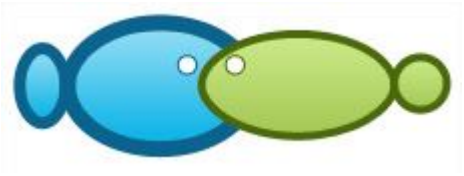

Phytoplankton *Ankistrodesmus* sp. as an alternative tool in controlling fish disease

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Abstract. The ability of the freshwater phytoplankton, *Ankistrodesmus* sp. to inhibit the growth of the fish pathogenic bacterial, *Aeromonas hydrophila* and *Streptococcus agalactiae* was tested. The inhibitory activity of *Ankistrodesmus* sp. against these bacterial were observed in (1) phytoplankton culturing control medium, Bold Basal Medium (BBM) and (2) filtrate prepared from *Ankistrodesmus* sp. cultured in BBM. The cell densities of *A. hydrophila* when cultured in *Ankistrodesmus* sp. filtrate medium showed no significant difference ($P>0.05$) from those cultured in control medium. In contrast, there was inhibition activity on the growth of *S. agalactiae* observed in *Ankistrodesmus* sp. filtrate medium against those cultured in control medium. The cell density of *S. agalactiae* was significantly lower ($P<0.05$) in *Ankistrodesmus* sp. filtrate medium compared to the control medium started from day 4th until the 14th day. Thus, the present study concluded that *Ankistrodesmus* sp. can be an essential tool for inhibiting the growth of *S. agalactiae*.

Key Words: aquaculture, microalgae, streptococcosis, MAS, fish pathogen, disease control.

Introduction. In order to support the global market demand and maintain the continuous supply of fish products, the fish are cultured in mass condition. However, the high mortality in fish culture is the main issues and problems in this industry and fish diseases is one of the major factors in limiting the production of fishes (Toranzo et al 2005). The presence of microbial pathogens in the environment especially in water body is one of the most significant factors affecting fish culture (Zorilla et al 2003). The appearance and development of a fish disease is the result of the interaction among pathogens, hosts and environment. Disease occurrence in the ecosystem is influenced by various environmental factors including infectious organisms which are the pathogens and stressors (Nils et al 2000).

Fish disease is the main issue in aquaculture industry and responsible for financial loss to the farmers. *Aeromonas hyrophila* and *Streptococcus agalactiae* are the most common fish pathogens and highly concerned by the aquaculturists. Motile aeromonas septicemia (MAS) caused by *A. hydrophila* is responsible for heavy losses in fish culture (Groff & Lapatra 2000). In Philippines, it has been reported that this pathogen caused high mortality in reared *Oreochromis mossambicus*, *Oreochromis niloticus* and *Tilapia zilli* (Lio-Po et al 1983). Streptococcosis is one of the major fish disease that have been reported widely in aquaculture sector and cause high economic loss to fish farmers. *Streptococcus* spp. are responsible to cause disease in fish include *Streptococcus agalactiae* (Suanyuk et al 2005). *Streptococcus* spp. are zoonotic which can cause infections in human associated with the preparation and handling of infected fish.

In order to overcome the problems and avoid continuous mortality in fish culture due to the fish disease, antibiotics have been used but it is debated and banned in many countries (De Paola et al 1995; Watson et al 2008). It is believed that the overuse and misuse of these chemicals to combat bacterial diseases in aquaculture have led to an increase of antimicrobial resistance cases (Watson et al 2008). Therefore, another alternatives strategies should be introduced to reduce the widely use of antibiotics in aquaculture industry.

