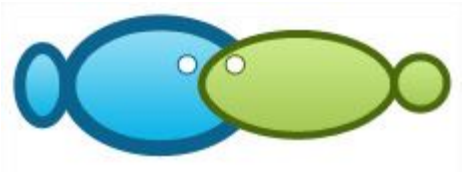

Quorum quenching activity of lysates from bioluminescent bacteria isolated from selected Philippine marine and freshwater organisms

[1,2]Roja G. Angeles-Gumban, [1,3]Mudjekeewis D. Santos, [3]Minerva Fatimae H. Ventolero, [1]Janice A. Ragaza, [1,4]Ronie J. Calugay

[1] Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Quezon City, Philippines; [2] Elementary and High School Department, College of Arts and Sciences, University of the East, Caloocan City, Philippines; [3] Genetic Fingerprinting Laboratory, National Fisheries Research and Development Institute, Quezon City, Philippines; [4] Department of Biology, College of Science, University of the Philippines Baguio, Baguio City, Philippines. Corresponding author: R. J. Calugay, rjcalugay@up.edu.ph

Abstract. Pathogens use cell density dependent gene expression known as Quorum Sensing (QS) to launch virulent factors. Targeting the QS circuit without killing the pathogens is a very promising alternative to using antibiotics which generate antibiotic resistance. The identity and diversity of bioluminescent bacteria associated with Philippine aquatic organisms, and their potential as sources of anti-QS or quorum quenching (QQ) molecules remain unknown. In this study, out of 43 organisms, 30 were observed to harbor bioluminescent bacteria with 26 from marine and four from freshwater sources. Purified bioluminescent bacterial cultures were screened for QQ activity using motility assay and the membrane fractions (MF) of 5 isolates from marine organisms namely *Johnius* sp. (croaker), *Sillago* sp. (golden-lined sillago), *Variola louti* (yellow-edged lyretail), *Strombus variabilis* (variable conch), *Cistopus indicus* (octopus), and one from a freshwater host, *Chanos chanos* (milkfish), inhibited the QS-mediated swarming of *Pseudomonas aeruginosa* strain NSRI based on statistical analysis using Paired t-test and Univariate Analysis of Variance ($p < 0.05$). Despite the host differences, the bacterial isolates shared evolutionary relationship with commonly known bioluminescent genera based on 16S rRNA gene sequence analysis with five strains belonging to the genus *Photobacterium* (*P. leiognathi and P. mandapamensis,* 99-100% identity), and two to the genus *Vibrio* (*V. harveyi* and *V. campbellii*, with 99% identity).

Key Words: bioluminescent bacteria, quorum sensing, quorum quenching.

Introduction. Bioluminescence is the production of observable light by organisms, a phenomenon occuring across different taxa (Popham & Stevens 2006) but most commonly observed in microorganisms known as bioluminescent bacteria which exist as symbionts or transiently associated in marine fishes and squids (Dunlap & Kita-Tsukamoto 2006; Davis et al 2016). Bioluminescent bacteria emit light for attraction of mates and evasion of predators for their hosts. The skin, gills, intestinal tract or light organ of associated hosts in turn provide nutrient-rich environments for the bacteria (Widder 2010). The expression of bioluminescence in bacteria is induced at high cell density condition which accumulates a sufficient concentration of autoinducers called acyl homoserine lactones (AHLs) to exchange on the lux operon contained of the luxCDABEG genes which include the expression of luciferase and other significant enzymes for the production of light, this cell-density dependent phenomenon is called 'quorum sensing' (QS) (Dunlap & Kita-Tsukamoto 2006). Remarkably, QS also mediates the production of virulent factors in pathogens such as the formation of biofilms or swarming motility in *Pseudomonas aeruginosa* (Pearson et al 1997). Disrupting the QS circuit by targeting the autoinducers, a scheme called 'quorum quenching' (QQ), therefore is a very promising

and novel strategy of disarming pathogens without the use of antibiotics which only generates antibiotic resistance (Roy et al 2011).

