The Application of Ozon and Chitosan as Microbial Inhibitor Prawn Larvae Rearing

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Abstract. The application of ozon and chitosan solution was found to be effective means in hampering microbial growth since the introduction of ozon can inhibit microbial growth in aquatic environment and the fact that chitosan can act as a coagulant, effectively increasing water quality as prawn breeding medium and increasing the survival rate of prawn larvae. This research aims to measure the efficacy of ozon and chitosan usage in prawn larvae rearing, and to measure the survival rate of prawn larvae during the rearing stage. The study was carried out using experimental method in laboratory, with factorial research design using 3 treatment combinations and 1 control groups. The chitosan dose administered in this research was 25 ml in 25 L of sea water, equating to 10 ppm. The dissolved ozon in this research was measured at the concentration of 8.245 – 13.748 ppm. Weekly measurement of water quality in terms of temperature, pH, salinity, dissolved oxygen and dissolved ozon were carried out throughout the course of the research. Total microbial population in the water was measured by means of Total Plate Count (TPC) method in the Institute for Natural Medicines of Diponegoro University. Statistics figure from “ANOVA” test suggested that the application of ozon and the introduction of chitosan in prawn larvae rearing media gave impact to the microbial population in the media. Results of BNT test showed that there was a significant difference between the measurement results of the 3 treatment combination groups and that of the control group. The highest prawn larvae survival rate was found in the media with combined ozon and chitosan treatment, which was recorded at 100%. The second highest survival rate was recorded in the treatment combination group of ozon and chitosan with 80%, and the lowest survival rate was attributed to the control group with 20% prawn larvae survival rate. It is concluded that treatment combination of 10 ppm chitosan and 8.245 – 13.748 ppm of ozon showed significantly positive results in its application in giant tiger prawn farming media.

Keywords: Ozon, Chitosan, Microbe, Prawn Larvae.

1. Introduction
Bacteria can hamper aqua farming process, as exemplified in prawn aqua farms. Prawn, as an export commodity, can contribute to national income from the non-energy sector. In addition to being sought after by many for its taste, prawn has also been proven to have high protein content, making it one of the major export commodities in fisheries. Recently, however, prawn aqua farm suffers due to widespread pathogenic
bacteria infestations. To suppress the growth of harmful microbial, formalin, antibiotics, ozone as well as chitosan are commonly used. The use of formalin and antibiotics is highly discouraged considering the detrimental impact caused to environment and natural aquatic ecosystems. Therefore, ozone and chitosan provide viable alternatives to the former antimicrobial agents since the latter are organic and environmentally safe.

Based on the study by Summerfelt et al.,[1], ozone is formed from nonthermal plasma, raised by feeding air or oxygen gas through coronal discharge as well as dielectric barrier discharge or silent plasma. Ozone is beneficial in that it can increase water quality, suppress the growth of pathogenic microbes, save for fish, has high reaction time, and its reaction results merely in oxygen which is safe for farm ecosystem. The use of ozone also do not produce malodorous or foul residue compared to the use of chlorine [2]. However, utilization of ozone still has drawbacks which are attributed to the inability of the treatment process in transferring diluted minerals and salt, high cost from electricity consumption of the device, and the dangers from excessive concentration of bromate from the treatment process used ozone in water sterilization, fruits and fresh product [3]. In the experiment, tomatoes washed with water could only be kept for a week, whereas those which were sprayed with ozone could be stored for 27 days and was proven to be free of harmful bacteria.

In addition to ozone, chitosan has been proven to suppress the growth of harmful bacteria and has seen commercial application in commercial biomedicines. Chitosan is fiber made of polysaccharides derived from marine animals such as oysters, shrimps, crabs and etc. The benefits of chitosan are attributed to its organic nature, efficiency in usage (concentrated extract) making it economical in its use, its efficient application in detoxification, its use in bacterial inactivation, its non-toxic nature and its biodegradability [4].

Based on the information on ozone and chitosan above, that both agents has been proven to inhibit the growth of harmful bacteria, this research aims to measure the efficiency of the application of ozone and chitosan in prawn larvae rearing treatment, as well as to measure the survival rate of prawn larvae during the experiment.

