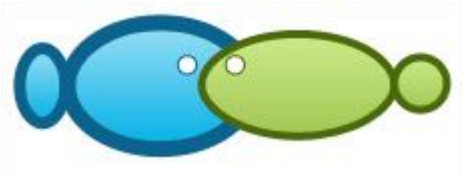


Antibiotic-resistant bioluminescent vibrios from Philippine aquacultured *Chanos chanos* and *Oreochromis niloticus*

[1]Andrianne F. Langaoen, [1]Vernon Josh V. Manzano, [1]Enrico Miguel R. Requilman, [2]Joselito M. Tabardillo, [2,3]Mary Beth B. Maningas, [1]Ronie J. Calugay

[1] Department of Biology, College of Science, University of the Philippines Baguio, Baguio City, Philippines; [2] Research Center of the Natural and Applied Sceinces, University of Santo Tomas, Manila, Philippines; [3] Department of Biology, College of Science, University of Santo Tomas, Manila, Philippines, Corresponding author: R. J. Calugay, rjcalugay@up.edu.ph

Abstract. The global contribution of the Philippines in aquaculture production has fallen steadily from 5% to only a little over 1%. The increase in the utilization of antibiotics for aquaculture health management induces selective pressure, and consequently, the emergence of antibiotic-resistant pathogens. There are virtually however very few studies on the presence of antibiotic-resistant bacteria harbored by Philippine aquacultured organisms despite the alarming consequence to Filipino consumers. To determine the presence of antibiotic-resistant bacteria in two top Philippine aquacultured fishes, bacteria were isolated through swabbing of the inside of the vitreous sac of the eyes, gills, stomach, and intestines from 10 samples each of *Chanos chanos* (milkfish) and *Oreochromis niloticus* (tilapia), purchased from an aquaculture farm in Lingayen, Pangasinan, Philippines. The obtained bioluminescent bacterial isolates designated here as strains UPB-01 to UPB-03, were isolated from *C. chanos*, and strains UPB-04 to UPB-07 isolated from *O. niloticus*, were resistant to ampicillin (10 µg). Furthermore, bacterial strains UPB-01, UPB-06, and UPB-07 were found to be multiple-drug resistant, UPB-01 was also resistant to tetracycline (30 µg) while UPB-06 and UPB-07 were also resistant to polymyxin B (300 µg). Based on 16S rRNA gene sequence analysis, the isolates were determined to be under the genus *Vibrio*. The results of this study may provide grounds for the government to thoroughly regulate the use of antibiotics in aquaculture farms for risk assessment and to ultimately protect Filipino consumers from acquiring antibiotic resistance.

Key Words: *Vibrio*, quorum sensing, tilapia, milkfish, antibiotic-resistance.

Introduction. Aquaculture, the farming of aquatic organisms in both saltwater and freshwater areas involving interventions to enhance the growth of the organisms and their population, has a large contribution to the country's food security, employment and foreign exchange earnings (Food and Agriculture Organization of the United Nations 2015) and is therefore an important asset to the national economy (Yap 1999). Based on the 2012 statistics by the Bureau of Fisheries and Aquatic Resources (BFAR), about 52.4% of total fish production came from the aquaculture sector, compared to the 26.3% and 21.4% contribution of municipal and commercial fisheries, respectively (Philippine Statistics Authority 2016). The major aquacultured species in the Philippines are milkfish, tilapia, carp, seaweeds, shrimp, oyster, and mussel (Food and Agriculture Organization of the United Nations 2015; Bureau of Fisheries and Aquatic Resources 2014b).

The expansion of aquaculture in the Philippines preceded the increase in trend of utilizing chemicals and antibiotics in managing aquatic health. Eliminating opportunistic pathogens such as vibrios (Inglis 2000) deemed necessary to promote healthy aquacultured organisms. In an attempt to prevent and avoid infectious diseases, massive use of antibiotics became prevalent (Serrano 2005). The use of antibiotics in aquacultured organisms eventually leads to the development of antibiotic-resistant

bacteria through resistance genes. These genes can be transferred vertically or disseminated through horizontal gene transfer to other bacteria and ultimately reach human pathogens (Park et al 2012).

In this study, bioluminescent bacteria were isolated to facilitate and limit the type of bacteria isolated. Bioluminescence provides the visual tracking of fish-associated bacteria during the isolation process, and hence the study also provides additional data to the very few reports on bioluminescent bacteria in Philippine aquacultured organisms (De la Peña et al 2001). Bioluminescence is the emission of visible light by an organism through a chemical reaction (Haddock et al 2010). It is exhibited by a broad range of organisms from bacteria to cephalopods, crustaceans, fish, and some terrestrial organisms (Haddock et al 2015c). Bioluminescent bacteria exhibit bioluminescence through a system called quorum sensing when a certain high population density is achieved. In this system, secreted signaling molecules or autoinducers bind to cell surface receptors, which turn on several genes which eventually express bioluminescence (Haddock et al 2015a).

