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The effects of contaminant microorganism towards *Chelonia mydas* eggs hatchery results in Pangumbahan Green Sea Turtles Conservation, Sukabumi, Indonesia

TOUFAN GIFARI[1,♥], DEWI ELFIDASARI[1], IRAWAN SUGORO[2]

[1]Department of Biology, Faculty of Sciences and Technology, Universitas Al-Azhar Indonesia. Jl. Sisingamangaraja (Komplek Masjid Agung Al-Azhar), Kebayoran Baru, South Jakarta 12110, Jakarta, Indonesia. Tel./fax.: +62-21-72792753, ♥email: gifari06@gmail.com.

[2] Microbiology Laboratory, Center for Application of Technology of Isotope and Radiation, National Nuclear Energy Agency of Indonesia (PAIR-BATAN). Jl. Cempaka Lestari II, Lebak Bulus, South Jakarta 12440, DKI Jakarta, Indonesia.

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Abstract. *Gifari T, Elfidasari D, Sugoro I. 2018. The effects of contaminant microorganism towards* Chelonia mydas *eggs hatchery results in Pangumbahan Green Sea Turtles Conservation, Sukabumi, Indonesia. Biodiversitas 19: 1207-1212.* Green sea turtles (*Chelonia mydas*) is an endangered species, in which its population is continuously decreasing caused by a number of factors. Microorganisms are suspected to have important role in declining eggs hatching success. The aim of this study was to investigate the role of microbial contaminant on the egg hatching success at the green sea turtles conservation area in Pangumbahan needs. The microbial contaminants detection was carried out on the eggs at the semi-natural hatching nest. RAL with three times repetitions was selected to figure out the correlation between microbial contaminants and hatching success was. The eggs were incubated at three different depths, i.e., 40, 60, and 80 cm. The results showed that the Gram-negative microorganisms were from the coliform bacteria such as *E. coli*, *Salmonella* and *Shigella*. Meanwhile, the fungi were dominated by mildew and yeast. The hatching success rate was 93.34% in 80 cm depth, 85% in 60 cm depth and 60% in 40 cm depth. *HSD Tukey test* shows the effects of various nesting depths towards the hatchery percentage ($p \leq 0.05$) with the correlation test of microorganisms contaminant shows negative value meaning the level of contaminant microorganism is an inverse proportion to the hatchery results.

Keywords: *Chelonia mydas,* contaminant microorganism, eggs hatchery, green sea turtles, Pangumbahan

INTRODUCTION

Green sea turtle (*Chelonia mydas*) characterized by dark brown carapace with dark green spots and brown as well as yellow-white patterns (Wyneken 2001) is listed as one of the endangered species (Seminoff 2004). This shows that conservation is needed to protect green sea turtles from extinction. A number of factors are known as the causes of, the green sea turtles population decline in the world to this long and slow life cycle species including poaching, damaged habitats and climate change (Prasetyo 2014). The high demand for green sea turtles' eggs and the souvenir of green sea turtle's carapace in the black market makes poaching hard to stop (Spotila 2004). In addition, the conservation effort that had been done in the past years did not run optimally due to limited human resources and infrastructures.

Another important factor causes the decline of its population is the reduced hatching success of green sea turtle eggs due to bacteria contamination (Al-Bahry et al. 2009). *Bacillus* sp., *Salmonella* sp., *Citrobacter freundii* and *Mucor* sp were often found contaminating the eggs of green sea turtles in conservation area in Indonesia, such as in Bilang-bilangan Island conservation area, Balikukup Islands, Berau District, East Kalimantan, Indonesia (Estika 2013). *Hafnia alvei, Salmonella choleraesuis, Escherichia fergusonii, Serratia odorifera, Serratia marcescens, Acinetobacter calcoaceticus* and *Shigella* sp. were also reported contaminated the eggs of green sea turtles at Taman Pesisir Pantai (Coastal Park) Pangumbahan Green Sea Turtles conservation area (Wicaksono 2013).