2. Research Method
2.1 Research Materials
Giant tiger prawn *Penaeus monodon*, numbering forty in total, in post-larval stage (PL 30) was selected as the test species. The containers used in the experiment were 40-Liter glass aquariums, with 25 Liters of water as rearing media in each aquarium. Four aquariums were used in this research, with each aquarium subjected to one type of treatment. Ten prawn larvae were placed in aquarium.

Ozone acted as antibacterial agent in this study. During the course of the experiment, ozone was discharged into the rearing media by means of circulation. Ozonizers were turned on for 10 minutes to discharge the ozone, with 40 minutes interval for each discharge. One of the main ozone discharging devices was the ozone reactor. The main characteristics of the ozone reactor is spiral configuration of its electrodes. Each spiral electrode is made of copper wire and aluminium foil cylindrical electrode. Dielectric materials used in this research were 500 mm Pyrex tubes. A schematic of the ozone reactor used in this study is displayed in Figure 1 and the configuration of the devices can be seen in Figure 2.
In order to form nonthermal plasma, the spiral electrodes were connected to a high-voltage AC power source, whereas the cylindrical electrodes were connected to ground. Water was fed from the intake of the reactor simultaneously as the reactor form ozone which resulted in diluted ozone. The diluted ozone was then dispersed through the output port.

The chitosan used in this study was made of chitin taken from crabs of Portunidae family. The chitosan materials were procured from Laboratory of Microbiology, Institute to Natural Medicines, Diponegoro University [5], with 25 ml of chitosan diluted in 25 L of sea water. The mixture resulted in a diluted chitosan in sea water with 10 ppm concentration. Chitosan in this research was given as bacterial inactivation agent in the rearing media. The administration of chitosan was performed every two days, after replacement of rearing media.

The samples were fed with Artemia brine shrimp. The food was given in the ration of 20 : 1, or 20 brine shrimps were provided for each sample for every feeding [6]. Feeding was performed three times in one day, at 06:00 hrs (after media replacement), at 12:00 hrs and at 18:00 hrs [7].
The experiment was divided into two phases, namely the preparation and the execution phases. The preparation process of the experiment is explained in the preparation phase sub-heading, and the series of experiments conducted are explained in the execution phase sub-heading.

2.1.1 Preparation Phase
The preparation phase began with procuring the rearing container; 4 aquariums with 45 cm of length and 30 cm of width. The placement layout of the giant tiger prawn (*P. monodon*) larvae was determined randomly by drawing lots. Sea water as rearing medium was treated with 5 gr of chlorine to kill harmful microbes and parasites. One to two days after the chlorine treatment, the medium was further treated with 10 gr of thiosulfate to remove chlorine odor and improve the usability of the media. Nutrient Agar was provided as bacterial culture media.

2.1.2 Execution stage
Each rearing container was filled with 25 liter of sea water, after which 10 giant tiger prawn larvae were placed. Chitosan was diluted every two days during the course of the experiment. Ozonizer was regularly operated for 10 minutes with 40 minutes interval between ozonization of media. Measurements of the water quality were taken every week for 6 times (45 days). Temperature, acidity and salinity of water were measured using mercury thermometer, pH paper, and refractometer respectively. Dissolved oxygen concentration in the rearing media with chitosan and ozone was measured weekly, using Winkler titration method. Before being tested in the UV-Vis spectrophotometer, rearing media sample was added with binding solution KH$_2$PO$_4$, Na$_2$PO$_4$ and KI 0.06 M. Absorbance value of the rearing media was then calculated. Growth of colony bacteria was measured weekly by dilution method factoring 10$^1$ to 10$^4$. Rearing media was replaced by water siphoning, as much as one third of the initial volume. *Artemia* brine shrimps as food were given three times a day, everyday during the course of the experiment, with regular feeding times in the morning, at noon, and in the evening. At the end of the experiment, the number of living larvae was recorded to determine survival rate. The rearing media was also examined to determine microbial capable of surviving the treatment used.

2.2 Identification of Bacteria
Biochemical tests performed in this study referred to the methods in *Cowan and Steel's manual for the identification of medical bacteria* [8]. Based on the observation made on bacterial shape during the incubation period, 20 bacterial isolates were identified in control media and 8 bacterial isolates were found in the treated media. However, in this study only the most dominant species will be identified.