Bioluminescent bacteria are mostly observed in marine organisms caught in the wild. *Vibrio harveyi* and *Vibrio fischeri* are two species of luminous bacteria that can be found in the marine environment (Bassler et al 1997). The former is predominantly a free-living organism and can also be found in the gut of its host that can co-exist with other bacterial species in many marine organisms such as penaeids, sea horses, bivalves, cephalopods, and marine teleost (Haddock et al 2010; Hashem & El-Barbary 2013). The latter is found mainly in the light organ of its host namely sepioloid and loliginid, and monocentrid fishes in which it is the only bacterial species present (Haddock et al 2010; Hashem & El-Barbary 2013). Bioluminescent bacteria are also found in terrestrial organisms, *Photorhabdus luminescens* is found in the gut of nematodes infecting insects such as caterpillars, and rarely in freshwater limpets (Lin & Meighen 2009; Haddock et al 2015b).

Because of the lack of sufficient studies on the presence of antibiotic-resistant bacteria in Philippine aquacultured organisms, this study therefore aimed at the following objectives: to isolate bioluminescent bacteria from aquacultured *Chanos chanos* (milkfish) and *Oreochromis niloticus* (tilapia), to screen the bioluminescent bacterial isolates for antibiotic resistance, and to identify the antibiotic-resistant isolates through DNA sequence analysis.

Material and Method

Collection of aquacultured fish samples. Ten samples each of *C. chanos* and *O. niloticus* from an aquaculture farm in Lingayen, Pangasinan, Philippines, were purchased from January to February 2016 and packed in sterile polybag, sealed airtight, placed in an icebox, and were transported to the Microbiology Laboratory of the University of the Philippines Baguio for further processing (Ruangpan & Tendencia 2004; Eze et al 2011).

Isolation and purification of bioluminescent bacterial isolates. The inside of the vitreous sac of the eyes, gills, stomach, and intestine of the fish samples were swabbed with sterilized cotton swab and streaked onto luminescent agar (American Type Culture Collection 2014). The inoculated plates were wrapped in sterile paper and incubated at room temperature. After 8-12 hours, the plates were checked for the growth of bioluminescent colonies. Bioluminescent bacteria were isolated to limit the type of bacterial isolates. The colonies were purified using standard microbiological streak plate method (Singh et al 2011).

Screening of antibiotic resistance. The isolates were screened for antibiotic resistance using Kirby-Bauer disk diffusion method in triplicates. Pure cultures of bioluminescent bacteria were inoculated in nutrient broth and incubated at 35^{o}C until turbidity of 0.5 McFarland standard is achieved (Coyle 2005) with approximate cell density of 1 x 10^{8} CFU mL^{-1}. Fifty microliters of bacterial suspension per isolate were inoculated on Mueller-Hinton agar (MHA). Inoculation was done by streaking an inoculating loop in a back and forth

motion very close together as it is streaked across and down the plate (Hudzicki 2013). The MHA used had a pH between 7.2-7.4 and contained beef extract powder, an acid hydrolysate of casein, starch, and agar (Neogen Corporation 2011). The antibiotics tetracycline (30 µg), kanamycin (30 µg), polymyxin B (300 µg) and ampicillin (10 µg) were tested for the bacterial resistance test with sterile distilled water as the negative control (Inglis 2000; Faruk et al 2008). The isolates were determined to be antibiotic-resistant by comparing the mean diameter of zone of inhibition of each bacterial strain from the different antibiotics based on the Zone Diameter Interpretative Standards (French Society of Microbiology 2001), in which for tetracycline (TE; resistance: < 17 mm), ampicillin (A; resistance: < 14 mm), kanamycin (K; resistance: < 10 mm) and polymyxin B (PB; resistance: < 15 mm).

Genomic DNA extraction. Extraction of genomic DNA from the bioluminescent bacteria isolates was done through the Xanthogenate method (Tillett & Neilan 2000). Ten milliliters of each bioluminescent bacterial isolate grown overnight was harvested by centrifugation at 4000 rpm for 3 minutes. The supernatant was removed after centrifugation and the pellet was resuspended in 600-µL XS buffer. The mixture was transferred to a 1.5 mL microcentrifuge tube before incubation at 70°C for 30 minutes. The mixture was vortexed for 10 seconds before incubation on ice for 30 minutes. After incubation, the mixture was centrifuged at 14000 g for 10 minutes. The supernatant was transferred into a new microcentrifuge tube before addition of 750 µL of 100% isopropanol. The mixture was incubated at room temperature for 5 minutes before centrifugation at 14000 g for 10 minutes. The supernatant was discarded. One mL of 70% ethanol was added to the pellet. The mixture was once again centrifuged at 14000 g for 10 minutes. The supernatant was discarded again and the pellet was air-dried before resuspension in 50 µL TE buffer.

