The structure of thallus was complete; the texture was solid.	Round shaped, thick cell walls; the cell size was homogenous.
pH 9	The air bladder was separated from thallus; the texture was soft and could easily be broken.	Irregular, thin and damaged cell walls. The cell size was various.

Table 2 presents the structure and texture of the thallus in each treatment group. The treatment groups with low and high pH have similar characteristics, the air bladder being separated from the thallus, with soft and easily to break texture. In the control group, the thallus structure was complete, round shaped and with a solid texture. Different cell

morphology compared with the control group was also observed in the treatment groups, consisting in irregular cell forms and thin and damaged cell walls. The cell morphology of the control group did not experience changes. The cell morphological state from plant tissues in the pH 5 group can be observed in Figure 2.

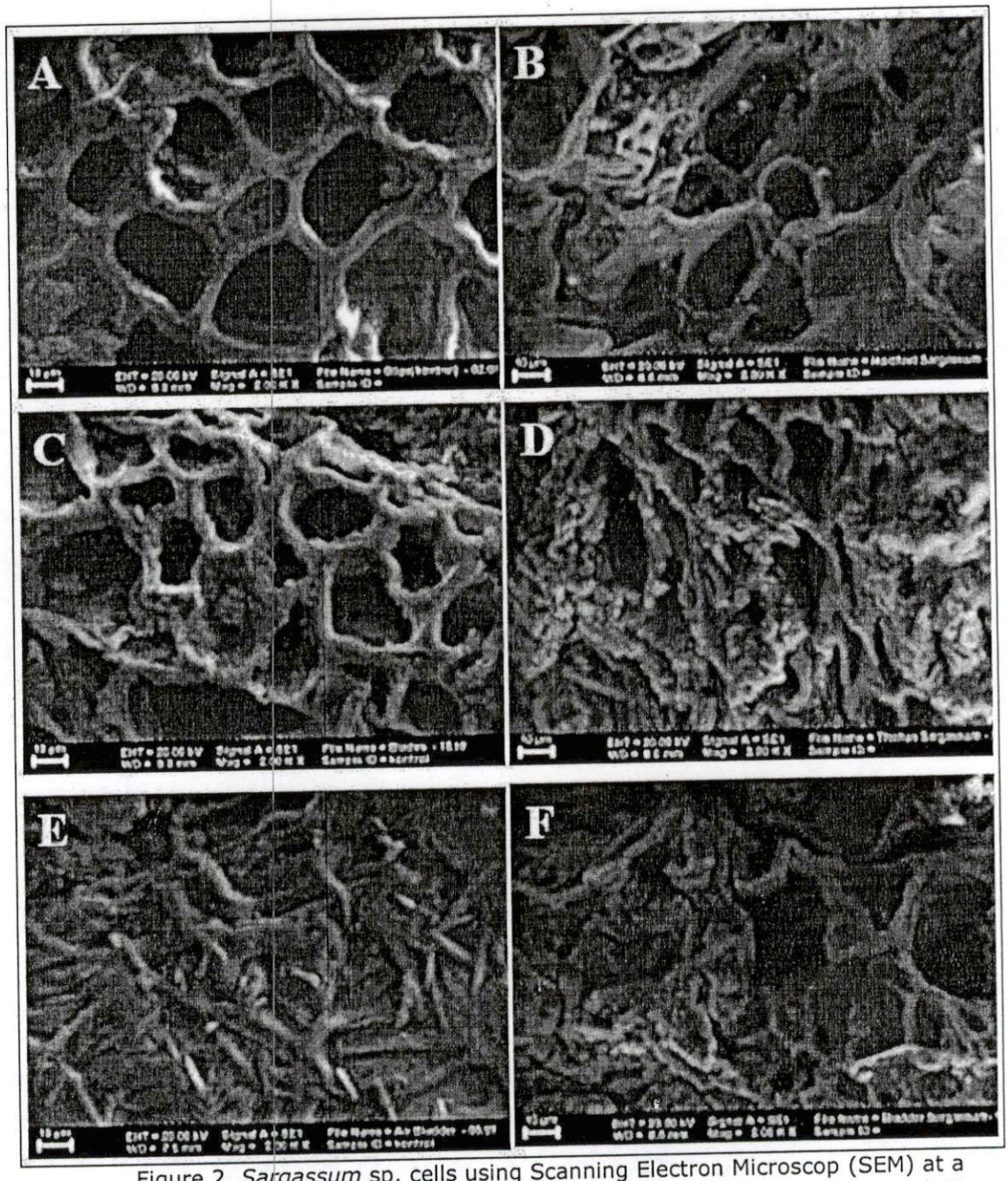

Figure 2. *Sargassum* sp. cells using Scanning Electron Microscop (SEM) at a magnification of 2000x. A - horizontal stipe section on day 1, pH 5; B – horizontal stipe section on day 14, pH 5; C - horizontal blade section on day 1, pH 5; D – horizontal blade section on day 14, pH 5; E - horizontal bladder section on day 1, pH 5; F – horizontal bladder section on day 14, pH 5.

The stipe, blades and bladder parts were observed using SEM with magnification of 2000x on the seaweeds from pH 5 group, because *Sargassum* sp. had very severe bleaching in this treatment. A change in cell size and shape was clearly observed. In the

SEM observations on the first day, the horizontal stipe section was clearly visible, the shape and size of cells looked regular and homogenous, whereas in the 14th day, there were irregular shaped cells and narrowed cell sizes because of the damage. The cell shape of the blade was clear; the size of the cells was regular and looked homogenous in the first day, while in the 14th day the cell shape looked narrow and the size of the cells shrunk. The bladder seemed to be dense on the first day and the cell size was homogenous, whereas the cell size has shrunken and the cells were loose on the 14th day; the intercellular space hollowed.