Phytoplankton is commonly used in the rearing of fish. They usually added directly to the water in the rearing tanks, when applying the green water technique (Reitan et al 1997). The use of phytoplankton in aquaculture is also popular due to their antibacterial effect. Naviner et al (1999) has demonstrated the antibacterial activity of the marine diatom, *Skeletonema costatum* against aquacultural pathogens including several species of the genus Vibrio. Besides, Austin et al (1992) have proved that the extracts from *Tetraselmis suecica* able to inhibit bacterial activity within 15 minutes upon addition to the fish tanks and for periods up to 4 hours which managed to prevent the outbreak of infections with *Vibrio anguillarum*, *V. salmonicida* and *Serratia liquefaciens* among Atlantic salmon. Lio-Po et al (2005) in his study on the anti-luminous Vibrio factors in the grow-out culture of the tiger shrimp, *Penaeus monodon* have shown the ability of phytoplanktons in inhibiting those bacterial infections. In another example, compounds or substances excreted by phytoplankton play role in enhancing the effectiveness of certain probiotics in the prevention of fish diseases including Vibriosis (Sharifah & Eguchi 2011, 2012a, 2012b). Hence, the uses of microalgae can be an alternative since most of the farmers nowadays tend to depend on antibiotics and due to high awareness of people to the side effects of short and long term of these chemicals. Therefore, the objective of this study is to investigate the effect of *Ankistrodesmus* sp. a potential fish disease controlling tool in inhibiting growth of different fish pathogens as *A. hydropila* and *S. agalactiae*.

Material and Method

Phytoplankton culture condition. Freshwater *Ankistrodesmus* sp. which was isolated from Terengganu was derived from Dr. Helena Khatoon. *Ankistrodesmus* sp. was transferred and cultured in freshly prepared freshwater phytoplankton medium, Bold Basal Medium (BBM) for 7 days at 25°C (Nichols 1973; Andersen 2005). The density of the phytoplankton was measured daily using a Neubaer haemocytometer and once it reached 10^6 cells/mL in the late log phase, the culture was harvested and filtered as described below.

Bacterial culture condition. *A. hyrophila* and *S. agalactiae* were derived from infected tilapia. The bacteria were cultured in tryptic soy broth (TSB) for at least 16 h with shaking 120 rpm. The growth phase of each bacterium was checked frequently for 24 hours. Each of the bacterial culture was harvested and centrifuged after incubation at 8000 × g for 5 minutes and washed twice with sterile 0.75% NaCl.

Phytoplankton filtrate and media preparation. Two types of media were used to observe the growth of *A. hyrophila* and *S. agalactiae*. Sterilized BBM without phytoplankton was used as control medium. BBM medium with filtrate containing substances excreted by *Ankistrodesmus* sp. were prepared by culturing those phytoplankton for 7 days in BBM until they achieved their late log phase. Next, the phytoplankton culture was centrifuged at 5000 × g for 15 min and the supernatants were filter-sterilized (Whatman, 0.22 µm; Millipore). Fifteen milliliter of all media was prepared in triplicate.

Phytoplankton and pathogenic bacterial interactions. Fish pathogens, *A. hyrophila* and *S. agalactiae* were inoculated individually into *Ankistrodesmus* sp. filtrate and BBM medium (control) of 10^8 cells/mL of each bacterium. All cultures were incubated under shaking (120 rpm) throughout the experiment. 1 mL of aliquots were taken from each culture daily from day 0 to day 6 and every two days from day 8 to day 14. Ten microliter of samples containing *A. hyrophila* or *S. agalactiae* was diluted serially tenfold and dropped (three drops per dilution) onto tryptic soy agar (TSA) plates using the drop plate method (Herigstad et al 2001). The plates were incubated overnight in the dark at 37°C, and then the colonies were counted. Experiments were carried out in triplicate.

Statistical analysis. Significant differences between samples of control (without phytoplankton) and mixed cultures (with phytoplankton) in each medium were analyzed

by the independent sample T test. Statistical analysis was done using SPSS v16.0 software.

Results and Discussion

Effect of Ankistrosdesmus sp. filtrate on the inhibition of A. hydrophila. There were no significant differences (P>0.05) between the cell densities of *A. hydrophila* in *Ankistrosdesmus* sp. filtrate medium and control medium, BBM without *Ankistrodesmus* sp. when co-culturing for 14 days with exceptionally of day 4 and 8 (Figure 1).