The isolation and identification of bioluminescent bacteria have several applications, bioluminescent bacteria are used as biosensors for the detection and measurement of contaminants in soil and water samples (Nunes-Halldorson & Duran 2003), potential sources of antibiotics against known nosocomial pathogens (Molina et al 2016), for non-invasive imaging technology known as bioluminescent imaging (BLI) to monitor bacterial infections and proliferation of tumor cells *in vivo* (Heuts et al 2009) to name a few. So far, bioluminescent bacteria are not yet investigated as possible new bioresources of QQ molecules to battle the global health threat by antibiotic resistance. Given the vast aquatic ecosystems and untapped rich microbial diversity of the Philippine archipelago, this study was undertaken to isolate bioluminescent bacteria associated with the diverse aquatic organisms of the Philippines and screen the extracts of the isolates for QQ activity.

Material and Method

Collection and morphological identification of marine and freshwater samples. Twenty (20) samples of each of the 43 randomly selected freshwater and marine organisms sold in public city markets of Navotas, Malabon,Valenzuela, Batangas, Rizal and Iloilo, were purchased from February to August 2014 at 4:00 AM to obtain fresh-catch samples. The taxonomic key by Carpenter & Niem (1998) was the basis for the morphological identification of the samples.

Isolation and purification of bioluminescent bacteria. Bioluminescent bacteria were isolated from the marine and freshwater samples through standard microbiological procedures. The head, vitreous sac of the eyes, skin, stomach and intestines of the samples were swabbed using sterile cotton swabs and streaked on bioluminescent agar (Nealson & Markovitz 1970). Plates were incubated overnight at room temperature and were observed in a dark room for the presence of bioluminescent colonies and subsequently picked, and purified by re-streaking colonies onto fresh bioluminescent agar plates.

Preparation of membrane and cytosolic fractions from bioluminescent bacterial isolates. Pure cell cultures incubated overnight in 250 mL broth and 3.50 x 10^8 cells were harvested through centrifugation at 6000 rpm at room temperature for 10 min. The pellets were sonicated at 40 amps for 15 min on ice. The pellets were centrifuged at 8500 rpm at room temperature for 15 min. The cytosolic fraction (CF) was the resulting supernatant. The pellet products after centrifugation were washed 3X with sterile distilled water to remove carry overs from the CF. The washed pellets were sonicated and centrifuged as above and the liquid were collected as the membrane fraction (MF). The CF and MF samples were stored at -20°C until use for the Kirby Bauer and swarming motility assays.

Screening for antibacterial activity. An efficient quorum quencher must not have an antibiotic activity because the latter only generates antibiotic resistance to pathogens. Hence the bacterial CF and MF were subjected to screening for antibiotic activity using the Kirby-Bauer assay against *Pseudomonas aeruginosa* NSRI, a bacterium with QS-mediated biofilm formation. An overnight *P. aeruginosa* culture at 10^6 cells mL^{-1} were spread on Mueller-Hinton agar (MHA) plates. Ten microliters of the following test samples were added to sterile filter paper discs: (1) CF; (2) the MF; (3) 10 mg mL^{-1} gentamicin (positive control), and (4) sterile distilled water (negative control), and laid on MHA plates. The zones of inhibition were measured in millimeter (mm) unit.

Screening of quorum quenching potential from the bioluminescent bacterial CF and MF. To determine the quorum quenching potential from the bioluminescent bacterial CF and MF, the swarming motility assay used by Norizan et al (2013) was followed.

Swarming agar was used with the following composition: glucose (1% w/v), bacteriological agar (0.5% w/v), peptone (0.5% w/v), and yeast extract (0.2% w/v). A volume of 1 mL each of CF, MF, sterile distilled water (negative control), and 0.15 mg mL^{-1} caffeine (positive control), were sowed into 44 mL of molten swarming agar, then mixed up well lightly and distributed onto petri plates in triplicates and 1 µL of overnight culture of *P. aeruginosa* (1.5 x 10^6 cell mL^{-1}) were stab-inoculated by at the center of the agar plate and incubated for 16 hours at 37°C. The diameters of the swarming of the *P. aeruginosa* were measured in millimeter (mm) unit.

Statistical analysis. The zones of inhibition and diameters of swarming measured were obtained as the mean±standard deviation. The measurements were statistically analyzed using the Paired t-test and Univariate Analysis of Variance. Paired t-test was used to compare the general MF and CF of the isolates in quorum quenching and antibiotic activities. Univariate was used to determine the activities of quorum quenching and antibiotic properties of the fractions. Finally to confirm the assumption and conclusions, Post hoc (Tukey HSD) was used.

