Multivariate Analyses of Selected Hydro-bacterial Variables along the Longitudinal Gradient of Orani River, Philippines

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Abstract. There is paucity of environmental data for Orani river systems in Bataan (Philippines) despite its regional economic and ecological significance. The present study conducted multivariate analyses of various water quality parameters and selected bacterial communities to evaluate the river’s health integrity. River data exhibited spatial and seasonal variation, with observed significantly distinct heterotrophic and Gram-negative bacterial counts in the midstream and downstream against upstream samples. Fecal coliform concentrations were statistically different in the sequence: upstream < downstream < midstream. Multivariate Principal Component Analysis identified the most important variables affecting the variability in bacterial concentrations among the sampling groups (81.64% variation). Cluster analysis also revealed the associations and similarities of sampling groups based on abundance of bacterial loadings. Further, water quality indices were classified within “marginal” class (54.77 for wet season; 57.67 for dry season). The hydrological index estimated a score of 47.97 for wet season that described as “suitable with high restriction” class (30<(HI)c<55), and 62.24 for dry season in the “suitable with medium restriction” class (55<(HI)c<75).

1. Introduction
The discharge of untreated sewage and overland flow into surface waters, such as rivers, are common to highly populated human settlements that are unequipped with sewage treatment facilities. Sources of effluents include wastes coming from residential areas, industries, and even manufacturing plants [1]. The demographics of Orani, Bataan (Philippines) are characterized with high dependence to Orani river system [2], hence may be susceptible to threats posed by polluted and contaminated water. Similar to other Philippine streams, these threats are usually downplayed due to limited riverine studies [3] [4] that may serve as impetus for improved management and basis for the creation of tailor-fit solutions. Bacterial populations are reactive and sensitive to environmental conditions. The detection and enumeration of facultative and obligate bacteria are of primary importance for monitoring the sanitation and hydro-bacterial quality of riverine waters. Escherichia coli is one of the specific indicators of fecal contamination. The pathogenic E. coli are known to cause many types of infection and are spread to humans in a variety of ways. Unfortunately, the negative implications brought about by the presence of E. coli to the Orani river is magnified as it is utilized as a water source for shrimp culture, a very important industry in the area [5]. Further, it has been observed that shrimp producers in the region are rated “poor” on the implementation of biosecurity measures [6].
Several ecological studies in rivers have employed multivariate ordination analyses to determine the riverine health status and fish integrity [7] [8]. Similarly, the use of multicriterial hydrological assessment as a management tool for both brackishwater and marine ecosystems has been reported [9] [10]. Using the abovementioned concepts, the abiotic hydrological parameters complemented bacterial populations may provide a more precise evaluation of the water quality. Hence, this study assessed the bacterial and environmental parameters in Orani river, with emphasis on the spatiotemporal concentrations of heterotrophic and fecal coliform bacteria from Orani river water samples. Moreover, the study evaluated the association between microbial data with several key ecological parameters.

2. Materials and methods

2.1 Study sites
Water samples were collected in the Orani rivers (Tala-Pantalan Luma continuum), and in the two experimental fishponds of BPSU Orani Campus (Bataan, Philippines) (14° 47’ 4.1” N; 120° 33’ 12” E). The upstream portion of the river system was generally known for its natural riverscape serving as a tourist attraction in the municipality and a protected area inside the Bataan National Park. The midstream portion (site 2), and the downstream portion (site 3) are demographically surrounded by communities where the river serves as a catch basin for untreated sewage directly from household, agricultural or industrial sources (figure 1).

2.2 Water sample collection and analyses
In the river, the sampling points were setup along the upstream-downstream transect of the river [11]. For the quantitative analyses, physico-chemical water quality parameters were measured in situ. Instruments and test kits were standardized and calibrated at the Water Quality Laboratory of the Department of Chemistry, Ateneo de Manila University, Quezon City, Philippines.