2.3 Data Collection
After 45 days of experiment, the data collected were:
- Survival rate data from observation of each groups [9]
- Water quality and dissolved ozone which was measured throughout the course of the experiment, data on bacterial colony growth and identified bacterial species.

2.4 Data Analysis
The primary aim of this research was to prove whether the treatment can yield significant or insignificant results. The results of this research was tested using analysis of variance (ANOVA) method, after they went through normality test by Kolmogorov-Smirnov method.
1.5. Analysis of Variance (ANOVA)

The hypothesis formed were $H_0$: The four different treatments (ozone, chitosan, ozone and chitosan, and control) do not give significant difference in resulting bacterial population. $H_1$: The four different treatments (ozone, chitosan, ozone and chitosan, and control) (ozone, chitosan, ozone and chitosan, and control) give significant difference in resulting bacterial population.

If $H_0$ was rejected and $H_1$ was accepted, then the next step was to conduct multiple comparative test (further testing) to determine which treatment attributed to the rejection of $H_0$. One of the suitable test method to determine the validity of the result is the LSD (Least Significant Difference) test.

3. Results

3.1 Water Quality Parameters

Dissolved oxygen measurement results (in ppm) from each treatment, which were regularly taken from week one, showed the following results: 6.12; 6.51; 5.89; 4.8; 5.12; 5.78 for control; 6.14; 6.65; 6.61; 5.37; 6.39; 6.64 for chitosan treatment; 7.68; 7.52; 7.24; 7.13; 7.89; 7.76 for ozone treatment; and 7.48; 7.29; 7.5; 7.07; 7.64; 7.66 for chitosan & ozone treatment. Figure 4a below provides the temporal graphs for the results.
Measurement results of water acidity for each treatment during the course of the experiment showed the following figures: 6 for control media, 7 for chitosan media, 7 for ozone media, and 7 for chitosan & ozone media in the first two weeks, in which between the third and the sixth week rose to 8, as seen in Figure 3b.

![Figure 3b](image)

**Figure 3b.** Graphic charts of water temperature measured during the experiment (a), and Graphic charts of dissolved ozone measured during the experiment (b)

The water temperature measurements for control and all treatment groups showed steady results between 24 – 25 °C, as seen in Figure 4a. Measurements for dissolved ozone could only be retrieved from media with ozone treatments, making this measurement not applicable for chitosan and control media. The dissolved ozone (in ppm) through the course of the experiment were measured at 9.316; 10.588; 11.065; 11.998; 13.127; 13.748 for ozone treatment and at 8.245; 9.312; 9.798; 10.842; 11.857; 12.478 for chitosan & ozone treatment. Figure 4a above details the graphic charts of these results.

### 3.2 Growth of Bacterial Colony in Each Media

The observation on the growth of bacterial colony for control and all treatment media showed that the highest Colony-forming Unit (CFU) found in control media was 1.5 x 10^6, in chitosan & ozone media was 1 x 10^5, in chitosan media was 2.8 x 10^4, and in ozone media was 1 x 10^4, as described in Figure 6.
3.3 Statistical Calculation of Bacterial Colony

To prove that the data distribution was normal, a Kolmogorov-Smirnov test was carried out on the data. The test criteria were reject $H_0$ if $\sigma$- or $P$-value at $< \alpha = 5\%$. The test result proved that $H_0$ was accepted which means that the data residue showed normal distribution, since data output displayed Kolmogorov-Smirnov value of $\sigma$-$P$ from the transformation logarithm = 0.150. The result of Kolmogorov-Smirnov test can be seen in Figure 7.

Figure 6. Bacterial seeding in the first week of observation
3.4 ANOVA test and LSD (Least Significant Difference) test
Statistical test using ANOVA showed significant difference between each treatment group. With significance rate $\alpha = 0.05$, F-Value = 106.29 with Pr>F = 0.0001 was obtained.

Figure 7. Hypothesis testing on variance similarity by LSD method
The results yielded a P-Value for Levene’s test $= 0.019 < \alpha = 0.05$, thereby $H_0$ was rejected. The difference in median of all the treatment groups were shown to be less than the BNT value of 5%. Hypothesis test results on similarity between variance and BNT is presented in Figure 8.