Previously, it was assumed that the microbial contamination of the green sea turtle eggs due to the interaction between microorganisms and green sea turtles eggs occurred from external environments such as the sand and the sea. The microbial contamination of green sea turtles eggs might cause the decline in the survivability of the eggs so that the eggs would potentially not hatch successfully (Phihlott and Parmenter 2001). Thus, microbial contamination on the eggs of green sea turtles might reduce. Some green sea turtles conservation areas in Indonesia.

The Local Technical Implementing Unit (UPTD) of Pangumbahan Sea Turtles conservation is a conservation area which focuses on Green Sea Turtles species (*Chelonia mydas*). The conservation activities are started from collecting eggs at the nesting areas, eggs relocation, and eggs incubation at the semi-natural hatching nest, preservation and hatchlings release. The research on contaminant microorganisms conducted at this location was in 2013, did not describe the source of the contaminant microorganisms as well as its effect on hatchery results. It was hypothesized that the contaminating bacteria on the sea turtles eggs were from the sea water and polluted sand at

the Pangumbahan sea turtles conservation area. Therefore, a further study is needed to detect the source of contaminant microorganisms in the area and its correlation with the hatchery results. This study was aimed to figure out whether the microorganisms affect the decline in green sea turtles' eggs hatchery results in Pangumbahan sea turtles conservation area.

MATERIALS AND METHODS

Study area

The research was carried out from March to July 2017. The turtle egg samples were collected from the Local Technical Implementing Unit (UPTD) of Pangumbahan sea turtles conservation, Sukabumi, West Java, Indonesia. The microorganism presence on the egg samples was carried out at the Microbiology Laboratory, the Centre of Isotope and Radiation Application, National Nuclear Energy Agency (BATAN), South Tangerang, Indonesia.

Procedures

Research objects

The research objects were seawater samples, sand in natural nest, the cloaca's mucous of the sea turtles after hatchery, the sand at the semi-natural nesting pre and post hatchery with different depths, and the fresh eggs and unhatched eggs at the Pangumbahan sea turtles conservation area.

Tools and materials

The tools used in this research were cooling box, laminar air flow, autoclave, vortex, centrifuge, magnetic stirrer, microcentrifuge tube, analytical balance, microscope, incubator, sample tube, SV column, Erlenmeyer, petri dish, beaker glass, pipette tips, micro pipette, L rod, ose, spoon, tube and sterile bottle, pH meter, multimeter, Bunsen burner, refrigerator, DSLR camera, stopwatch, rope, tissue, cotton, gloves, mask, labelling paper, plastic wrap and aluminium foil. The materials used were *Nutrient agar* (NA) media, Mac Conkey Agar (MCA) media, *Salmonella-Shigella* Agar (SSA) media, *Potato Dextrose* Agar (PDA) media and *E. coli* Agar (ECA) media, NaCl 0.85%, aquadest, coloring Gram material and alcohol 70%.

