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The Effect of Ultrasound Frequency on the Harmful Algal Species: *Pyrodinium bahamense* var. *compressum* and *Margalefidinium polykrikoides*

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ABSTRACT

Harmful algal blooms (habs) in Sabah, particularly *Pyrodinium bahamense* var. *compressum* (*Pbc*) and *Margalefidinium polykrikoides (M. polykrikoides)*, have been the subject of research due to their deleterious effects on the aquaculture industry and human health. Several methods have been established to mitigate the habs' cell, including using ultrasound. This study was conducted to identify the effect of different frequencies of ultrasound on the removal rate of habs species. The harmful dinoflagellate, *Pbc* and *M. polykrikoides* were cultivated in f/2 media. The established culture was then exposed to ultrasound with various frequencies consisting of 20khz, 40khz, 512kHz, 816 kHz and 1100 kHz. Results showed that, upon increasing the frequency, the habs' cell removal rate was highly targeted, associated with an increase in both habs species. At the lowest frequency (20kHz), the cell removal rate was 18±3% for *M. polykrikoide* and 20±2% for *Pbc.* Whereas, at the highest frequency (1100 kHz), the rate of cell removal was up to 96 ±2% for *M. polykrikoides* cells and 86 ± 3% for *Pbc*. In addition, the cell removal rate for *M. polykrikoides* was significantly higher (P<0.05) compared to *Pbc* cells. It was observed that the rate of cell removal is affected by the size of harmful algae cells. Findings from this study can be utilized as a starting point for eliminating future harmful algae blooms in Sabah, Malaysia

INTRODUCTION

Harmful algae blooms (habs) defined as phenomenon of the excessive presence of any harmful algae species that can lead to negative effects on health, economy and sociology **(Albay & Akçaalan, 2003** ; **Hallegraeff, 2004** ;**Yñiguez *et al.*, 2021)**. Generally, this phenomenon occurs with the triggering of excessive micronutrients such as nitrogen (N) and phosphorus (P) inputs inside the water column. Thus, the presence of those micronutrient causes a rapid division and an uncontrollable growth for the harmful algae cell **(Hallegraeff, 1993)**.

Up till now, problems related to habs have only been documented along the coastal waters of Sabah's western shore. While, no reports were conducted on habs in the east coast of Sabah. **(Roy, 1977; Ming & Wong, 1989** ; **Adam *et al.*, 2011; Jipanin *et al.*,**

2019). Two main species of habs were recorded in Kota Kinabalu water, including *Pyrodinium bahamense* var. *compressum* and *Margalefidinium polykrikoides* (previously named as *Cochlodinium polykrikoides*). Pyrodinium bahamense var. compressum blooms were first detected in the coastal waters of Sabah in 1976 (**Roy, 1977**). Since then, many human illnesses have been recorded **(Ting & Joseph, 1989)** due to the cellular capability that can cause paralytic shellfish poisoning (PSP). *M. polykrikoides* were first reported in 2005, the occurrence of which has been associated with fish mortality in aquaculture **(Adam *et al*., 2011)**. In addition, almost every year *M. Polykrikoides* blooms have been recorded along the Kota Kinabalu, Sabah water since this occurence **(Jipanin *et al*., 2019)**.

The most common treatments to mitigate the harmful algal blooms include coagulation, flotation, clarification, filtration, algicides, ozone and photolysis **(Kim *et al*., 2008; Yu *et al*., 2017)**. However, some of the methods are usually expensive, complicated, and can cause further pollution due to the use of chemicals and pollutants **(Lee *et al*., 2000)**. In the past decade, sonication was considered as a simple and potentially environmentally friendly approach. Ultrasound refers to sound waves with a frequency greater than 20kHz **(Carovac *et al.*, 2011)**. In water, ultrasonic radiation induces a sequence of compression and rarefaction cycles that result in the formation of cavitation bubbles (acoustic cavitation). Millions of these bubbles implode, resulting in temperatures as high as 5,000 degrees Celsius, pressures as high as 2,000 atmospheres, and free radicals **(Manson, 2000)**. Fig. (1) provides an overview of this process, adapted from the studies of **Manson (2000)** and **Purcell *et al.* (2013)**. This severe condition can disrupt the buoyancy of algae by bursting the gas vacuoles in microorganism and preventing photosynthesis by sedimentation, cell membrane breakdown, and the formation of free radical species **(Rodriguez-Molares *et al*., 2014; Schneider *et al*., 2015**).

