Short Communication

Reduction of Antibiotic-Resistant Bacterial Counts in a Mangrove-Riverine System in Capiz, Philippines - Preliminary Data

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Key words: antibiotic resistance, mangrove riverine system

Abstract
The anthropogenic impact of effluents and indiscriminate use of antibiotics in aquaculture promotes the emergence and proliferation of antibiotic resistant (ABR) bacteria. Comparative total bacterial and ABR counts were determined from 3 locations of the Cadimahan river of Capiz province, Philippines. The upstream station showed lower total bacterial but higher ABR counts, which is the inverse of the downstream station which showed higher total bacterial but lower ABR counts. The ABR counts accounted for 0.0173%, 0.0043% and 0.0002% of the total bacterial counts for upstream, midstream, and downstream, respectively, showing a strong inverse correlation of -0.84 in the Pearson correlation coefficient. This result suggests a remediating effect potentially mediated by microbial dynamics in the Mangrove-riverine system.

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Introduction

CoVID-19 has shown how devastating a global pandemic can be to loss of lives, global economic output, and productivity (Turner, 2014). One potential cause of a pandemic may be the resurgence of highly pathogenic bacteria due to antibiotic resistance (ABR) (Anderson, 1999); a subset of antimicrobial resistance (AMR), which has been declared by the World Health Organization as one of the top 10 global public health threats facing humanity ("Antimicrobial resistance," n.d.). Anthropogenic impact of effluents from human habitation and human activities like aquaculture are some of the sources of antibiotics in the environment which in turn drive up occurrence of ABR (Tipmongkolsilp et al., 2012; Zdanowicz et al., 2020; Canteón, 2009).

In aquaculture, antibiotics has been used to treat diseases, optimize feed efficiency and improve growth rate (Sarmah et al., 2006). Majority of the antibiotics introduced in aquaculture are not assimilated by the culture organisms and are released into the environment (Thuy et al., 2011). The presence of antibiotics below lethal concentrations promotes resistance while changes in the bacterial community results in the alteration of the biogeochemical cycles (Balcázar et al., 2015). From an environmental perspective, aquatic ecosystems are major reservoirs of resistant bacteria and genes because of the uncontrolled use of antibiotics and lack of treatment facilities for effluents (Michael et al., 2014); however, a healthy ecosystem may attenuate the presence of these antibiotic resistant microorganisms (Levy, 2007) by restoring the competitive balance between them and non-resistant microorganisms.

Mangrove forests are important coastal resources that play a major role in socio-economic development (Kathiresan, 2012), mitigation of disasters (Osti et al., 2009) and protection of biodiversity (Mmom, 2010). The constant change in environmental factors such as salinity, temperature, and pH, leads to the extreme diversification of microbial communities in mangrove areas. Thus, knowledge of microbial activity is important for understanding the dynamics of mangrove ecosystems and the formulation of effective management strategies (Holguin et al., 2006). The unique composition of mangrove estuaries allows it to act as catch basins for anthropogenic wastes without suffering disturbance in its fundamental structure and functions (Bouchez et al., 2013). Toxic chemicals that are released into estuaries endanger coastal ecosystems and human health. Besides conventional pollutants such as organochlorine, organophosphates, and heavy metals, antibiotics and antibiotic resistant genes (ARGs) are also identified as contaminants that affect stability of coastal ecosystems and have become a serious issue (Zhang et al., 2015)(Zhu et al., 2017).

The Cadimahan-Libutong river covers approximately sixteen hectares of area, surrounded by seven barangays (an administrative division in the Philippines equivalent to a village, district, or ward). It is recognized as one of the biggest among the sixteen river systems in Capiz province. Aquaculture in the form of fish cages and fishponds are prevalent. In the Capiz province, total Aquaculture in 2019 was tagged at 62.78 thousand MT (*CAPIZ FISHERY PRODUCTION IN 2019*, 2020). Local conservation and reforestation of the mangrove area has also been a highlight of the river system (Primavera et al., 2012).