Molecular identification and phylogenetic analysis of bioluminescent bacterial isolates. For PCR templates, the genomic DNA of each bioluminescent bacterial isolate was extracted using the potassium ethyl xanthogenate method (Jhingan 1992). The universal 16S rRNA gene primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' GGTTACCTTGTTACGACTT-3') (Abulencia et al 2006) were used to amplify the 16S rRNA gene with the following PCR conditions: an initial denaturation of 94°C for 5 min; 25 cycles: denaturation of 94°C for 30 s, annealing of 50°C for 30 sec and extension of 72°C for 1 min; final extension of 72°C for 4 min; and storage at 4°C. The ~1500 kb amplicons were sent to Macrogen Korea Incorporated (Seoul City, South Korea) for sequencing. With the use of software Chromas (Goodstadt & Ponting 2001) and CAP3 (Huang & Madan 1999), the sequences were edited and assembled. The Database Enabled Code for Ideal Probe Hybridization Employing R (DECIPHER) (Wright et al 2012) was used for analyzing the presence of chimera and compared against to the deposited sequences using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul et al 1990). Then for the alignment of rRNA gene sequences of the bioluminescent bacterial isolates and the 16S rRNA gene sequences of known species of bioluminescent bacteria which were downloaded from the NCBI database (accession numbers:JN380344.1, AY341442.1, AY204490.1, KJ508002.1, KC534273.1, KJ022620.1, AM930501.1), ClustalW was used. Molecular Evolutionary Genetics Analysis version 6 software (MEGA 6) (Tamura et al 2013) was used for the construction of the molecular phylogenetic tree of the bioluminescent bacterial isolates and for the Evolutionary history, Neighbor-Joining method (Tamura et al 2004) was used.

Results and Discussion

Isolation and morphological identification of bioluminescent bacteria from marine and freshwater organisms. This study conducted a mini-survey of freshwater and marine fish hosts harboring bioluminescent bacteria. The samples were purchased fresh from selected public wet markets of the Philippines. This study showed that bioluminescent bacteria have a wide range of hosts in Philippine waters. Out of 43 organisms, 30 or 69.77% were observed to harbor bioluminescent bacteria; 26 or 86.67% hosts were from marine and four or 13.33% were from freshwater sources.

Only 13 or 43.33% out of the 30 isolated colonies obtained from various marine and freshwater organisms, designated here as strains ADMU-UE-01 to -13 maintained their bioluminescence from generation to generation in the repeated streaking during the purification process. Nealson & Markovitz (1970) explained that this process happens when lesions block the synthesis of luciferase and that, repeated streaking during the purification contributes to the curing of genetic materials during conjugation generating daughter cells which multiplied into colonies lacking some *lux* genes. Colonies are randomly picked when purifying and mutant colonies may have been randomly picked

and streaked for the next series of streaking, generating dark mutants. This is also supported by the study conducted by O'Grady (2008) where the non-luminescence is caused by the disruption of the *lux* genes through mutation. All of the bioluminescent bacterial isolates emit light in the blue-green spectrum approximately 6 to 8 h after incubation at room temperature. The thirteen samples were further characterized and the respective marine and freshwater hosts were identified based on morphology (Figure 1) as (A) BLB-ADMU-UE-01: *Johnius* sp. (commonly known as croaker), (B) BLB-ADMU-UE-02: *Sillago* sp., (golden-lined sillago), (C) BLB-ADMU-UE-03: *Valamugil buchanani* (half fringelip mullet), (D) BLB-ADMU-UE-04: *Chanos chanos* (milkfish), (E) BLB-ADMU-UE-05: *Cynoglossus punticeps* (tonguesole), (F) BLB-ADMU-UE-06: *Variola louti* (yellow-edged lyretail), (G) BLB-ADMU-UE-07: *Upeneus* sp. (ochrebanded goatfish), (H) BLB-ADMU-UE-08: *Cardites bicolor* (cockles), (I) BLB-ADMU-UE-09: *Crassostrea iredalei* (Philippine cupped oyster), (J) BLB-ADMU-UE-10: *Strombus variabilis* (variable conch), (K) BLB-ADMU-UE-11: *Terebralia palustris* (mud creeper), (L) BLB-ADMU-UE-12: *Cistopus indicus* (octopus) and (M) BLB-ADMU-UE-13: *Harpiosquilla anandalei* (tropical mantis shrimp). To date, marine fish hosts such as the *Johnius* sp., *Sillago* sp., *V. louti* and *C. punticeps* were never before identified and reported to be associated with bioluminescent bacteria.