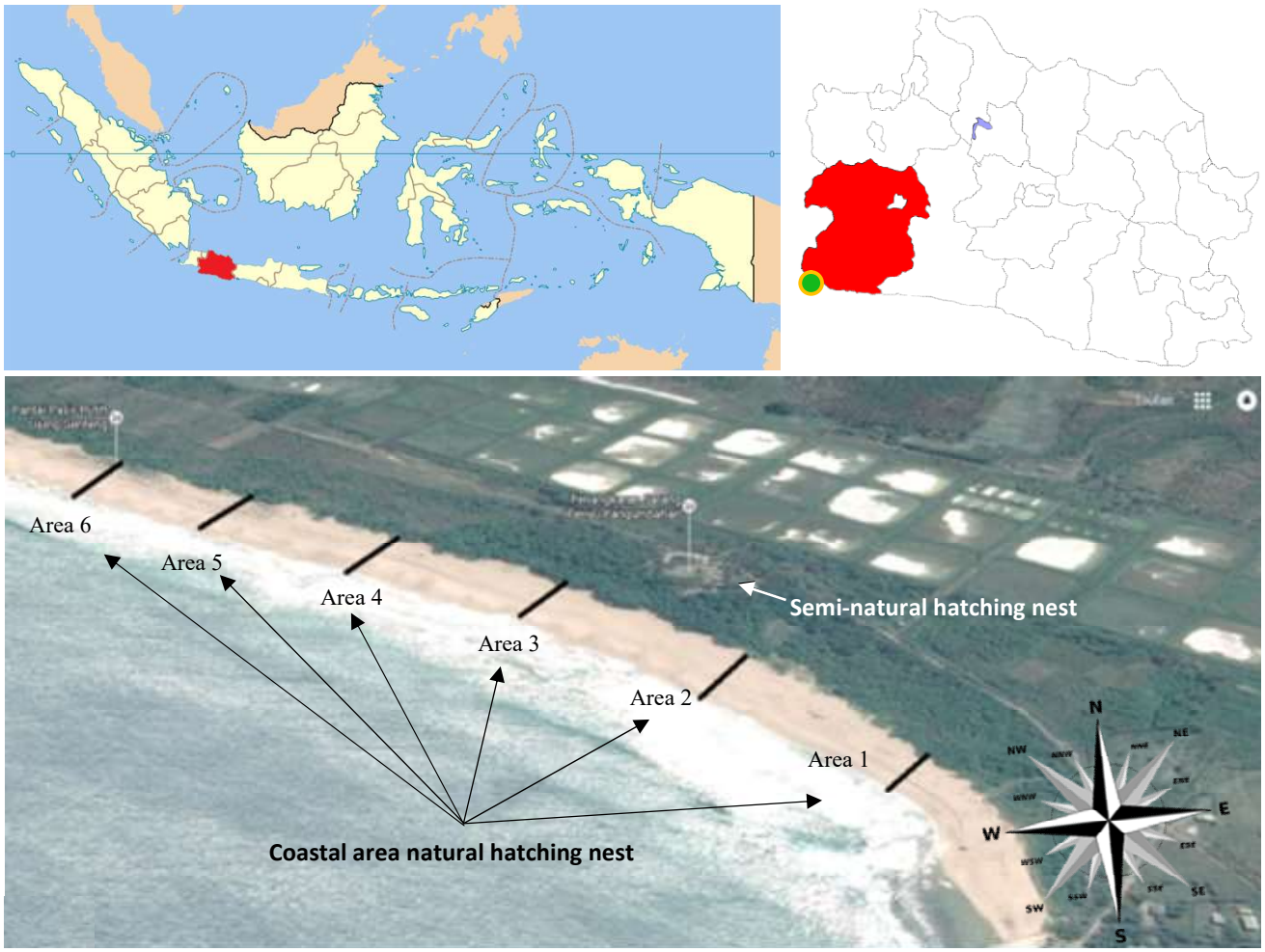

Figure 1. The location of semi-natural hatching nest at the Pangumbahan Sea Turtles Conservation area, Sukabumi, West Java, Indonesia at $106^{0}19'37"$-$106^{0}20'07"$S, $07^{0}19'08"$-$07^{0}20'52"$ W

The process of eggs incubation and hatchery

The eggs were taken from three female parents which produced more than 60 eggs per female at natural hatching nest in coastal area 2 and 3. The eggs were taken using the *ditande* method (taking the eggs at the same time during hatchery process). Then the eggs were relocated using a bucket to a semi-natural hatching nest and were buried in less than two hours. The eggs were then randomly selected and divided into nine clutches of eggs which then laid at three artificial nest depths; they were 80 cm (A), 60 cm (B), and, 40 cm (C) depth. There were three replications of nests in each depth. Thus there were 9 clutches of 20 eggs were incubated.

Seawater samples were taken from three sea turtles drop-zone locations and were kept in the sampling bottles. Samples of cloaca mucous weres taken from the three sea turtle female parents. Natural sand from three hatchery locations and the semi-natural sand from the nests was sampled in varieties of depth, i.e., 40, 60, and 80 cm (pre and post hatchery). The eggs were inspected for their condition as the samples, which comprised of three fresh eggs, three unhatched eggs, and three inner-part of the eggs. The collected types of samples were 12 samples with the total samples for 36 samples (sea water = 3; cloaca mucous = 3; natural sand = 3; pre semi natural sand 80 cm = 3; pre semi natural sand 60 cm = 3; pre semi natural sand 40 cm = 3; post semi natural sand 80 cm = 3; post semi natural sand 60 cm = 3; post semi natural sand 40 cm = 3; fresh eggs = 3; unhatched eggs = 3; unhatched eggs content = 3).