It is universally acknowledged that ABR bacteria are present in the natural environment. Conditions in aquatic ecosystems promote the acquisition and dissemination of ARGs (Zhu et al., 2017). The presence of these contaminants in the Cadimahan-Libutong river would suggest a fundamental, functional, and spatiotemporal shift in the microbial community. The main objectives of the current study are to quantify the presence of ampicillin-resistant bacteria in the Cadimahan-Libutong river as an indicator and a proxy for ABR bacteria and to explore spatial dynamics between total and ABR bacteria.

Materials and Methods

The study was conducted along the Cadimahan River, Capiz Province, Panay Island, Republic of the Philippines on October 2019. Sampling was conducted only once for gathering preliminary field data to determine antibiotic resistance in the area. Three

sampling points collected mid-morning during low tide were selected, representing the upstream, midstream and downstream regions of the river (**Figure 1**). The Upstream (US) point was located at 11°35'37.6" N 122°44'28.1" E; Mid-stream (MS) was 11°35'47.5" N 122°43'57.8" E; Downstream (DS) was 11°36'04.0" N 122°43'4104" E.

Water samples were collected and stored on ice. For each sample point, salinity, temperature, pH and dissolved oxygen concentrations were recorded. A 9 mL aliquot of the water was immediately treated with 1ml of antibiotic solution of 5ug/ml ampicillin (ABPC) for a total concentration of 0.5ug/ml ABPC. Ampicillin was chosen as the proxy for studying antibiotic resistance because it is commonly used in the area, it is a broad-spectrum antibiotic and is stable against hydrolysis. An untreated sample was collected and stored in the same conditions (**Figure 2**).

Modified marine nutrient agar was formulated using 1.5% agar in nutrient medium composed of polypeptone 5g/L and yeast extract 1g/L in UV treated seawater. The medium was autoclaved for sterility and poured on petri plates. Dried plates were stored in 4°C until use.

Samples were serially diluted and spread out on prepared modified agar plates in triplicates. The development of bacterial colonies was monitored for a 7-day period. Individual bacteria were not further examined; only collective microbial content for both antibiotic-treated and untreated samples were considered. The ABPC-resistant count was divided by 0.9 to compensate for the introduction of antibiotics.

Results were parsed through a Pearson correlation coefficient test (Pearson, 1895) which measures the strength of the relationship between two variables and their association with each other.
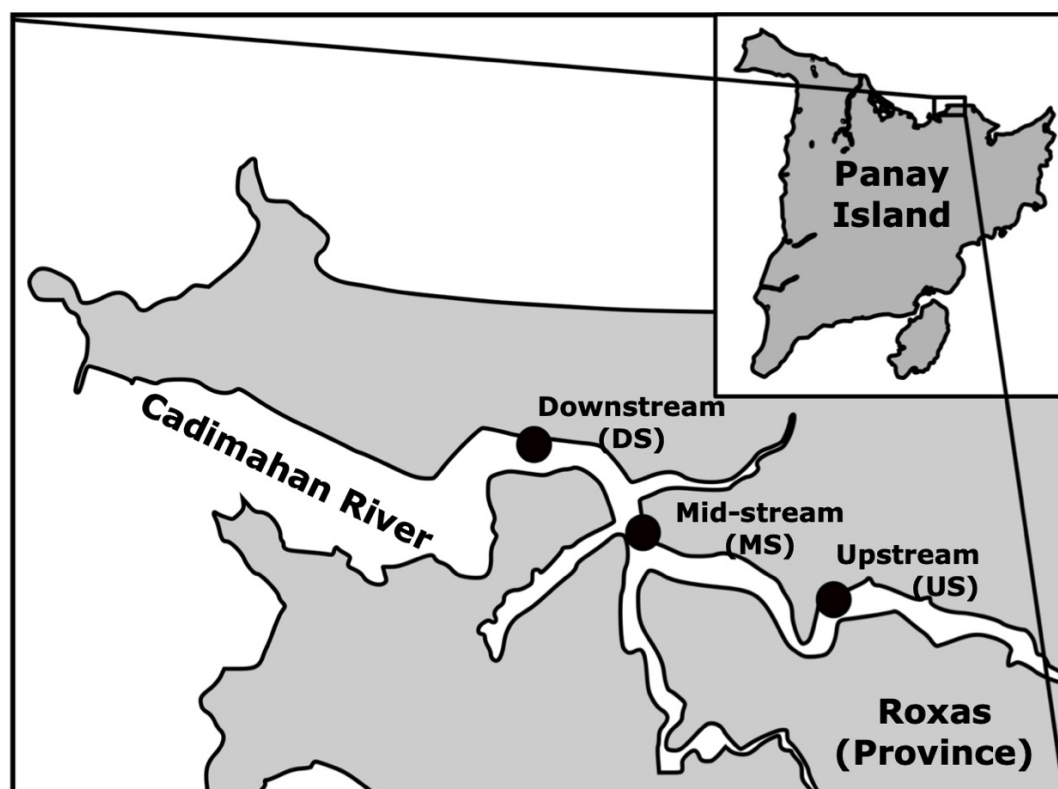