3.5 Survival Rate of Giant Tiger Prawn (P. monodon)
The final survival rate was obtained at day 45 of the rearing stage. Ten prawn larvae were introduced into each container at the beginning of the experiment. The highest survival rate was observed in two treatment media, chitosan treatment and ozone treatment, with both media yielding 100% larvae survival rate. The ozone and chitosan combination treatment was capable of resulting 80% of survival rate, whereas the control media larvae population had the lowest survival rate of all with 20% rate (Table 1)

| Treatment                  | No (prawn) | Nt (prawn) | SR (%) |
|---------------------------|------------|------------|--------|
| Ozone Medium              | 10         | 10         | 100%   |
| Chitosan Medium           | 10         | 10         | 100%   |
| Chitosan and Ozone Medium | 10         | 8          | 80%    |
| Control Medium            | 10         | 2          | 20%    |

Table 2. Biochemical test of bacterial species in control and ozone media

| Biochemical Test       | Bacterial Isolate Code |
|------------------------|------------------------|
|                        | E. 09.166              |
|                        | E. 09.167              |
| Gram                   | -                      |
|                        | +                      |
| Shape                  | Rod                    |
|                        | Rod                    |
| Acid Fast              | v                      |
|                        | -                      |
| Spore                  | -                      |
|                        | +                      |
| Spore position         | v                      |
|                        | VX                     |
| Cell length $> 3\mu$m   | v                      |
|                        | -                      |
| Motility               | +                      |
|                        | +                      |
| Aerobic                | +                      |
|                        | +                      |
| Anaerobic              | -                      |
|                        | +F                     |
| Catalase               | +                      |
|                        | +                      |
| Oxidase                | +                      |
|                        | +                      |
| Glucose Acid           | +                      |
|                        | +                      |
| Carbohydrate (OF)      | O                      |
|                        | O                      |
| Nitrate reduced        | +                      |
| Gas from glucose       | -                      |
| Indol                  | -                      |
| ONPG                   | v                      |
|                        | -                      |
| VP                     | +                      |
| Hydrolysis of:         |                        |
| - starch               | +                      |
| - urea                 | v                      |
| - casein               | -                      |
|                        | +                      |
Moller’s decarboxylase:
- Arginine
- Lysine
- Ornithine

Gelatinase
- Growth on 50°C
- Growth on 42°C

Acid from ASS medium
- L-arabinose
- Salicin
- Sucrose
- Xylose
- Cellobiose
- Galactose
- Raffinose

Pigment production

Utilization of Citrat

Flagellum

Growth with 10% NaCl

Legend: VX = central oval, v= n/a, + F = Facultative anaerobic, O = Oxidative

3.6 Identification of Bacteria
The identification of bacteria in the control media and ozone media was carried out up to the species level. The biochemical test method used in the identification referred to Cowan and Steel’s Manual for The Identification of Medical Bacteria. Biochemical test results are displayed in Table 2.

The biochemical test result showed that one of the two most dominant bacteria was identified as *Pseudomonas stutzeri* with code E.09.166. This species is gram negative, rod-shaped, with no spore, has polar flagella, motile, aerobic, capable of producing catalase and oxidase enzymes, capable of using glucose, capable of oxidizing carbohydrate, incapable of forming pigment, capable of reducing nitrates, incapable of producing gas from glucose, incapable of forming indole, capable of hydrolyzing starch, incapable of hydrolyzing casein, incapable of producing L-arabinose acid, incapable of synthesizing acid from salicin, incapable of synthesizing acid from sucrose, capable of synthesizing acid from xylose, incapable of synthesizing acid from cellobiose, capable of synthesizing acid from galactose, incapable of synthesizing acid from rafinose, and do not survive at 42 °C temperature.

