Microalgal profile and anti-Vibrio activity of crude extracts from green water reservoir stocked with tilapia

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Abstract— The microalgal profile of the green water from brackish water pond reservoir stocked with adult Tilapia (mosambique and nile hybrid) was conducted weekly for a month cycle. Five ten liters composite samples were collected per sampling and mixed thoroughly. Of these, ten liters were filtered and preserved with buffered formalin for plankton composition and the remaining filtered samples were extracted with ethyl acetate for anti-Vibrio activity. Four microalgae were identified from the composite samples: *Nitzschia, Pleurosigma, Nannochloropsis* and *Oscillatoria*. *Nitzschia* dominated the first sampling with a density of 1.54E+06 cells ml^{-1} and relative abundance of 99.99%. The subsequent sampling was dominated by *Nannochloropsis* with increasing density from 1.03E+06 cells ml^{-1} (56.64%) to 2.32E+06 cells ml^{-1} (99.99%), respectively. *Pleurosigma* was only observed in the early culture and *Oscillatoria* in the latter culture.

Anti-Vibrio activity of extracts using disc diffusion techniques revealed that only non-polar fractions exhibited inhibitory activity against *Vibrio harveyi*. The minimum inhibitory concentration (MIC) of the extract dominated by *Nitzschia* was 25μg ml^{-1}, significantly higher (P=0.0083) compared to the MIC for extracts of the algae in the samples dominated by *Nannochloropsis* in subsequent sampling (12.5μg ml-1). Subculture of MIC tubes in fresh media did not show growth after 24 hours indicating bactericidal activity, a comparative advantage to the control, chloramphenicol, which is only bacteriostatic at 12.5μg ml^{-1}.

***Keywords*—**microalgal profile, green water, anti-Vibrio activity.

I. Introduction

THE farming of black tiger shrimp (*Penaues monodon*) contributes significantly to the Philippine economy and other countries in the Asia-Pacific region. Production in many of these countries has declined due to luminous bacteria, *Vibrio harveyi*, which has caused death to nearly 100% of shrimp stock in hatcheries and ponds. White Spot Syndrome Virus (WSSV), Infectious Hypodermic Haematopoetic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV), and Yellow Head Virus (YHV) has slumped the industry further [2, 3]. Luminescent vibriosis occurred during the first 10-15 days after stocking of shrimp post larvae in grow out ponds [4]. The outbreak of the disease is found to be preceded by substantial increase in the number of *Vibrio* in the pond water following pond disinfection and was associated with perturbed microbial community in combination with the presence of nutrients The use of antibiotics and/or disinfectants destroyed matured microbial systems and were also proven ineffective in treating diseases caused by luminescent vibrios (*V. harveyi*) and closely related bacteria causing early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) [5, 6]. Alternative methods and technologies have been proposed to control this disease [7, 8 and 9]. One of the techniques reported to work against luminous bacteria in the Philippines is the green water culture system (or finfish–shrimp integrated culture system [10, 11 and 12]. The ability of the "green water" grow-out culture of the tiger shrimp *P. monodon* to prevent outbreaks of luminous vibriosis was investigated by screening associated isolates of bacteria, fungi, phytoplankton and fish skin mucus for anti-luminous *Vibrio* metabolites [2,7]. Although a number of studies has been done [3, 7, 10, 11and 12] on the green water, there are still a lot of information gaps that needs to be explored such as information on algal composition of green water from reservoirs with Tilapia or in the ponds stocked with prawn and which of these algae really show anti-inhibitory activity or are these algae acting in cohorts?

II. Methods

The study was conducted in water reservoir stocked with adult tilapia (cross-breed of nile and mosambique tilapia) that supplies green water to prawn farms operated by BFAR-NFRDI (fig 1). The reservoir is an earthen pond with an area of 3,000 m^2.

Fig. 1.The water reservoir stocked with adult tilapia that supplies water to prawn ponds

A. Sampling

Weekly sampling of green water microalgae for composition and density and anti-Vibrio activity were

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undertaken in one month cycle. Sampling points were established in the reservoir following an S-pattern that is four points at each corner and one at the center. Five-ten litres composite samples were collected per sampling and mixed thoroughly. Twenty five litres was filtered and preserved with buffered formalin for algal composition analysis and the rest of the samples were filtered and extracted with ethyl acetate for anti-Vibrio activity.