Figure 1 Map of the sampling location. Three locations were sampled in Cadimahan River, Roxas Province, Panay Island, Republic of the Philippines. The Upstream (US) point was located at 11°35'37.6"N 122°44'28.1"E; Mid-stream (MS) was 11°35'47.5"N 122°43'57.8"E; Downstream (DS) was 11°36'04.0"N 122°43'4104"E.
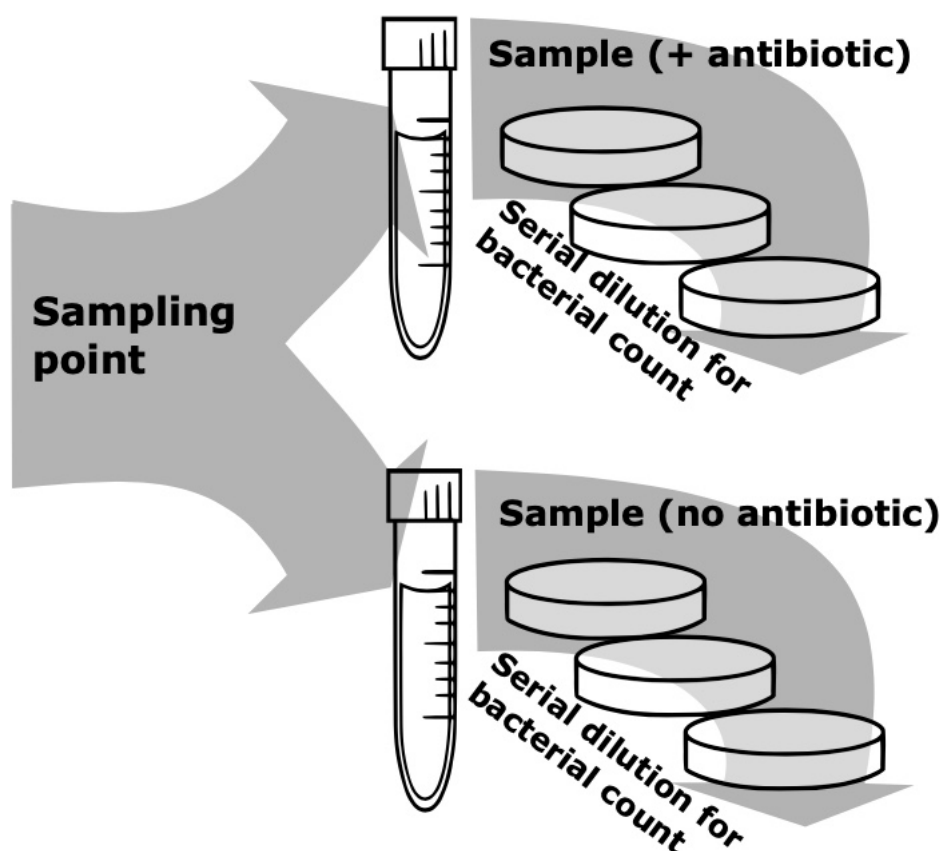
Figure 2 Sampling method. Two samples were retrieved from each sampling point. One sample was treated with an antibiotic, another sample was not. Samples were serially diluted to quantify the viable bacterial count.