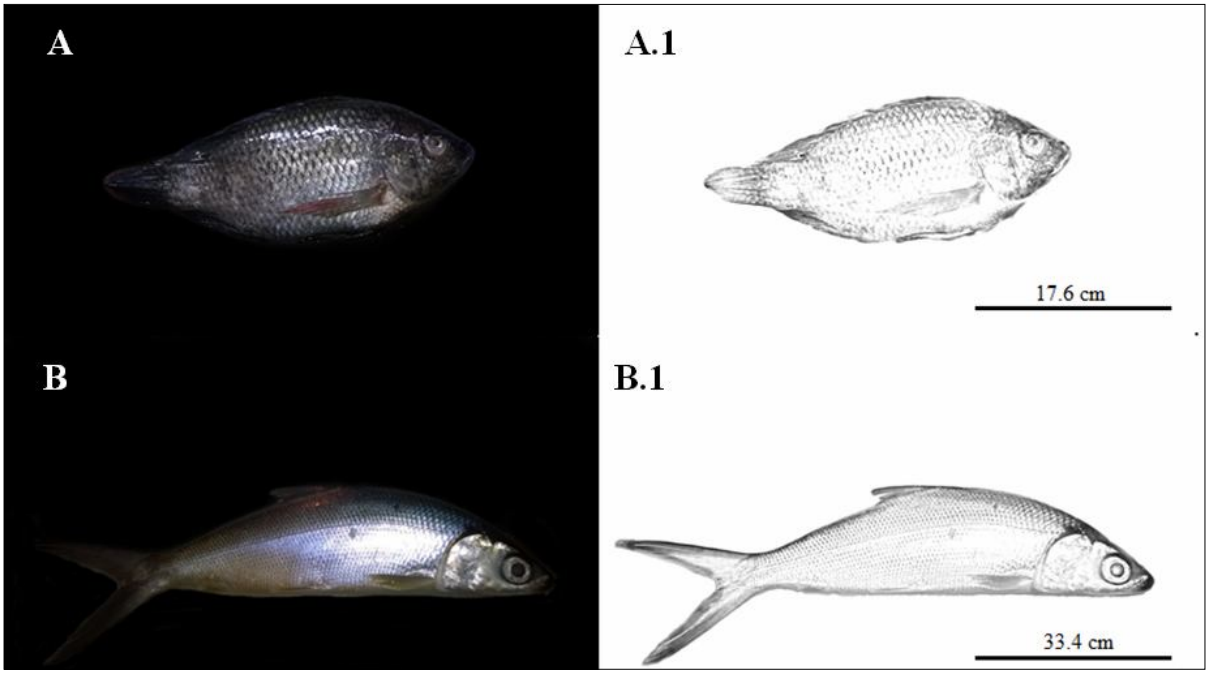
Amplification of 16S rRNA gene. The extracted genomic DNA from the bioluminescent bacterial isolates were subjected to Polymerase Chain Reaction (PCR). The universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT-3') were used to amplify the 16S rRNA gene (Abulencia et al 2006). The PCR cocktail consisted of 1X PCR Buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM forward primer, 0.5 µM reverse primer, 0.5 U μL^{-1} Taq polymerase, and 1 ug DNA template. PCR was done under the following thermal cycling conditions for 20 cycles: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 4 minutes.

Molecular identification and phylogenetic analysis. The PCR products were sent to the sequencing service laboratory of Macrogen Incorporated in Seoul, Korea. The chromatogram editing of the forward and reverse sequences was performed using the software Chromas (Goodstadt & Ponting 2001). Sequence assembly and chimera identification were performed using the CodonCode Aligner and Database Enabled Code for Ideal Probe Hybridization Employing R (DECIPHER), respectively (Huang & Madan 1999; Wright et al 2012). The edited, non-chimeric sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) algorithm. A phylogenetic tree that indicates the relationships of the isolates with previously known bioluminescent bacteria was constructed using the Neighbor-Joining method in the Molecular Evolutionary Genetics Analysis (MEGA) 6 software (Tamura et al 2013). The sequences of related species were obtained from the public database of GenBank.

Results

Isolation of bioluminescent bacteria from aquacultured fish samples. Three bioluminescent bacterial strains were isolated from *C. chanos,* designated here as strains UPB-01 to -03, and 4 strains from *O. niloticus* designated here as strains UPB-04 to -07 (Figure 1). All strains exhibited bioluminescence (Figure 2), which helped in limiting the

number and types of bacteria isolated and in the purification process. All strains were



tested for antibiotic resistance using the Kirby-Bauer disk diffusion assay.

Figure 1. The aquacultured fish species sampled. (A) *Oreochromis niloticus* (tilapia) and (B) *Chanos chanos* (milkfish) harbored bioluminescent bacteria. The sketches of the aquaculture fishes were also included (A.1 and B.1).