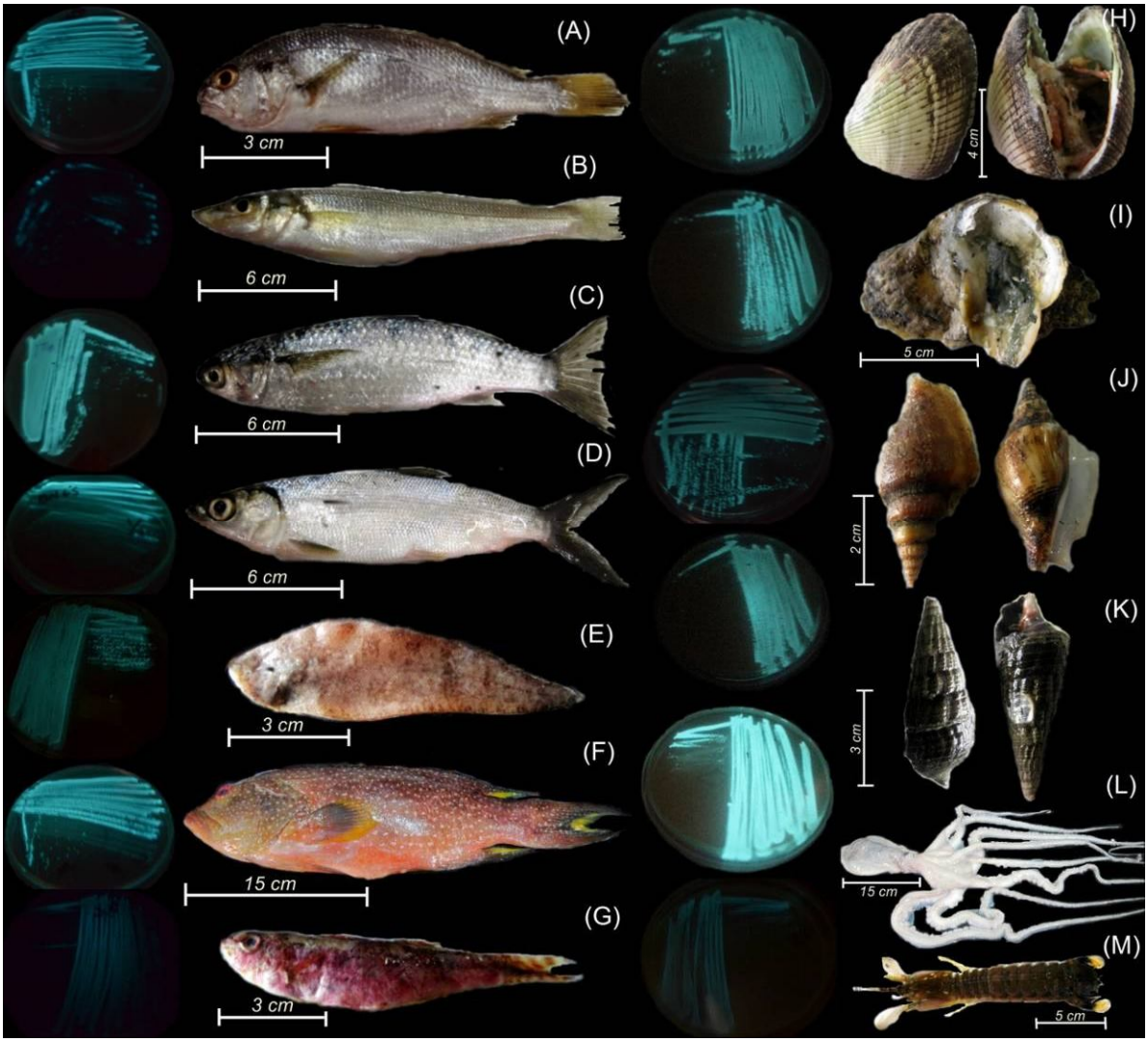

Figure 1. Isolated BLB from selected marine and freshwater hosts.