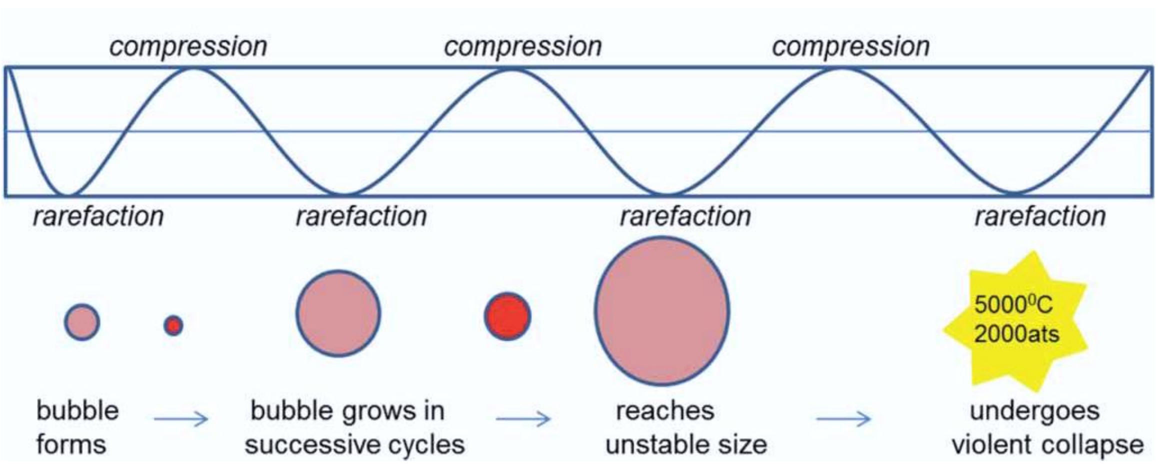

Fig.1. The development and destruction of cavitation bubbles (adapted from **Manson, (2000)** and **Purcell *et al*. (2013)**).

It has been demonstrated that ultrasound has a detrimental effect on organism structural and functional status, and hence, sonication is applied to reduce cyanobacterial blooms in eutrophic water (**Phull *et al*., 1997; Lee *et al*., 2010)**. Research has found that sonication inhibits the growth rate of cyanobacteria by causing the rupture or collapse of gas vesicles due to cavitational effects, membrane disruption, damage to photosynthetic activity, and inhibition of cell division and cell cycle; the extent of the damage and thus bloom control is dependent on parameters such as ultrasonic frequency, power intensity, and exposure duration (**Chen *et al*., 2022**). Although it may lead to the release of toxins due to cyanobacterial cell lysis (**Song *et al*., 2005** ; **Purcell *et al*., 2013**), sonication has also been reported to be effective in degrading the cyanotoxins (**Song *et al*., 2005**).

The previous study, more than 90% of all reported research, have focused on *Microcystis* (**Jong *et al*., 2000** ; **Nakano *et al*., 2001**; **Lee *et al.*, 2002** ; **Ahn *et al*., 2003** ; **Hao *et al*., 2004** ; **Tang *et al*., 2004** ; **Song *et al*., 2005** ; **Ma *et al*., 2005** ; **Zhang *et al*., 2006 ; Huang *et al*., 2020**) but the utility of widespread application of ultrasound for other algal species especially harmful dinoflagellate is remain unclear. Therefore, this study was conducted to identify the effect of different frequency of ultrasound toward the removal of harmful algae specifically harmful dinoflagellates : *Pyrodinium bahamense* var. *compressum* (*Pbc*) and *Margalefidinium polykrikoides* (*M. polykrikoides*)