Isolate code E.09.167 was identified as gram positive, rod-shaped, has spore with central oval position, motile, aerobic and facultative anaerobic in nature, is capable of producing catalase and oxidase enzymes, capable of utilizing glucose, capable of oxidizing carbohydrate, capable of reducing nitrate, incapable of synthesizing gas from glucose, incapable of forming indole, capable of hydrolyzing starch, capable of hydrolyzing casein, incapable of synthesizing acid from salicin, incapable of synthesizing acid from xylose, capable of synthesizing acid from cellobiose, capable of synthesizing acid from galactose, incapable of synthesizing acid from rafinose, incapable of decarboxylation of arginine, incapable of decarboxylation of ornithine and cannot thrive in 50 °C of temperature and in 10% concentration of NaCl. This species was later identified as *Bacillus thuringiensis*. 
4. Discussion

The water quality parameters being measured covered dissolved oxygen, pH, temperature, salinity and dissolved ozone. Results of the measurements showed that the rearing media were capable of supporting the ecosystem of the test subjects. Generally, water quality is a determining factor in survival and immunity formation respond rate. Despite the fact that only temperature directly influence the prawn population, DO, pH, and salinity also play important roles in immunity system formation of the prawns. According to Amri (2003) [10], the water quality early in the experiment was still suitable for prawn rearing, because salinity levels were at 19 – 25 °/00 pH, temperatures were in 25 – 32 °C, and DO rates were at 4 – 8 ppm. All these readings met the requirement for the suitable rearing media.

The dissolved oxygen (DO) parameter was found to be a limiting factor in the survival rate of the prawn larvae. The minimum DO rate for larvae survival is 3 ppm [11]. Dissolved oxygen from each treatment media during the course of the study was measured at between 4.8 – 7.89 ppm. Ideal DO rate spans around 4 – 7 ppm. However, minimum DO during night time is suggested to be more than 3 ppm [6].

The highest DO rate was found in ozone-treated media, measured at 7.13 – 7.89 ppm. The capability of ozone to enhance the availability of oxygen in water was attributed to the high DO in the media. This was made possible due to the oxygen released by ozone reactor whenever silent plasma was formed in dielectric barrier-equipped plasma discharge, which was dissolved in the water. When free electron has sufficient energy, ozone molecule is believed to undergo dissociation into oxygen molecule and atom [12]. The formed oxygen atom collides with ozone to form two other oxygen molecules. In addition, ozone is a highly reactive and unstable gas, with fast decay rate before reforming into oxygen [13].

The dissolved oxygen levels of the chitosan and ozone media placed second after that of the ozone-treated media, measured at 7.07 – 7.66 ppm. Chitosan is a natural coagulant, which precipitate all particles both essentials and pollutants dissolved in the water. This nature of chitosan was believed to be the cause of increasing thickness of the water, forming sediments that slows oxygen reaction in the media. Therefore, it was concluded that the combined usage of chitosan and ozone is not the best treatment to support optimum dissolved oxygen. The majority of chitosan usage is aimed at water treatment to separate radioactive heavy metal. Chitosan is one of the most efficient chemical agents to bind dissolved substances such as dissolved solids, dissolved colloids, and suspended solids in a body of water by separating and forming sediments or floating crusts.

Chitosan was shown to lower dissolved oxygen in the research, and to that extent the use of chitosan was limited to antimicrobial agent for media treatment. Furthermore, that the dissolved oxygen figures in chitosan media was measured at 5.37 – 6.65 ppm supported the statement that chitosan is capable of dissolving organic contaminants [5].

The control medium had the lowest dissolved oxygen rate of all the media in the research, measured at 4.8 – 6.51 ppm. The use of only two aerators was believed to be the main cause of this condition. These aerators might not supply sufficient oxygen in the water, which in turn reduced the availability of dissolved oxygen in the water. The lack of oxygen in the water was also exacerbated by oxygen consumption by the prawn larvae. Romimohtarto [14] stated that oxygen is created from photosynthesis process of plants and animals and that its availability is vital for respiration. Low larvae survival rate in the control medium may also be attributed to the cannibalistic nature of prawn larvae. It is known that larvae may cannibalize their own during the molting state, where larvae are in their weakest physical condition.

Judging from the DO rate of the control medium, it was concluded that control medium was not the most efficient medium to be used in larvae rearing. Romimohtarto [14] stated that low DO rate can reduce oxygen absorption efficiency of marine animals, reducing their survival rate in such environment.

Acidity (pH) of the rearing media of this research was still within the acceptable limits, measured at approximately 6 – 8. According to Amri (2003) [10], water acidity for prawn rearing ecosystem should
ideally be kept between 6 – 9. This is also supported by Anggoro [15] who stated that the ideal pH value for post larval stage rearing media is between 6.5 – 8.5.