An acidic environment, like the one from the pH 5 group, can involve double bond oxidation reactions to chromatophores in *Sargassum* sp. cells and damage one or more of the double bonds in the conjugated system. Oxidation reactions cause damage to the chromatophores, thus the pigments in the cells will be oxidized and degraded (Wisnuaji & Rochima 2015). The degraded pigment causes the product to become brighter or colorless, so the thallus color degree lowers. An increase of 50% ammonia in seaweed has been observed in the alkaline group (pH 9). The optimal cytoplasmic pH of seaweed is 7-7.5. Because of this, a quick diffusion into the cells occurs (Hurd et al 2014). Most ammonia is protonated to be ammonium, which cannot spread back across the cell membrane, resulting in the passive accumulation of ammonia by diffusion and trapping acids in cytoplasm and vacuoles (Hurd et al 2014). This results in the disruption of the permeability of the cell wall.

This permeability disorder triggers the activation of enzymes that destroy the cell wall. Enzymes break the bonds of polysaccharides in the cell wall and cause cell walls to become loose (Salisbury & Ross 1992). Damage to the permeability of cell walls also causes non-selective substance exchanges from inside and outside the cell. If damage to the cell wall occurs continuously, then the cell will experience a slow death due to the disruption of the process of photosynthesis and the decrease of pigments in cells (Widyartini et al 2017).

The color gradations of *Sargassum* sp. thallus in the pH 5 and pH 9 groups were similar, showing no significant differences between each other. This is because high and low pH can lead to the disruption of cell membrane permeability. Disruption of cell membrane permeability results in stressing *Sargassum* sp. and in color loss. The color gradation of *Sargassum* sp. visually showed that in a low pH (pH 5), the seaweed had more consistent bleaching than in high pH (pH 9). The thallus color in the pH 5 treatment looked more faded and pale when compared with the thallus color from the pH 9 treatment. Moreover, the texture of the thallus is softer and more easily to break. A lower pH (pH 5) caused the chlorophyll to become unstable, while in alkaline condition (pH 9) chlorophyll became very stable in high temperatures. The color of chlorophyll fades after heating, due to a decrease in pH caused by acid release (Arfandi 2013).

The highest content of chlorophyll-*a* in *Sargassum* sp. was observed in the control group (pH 7), followed by pH 9 group, the lowest being in the pH 5 group. Chlorophyll-*a* is a pigment that has a role in receiving and transferring light energy to the center of photosynthetic reactions in brown algae (Kosumi et al 2012). Each treatment had an effect on the chlorophyll-*a* content of *Sargassum* sp., the difference being significant ($p<0.05$). Acidity degree seems to affect the chlorophyll-*a* content of *Sargassum* sp.