![Figure 1. Satellite image showing plotted sampling sites (1,2, and 3) along the stream of the Orani river and the experimental ponds (Site 3) located at the downstream portion (photo modified from Google Earth).](image)

2.3. Sample collection and water quality measurement
The samples were collected in triplicates from the three different sampling areas at the Orani river in a monthly basis during dry months (February – April 2019) and wet months (August – October 2019). Samples were aseptically obtained from the mid-surface part of the water column using sterile polyethylene containers. Water quality parameters including dissolved oxygen (DO), salinity, turbidity, column depth, temperature, pH, hardness, nitrate (NO₃⁻), nitrite (NO₂⁻), and total ammonia (NH₃) were determined in situ. The samples were immediately stored in ice boxes and transported back to the laboratory for further bacterial analyses.
2.4. Bacterial isolation and culture
The bacterial concentrations were assessed based on indicators for microbial safety for public use and food safety. General culture media (NA) and MacConkey (MC) plates were prepared according to the manufacturers’ specifications (HiMedia®). The glass wares, micropipettes, and other materials (heat resistant) for microbiological methods including the prepared media were autoclaved under 121°C; 15 psi for 15 min. A 1-ml inoculant from the pooled water samples from each of the three sites were serially diluted following ten-fold dilution (10^{-1} to 10^{-5}) using sterile dH2O. One to two dilution factors were utilized for the enumeration of the total heterotrophic (THC) and fecal coliform (TC) bacteria counts. A minute volume of 100 μl from each of the chosen dilution factors were inoculated through standard spread plate method on the prepared culture plates. Each dilution factor utilized were spread-plated in triplicates. Thereafter, the inoculated plates were incubated in an inverted position at room temperature.

2.5. Bacterial quantification
The NA plates were incubated for 24 h before conducting the quantification (CFU ml^{-1}) of the THC, whilst the MC plates were incubated for 48 h for the enumeration (CFU ml^{-1}) of the total gram-negative bacteria counts (TGNC). The MC plates were further observed for the morphology and color of the growth colonies. Presumptive identification of E. coli was based on colonies with pinkish red growth having a metallic sheen. Further characterization of fecal coliforms was confirmed through rapid lactose fermentation. The total plate counts were obtained through manual counting using grid method. Total plate counts were used to determine the CFU ml^{-1} of samples using the formula:

\[
\text{CFU ml}^{-1} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{final volume plated}}
\]

2.6. Data analyses
2.6.1. Statistical analyses. Normality of data (Shapiro-Wilk test) and homogeneity of variance (Levene’s test) were verified. Variables did meet the assumptions and were subjected to univariate parametric tests. Mean bacterial count was subjected to analysis of variance (ANOVA), followed by Tukey’s post-hoc test if found significantly different (p < 0.05). Principal component analysis (PCA) was used to examine the association of environmental parameters with bacterial abundance based on spatio-seasonal variation. PCA also identified the most important variables that contribute the most to group differences. Similarity among stream sections at two collection seasons was measured using Bray-Curtis index, and the unweight pair group average method (UPGMA) was used to cluster similar group (sites) according to bacterial load data. All data analyses were performed using PaST® v 3.0 and SPSS v 21.

2.6.2. Shrimp culture hydrological index (HI)c. The (HI)c values were based on the method [9] developed to evaluate and pre-select areas for shrimp culture based on four parameters: salinity, turbidity, pH, and dissolved oxygen. The variables were selected based on their correlation to hydrodynamic dominant characteristics of the water masses and the spatial similarity of their distribution pattern in relation to other investigated properties that are more difficult to collect [10]. The (HI)c values were computed using equations 2 and 3. The exact weight values (WV) were calculated for each parameter and multiplied by the corresponding variable weight (VW) based on the methods [11] to obtain the score for each parameter per sampling station (S). The final score for each sampling site (FSs) was obtained from the multiplication product of the four WVpar. Values for FS range from 0 to 18,750, hence was recalculated to values from 0 to 100 (HI) based on the equation (4) with modification [10].