Samples analysis

All of liquid samples (sea water, cloaca mucous, unhatched eggs content) were taken for 2 mL then adjusted using NaCl 0.85% to 10 mL, solid samples (sands) for 0.5-1 g and were diluted using NaCl 0.85% up to 10 mL. The samples of eggshells (fresh and unhatched) were diluted by rinsing the eggs using NaCl 0.85% and was fixed exactly up to 100 mL. All samples were then diluted for eight times. The spread plate method was used to distribute bacterial cells evenly across the surface of an agar plate. Bacterial cells suspended in a small drop of liquid were distributed using a sterile spreader for all samples on the NA, MCC, ECA, SSA and PDA media. The growing microorganisms were then isolated and counted using the TPC method. The Gram bacteria coloring was performed to each successfully isolated colony.

Data analysis

Data of percentage of hatched eggs were analyzed using one-way ANOVA followed by Tukey HSD *test* ($p \leq 0.05$) using SPSS software (version 17). Descriptives results of data about the microorganisms in the water, sand, eggs, and cloacae mucous were analyzed using Microsoft Excel. The correlation between contaminant microorganisms and hatched eggs was analyzed using Pearson correlation formula in SPSS software.

RESULTS AND DISCUSSION

The detection of contaminant microorganisms at the conservation area

Based on the microscopic observations, six different bacteria colonies were identified. Morphologically, all of the colonies were round in shape, and in term of color, it was found that most of the colonies were white, then followed by green and blue. White colonies have such two size classes as big and small (Figure 2). Results from the Gram coloring, showed that all of the colonies were Gram-negative and pathogenic. The cell shape was basil with different sizes.

The result of fungi identification on the PDA media from the overall samples showed that the fungi were from mildew and yeast. The observation on the mildew colonies was conducted after the samples were incubated for 24 hours. Thus the mildew could be observed after 72 hours. Macroscopically, the mildew that was found out has circular hypha and mucus. Meanwhile, yeast has smaller size and mucus (Figure 2).

Results from enumeration of microbes, coliform, *E. coli, Salmonella-Shigella* and fungi on cloaca's mucous samples, fresh eggs, sea water and natural nest sand, revealed that cloaca's mucous and eggs were contaminated the most (Figure 3). The higher contaminant microorganisms on cloaca's mucous were probably due to the digestive condition of sea turtle mothers. Alkindi et al. (2006) suggested that the microorganisms contamination was probably from the digestive system of the sea turtle mothers including from their cloaca channels. It is basically because this mucous is a clear liquid containing glycoprotein that is secreted from cloaca to coat the eggs during hatchery process occurred (oviposition). The microorganism's displacement on to the eggs may occur through cloacae mucous due to the sea turtle mothers' reproduction system that has been previously infected by bacteria and fungi (Keene 2012). Therefore, this mucous may have been infected with bacteria and fungi originating from the turtle mothers' cloaca that had been contaminated earlier, causing the transfer of microorganisms to the eggs (Keene 2012).

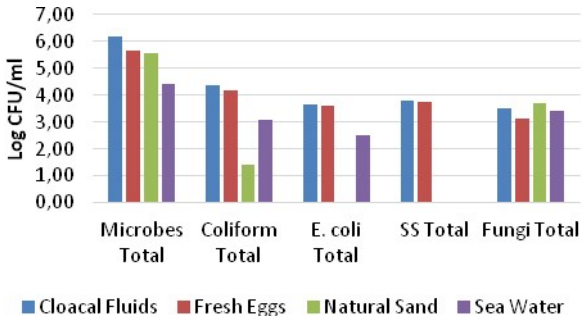

Figure 3. The quantification result of microorganisms on the sample taken during the sea turtles' hatchery process