Changes in pH may also affect the survival rate of prawn larvae. During the course of the experiment, the lowest pH was measured at 6, in the control media. Low acidity was identified with very low survival rate, at 20%, of the medium, followed by the 2nd lowest survival rate measured from that of the chitosan medium. Royce [16] stated that for medium with pH 6.4, although the prawn larvae managed to survive, the growth of the larvae was slowed down to 60%. Furthermore, Romimohtarto [14] revealed that changes in acidity may be directly and indirectly detrimental to marine ecosystem. Change in water acidity directly impact marine animals in that fish, spawn, eggs and other kinds of animals may die from the change. Another direct impact is that the change in acidity may reduce primary productivity. Even a slight change from the natural acidity may hint disruption in the biology support system.

The temperature parameter for all media in this research showed almost similar readings at 24 – 25 °C. The data obtained from all media showed no significant difference in temperature between each medium. According to Nurdjana, M. L., Martosudarmo, B dan Anindyastuti [17], the optimal water temperature for prawn rearing is identified at 25 – 32 °C. However, temperature is also believed to have a significant relationship with dissolved ozone.

Lentech [18] explained that water temperature is a determining factor in the levels of dissolved oxygen in water. Generally, rate of oxygen dissolution will slow down in 40 °C of water temperature. Starrs. D et al. [19] stated that within certain limits, the rate at which prawn grows is linear with the increase of temperature, albeit conversely, the survival rate of prawn is inversely proportional o the rise of temperature.

The concentration of dissolved ozone is highly determined by the amount discharge, reaction speed and contact time of ozone in the water, and water quality. One of the parameters of water quality which determines dissolved ozone is temperature. The lower the water temperature, the more ozone concentration there is in the water and vice versa. Other variable which may affect dissolved ozone in water is acidity (pH must be close to 7) [20].

Salinity of all the media during the treatment were measured between 30 to 31 %/0. According to Amri [10], the most ideal water salinity to support prawn growth is around 19 – 35 %/0. On the other hand, Tricahyo [19] explained that the most ideal salinity for prawn ecosystem is at 15 – 30 %/0. Sudden change in salinity may result in high prawn mortality rate. Prawn is generally more resistant to low salinity during its larval stage compared to its adult stage [21].

Medium treated with combination of chitosan and ozone showed significant increase in dissolved ozone from week to week during the experiment. The most effective dissolved ozone rate in water obtained was between 9.316 – 13.748. The highest dissolved ozone concentration was found in ozone-treated medium, a condition believed to be the result of reaction between formed oxygen atoms with other oxygen atoms which in turn formed more ozone. Kogelschatz et all [22] stated that ozone is created by feeding oxygen gas through electrical discharge. Oxygen ions types are \( O^+ \), \( O_2^\cdot \), \( O^- \), \( O^{2-} \) and \( O_3^- \), which all of their combination results in ozone. Nitrogen molecule can also react into nitrogen dioxide which is unstable in nature and can react with oxygen to produce ozone. Combined chitosan and ozone medium had dissolved ozone concentration of 8.245 – 12.478. This media showed the lower dissolved ozone figure out of the two ozone-treated media, with larvae survival rate observed at 80%. Low dissolved ozone in combined chitosan and ozone treatment was believed to be caused by high water turbidity, owing the the nature of chitosan as coagulant, which prevented the collision of oxygen molecules with electron, other atoms or molecules. Chitosan is one of the substances capable of binding other fine materials such as dissolved solids, colloidal solids, and suspended solids in a body of water which it then separates by forming sediment or crusts. Furthermore, Lentech [18] stated that measurement of ozone can only be taken in contact with water. This is made possible by the fact that contact with water made it safe for surface contact for
both human and prawn. Survival rate in the ozone-treated water was also found to be the highest of all the media, at 100%. The most immediate hint for ozone safety levels is whenever water becomes malodorous, then the ozone levels in the water has become unsafe [23].