Screening of CF and MF from bioluminescent bacteria with antibacterial and quorum quenching potential. An efficient quorum quencher must not have an antibiotic activity because the selective pressure applied through antibiotics only generates resistance in pathogens. Hence the bacterial CF and MF were subjected to screening for antibiotic activity using the Kirby-Bauer assay. Only strain BLB-ADMU-UE-

05 showed the presence of zones of inhibition against *P. aeruginosa* but smaller than the diameter of the zone of inhibition exhibited by the positive control gentamicin. The results suggested that there was significant ($p > 0.05$) difference in the CF and MF of the ADMU-UE-05 and the negative control on the zone of inhibition against *P. aeruginosa*, showing that it has antibacterial activity but lower than the zone of inhibition exhibited by the positive control. These suggest that the lysates of the said strain has weaker antimicrobial activities.

To screen for QQ potential using the swarming motility assay, the swarming of *P. aeruginosa* was inhibited by the MF of the strains BLB-ADMU-UE-01, 02, 04, 06, 10 and 12. On the other hand, the CF did not have an effect on the swarming motility of the *P. aeruginosa* (Figure 2, Table 1). The Paired t-test ($p < 0.05$) strongly suggested that the MF of the said strains were statistically different from the CF and have shown high QQ activity against *P. aeruginosa* based on the swarming diameter. Based on the results of Univariate Analysis of Variance and Post Hoc-Tukey HSD also indicated that there was significant ($p < 0.05$) difference on MFs of ADMU-UE-10 as compared to all MFs and caffeine (positive control), showing that it has a very high QQ potential.

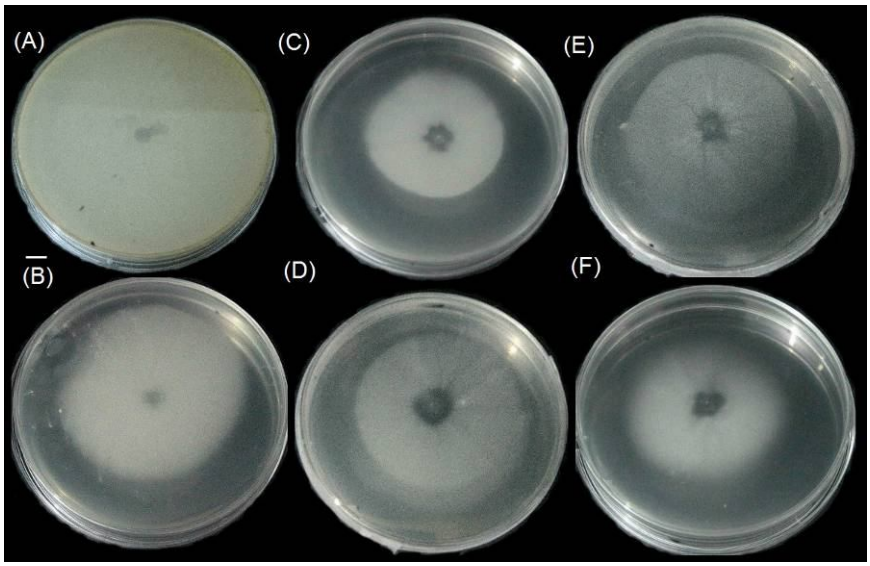

Figure 2. Anti-Quorum sensing potential of Membrane Fraction (MF) of BLB Isolates. (A) sterile distilled water (negative control), (B) 0.15 mg mL^{-1} caffeine (positive control), (C) MF of BLB-ADMU-UE-01, (D) MF of BLB-ADMU-UE-02, (E) MF of BLB-ADMU-UE-06, (F) MF of BLB-ADMU-UE-10 (Scale bar = 10 mm).

The data gathered on the Kirby-Bauer assay complements the data of the swarming motility assay for the MF. Since the strains did not have any antibacterial activity against the *P. aeruginosa*, the inhibition of swarming of the same bacteria in the swarming motility assay is not due to the destruction of the bacterial cells. Instead, the swarming inhibition is more possibly due to an inhibition of the QS-mediated swarming of *P. aeruginosa*.