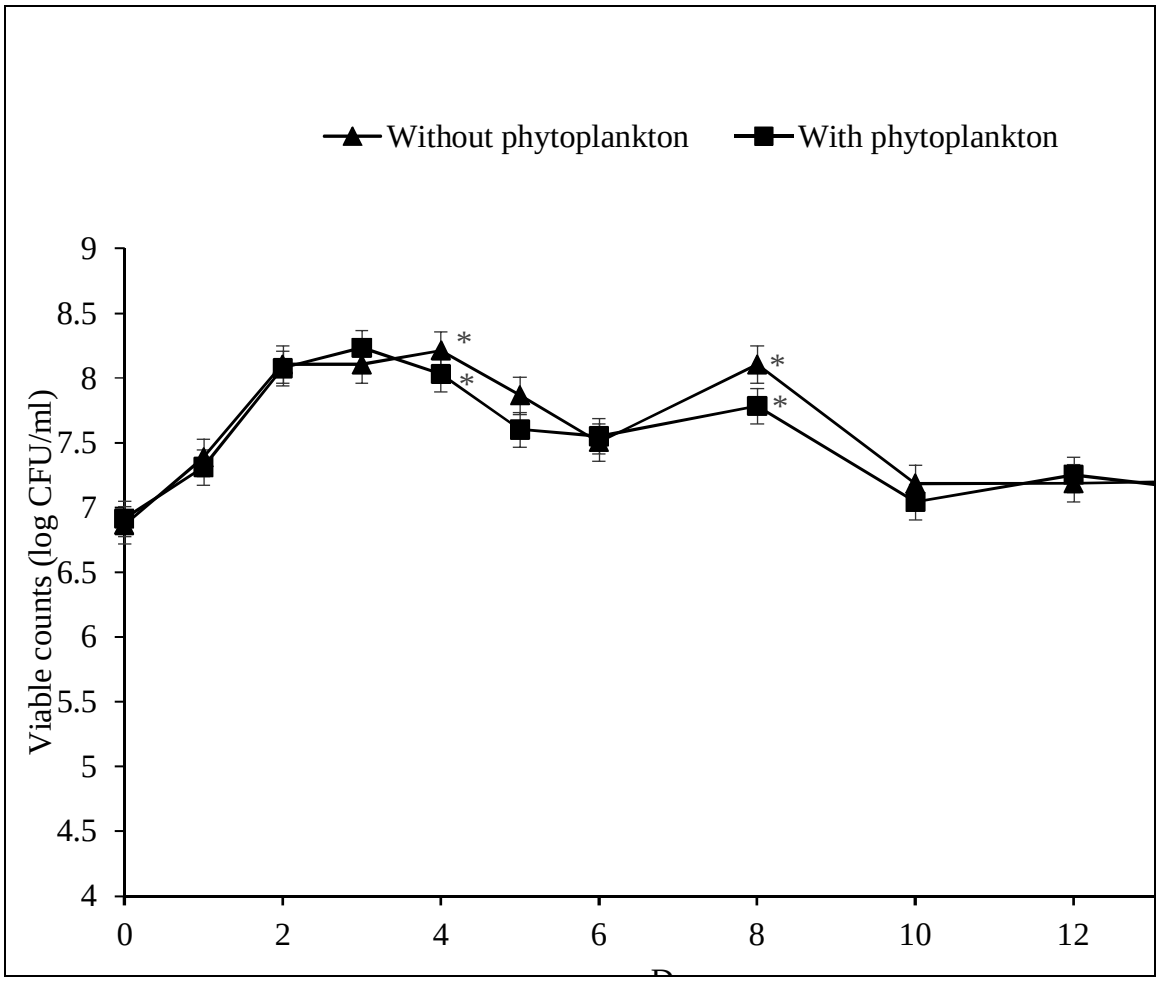

Figure 1. Cell densities of *A. hydrophila* in BBM medium. Close triangles, *A. hydrophila* cultured in control medium, BBM medium without *Ankistrodesmus* sp. filtrate. Close squares, *A. hydrophila* cultured in *Ankistrodesmus* sp. filtrate medium. Error bars - standard deviation; n - 3; (*) - significantly different, (P<0.05).