Control medium showed the highest microbial population among the media in this research. Weekly interval of microbial population count of the control media was measured at 1.5 x 10^6 – 3.0 x 10^6. This was believed to be caused by aeration treatment of the control medium, by simply discharging oxygen into the water. The bacterial colony count of ozone media was significantly different to that of the control media. This result is in line with Lentech [18], which mentioned germicidal, bactericidal and fungicidal properties of oxygen is far weaker than that of ozone. The media with combined chitosan and ozone treatment had the second highest bacteria colony count, whereas the weekly interval growth of the colony was measured at 1 x 10^5 – 3.7 x 10^5. The result shown by chitosan media was not as good as that shown by ozone media. This is believed to be caused by concentration of dissolved chitosan in chitosan treated media, at 10 ppm (25 ml in 25 L of sea water), might not be comparable to the dissolved ozone concentration in the ozone treated media, at 9.316 – 13.748 ppm, 10 ml of chitosan solution in 1 L of water resulted in 10 ppm of dissolved chitosan.

The relatively low growth of bacterial colony found in chitosan media might be attributed to the nature of chitosan as antimicrobial agents, which is capable of attacking the membrane of bacteria. Roberts [24] explained that chitosan is cationic polyelectrolyte (positively charged) and readily attract opposite negative ions. In addition, the positively charged polycation of chitosan is proven to be capable in hampering bacterial growth [5]. These properties play an important part in keeping the bacterial growth to a very low level and was believed to be the main factor in the lowest bacterial growth rate compared with control and chitosan and ozone combination treatment media, being measured at 2.8 x 10^3 – 7.4 x 10^5 in weekly interval. Ozone treatment showed the most efficient result. From week to week, colony in the media showed interval of 1 x 10^4 – 3 x 10^5. Ozone treatment and the high concentration of dissolved oxygen was believed to keep bacterial growth at minimum. In aquarium, these properties are also utilized to kill various parasites, such as bacteria, virus, and spores as well as to neutralize other pollutants. Furthermore, the efficacy of ozone in clearing away odor and turbidity is one of the reasons why ozone is preferable as sea water aquarium treatment [18].

Through oxidation, ozone is capable of killing various microorganisms such as Eschercia coli, Salmonella enteriditis, Hepatitis A and many other pathogenic bacteria. Direct oxidation is highly damaging to outer membrane of cells (cell lysis), which result in death of the cell [25]. A Team of Unibraw Medical School [26] wrote that in the event of cell death, important metabolites escape. In addition, ozone technology is time and cost efficient as well as practical in that it does not require much space for its installation. The bacterial growth in ozone medium was observed at 1 x 10^5 on the initial week, due to lag phase of the bacterial growth. On the second week, bacterial growth in the medium was measured at 1.2 x 10^4, which was believed to be the initial phase of growth. Measurements on the third week showed 3 x 10^4, a relatively rapid growth which was believed to be the exponential phase. Between the fourth and the final week of the experiment, bacterial growth in the medium showed a constant value of 2 x 3 x 10^4, which was believed to be the static phase. These results are in line with Lud Waluyo [27] who proposed four phases in bacterial growth. Soon after being dispersed in a medium, bacteria begin to adapt with substrate and environment. No reduplication takes place in this phase since several enzymes may not be synthesized yet. This phase is also called lag phase. After the adaptation has been completed, cells begin to multiply in low rate. This phase is called initial growth phase. After the previous phase, cells begin to multiply rapidly at an exponential rate, often following logarithmic patterns. It is believed that this phase is highly affected by the properties of the environment in which the bacteria grows, such as acidity, nutrient contents and temperature. This phase is also known as logarithmic growth phase (exponential phase or rapid growth phase).
Between the fourth and the final week of this research, there was little or no bacterial growth observed in the ozone medium. This phenomenon is believed to be caused by the change in the environment which may be disadvantageous to bacterial growth and development. Bacterial cell population was observed to be constant, which might also be attributed by the balance in the amount of new and dying cells. This phase is referred to as the static growth phase. When observed by visual plot residual (respond of the bacterial population), linear reading was obtained. It was assumed visually that at this phase the growth had met normality pattern (normal distribution). Mathematical calculation of P Value (significant) for Kolmogorov-Smirnov method yielded $> \alpha$, in which $\alpha = 5\%$. It was calculated that $P$ Value $= 0.150$ making $H_0$ accepted and reinforced the assumptions for residual respond (bacterial population normality). The result was then further analyzed by ANOVA method.