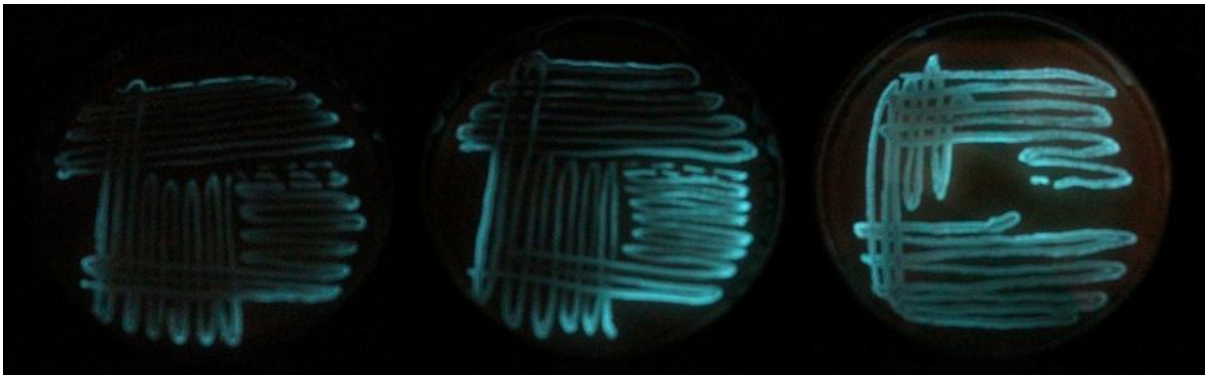


Figure 2. Bioluminescent bacterial swab samples from aquacultured fishes. From left to right, strain UPB-02 from *Chanos chanos* (milkfish); strains UPB-05 and UPB-07 from *Oreochromis niloticus* (tilapia).

Screening for antibiotic resistance of isolated biolumonescent bacteria. All seven isolates exhibited antibiotic resistance (Figure 3). Furthermore, bacterial strains UPB-01, UPB-06, and UPB-07 were found to be multiple-drug resistant. These strains were not only resistant to ampicillin but isolate UPB-01 was also found to be resistant to tetracycline while UPB-06 and UPB-07 were also resistant to the antibiotic polymyxin B. These results were determined by comparing the mean zone diameter of inhibition (Figure 4) of each bacterial strain from the different antibiotics based on the Zone Diameter Interpretative Standards (French Society of Microbiology 2001). The Venn diagram summarizes the Kirby-Bauer disk diffusion assay result to show isolates which were multiple-drug resistant (Figure 5).