MATERIALS AND METHODS

The strain of *Pbc* and *M. polykrikoides* cells, was originally isolated from the blooms occurred in Sepanggar Bay, in December 2018 and February 2021 respectively. Both harmful species were cultured in f/2 medium. The strain was kept at pH 7.7-7.8, salinity 33, and 25°C under cool white fluorescent lamps (light intensity 35 mol/m2/s) on a 12:12 LD cycle as a source of cells for the experiments. The isolated strain of *Pbc* was preconditioned at 25°C, and the cells in logarithmic phase were inoculated in triplicate into flasks containing 3 L of f/2 medium, at approximately 200 cells/ml. They were grown in the same conditions as stock culture, with no stirring. Cultures were maintained for 30 days (for *M. polykrikoides*) and 60 days (for *Pbc*) before the experiments, depending on the species. The cultures were maintained in exponential growth phase until reach the cell concentration about $3x10^3$ cell /ml (*M. polykrikoides*) and $4x10^3$ cell /ml (*Pbc*) during all experiments.

For ultrasonic test experiment will, ultrasonic probe with multifrequency unit was used follow method suggested by **Purcell *et al*., (2013)** with some modifications. The probe immitted frequencies 20khz, 40khz, 512kHz, 816 kHz and 1100 kHz with constant power of 100 W. Sample consist of 1000 ml harmful algae ; *M. polykrikoides* and *Pbc* were exposed to the different ultrasound frequencies. Exposure period were ranged from 5-600 second for each tested frequencies. Along the exposure experiment, the temperature was maintained within ± 3^oC to avoid sudden temperature change that can disturb the experiment result (**Purcell *et al*., 2013**). Sample are taken for every interval of time for cell count and cell morphology observation under microscope. For morphological observation the cell were classified into the main condition : firstly healthy cell (capable to move freely), secondly cell with damaged flagellate (immobile cell) and lastly burst cell (lysed cell).

Finally for the statistical analysis, a one-way analysis of variance (ANOVA) was conducted, with a significance level of $p \leq 0.05$, followed by a Tukey post hoc test using Statistical Package for Social Science (SPSS) ver.21.

RESULTS AND DISCUSSION

The exposure experiment's results, as shown in figure 2, clearly indicate that ultrasound has a positive reaction for habs cell removal. Removal of the harmful algae cell was observed to increase simultaneously with increased frequency. In the case of *Pbc* cell, the removal percentage is quite lower compared to *M. polykrikodes*. Maximum cell removal of 96% and 84% was recorded for *Pbc* and *M. polykrikodes* respectively, when being treated with 1100 kHz of ultrasound frequency. For the lowest frequency (20kHz) shown, the removal rate was below 20%. Recent study found that the morphological size of algae also contribute to the efficiency of ultrasound for cell removal. In this study diameter size of cultured *M. polykrikode* were relatively small (25 - 38 µm average cell size) as compared to *Pbc* (33-47.9 µm average cell size). In principal small size organism will have bigger surface area (**Ray, 2016**). The larger the surface, the greater the chance that an alga will come into touch with a bubble formed by ultrasound; this contact causes the cell to destroy as explained in figure 1. This finding has also been proved by the previous study done by **Yamamoto *et al*., (2015)** and **Dehghani, (2016**).

In this study, if the removal cell is less than 20%, it considers as ineffective to apply in natural blooms cases. This is because the exposure time needs to be increased to remove higher cells with low ultrasound frequency. Increasing the exposure time will be caused the increase in power input that leads to higher electrical power consumption and will affect the electrical cost increase (**Chen *et al*., 2022**). So this lowest frequency is not economical for harmful algae removal.