Caffeine inhibited swarming and AHL production of *P. aeruginosa* according to the study of Norizan et al (2013). Hence, the MFs of these strains which were added to the swarming agar, may have interrupted the rhamnolipid synthesis of the *P. aeruginosa* either through the destruction of the autoinducers, N-butyryl-homoserine lactone, which is produced by the bacterial cells, or through the production of molecules with structures analogous to the said autoinducers (Verstraeten et al 2008). Consequently this study reports that the MFs of the said strains may contain molecules that can interrupt the AHL mediated QS-controlled systems such as swarming motility of *P. aeruginosa*.

Table 1

Swarming activity of membrane fraction and cytosolic fraction of lysates of isolated BLB from marine and freshwater hosts against *P. aeruginosa*

No.	Scientific name of host	Bacterial identification based on 16S rRNA	Swarming diameter (mm)	
			Membrane fraction	Cytosolic fraction
Negative Control	Sterile distilled water	-	80.000±0.000	80.000±0.000
Positive Control	Caffeine	-	52.067±1.550	52.067±1.550
BLB-ADMU-UE-01	*Johnius* sp.	*Photobacterium* sp.	47.333±1.041	80.000±0.000
BLB-ADMU-UE-02	*Sillago* sp.	*Photobacterium* sp.	49.833±2.021	80.000±0.000
BLB-ADMU-UE-03	*Valamugil buchanani*	Unidentified 1	80.000±0.000	80.000±0.000
BLB-ADMU-UE-04	*Chanos chanos*	*Photobacterium* sp.	54.433±0.513	80.000±0.000
BLB-ADMU-UE-05	*Cynoglossus punticeps*	*Vibrio* sp.	80.000±0.000	80.000±0.000
BLB-ADMU-UE-06	*Variola louti*	*Photobacterium* sp.	49.267±1.250	80.000±0.000
BLB-ADMU-UE-07	*Upeneus* sp.	Unidentified 2	80.000±0.000	80.000±0.000
BLB-ADMU-UE-08	*Crassostrea iredalei*	Unidentified	80.000±0.000	80.000±0.000
BLB-ADMU-UE-09	*Cardites bicolor*	*Vibrio* sp.	80.000±0.000	80.000±0.000
BLB-ADMU-UE-10	*Strombus variabilis*	*Vibrio* sp.	43.500±1.947	80.000±0.000
BLB-ADMU-UE-11	*Terebralia palustris*	Unidentified 3	80.000±0.000	80.000±0.000
BLB-ADMU-UE-12	*Cistopus indicus*	*Photobacterium* sp.	49.600±2.022	80.000±0.000
BLB-ADMU-UE-13	*Harpiosquilla anandalei*	Unidentified 4	80.000±0.000	80.000±0.000

Some potential targets of quorum quenching compounds in the *P. aeruginosa* QS system could be (a) inhibition may occur via competitive inhibition by an AHL mimic, (b) interruption of the AHL signal, (c) signal degradation by lactonases, (d) inhibition of AHL synthesis, (e) blocking of upstream regulation, and (f) interference by antisense RNA (Smith & Iglewski 2003).

The QQ potential of these isolates may be due to certain molecules such as furanones (Manefield et al 2002) present in or on the membrane of the bioluminescent bacteria and absent in the cytosolic fraction. These QS antagonists could be based on N-butyryl-homoserine lactone (C(4)-HSL) structure and cause a reduction in RhlR activity (Smith & Iglewski 2003) and RhlI, an autoinducer synthase which is important in synthesizing of C(4)-HSL needed for the production of rhamnolipids. This rhamnolipid is a biosurfactant which is significant for the swarming motility of *P. aeruginosa* (Pearson et al 1997).

In this study, the MF of the lysates of some of the bioluminescent bacterial isolates were positive for QQ activity and not the CF, since the membrane organizes selected structural and biochemical components to modulate the efficiencies of cytoplasmic biochemistries as well as a source of biological substrates that can be enzymatically manipulated to generate therapeutically relevant signaling molecules - a key target in drug discovery research (Lunn 2010). The results therefore of this study suggested that the MFs of the bioluminiscent strains may pose as candidates for novel sources of quorum quenchers against bacterial pathogens.