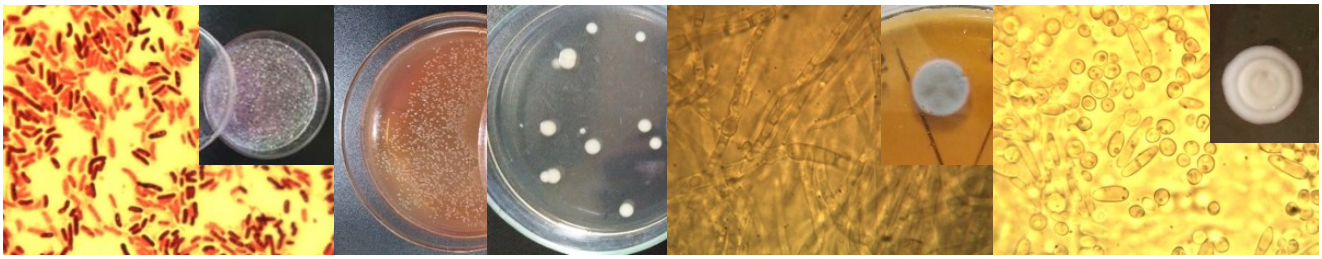

Figure 2. The results of microorganism isolation, in general, using spread plate method. A. Total coliform, B. *Salmonella-Shigella*, C. *E. coli*, D. Mildew, E. Yeast

Escherichia coli and *Salmonella-Shigella* colonies were not detected on the natural nesting sand, and *Salmonella-Shigella* colony was also not detected on the seawater samples. Thus it suggests that the contamination of *Salmonella-Shigella* was from the sea turtles' digestive system which was brought by the eggs and cloaca's mucous. Berrang et al. (1996) suggested that there was a possibility that bacterial infection towards the eggs' components prior to the eggshells construction, since the ovary and the uterus of the sea turtle mothers were infected by bacteria beforehand. Gantois et al. (2009) inferred that the reproduction organ of sea turtle mothers that has been infected by *Salmonella* might contaminate the eggs through filtration on the eggshells and intestine (feces contamination) and direct contamination on the yolk, albumin, and eggshells before oviposition occurred.

The results showed that the number of successful colonies was detected in each sample, especially in post-hatch samples, including total microbes, coliforms, E. coli, Salmonella-Shigella and fungi (Figure 4). The total of microorganisms on semi-natural post-hatchery nesting sand was higher compared to semi-natural pre-hatchery nesting sand, except on the total growth of microbes and fungi. According to Simbolon (2008), this happened due to competition between the microorganisms themselves; it was thought that the fungi were defeated in taking advantages of the nutrients to grow and to develop. Furthermore, the decrease in total microbes on semi-natural nesting sand might occur due to competition with coliform brought by the eggs. Coliform in the eggs would easily develop because of its position was closer to the eggs that have a source of nutrients richer than the nutrients in semi-natural sand. The closeness of coliform to the nutrient sources probably caused the *E, coli* colony and*Salmonella-Shigella* on the semi-natural post-hatchery nesting sand was higher. The excavation and nest closure during relocation process may also cause microorganisms to be dispersed to the entire parts of the nests, so that in the 80 cm depth, the coliform colony and *E. coli were* detected which were probably from the sand surface.

The correlation of contaminant microorganisms and the hatching success

Nest depth significantly affected the hatching success rate ($F = 8.249$; $P = 0.004$; Figure 5). The highest hatching rate was found in the nests located at 80 cm depth, which was higher ($p \leq 0.05$). The further test (*Tukey HSD*) displayed that the depths of 80 and 60 cm did not have different results. In accordance with the previous research, the depth of 60 cm and above showed better hatchery due to stable temperature fluctuation. The optimum temperature for the development of sea turtles embryo is between 28-30^{0}C and will be impeded if it is outside the approximate temperature (Nuitja 1992).