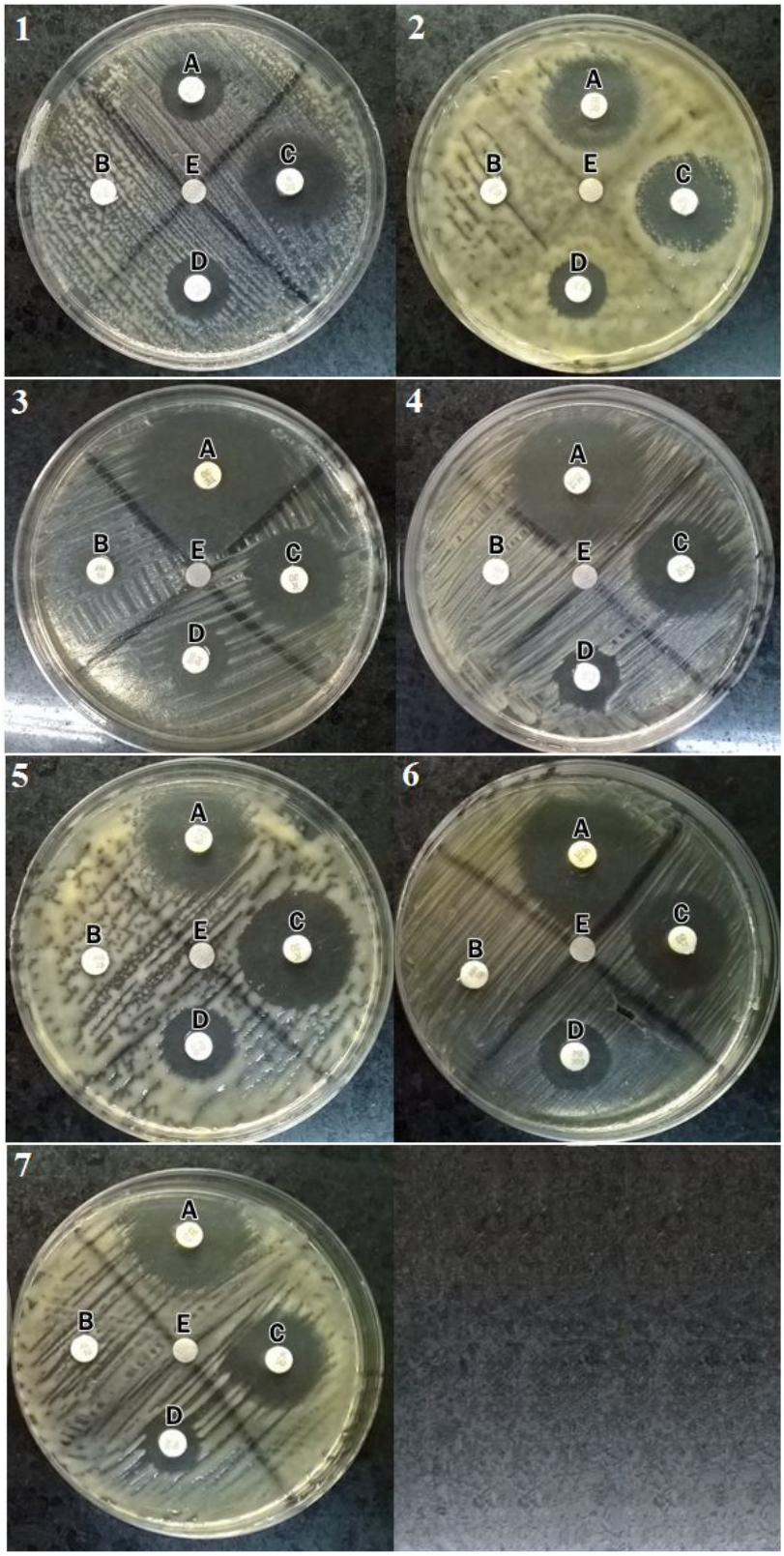


Figure 3. Kirby-Bauer disk diffusion assay plate samples of bioluminescent bacterial strains. The assays were performed in triplicates. Strain UPB-01 (1), UPB-02 (2), UPB-03 (3) were isolated from *C. chanos* (milkfish), and strain UPB-04 (4), UPB-05 (5), UPB-06 (6), and UPB-07 (7) were isolated from *O. niloticus*. A = tetracycline (30 µg), B = ampicillin (10 µg), C = kanamycin (30 µg), D = polymyxin B (300 µg), E = distilled water (negative control).

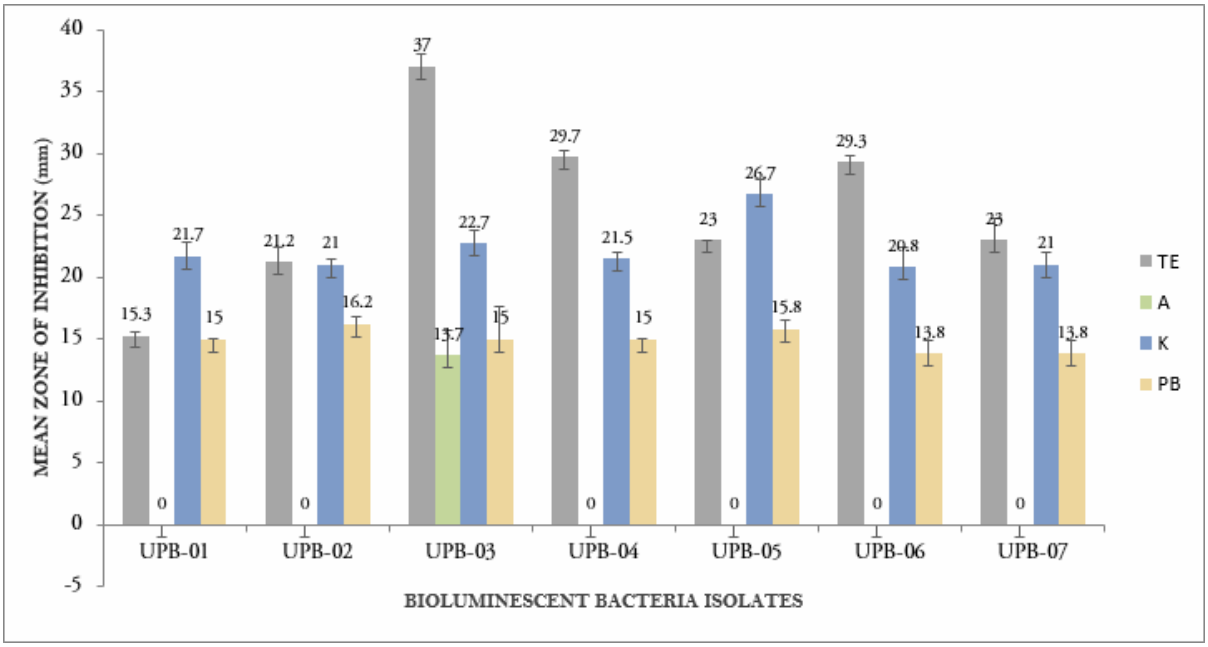


Figure 4. Measured mean diameter (in mm) of zones of inhibition per bacterial isolate. Resistance is based on the Zone Diameter Interpretative Standards (French Society of Microbiology 2001). For tetracycline (TE; resistance: < 17 mm), ampicillin (A; resistance: < 14 mm), kanamycin (K; resistance: < 10 mm) & polymyxin B (PB; resistance: < 15 mm).