Molecular identification and phylogenetic analysis of the bioluminescent bacterial isolates. Based on the BLAST analysis of 16S rRNA gene sequences, ADMU-UE-01 was identified as either *Photobacterium leiognathi* strain lbind 1.1/ AY204490.1 (100% identity) or *Photobacterium mandapamensis* strain ATCC 33981(100% identity). Strains BLB-ADMU-UE-02, 04, 06, and 12 were identified as either *P. leiognathi* strain gachl1.1/ AY204488.1 (99% identity) or *P. mandapamensis* strain ATCC 33981 (99% identity). This is because *P. leiognathi* is very closely related to *P. mandapemensis*. Both species are phenotypically similar and symbionts of marine hosts. However, they can be separated by the sequence divergence of their luminescence genes *luxCDAB(F)E*; *luxF* is absent in *P. leiognathi* while present in *P. mandapamensis*.

The bioluminescent strains isolated from the gut of *Johnius* sp., *Sillago* sp., *C. chanos* and *V. louti* were closely related to the bioluminescent bacterium isolated from the head of *C. indicus.* One possible reason for their close relationship despite being found in different hosts is that these isolates may be free-living to begin with and are not

permanent symbionts of the host. Previous studies have shown that planktonic *P. leiognathi* exists between the depths of 200 to 600 m in tropical waters. Attracted by the light produced by these microorganisms, potential hosts are lured into ingesting an inoculum of free-living bioluminescent bacteria from their surrounding environment (Zarubin et al 2012; Naguit et al 2014). The ingestion of bioluminescent bacteria then allows easier acquisition of nutrients by the bacteria in the host gut. After having survived ingestion, the bioluminescent bacteria colonized the host organs and have developed a close relationship with their respective hosts due to easier access of nutrients by these bacteria.

It is also important to note that while the isolates reside in different organs of fish and cephalopod hosts, they were still found to be phylogenetically related to each other on the basis of their 16S rRNA gene sequence (Figure 3). This may be due to the similarity in the microenvironments of the different organs. Environmental conditions such as temperature, osmotic pressure, and water activity might not differ significantly among the different organs in cold-blooded, aquatic organisms. Due to probable consistency of the environmental conditions, the identities of the isolates from different organs are similar.