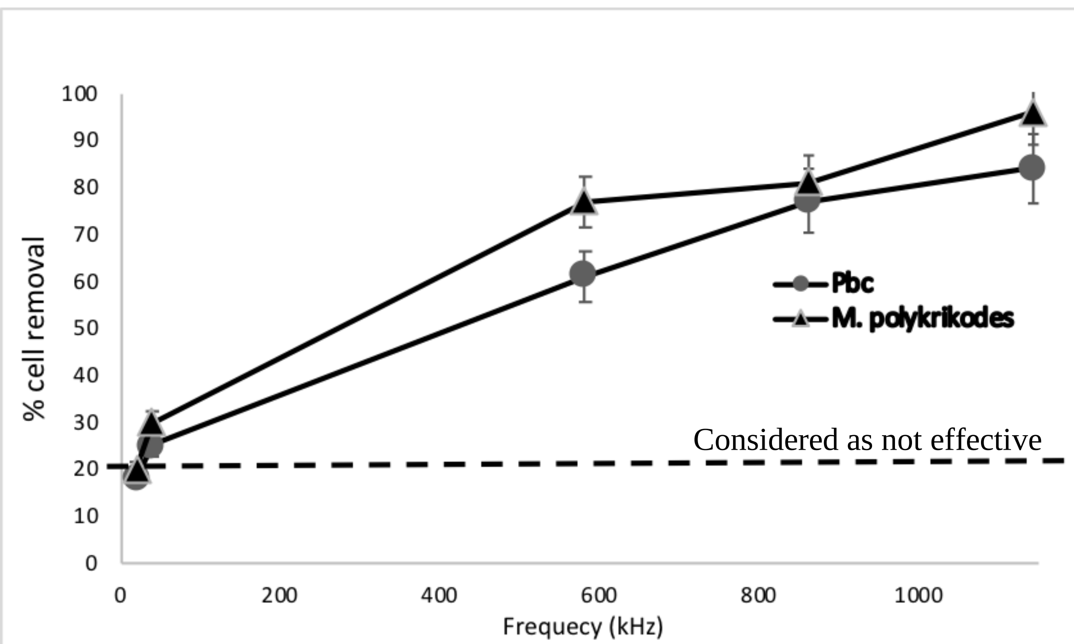

Fig. 2. Effect of difference frequency on removal rare (expressed in %) of *Pbc* and *M. polykrikoide*

The data from Figs. 3 and 4 show that with the increase of ultrasound frequency will cause the percentage of healthy cell decreased. The percentage of healthy cell (defined freely move) was minimum (*Pbc* 16% and *M. polykrikoide* 4%) at the highest frequency (1100khz). Whereas, the percentage of break cell and burst cell was rapidly increase with the increase of ultrasound frequency. Deformation of cell morphology at frequency 20kHz and 40 kHz was marked by deactivation of cell movement whereas at higher frequencies 512 kHz and above the broken and lysis cell discovered more often. The maximum burst cell recorded was 78% and 93 % for *Pbc* and *M. polykrikoide* respectively This results indicated that destroying of filament structures may be the main mechanism affecting algal activity for these two dinoflagellate species. A study done by **Purcell *et al*., (2013)** found that filamentous algae were highly affected as compared to none filamentous when exposed to ultrasound.

Besides that, ultrasound can collapse gas vacuoles that control algal locomotion during cavitation. When the size of gas vacuoles and the resonance size of cavitation bubbles are of the same magnitude order, gas vacuoles are more likely to resonate, experience acoustic cavitation, and then collapse (**Ahn *et al*., 2003**). Under these conditions, the inactivation of algae is caused by the formation and collapse of cavitation bubbles. As sonication frequency increases, rarefaction time throughout the acoustic cycle decreases. This means that it gets more difficult to produce cavitation bubbles in the available time, necessitating increased sound intensities (power) to induce cavitation. Increasing the frequency of sonication increases the formation of free radicals from the decomposition of water caused by cavitation collapse. Therefore, sonication at higher frequencies can inactivate harmful dinoflagellate by this mechanism in addition to the mechanical effects of cavitation collapse (**Joyce *et al*., 2010**).

Filamentous species with a higher surface area seemed to be more vulnerable to ultrasound than unicellular/colonial and non-filamentous species (**Purcell *et al*., 2013**). The destruction of flagella that acts as a propeller for the locomotion of algae also caused the algae to lose it capability to mobilize and lead to cell sink and cell death.