Neither medium with phytoplankton nor without phytoplankton medium affected the growth of the bacteria. The bacterial concentration of *A. hydrophila* in both control medium and *Ankistrodesmus* sp. filtrate medium kept increasing from day 0 until day 2 with no significant differences (P>0.05) between them. The cell densities of the bacteria increased from range 6.5 log cfu/mL in day 0 to 8 log cfu/mL in day 3. In day 4, *A. hydrophila* population in *Ankistrodesmus* sp. filtrate dropped after reaching peak density in day 3 with significant differences (P<0.05) between the cell densities for bacterial in both media. Even though the bacterial population in *Ankistrodesmus* sp. filtrate medium for day 5 are slightly lower number compared to those in control medium as shown in Figure 1, there was no significant different between them. In day 8, the bacterial population in *Ankistrodesmus* sp. filtrate showed significantly lower (P<0.05) number of

bacterial densities compared to control medium without *Ankistrodesmus* sp. filtrate but no continuous pattern showed for day 10 until end of the experiment.

Effect of Ankistrosdesmus sp. filtrate on the inhibition of S. agalactiae. In contrast, the growth of *S. agalactiae* was inhibited in the *Ankistrodesmus* sp. filtrate medium compared to the control medium (Figure 2). The cell densities of *S. agalactiae* cultured in the *Ankistrodesmus* sp. filtrate medium were significantly lower ($P<0.05$) than the control medium started from day 4 until the end of the experiment in day 14. No significant differences was observed ($P>0.05$) between the cell densities of the bacteria in *Ankistrodesmus* sp. filtrate medium and the control medium from day 1 until day 3. After reaching peak intensity at range of 8 log cfu/mL in day 3, the cell densities of *S. agalactiae* for both media dropped which the bacterial concentration in *Ankistrodesmus* sp. filtrate medium showed largest drop compared to those in control medium. *S. agalactiae* population in *Ankistrodesmus* sp. filtrate medium maintained significantly lower concentration ($P<0.05$) compared to the control medium started day 4 even though there was an increase in the bacterial concentration for the filtrate in day 8 as shown in Figure 2.