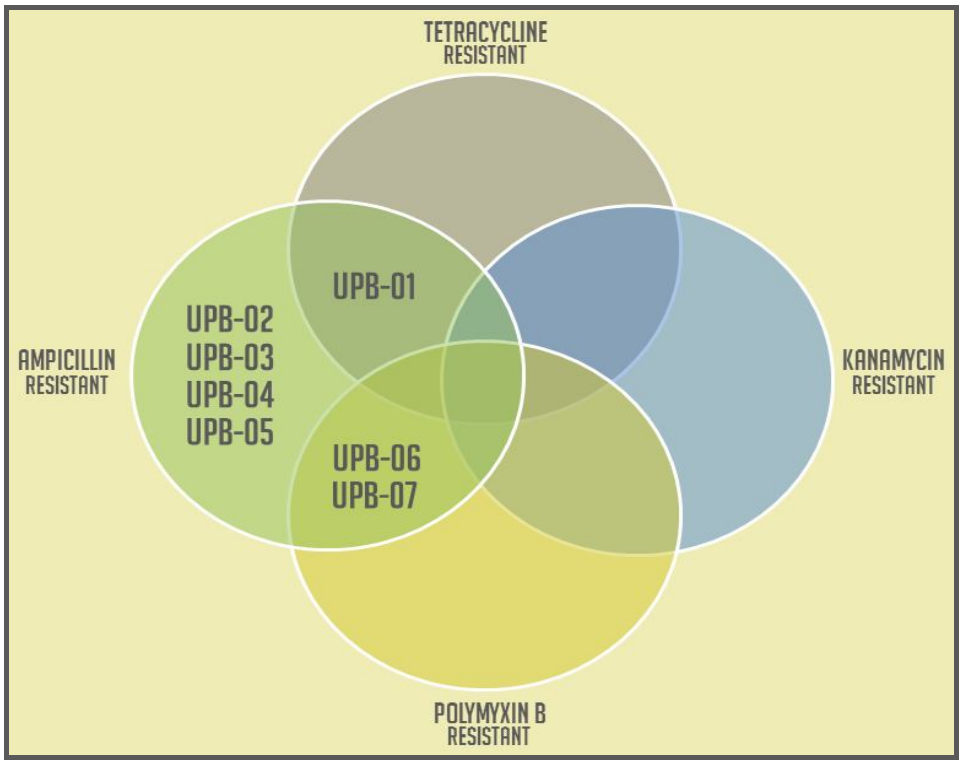


Figure 5. Venn diagram showing antibiotic-resistant bioluminescent bacterial isolates from Philippine aquacultured *C. chanos* and *O. niloticus*. Strains UPB-02 to -05 were found to be ampicillin resistant and strains UPB-01, UPB-06 and UPB-07 were resistant to both tetracycline and polymyxin B.

Molecular identification and phylogenetic analysis. The 16S rRNA genes of all the seven bioluminescent bacterial strains were sequenced. However, when the edited forward and reverse sequences of strain UPB-05 were assembled in CodonCode Aligner, no consensus sequence was formed. When the edited forward and reverse sequences of UPB-05 were subjected to BLAST, the result showed similarity to the *Vibrios* sp. The

other six isolates were successfully sequenced and were subjected to molecular analysis. Chimera analysis was also performed. Using DECIPHER, the sequences were revealed to be not chimeric. A phylogenetic tree was constructed by using the Neighbor-Joining method in MEGA6 and incorporating the related species, whose sequences were obtained from the public database of GenBank, to the tree. The molecular analysis of the six isolates revealed that the isolates all belong under the genus *Vibrio* (Figure 6). In particular, the isolates UPB-06, UPB-01 and UPB-02 were determined to be related to each other and to the strains *Vibrio neocaledonicus* CZN-17 (GenBank accession number: KR347255.1, max. identity = 100%), *V. campbellii* CAPL_B_07 (GenBank accession number: KT375318.1, max. identity = 100%), *V. harveyi* KVH (GenBank accession number: KU951243.1, max. identity = 100%), and *V. alginolyticus* CHB-14 (GenBank accession number: KR347282.1, max. identity = 100%). The closest match for strain UPB-07 was *V. harveyi* strain HM-10 (GenBank accession number: KX156952.1, max. identity = 100%) and *V. parahaemolyticus* strain CHB-40 (GenBank accession number: KR347297.1, max. identity = 100%). UPB-03 and UPB-04 are clustered together with *V. azureus LZ8* (GenBank accession number: KX037099.1, max. identity = 100%), *V. rotiferianus* MCCB 283 (GenBank accession number: KR921928.1, max. identity = 100%), *V. owensii* t11_2b (GenBank accession number: KU725826.1, max. identity = 100%), and *V. natriegens* strain CZN-19 (GenBank accession number: KR347257.1, max. identity = 100%), with *V. azureus* LZ8 being the closest to them.