Observation under microscope also found that, at frequency of 40 kHz and above, no chain of *Pbc* and *M*. *polykrikoide* observed. All the moving or broken algae was observed in a single unit instead of in a chain form. Commonly both species will form chain under normal conditions. *M*. *polykrikoide* form a chain with combination of 2-8 cells whereas *Pbc* form a chain up to 32 cells (**Maclean, 1977**). Previous study done by **Jiang *et al***.,(**2010**) revealed that under stress condition of *M*. *polykrikoide* will inhibit the formation of chain. Therefore the deformation of chain in both harmful species were suspected due to the stress condition caused by the ultrasonic frequency.

Besides, the removal of habs cell also related with the interruption of photosynthesis proses. Previous study found that with the exposure of ultrasound with high frequency will cause damages to the chlorophyll pigment of the algae **(Zhang, *et al.*, 2006 ; Dehghani, 2016**). As known, both dinoflagellate tested in this study is a photosynthetic species. *M*. *polykrikoide* consist of numerous yellowish-green to brown chloroplast and *Pbc* consist of golden reddish chloroplast (**Onda *et al*., 2014**). As a chemical result of cavitation from the ultrasound activity, the free radical reaction may disrupt algal photosystems and trigger lipid peroxidation of cell membranes (**Duan *et al.*, 2017**).

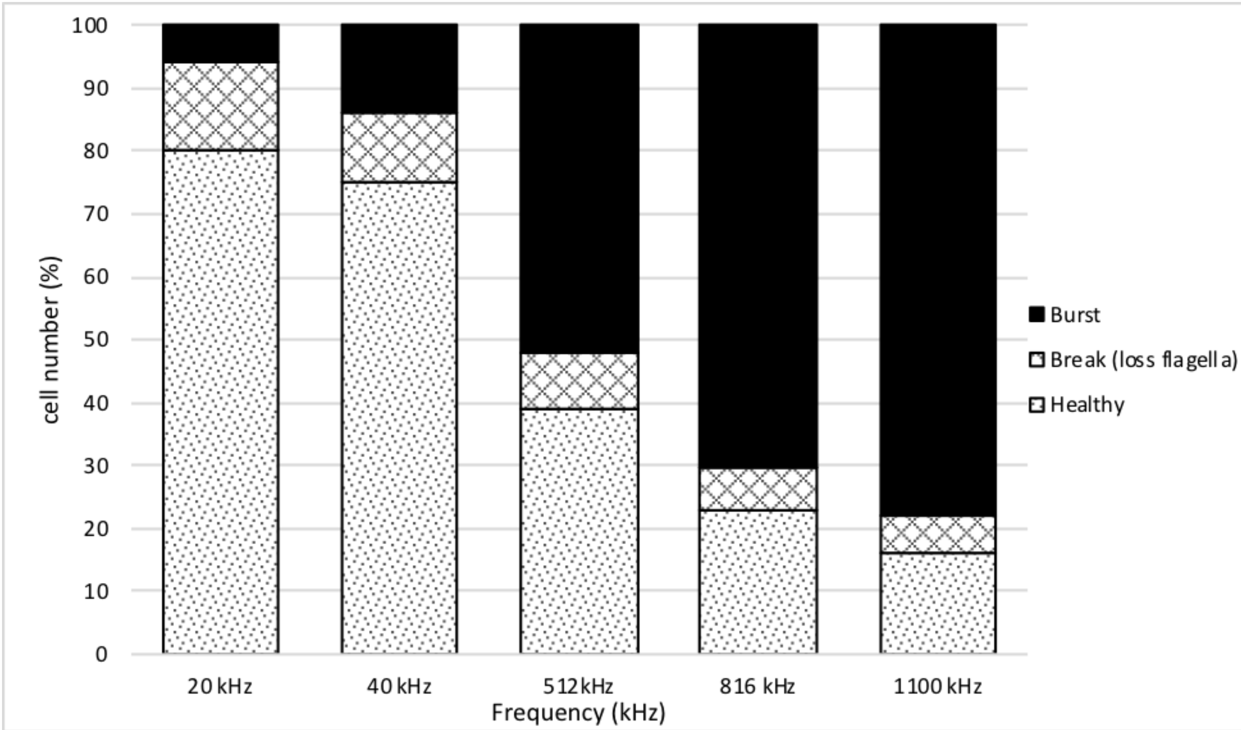

Fig. 3. Cell number condition (expressed in %) of *Pbc* exposed with different ultrasound frequencies