B. Plankton analysis

Counting of the microalgae was done using haemocytometer with the aid of a compound microscope and phytoplankton guides [13, 14].

C. Extraction of Anti-Vibrio activity fractions

The zooplankton was removed by filtering the green water through a 100µ mesh bolting cloth. The algae were concentrated by centrifugation for 15min and the residue was extracted with 30mL ethyl acetate and vortex-mixed for 15min and transferred to a column and allowed to separate in layers (fractions). The layers of extract were dried in rota-vapour [15].

D. Anti-Vibrio activity screening

The algal extracts from different fractions representing the different sampling periods were dissolved separately in exact amount of methanol necessary to come up with the right concentration of extract working solution. Exactly 30µl extracts was delivered to each 8mm DIFCO blank disk. Extra care was observed to avoid spattering during delivery. The discs were allowed to dry before use [16].

E. Preparation of V. harveyi

The test bacterium, *V. harveyi* was plated in seawater agar following standard operating procedure [16]. Five plates of bacterial culture used for sensitivity testing of the extract collected per sampling period. Crude extracts discs with 100µg representing the different layers and two controls: negative and positive controls were deployed per plate. A broad spectrum antibiotic Chloramphenicol (30µg) was used as positive control and methanol as the negative control.

F. MIC of green water extracts on V. harveyi.

Algal extracts showing anti-Vibrio activity were subjected for tube dilution to determine the minimum inhibitory activity concentrations [17]. The tubes that showed MIC were streaked on fresh seawater agar plates and incubated for 18-24h to determine growth of bacteria after assay. Growth of bacteria after 18-24hours indicated bacteriostatic activity and those without growth, a bactericidal activity or total eradication.

G. Statistical Analysis

One-way Analysis of variance (ANOVA) was used

III. Results and Discussion

The green water from the brackish water stocked with Tilapia is composed of multi-algal species. Four genera were identified during the four week sampling periods: *Nitzschia*, *Nannochloropsis*, *Pleurosigma* and *Oscillatoria*. *Nitzschia* and *Pleurosigma* belong to pennate diatoms. *Nannochloropsis* belong to Chlorophyceae *and Oscillatoria* belong to Cyanophyta. The density and abundance of the microalgae varied in every sampling period (fig. 2). *Nitzschia* dominated the algal population during the first sampling, four days after the introduction of water in the reservoir at density of 1.64E+06 cells ml^{-1} which is in contrast to the results in shrimp pond which was dominated by *Chlorella* [10, 11 and 12]. However, on the subsequent sampling *Nitzschia* decreased steeply while *Nannochloropsis* steadily increased up to the 4th sampling (table 1). The early bloom of the *Nitzschia* in the reservoir may have been attributed from the water source. The composition of *Nitzschia* in the coastal water of Panguil Bay was reported to be about 6% during the northeast monsoon and 5.5% during the southwest monsoon [18] which coincided with the pumping of water at the start of the cycle. Only *Nitzschia* and *Nannochloropsis* were observed throughout the cycle. The *Pleurosigma* was observed only in the early culture and *Oscillatoria* at the latter stages of growth.

TABLE 1. Mean microalgal densities and relative abundance in the different sampling period

S*	*Nitzschia* sp.		*Chlorella* sp		*Pleurosigma*		*Oscillatoria* sp	
	Cell density	Relative abundance	Cell density	%RA	Cell density	% RA	Cell density	% RA
1	1.64E+06	99.96	448	0.0130	6	0.0004	0	0
2	1.58E+05	13.36	1.02E+6	86.64	0	0	0	0
3	19	0.0009	2.17E+6	99.99	0	0	4	0.0002
4	10	0.0004	2.32E+6	99.99	0	0	15	0.0008

* Samples

Three separate distinct layers of extracts were observed in each sampling period. The first layer (fraction1) was light green in color, the middle layer (fraction 2) was very dark green and uppermost layer (fraction 3) was transparent, viscous with tinge of brown color. Table 2 showed the mean volume and mean percentage of liquid extracts generated per sampling period. The lowest fraction (fractions 1) obtained the highest volume, more than the volume of Fractions 2 and 3 combined.