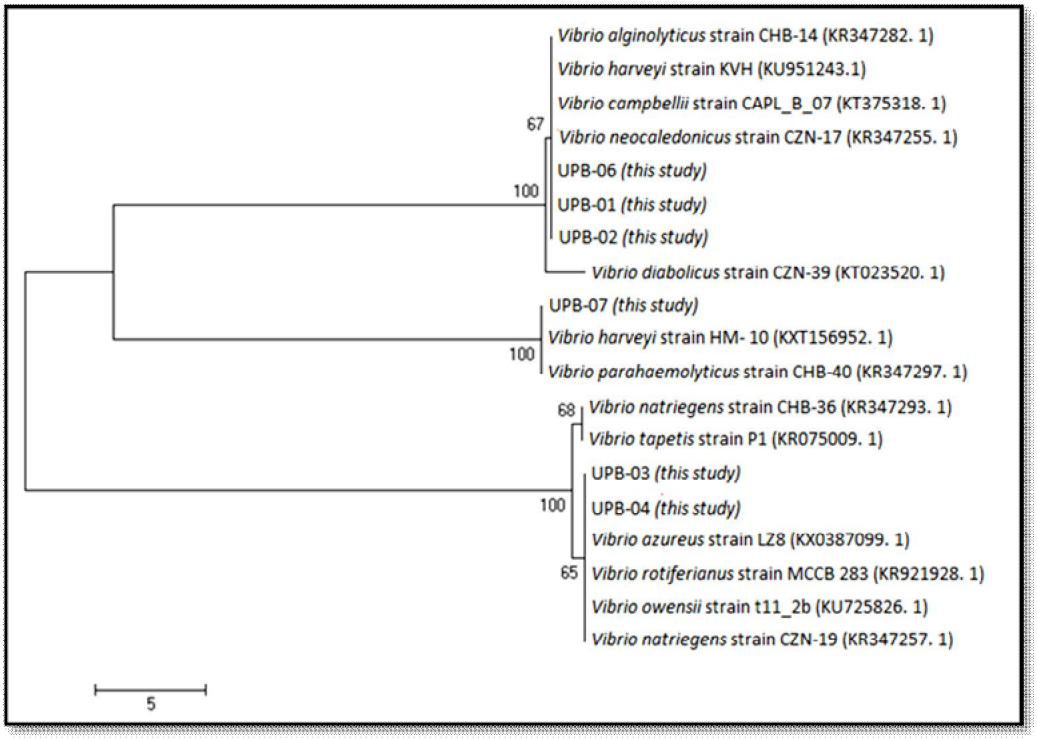


Figure 6. Molecular phylogenetic analysis of the bioluminescent bacterial isolates obtained from aquacultured *C. chanos* and *O. niloticus*. The evolutionary history was inferred using the Neighbor-Joining method.

Discussion. The Philippines has been known as one of the top exporter of aquaculture yields in Asia. The intensified aquaculture competition in the production has led to the indiscriminate use of antibiotics for maintaining aquaculture health. One consequence of inflicting antibiotic selective pressure to fish-associated bacteria is the emergence of antibiotic-resistant bacterial strains.

All of the seven bacterial isolates in this study initially exhibited bioluminescence, which helped in selecting and limiting the number and type of bacteria isolated during the purification process. One of the common groups of fish-associated bacteria are under the genus *Vibrio* under which are some of the bioluminescent bacterial groups (Zarubin et al 2012; Molina et al 2016; Angeles-Gumban et al 2017). Hence bioluminescence provides

the visual tracking of fish-associated bacteria during the purification process. The farm operators where the fish samples were collected declined to disclose the type of antibiotic used in the farm. The antibiotics tetracycline, ampicillin, kanamycin, and polymyxin, were utilized as assay disks for the succeeding antibiotic screening in this study. Tetracycline was used because it inhibits protein synthesis in a broad spectrum of Gram-negative and Gram-positive bacteria. Ampicillin was used to disrupt the cell membrane synthesis in Gram-positive and Gram-negative bacteria. Almost all bioluminescent bacteria share a common morphology of being Gram-negative and in view of this, the antibiotics kanamycin and polymyxin B were utilized because of their antibiotic effectiveness against many Gram-negative bacteria in broad-spectrum and narrow-spectrum, respectively.

All strains exhibited antibiotic resistance, specifically to ampicillin. Furthermore, bacterial strains UPB-01, UPB-06, and UPB-07 were found to be multiple-drug resistant. These strains are not only resistant to ampicillin but UPB-01 was also found to be resistant to tetracycline while UPB-06 and UPB-07 were also resistant to the antibiotic polymyxin B. These results were determined by comparing the zone of inhibition of each bacterial strain from the different antibiotics with the Zone Diameter Interpretative Standards (French Society of Microbiology 2001). The 16S RNA gene of all of the strains (except UPB-05) were sequenced and subjected to molecular analysis. The phylogenetic analysis of the isolates revealed the formation of three clades.