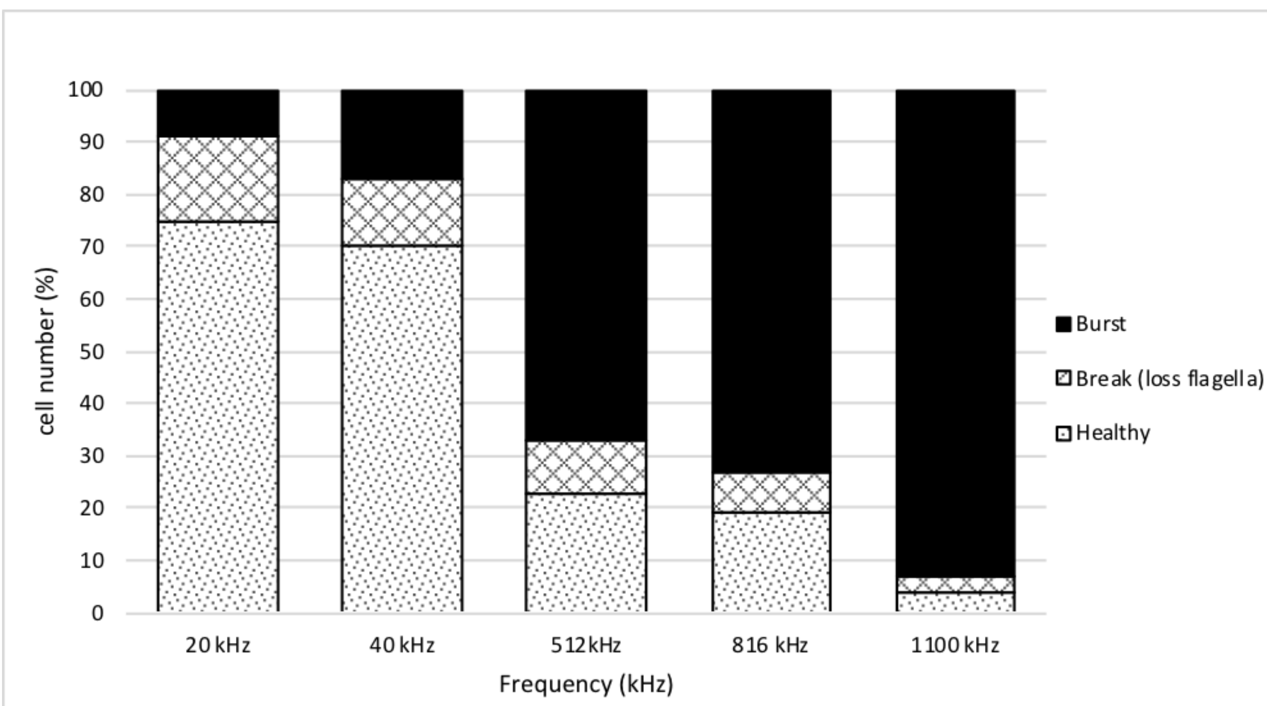

Fig. 4. Cell number condition (expressed in %) of *M. polykrikoide* exposed with different ultrasound frequencies

CONCLUSION

The removal of harmful algae species *Pbc* and *M. polykrikoide* depends on the ultrasound frequency. The higher the frequency, the higher the cell removal can be observed. The mechanism of ultrasound mitigates the habs cell was identified through the damages of flagella, lysis of cell membrane and alteration of chlorophyll pigments. The results indicate that ultrasound can be an effective tool for inhibiting or controlling algae, but the sonication factors must be considered.

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