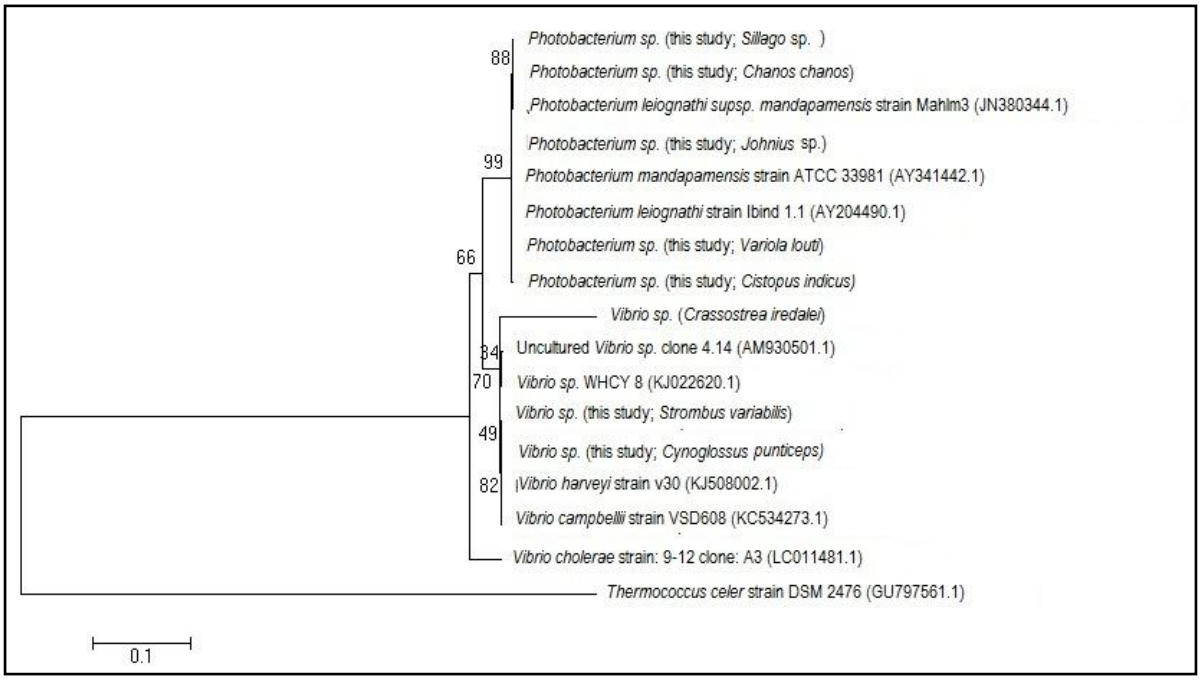

Figure 3. Molecular phylogenetic analysis of the BLB isolated from the gut of *Johnius* sp., *Sillago* sp., *C. chanos*, *V. louti*, *C. punticeps*, *C. iredalei*, *S. variabilis* and eye of *C. indicus*. The evolutionary history was inferred using the neighbor-joining method in MEGA 6. The optimal tree with the sum of branch length = 1.30015125 is shown.

However based on the BLAST analysis of 16S rRNA gene sequences of isolates, strains BLB-ADMU-UE-05 and 10 were identified as either *Vibrio campbellii* strain VSD608/ KC534273.1 (99% identity) or *Vibrio harveyi* strain v30/ KJ508002.1 (99% identity) because *V. campbellii and V. harveyi* share nearly 100% 16S rRNA gene sequence similarity hence these two species cannot be easily differentiated (Thompson et al 2007). Lastly, the strain BLB-ADMU-UE-09 was identified as either *Uncultured Vibrio sp.* Clone 4.14/AM930501.1 (85% identity) or *Vibrio sp.* WHCY8/KJ022620.1 (85%). This may indicate the possibility that this isolate is a new species of a bioluminescent bacterium. The use of a different gene marker is highly recommended.

Conclusions. Taken altogether, five bioluminescent bacterial strains from marine specifically BLB-ADMU-UE-01, 02, 06, 10, 12 and only one from freshwater which is the BLB-ADMU-UE-04 yielded lysates with QQ potential against *P. aeruginosa.* Only one isolate, strain BLB-ADMU-UE-05 showed antibacterial property against *P. aeruginosa*, but was not as effective as the positive control.

Using their respective 16S rRNA gene sequences, BLB-ADMU-UE-01, 02, 04, 06, and 12 belong to the genus *Photobacterium* (*P. leiognathi and P. mandapamensis,* 99-100% identity*)* while the BLB-ADMU-UE-05 and 10 belong to genus *Vibrio* (*V. harveyi* and *V. campbelli*, 99% identity).

This study provided the identity and diversity of bioluminescent bacteria and their associated Philippine aquatic organisms.

More significantly, this study presented the potential of bioluminescent bacteria as sources of QQ molecules which are very promising alternative to disarming pathogens without the use of antibiotics which only generates antibiotic resistant pathogens which pose a global health threat.

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Authors:
Roja Guia Angeles-Gumban, Elementary and High School Department, College of Arts and Sciences, University of the East, 105 Samson Road, Caloocan City, 1108 Philippines, e-mail: rojaguiagumban@gmail.com
Mudjekeewis D. Santos, Genetic Fingerprinting Laboratory, National Fisheries Research and Development Institute, Corporate 101 Building Mo. Ignacia Avenue corner Sct. Madriñan Street, Barangay South Triangle, Quezon City 1103, Philippines, e-mail: mudjiesantos@gmail.com
Minerva Fatimae H. Ventolero, Genetic Fingerprinting Laboratory, National Fisheries Research and Development Institute, Corporate 101 Building Mo. Ignacia Avenue corner Sct. Madriñan Street, Barangay South Triangle, Quezon City 1103, Philippines, e-mail: e-mail: mfventolero@gmail.com
Janice A. Ragaza, Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Katipunan Avenue, Quezon City 1106, Philippines, e-mail: jragaza@ateneo.edu
Ronie J. Calugay, Department of Biology, College of Science, University of the Philippines Baguio, Governor Pack Road, Baguio City 2600, Philippines, e-mail: rjcalugay@up.edu.ph

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