Results

The Cadimahan-Libutong River is a sedimentary environment characterized by dark fine to muddy substrates. It is an intertidal mangrove system suggesting that there are frequent fluctuations in temperature, salinity, dissolved oxygen, and other physico-chemical parameters. The 6 genera of mangroves found in the riverine ecosystem are Rhizophora, Avicennia, Bruguiera, Excoecaria, Sonneratia, and Nypa. The area is under anthropogenic pressure due to various human activities. In the upstream station, there is an open drainage system coming from 2 densely populated residential areas with no provisions for the treatment of human wastes and wastewater. The midstream station was predominantly used for the culture of oysters and brackish water fish species. Culture of marine fish species and artisanal fishing activities were observed more in the downstream station. The presence of ABPC-resistant bacteria was established in all three stations.

Water temperature was uniform at 32^0C in all three sampling stations. There was a difference in salinity, as expected. Lowest salinity was taken upstream at 22 ppt followed by 26 ppt midstream and 28 ppt downstream. Upstream and midstream stations had the same pH level at 7.5 while the pH level downstream was 7.7. A decreasing trend was observed in terms of dissolved oxygen concentrations with the highest being 4.92 ppm upstream, followed by 4.76 ppm midstream and 4.23 ppm downstream.

Total viable bacterial count increased from 8.0×10^7 to 1.8×10^8 then to 1.0×10^9 as the sampling points progressed downriver while antibiotic resistant bacterial viable counts decreased from 1.5×10^4 to 7.7×10^3 then to 2.2×10^3 (**Figure 3**). This is equivalent to 0.0173%, 0.0043% and 0.0002% of the total viable count for upstream, mid-stream, and

downstream, respectively. There was a strong inverse correlation (-0.84 in the Pearson correlation coefficient) between total viable bacteria count and antibiotic bacteria viable count as the sampling point progressed downriver. The total viable bacterial count had a trendline with a slope of $2.3 \times 10^7 \exp(+1.204x)$ with an R^2 of 0.943. ABPC had a slope of $4.5 \times 10^4 \exp(-0.973x)$ with an R^2 of 0.973.
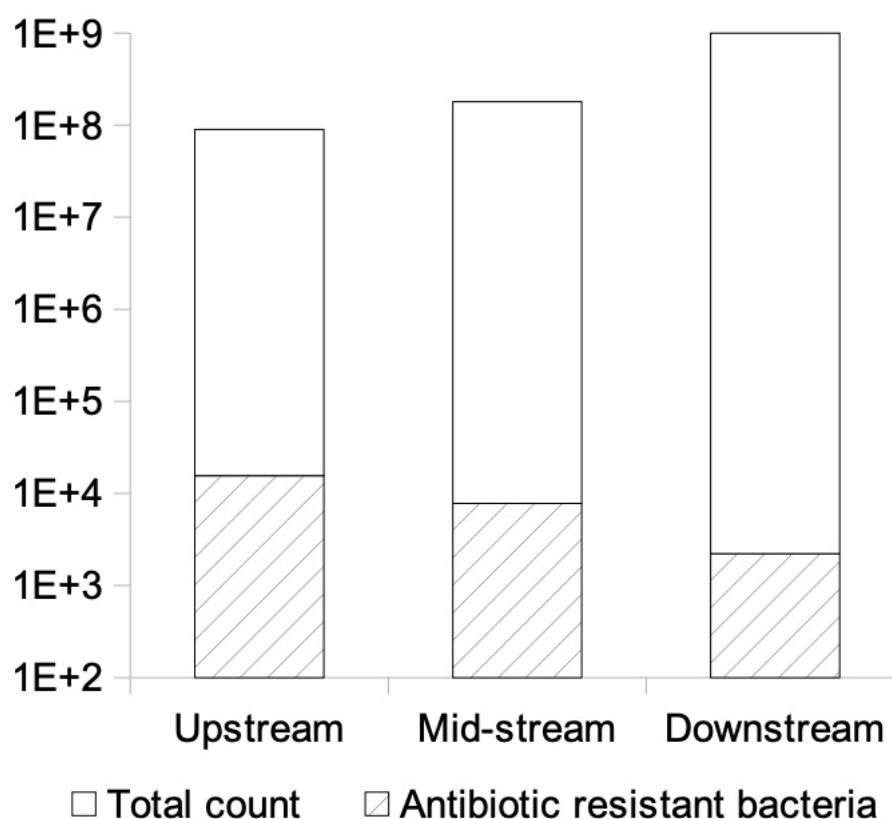