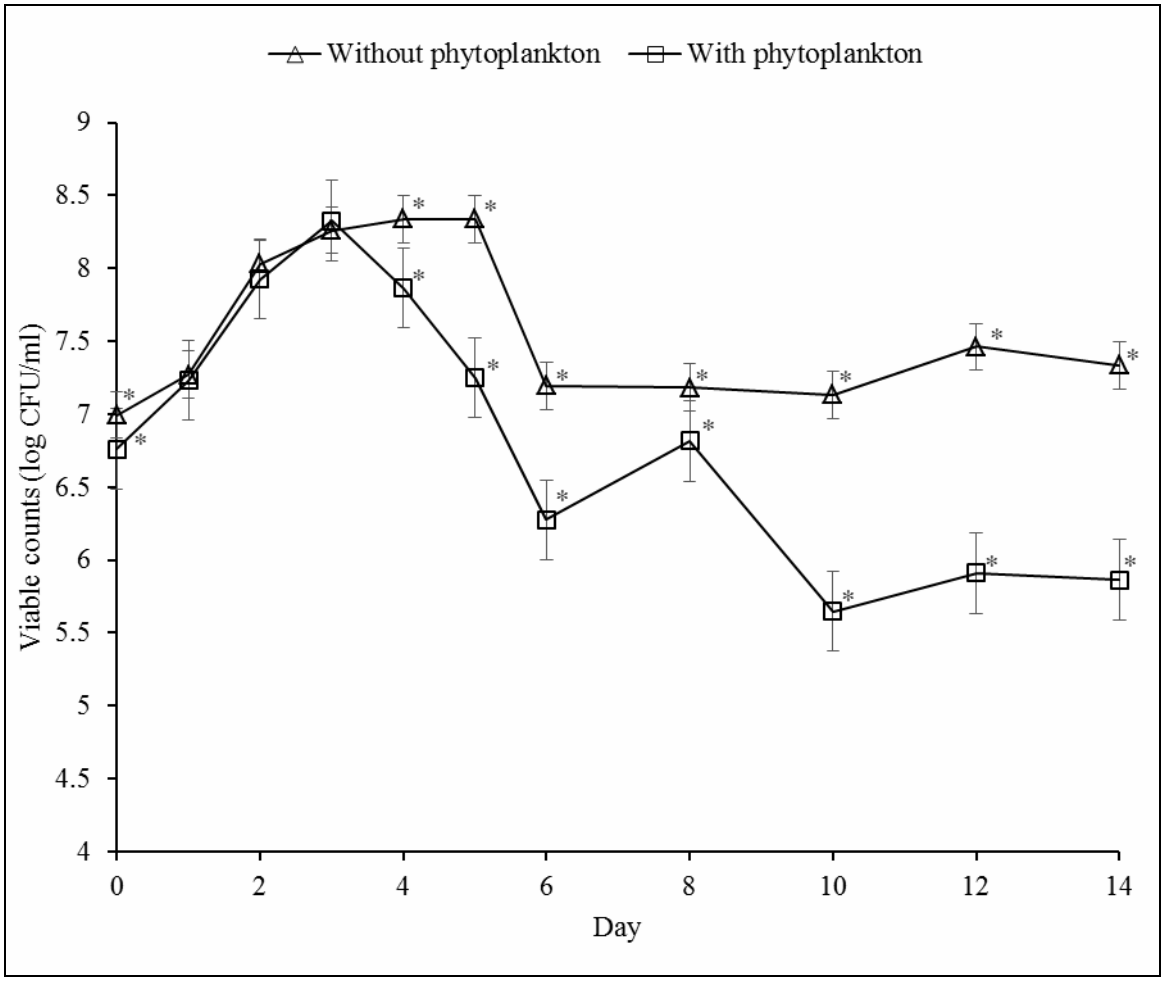

Figure 2. Cell densities of *S. agalactiae* in BBM medium. Open triangles, *S. agalactiae* cultured in control medium, BBM medium without *Ankistrodesmus* sp. filtrate. Open squares, *S. agalactiae* cultured in *Ankistrodesmus* sp. filtrate medium. Error bars - standard deviation; n = 3; (*) - significantly different, ($P<0.05$).

Application of phytoplanktons in aquaculture has been practiced widely for years ago, in the green water technique which is the addition of microalga into the intensive culture systems together with the zooplankton (Tamaru et al 1994). The introduction of microalga into the fish larval tanks improves the survival and growth of the fish larvae

(Nakase & Eguchi 2007; Nakase et al 2007). Hence, it is believed that the microalgae play significant role in modification of bacterial populations in the cultural tanks which contributes to better performances (Defoirdt et al 2011; Nakase & Eguchi 2007; Nakase et al 2007). Results of this study indicate the ability of *Ankistrodesmus* sp. to inhibit effectively the population of *S. agalactiae*. The cell densities of *S. agalactiae* cultured in medium containing *Ankistrodesmus* sp. filtrate are lower compared to those cultured in control medium. In other words, *Ankistrodesmus* sp. has produced antibacterial effects that cause reduction of the bacterial population. In previous studies, antibacterial effects have been noticed in many classes of phytoplanktons and microalgae especially in diatoms (Burkholder et al 1960; Duff et al 1966; Berland et al 1972).

In contrast, there are no inhibition effects on the growth of *A. hydrophila* neither in *Ankistrodesmus* sp. filtrate medium nor control medium. Even though there are significant differences between bacterial population in both media which those in *Ankistrodesmus* sp. filtrate medium were lower than the bacterial in control medium in day 4 and 8, the result is not reliable since it shows no continuous pattern. The inhibitory effect on the bacterias is only temporarily for those particular day.

It is suggested that the structure of the bacteria may play role to the effectiveness of the antibiotics or antibacterial substances to inhibit the pathogens. Antibiotics are generally less effective against gram-negative compared to gram-positive because of their complex and multilayer cell wall structure which makes the actives compound more difficult to penetrate the cell wall (Ordog et al 2004). As a result, the antibacterial activity is more potent against gram-positive than gram-negative bacteria (Ghasemi et al 2004, 2007). Thus, this will be a good explanation on why the antibacterial substances of *Ankistrodesmus* sp. are more effective against *S. agalactiae* compared *A. hydrophila*.