A reduction of the chlorophyll-*a* amount occurred because of the degradation reactions in the cells. The chlorophyll degradation reactions occurred due to magnesium dechelatase and chlorophyllase enzymes, which catalyze the ester hydrolysis reaction between propionic acid residues in macrocyclic rings with phytol in chlorophyll, causing a loss of Mg^{2+} ions. Chlorophyll degradation reactions take place in two ways. The first way is the change from chlorophyll-*a* to chlorophyllide-*a*, with the help of the chlorophyllase enzyme. The second way occurs due to the presence of magnesium dechelatase enzymes, that convert chlorophyll-*a* to feophytin-*a*. Both of these reaction pathways will produce feoforbide-*a*, which is formed due to feophytin-*a* chlorophyllase or magnesium dechelatase from chlorophide-*a*. Feofobida-*a* will undergo dioxygenase reactions and result into colorless, fluorescent and pigmented compounds (Heaton & Marangoni 1996).

Chlorophyll can be degraded by temperature, light, water, acid and alkaline medium and alcohol. The addition of HCl 13% to chlorophyll can cause the formation of chlorophyll derivatives, like feophytin (Gross 1991). The low pH can cause chlorophyll degradation to feophytin. Feophytin is one of the chlorophyll derivatives formed when the magnesium metal center in the chlorophyll is released. The acid with OH^- ions attracts Mg metal ions which are present in the chlorophyll macrocyclic ring, thus the ions are released (Kusmita & Limantara 2010). Enzymes that play a role in chlorophyll degradation are chlorophyllases, magnesium dechetalases and pheophorbide oxigenases. Chlorophyll-*a* enzymatic reactions are carried out by the chlorophyllase enzymes, resulting into chlorophyllide-*a* and phytol. Chlorophyllide is more polar than chlorophyll-*a* because it has lost phytol (Gaur et al 2006).

The structure and texture of *Sargassum* sp. thallus indicate the difference between treatment groups. Groups with pH 5 and pH 9 suffered major damages, the air bladder being separated from the thallus and the texture becoming soft and easily broken. The pH 7 group had a complete thallus structure, a rubbery, solid texture and dark brown color. The worst damage occurred in groups with pH 5 and pH 9, due to disruption of photosynthesis, resulting in slow growth. This involves the lack of regeneration capacity in damaged cells, thus the cells do not survive and die.

The cell shape and size of the thallus of *Sargassum* sp. indicate that there is a difference between the treatment groups. The control group (pH 7) presents round shaped cells, thick cell walls and homogenous cell sizes. *Sargassum* sp. cells from the pH 5 group had an irregular shape, thin cell walls, broken and damaged cells. The alkaline pH 9 caused damage to the medulla cell wall, as indicated by the irregular cell shapes. The medulla has an important role in water and metabolites transportation. Damage to medullary cells results in changes of the cell shape. The permeability of the cell walls suffers disruptions because of the high pH, which results in easier cell wall breakdown (Salisbury & Ross 1992). The cell wall has the main function of protection and cell order, so that if the cell wall becomes damaged, it will result in changes in the shape and health of the cells (Juwono 2002).

Conclusions. The difference in the acidity degree (pH) has different effects regarding the bleaching of *Sargassum* sp. Low and high pH can cause irregular shapes, thin cell walls and damages the thallus cells. Moreover, it can affect the color gradation of *Sargassum* sp. thallus, due to the reduced chlorophyll-*a* content. Acid pH 5 had the greatest effects on the bleaching of *Sargassum* sp.

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Authors:
Arini Fadhla Wahyuningtyas, Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Jl. Airlangga No. 4–6, Surabaya 60115, Indonesia, e-mail: arini.fadhla@yahoo.com
Afif Mufidah, Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Jl. Airlangga No. 4–6, Surabaya 60115, Indonesia, e-mail: afifmufida16@gmail.com
Mochammad Amin Alamsjah, Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Jl. Airlangga No. 4–6, Surabaya 60115, Indonesia, e-mail: moch.amin.alamsjah16@gmail.com
Agustono, Department of Fish Health, Faculty of Fisheries and Marine, Universitas Airlangga, Jl. Airlangga No. 4–6, Surabaya 60115, Indonesia, e-mail: agustono845@gmail.com
Pratiwi Pudjiastuti, Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Jl. Airlangga No. 4–6, Surabaya 60115, Indonesia, e-mail: pratiwi.pudjiastuti@yahoo.com

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