TABLE 2: MEAN VOLUME AND MEAN PERCENTAGE OF LIQUID EXTRACTS

Sampling period	Fraction 1		Fraction 2		Fraction 3	
	Vol (ml)	%	Vol (ml)	%	Vol (ml)	%
1	23.00	51.11	20.00	44.00	20.00	4.40
2	25.00	56.60	17.50	38.90	25.00	5.60
3	23.50	52.20	20.00	44.40	15.00	3.30
4	24.00	53.30	16.00	35.60	5.00	11.00

The mean weights and percentages of dried crude extract residues of the different samples (table 3) follow the trends in the mean volume and percentages of the extracts. Obviously, greater volume of fractions would yield greater yield of extracts.

TABLE 3. MEAN WEIGHTS AND PERCENTAGES OF DRIED CRUDE EXTRACTS RESIDUES OF THE DIFFERENT SAMPLES

Sampling period	Fraction-1 Crude Extract		Fraction-2 Crude Extract		Fraction-2 Crude Extract	
	g	%	g	%	g	%
1st	0.117	51.09	0.100	43.67	0.012	5.24
2nd	0.388	72.52	0.134	25.05	0.013	2.43
3rd	0.527	53.18	0.454	45.81	0.010	1.01
4th	0.596	0.596	0.3.13	33.13	0.035	3.70

Results on the anti-Vibrio activity showed that only the uppermost layers (fractions 3) were able to inhibit the growth of *V. harveyi*. Fractions 1 and 2 did not show any inhibition on the growth of the pathogen even at concentrations of 500µg ml^{-1}. These two fractions have higher molecular weights compared to Fraction 3. Substances with high molecular weights have limited capability to diffuse on a solid medium. The inhibitory activity of Fractions 3 varied with the concentrations of the disc and increased steadily with age of the green water (table 4). Statistical tests have shown significant differences between the three fractions with Fractions 3 as most distinct from other fractions. The minimum inhibitory concentration (MIC) of the extract dominated by *Nitzschia* on the pathogen was 25µg ml^{-1}, significantly higher (P=0.0083, α=0.05) compared to the MIC for extracts of the algae in the samples dominated by *Nannochloropsis* in subsequent sampling (12.5µg ml-1). The results have also indicated that anti-Vibrio activity is not only limited to Chlorophycea but also on Bacillariophycea (*Nitzschia*). The microalgae, *Chaetoceros calcitrans* and *Nitzschia* sp, consistently demonstrated complete inhibition of luminous *Vibrio* from 24h and 48 h post exposure, respectively, and during the 7-day experiment. Other plankton also possessed effective mechanism in preventing outbreaks of luminous vibriosis [7].

TABLE 4. MEAN INHIBITION ZONE OF THE F3 FRACTIONS AND THEIR POSITIVE AND NEGATIVE CONTROLS

Sampling Period	F3 layer	Positive Control (Chloramphenicol) (30µg)	Negative control (methanol) (30µg)
1	8.65	18.5	0.0
2	8.68	22	0.0
3	8.77	19.2	0.0
4	8.83	18.5	0.0

IV. SUMMARY AND CONCLUSION

The green water may be dominated by other plankton which exert inhibitory or bactericidal activity not just *Nannochloropsis*. The composition and percentage abundance of *Nitzschia* in the first sampling (table 1) clearly indicated that bioactivity of the extracts was due to *Nitzschia*. The bioactivity of both extracts in subsequent sample could either be additive effects or indifference rather than synergism because the effects of the extracts highly dominated by *Nannochloropsis* were far more potent. Had it been synergism the extracts in combination would have shown greater effects. The dominance of one alga may also have inhibited growth of other algae, hence the succession. The first inhibitory activity was exerted by the *Nitzschia* and this activity dissipated when *Nannochloropsis* started to dominate.

Although the MIC of F3 fractions of the green water concentration was higher (12.5µg) compared to that of Chloramphenicol (1.25µg), the effect of the extracts is bactericidal while that of Chloramphenicol is only bacteriostatic.

V. IMPLICATION AND RECOMMENDATION

The claim on the use of green water in controlling luminous bacteria is effective has been validated. This provides the scientific basis for using the green water technology in controlling luminous bacteria (*V. harveyi).*The data generated may help improve aquaculture management practice in this part of the country. Isolation and elucidation of the compound present in green water responsible for anti-Vibrio activity need to be investigated. The algal succession in reservoirs stocked with tilapia need to be fine-tuned. *Nitzschia* and *Nannochloropsis* and other species dominating in the culture need to be studied on other bacterial pathogens in pond and hatcheries.

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