There is a possibility that the mechanism of antibacterial effect of *Ankistrodesmus* sp. may related to the excretion of the antibiotics substances by the phytoplankton itself into the culture medium. As reported by Pratt et al (1944), freshwater species of Chlorella produced an antibacterial substance named chlorellin which is the first antibiotic to be isolated from an autotroph. Further study had been conducted and it is cleared that chlorellin, which is a liphophilic antibacterial substance produced by *Chlorella vulgaris* was excreted in the culture medium. It is possible that the antibacterial substances produced by *Ankistrodesmus* sp. may mimic the chlorellin since both *Ankistrodesmus* sp. and *Chlorella* sp. are under same group of Chlorophyta or green algae.

Lio-Po et al (2005), has conducted a study on anti-luminous Vibrio factors associated with the green water for grow-out culture of the tiger shrimp, *P. monodon*. Based on his study, Leptolyngbia sp. has cause reduction of the luminous Vibrio population from 10^4 down to 10^1 cfu/mL after 24 hours exposure. In the same study, both diatoms, *Chaetoceros calcitrans* and *Nitzchia* sp. secreted bactericidal metabolites against luminous Vibrio which bacterial population were eradicated at 24 and 48 hours post exposure. In contrast, *S. agalactiae* population dropped after 4 days co-culturing with *Ankistrodesmus* sp. filtrate. It is possible that Ankistrodesmus sp. may secreted extracellular metabolites that were inhibitory to *S. agalactiae*.

In addition, the antibacterial effects of microalgae are also related to its intracellular products. However, types of active compounds in *Ankistrodesmus* sp. remain unclear and not well-studied. Many reseachers have reported different types of active compounds contents in various species of phytoplanktons and microalgal which have antibacterial effect. Some microalgal including *Haematococcus pluvialis*, *Chlorococcum* sp., and *Phaeodactylum tricornutum* contain fatty acids and that able to inhibit selected bacterial such as *Escherichian coli*, *Staphylococcus aureus* and *Vibrio* spp. (Santoyo et al 2009; Desbois et al 2009).

Naviner et al (1999) in his study has demonstrated the antibacterial activity of the marine diatom, *S. costatum* against different types of aquacultural pathogens. The active compounds which contain unsaturated and saturated long chain fatty acids in the extraction of this phytoplankton were effectively inhibited the growth of *Listonella anguillarum* and *Vibrio* spp. For another example, Austin et al (1992), found that the extract from *Tetraselmis suecica* is able to inhibit the growth of certain bacterial fish pathogens in aquaculture. The exact mechanism of action of this antibacterial agent is

not well-studied. It is speculated that the compounds excreted from the phytoplankton may target on the cell wall of the bacteria and there is a possibility that the membrane of the bacteria have been damaged thus there will likely lead to the cell leakage and cause reduction of the nutrients uptake by the bacteria. As a consequence, the cellular respiration process occur in the cell will be inhibited (Desbois et al 2009). This study shows the potential of *Ankistrodesmus* sp. to be another alternative for prevention of the outbreak of fish diseases especially related to streptococcosis. The products secreted by *Ankistrodesmus* sp. are capable to retard bacterial development based on the positive results obtained from this study.

Conclusions. Due to increase of therapeutic resistance of the fish pathogens to the usual antibiotics and its potential antipathogenic actions, there appears to be a significant role for *Ankistrodesmus* sp. in the control of aquaculture diseases especially in cases related to *S. agalactiae*. This is the first study done on *Ankistrodesmus* sp. to show a great potential in controlling fish disease. However, further studies needs to be done in order to determine if this antibacterial action is due to extracellular products secreted by *Ankistrodesmus* sp. Further tests on different types of fish pathogens are recommended to be performed utilizing the potential of this phytoplankton and its antibacterial activity which is crucial in the prevention and treatment of fish diseases.

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