Figure 3 Total bacterial counts (cfu/ml) and antibiotic resistant bacterial counts (cfu/ml) from three locations: Upstream (US), Mid-stream (MS), and Downstream (DS). Values are in logarithmic scale.

Discussion

Although mangrove ecosystems are potential reservoirs for antibiotic resistant genes (Thuy et al., 2011), the rich and diverse microbial ecosystem (Gomes et al., 2011; Thatoi et al., 2013) may also serve to attenuate total numbers of these antibiotic resistant bacteria (Levy, 2007) that harbor these genes through a competitive balance between them and non-resistant microorganisms.

In the present study we have established the presence of Ampicillin resistant bacteria in upstream, midstream and downstream areas of the Cadimahan River. The river is a depositional coastal environment characterized by presence of fine sediments with accumulation of organic materials coming from sewage and fish cage operations. Sediments in mangrove communities consist of river and marine alluvium deposit resulting in dark, silty-clay with high organic matter and salts (Hossain and Nuruddin, 2016). The river previously had a problem with heavy siltation caused by the congestion of structures, such as fish cages, gill nets and bamboo stakes for oyster culture, that obscured the navigational paths and water flow. A rehabilitation effort was pushed for the clearing of the

river and the banning of the use of bamboo stakes. This rehabilitation effort also introduced mangrove reforestation in certain areas (Bering, 2010).

The presence of antibiotic resistance genes has been reported in mangrove systems (Ceccon et al., 2019; Imchen and Kumavath, 2020), but no spatio - temporal relationships have been demonstrated to surmise the role of mangrove riverine systems on the variance of antibiotic resistance bacteria. A study of microbial communities in a non-mangrove river estuary in Malaysia showed elevated levels of antibiotic resistance ESBL-producing bacteria in upstream portions of the river. However, the abundance of culturable bacteria were also elevated in the upstream portion and no correlation was observed in terms of abundance and location (Ho et al., 2021).

Other reported studies focus on ARGs on mangrove systems employed a metagenomics approach and focused on coastal and estuarine locations. In Kerala, South India no significant pattern of distribution of antibiotic resistant genes were found based on location (Imchen et al., 2019) and in the Gaoqiao Mangrove Wetland China the authors found no significant difference in antibiotic resistant bacteria based on location (Y et al., 2017). Variations in the distribution of antibiotic resistance may be due to a combination of factors such as temperature, tidal flow, surface runoff, agriculture and aquaculture wastes and other anthropogenic activities (Huang et al., 2019).

Since other riverine systems do not display these patterns, the currently observed reduction of Antibiotic resistant bacteria may be indicative of the remediation effect suggested for mangroves (Bouchez et al., 2013). However, more detailed analyses need to be done to the tested site and to other locations with varying spatio temporal dynamics to more fully understand the interaction of microbial communities in mangrove systems.

One of the problems in monitoring and detecting occurrence of antibiotic resistant bacteria is cost and accessibility. The method employed is a composite of the standard turbimetric assay (Kavanagh, 1968; Nascimento et al., 2020), in that the antibiotic was applied in broth, but quantified using spread plating method. It is a field expedient, rapid, high throughput, low-cost approach that has low equipment requirements. It is not meant to be a replacement for detailed and stringent approaches such as RAST (Rapid Antimicrobial Testing (RAST) recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Åkerlund et al., 2020; Valentin et al., 2021) which, however ideal for medical usage, may not be the best for environmental monitoring purposes. Although culture dependent methods exclude viable but non-culturable (VBNC) bacteria, and molecular methods can reveal more detail; molecular methods rely on expensive equipment that may not be readily available in the field, and may be beyond the financial capabilities of some researchers or monitoring agencies especially those in poorer countries.

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