By using ANOVA checking model, it was concluded that there were differences among the 4 treatment media, namely ozone, chitosan, chitosan and ozone, and control, in bacteria population (respond/dependent variable). This was shown by the results, where the value of $Pr > F = 0.0001$. The value $0.0001 < \alpha = 0.05$. This meant that $H_0$ was false and that there were significant influence among 4 treatment media toward the bacteria population (respond/dependent variable). $Pr > F$ is less than $\alpha$, then $H_0$ is rejected.

The value obtained on P-Value Levene’s Test $= 0.019 < \alpha = 0.05$, which meant that $H_0$ was rejected. This result validated the suspected difference in influence between the three treatment media and the control medium. According to Srigandono [28], P-Value Levene’s Test $< \alpha$ meant that there is a significant difference among 3 or more treatment groups. This also meant that there were differences in the variance of all four rearing media. In conclusion, the results showed that there was significant difference among the three treatment media and control medium, as well as between all the treatment media and the control medium.

When hypothesis zero ($H_0$) was rejected through Analysis of Variance procedure, then it can be concluded that there was significant difference in at least one of the observed treatment. Srigandono [27] stated that to identify the most significant difference, further test on mean value of the treatments must be carried out. Survival rate of the tiger prawn ($P. monodon$) showed different value for each medium. The highest survival rate was observed in both ozone treatment and chitosan treatment media with a perfect $100\%$ figure. The second highest survival rate was found in the combined ozone and chitosan treatment medium with $80\%$ and the lowest was identified in the control medium with $20\%$. However, the survival rate for each medium was deemed to be good. Biochemical test of 2 different test media, ozone treatment and control, showed different results. The most dominant surviving bacteria in control medium was identified as $Pseudomonas stutzeri$ and the dominant bacteria capable of survival in ozone-treated ecosystem was identified as $Bacillus thuringiensis$. Each species bacteria had different survival and adaptation capacity. Bacteria can quickly develop new defense mechanism against external threats that may harm them. Bacteria incapable of neutralizing harmful substances in an ecosystem cannot survive in the environment.

In the control ecosystem, the most dominant surviving bacteria was $Pseudomonas stutzeri$ which was identified to be gram negative, has polar flagella, and has no spore. The survival of this species was believed to be made possible by the abundance of oxygen in the water of the control medium. According to Rheinmer [29], $Pseudomonas stutzeri$ is a facultative anaerobic species which thrive in oxygen-rich environment.

On the other hand, the most dominant surviving bacteria on the ozone treatment medium was $Bacillus thuringiensis$, a species known to be both aerobic and facultative anaerobic. The survival of this species bacteria was made possible by the fact that the species is known to be resistant to low-oxygen environment, extreme shift in acidity, salinity or dissolved oxygen, such as during the ozone discharge into the medium, adapting by forming spores. Microbiology Team of Unibraw [26] reported that the formation of spores in this species takes place whenever the available nutrition can no longer sustain bacterial growth. According to Rheinmer [29], $Bacillus thuringiensis$ is a chemoorganotrophic species with fermentation metabolism or respiration that is sensitive to heat, acidity and salinity. The species is proven to be pathogenic.
only to a handful of vertebrates and invertebrates. Bacteria of the Bacillus species can be terminated on 0.2 ppm concentration of ozone with 30 seconds contact time, whereas Pseudomonas is easily affected or penetrated by ozone [30]. This fact was believed to be the main key in keeping the population of Pseudomonas low in ozone-treated medium, owing to the fact the species easily undergoes lysis by the introduction of ozone. However, this bacteria can still survive ozone treatment due to the fact that it has spores.

4. Conclusion
This research found that the most effective medium to be used in prawn larvae rearing is ozone-treated medium. In order of magnitude, the highest to lowest number of bacterial colony found was in control medium (1.5 x 10^6 CFU), in chitosan and ozone combined treatment (1 x 10^6 CFU), in chitosan treatment (2.8 x 10^6 CFU), and in ozone treatment (1 x 10^6 CFU). The use of ozone and chitosan proved to be significant in suppressing the bacterial population in the prawn larvae rearing media. Significant difference was found between the three treatment media and the control medium. The highest survival rate was found in ozone treatment and chitosan treatment, both measured at 100%, which was followed by combined chitosan and ozone treatment with 80% and control medium with 20%.

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