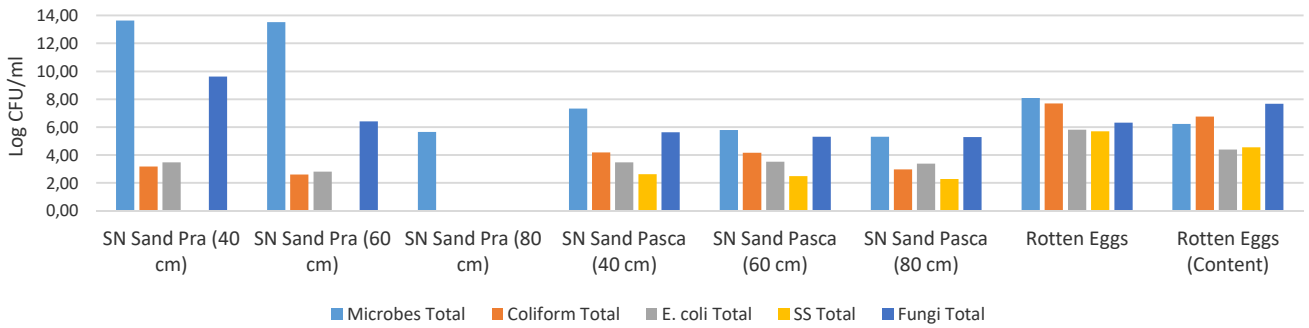

Figure 4. The number of microorganisms on samples was taken during the eggs incubation period until hatchery

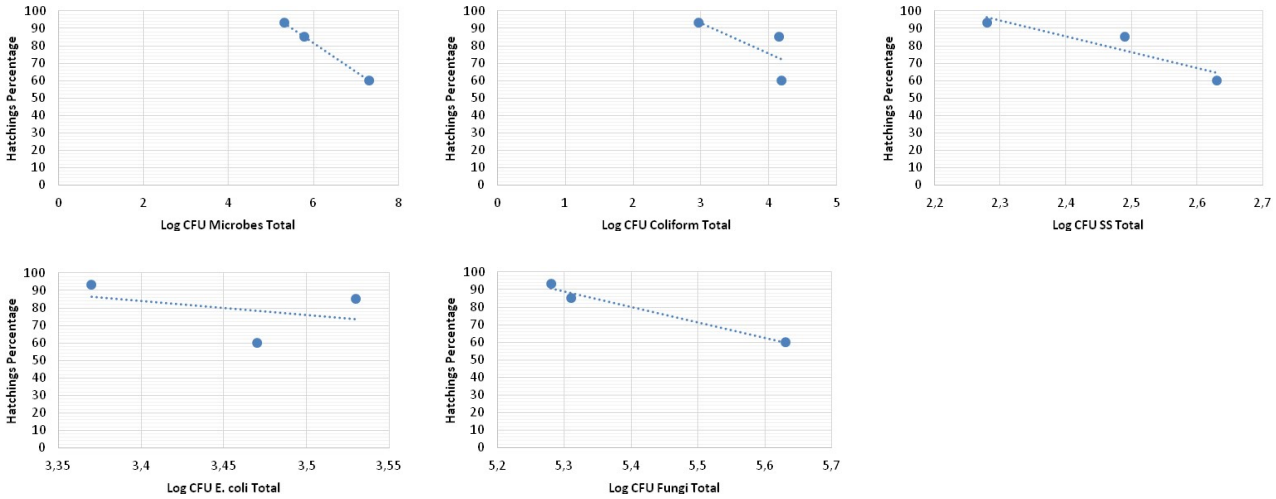

Figure 6. Scattered diagram of the correlation between log microorganism CFU and hatching success