The 16S rRNA gene sequence of the isolates UPB-06, UPB-01, and UPB-02 revealed that they are related to each other and to the strains *Vibrio neocaledonicus* CZN-17, *V. campbellii* CAPL_B_07, *V. harveyi* KVH, and *V. alginolyticus* CHB-14. The aforementioned isolates and vibrios constituted Clade 1. The closest match for strain UPB-07 was revealed to be *V. harveyi* strain HM-10 and *V. parahaemolyticus* strain CHB-40; these bacteria are grouped under Clade 2. UPB-03 and UPB-04 are found to cluster together with *V. azureus* LZ8, *V. rotiferianus* MCCB 283, *V. owensii* t11_2b, and *V. natriegens* strain CZN-19 in Clade 3 with *V. azureus* LZ8 being the closest to the isolates.

Vibrio species *V. harveyi, V. alginolyticus, V. parahaemolyticus, V. campbellii, V.rotiferianus, V. natriegens, V. azureus* and *V. owensii* which are present across the three clades are also known to be members of the established *Vibrio harveyi* clade wherein their common characteristic lies on their affinity and preference for habitat with high salinity (Ruwandeepika 2012; Ceccarelli & Colwell 2014; Ke et al 2017). Clade 1, which includes strains UPB-01, UPB-02 and UPB-06 are found to cluster together with *V. harveyi* and *V. campbellii,* both are well recognized as pathogens of aquatic organisms (Dong et al 2017; Ke et al 2017), and *V. alginolyticus*, an emerging pathogen associated with foodborne illness (Mustapha et al 2013; Citil 2015).

Strain UPB-07 was clustered under Clade 2, was found to be related to the pathogenic vibrioid *V. parahaemolyticus*, which commonly causes seafood-associated gastroenteritis which can lead to abdominal cramping, nausea, diarrhea, vomiting, chills and fever when ingested (Raszl et al 2016; Wang et al 2016). In some cases, vibrio infection can cause severe illness with chronic medical disease and may sometimes require hospitalization (Daniels & Shafaie 2000; Wang et al 2016; Letchumanan et al 2017). People become infected with vibriosis by consuming raw or undercooked seafood or by exposing an open wound to infected water (Centers for Disease Control and Prevention 2016; Hoi et al 2017). Strains UPB-03 and -04 in Clade 3 were found to be related to a pathogen of aquatic animals specifically *V. owensii* (Daniels & Shafaie 2000; Goulden et al 2012; Ke et al 2017).

Conclusions. In this study, the Philippine aquacultured *C. chanos* and *O. niloticus*, collected in Lingayen, Pangasinan, which is one of the major sources of aquacultured organisms in the northern region of the country, were discovered to harbour bacteria resistant to the antibiotic ampicillin, with strains UPB-01, UPB-06, and UPB-07 being multiple-drug resistant which are also resistant to tetracycline and polymyxin B. Based on 16S rRNA gene sequence analysis, the isolates were determined to be under the genus *Vibrio* which consists of free-living and pathogenic species.

Such presence of antibiotic resistance in the said aquaculture is the consequence of the selective pressure imposed upon by the use of antibiotics in aquaculture farms

over a period of time. Acquired resistance can spread across bacterial populations vertically, when bacteria inherit antibiotic resistance genes, and horizontally, when bacteria exchange genetic materials with other bacteria through conjugation. This creates an alarming consequence for human consumers, as the human gut flora is vulnerable to acquire antibiotic resistance genes and can lead to the alteration of gut flora in such a way that the susceptibility of a person to bacterial infections increases significantly.

To date, this study is one of the pioneering works that reports on the presence of antibiotic-resistant bacteria in Philippine aquacultured *C. chanos* and *O. niloticus*. This study may provide grounds for the government to thoroughly regulate the use of antibiotics in aquaculture farms for risk assessment, ultimately protecting the Filipino consumers from acquiring antibiotic resistance.

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Authors:
Andrianne F. Langaoen, Department of Biology, College of Science, University of the Philippines Baguio, Governor Pack Road, Baguio City 2600, Philippines, e-mail: aflangaoen@up.edu.ph
Vernon Josh V. Manzano, Department of Biology, College of Science, University of the Philippines Baguio, Governor Pack Road, Baguio City 2600, Philippines, e-mail: gerund16@gmail.com
Enrico Miguel R. Requilman, Department of Biology, College of Science, University of the Philippines Baguio, Governor Pack Road, Baguio City 2600, Philippines, e-mail: migsrequilman@yahoo.com
Joselito M. Tabardillo, Research Center of the Natural and Applied Sciences, University of Santo Tomas, Espana str., Sampaloc, Manila 1015, Philippines, e-mail: jratabardillo@gmail.com
Mary Beth B. Maningas, Department of Biology, College of Science, University of Santo Tomas, España str., Sampaloc, Manila 1015, Philippines, e-mail: marybethmaningas@gmail.com
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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