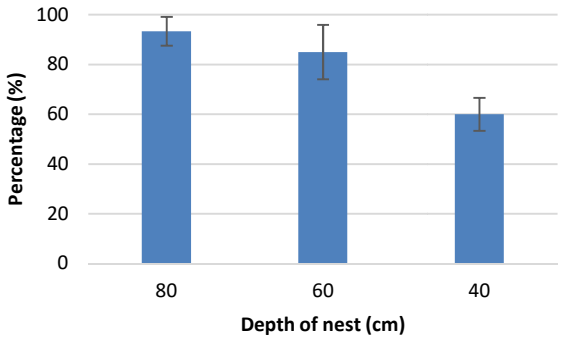

Figure 5. The effect of nest depth of the nests on the hatching success of the eggs of sea turtles using *Tukey* Test ($p \leq 0.05$) ($F = 8.249$; $P = 0.004$)

The previous experiment of depth variation of semi-natural nests which had been acknowledged to be the determinant factor of hatchery result was conducted to collaborate the data of microbial contaminant on the semi-natural sand. The data of contaminant used for the correlation test was only on the samples of pre and post-hatching in semi-natural nesting sand. This was because the sample of semi-natural sand was directly related to the incubation process until the hatching. *Pearson Correlation* analysis was performed on log-transformed data of Colony Forming Unit (log CFU)and the hatching percentage, and the result showed that there was a negative correlation value, i.e., an inverse relationship between log total microorganism CFU and the hatching success (Figure 6).

The existence of microorganism on both in the inner part and outer of the unhatched eggshells (Figure 4) indicated that the microorganism was successfully penetrated into the eggs through the eggshells' pores because the pores function as gas, water, and heat vapor exchange from the external environment (Limpus et al. 2008). However, the pores also become the filtration path for microorganism (Al-Bahry et al. 2011).

For the process of embryo growth eggshells should contain 43% of calcium (Bilinski et al. 2001). However, the fungi, exist on the eggshell, consumes the calcium on the eggshells surface causes calcium erosion (Phillot et al. 2006). In addition, the decrease of calcium on the eggshells coincides with the hypha growth covering the pores of the eggshells which affect the gas exchange. The hypha penetration continued into the embryo which may cause the death of the embryo (Phillot et al. 2006). of Microorganism penetration into the eggs components (yolk and albumin) happens when the layer structure of the eggshells has been damaged (Al-Bahry et al. 2004). This condition may worsen if within one nest a number infertile or fertile eggs do not grow as supposed to that they will trigger the colonization of microorganism and threatens the growth of all eggs (Robinson et al. 2003). Additionally, the growing microorganisms will take advantage of calcium ($CaCO_3$) on the eggshells as a cofactor for cytosol enzymes that play roles in creating cell wall as well as constructing endospore to keep growing (Brooks et al. 2013).

Environmental factors such as temperature and humidity may play important role in the process of microorganism filtration into the eggs. The soft structure of eggs with pores as the path for gas exchange will potentially be infected by bacteria if the nest temperature is too low and the humidity is high (Al-Bahry et al. 2011). Number of pores and sizes of the eggshells of sea turtle varied significantly (Ackerman 1972). For instance, the eggs of green sea turtles have high level of oxygen diffusion. Thus it is greatly possible for the eggshells to have more pores or bigger pores than other sea turtle eggshells. Consequently, they are prone to bacterial infection. Moreover, the pores on the eggshells surface become bigger and bigger towards the end incubation period which allows the microorganisms to penetrate and

colonize easier into the components of the eggs (Al-Bahry et al. 2004).

The hatching failure may be due to the microbial contaminants may be caused by a number of factors. During the hatching process sea turtle, the contaminated cloaca solution makes possible to transfer microorganism into the eggs and natural nesting sand (Keene 2012). The microorganism transfer continues when the process of eggs relocation to semi-natural nesting hatchery is conducted. This condition occurred spontaneously causing the contamination at the Pangumbahan Sea Turtles Conservation area, especially at the semi-natural nesting location. The contaminant microorganism might reduce the hatching success, and this explains the negative correlation between total of microorganism and hatching success. Thus, the conservation officers should be aware and cautious of the unintentional microorganism transfer by wearing Self-Protection Apparatus (APD) when they are relocating the eggs. The semi-natural nesting hatchery thus needs regular sand removals and replacement with new sand or heating the sand to sterilize microorganism. This effort is aimed at increasing the hatchery result and supporting the maximum conservation effort.

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