\[
S_{\text{par}} = WV_{\text{par}} \times VW_{\text{par}}
\]

\[
FS_{s} = S_{\text{salinity}} \times S_{\text{pH}} \times S_{\text{turbidity}} \times S_{\text{oxygen}}
\]
\[ HI = 8.546 \times (FS_2)^{0.25} \] (4)

2.6.3. Canadian water quality index (CCME-WQI). The Canadian water quality index were used to present a comparison of aquatic environments for seasonal variation in the river. This study utilized reference points based on recommended values of 8 water quality parameters for shrimp culture. The CCME-WQI was computed based on combined single value (ranging from 0 to 100) scale obtained from the CCME protocol.

3. Results and discussion

3.1. Spatial and seasonal hydro-bacterial dynamics

River parameters were expressed on extremes during the two seasons as shown in table 1. The spatial distribution of bacterial concentrations in the different stream sampling sections of Orani river are shown in Figure 2. The concentrations (CFU ml\(^{-1}\)) ranged between 100 to 1.7 \(\times\) \(10^4\) CFU ml\(^{-1}\) for THC, 0 (negative growth) to 1.5 \(\times\) \(10^4\) for TGNC, and 200 to 2.0 \(\times\) \(10^3\) for TC.

Table 1. Fluctuation of physicochemical characteristics of water during the study.

| Sampling period | Temp (°C) | DO (mg L\(^{-1}\)) | pH | Salinity (ppt) | NH\(_3\) (mg L\(^{-1}\)) | NO\(_3\) (mg L\(^{-1}\)) | NO\(_2\) (mg L\(^{-1}\)) |
|-----------------|-----------|--------------------|----|---------------|----------------|----------------|----------------|
| River           |           |                    |    |               |                 |                 |                 |
| Wet season      | 23.1–32.4 | 3.0–10.8           | 6.5–8.4 | 0–10.0        | 0–4.0          | 0–0.4          | 0–0.25         |
| Dry season      | 24.8–32.5 | 5.0–11.2           | 6.8–8.9 | 0–10.0        | 0–4.0          | 0–0.4          | 0–0.5          |

Figure 2. Mean values (SE = error bar) and differences of bacterial concentrations on river sampling points. Different superscripts represent significant differences at \(p < 0.05\) within bacterial groups. Sampling sites consist of upstream (1), midstream (2) and downstream (3). Bacterial concentrations were quantified for total heterotrophic counts (THC), total Gram-negative counts (TGNC), and total fecal coliform counts (TC).

The THCs of all the water samples at site 2 and 3 were mostly high, exceeding 10-folds of the limit of 5 \(\times\) \(10^2\) CFU ml\(^{-1}\) which is the standard set for heterotrophic counts on drinking water [12]. Isolated
bacterial communities in activated sludge samples obtained from the aerobic and anaerobic zones of a wastewater treatment plant were predominantly Gram-negative bacteria [13]. Similar observations in the occurrence of high TGNC associated in sewage itself and in relation to river pollution have been earlier established [14] [15]. The influence of anthropogenic pollution through waste inputs in Orani river may be observed in the corresponding TGNC levels obtained in this study. The high relative concentrations of TGNC in the total heterotrophic bacteria counts are also evident of river contamination. Midstream and downstream samples were confirmed to be contaminated with fecal coliform bacteria with generally high mean concentrations. High fecal coliform was very undesirable, and suggests that human sewage forms a significant portion of pollutants in the water. The lowest TC was recorded in the upstream samples and the highest was in the midstream samples. There exists a significant difference (p < 0.05) among the streams on the mean TC concentrations. The presence of fecal coliform bacteria in streams may indicate contamination from anthropogenic activities, hence could pose a serious risk to domestic users [5] [16].

The mean TC of samples from the mid- and downstream largely exceeded the $4 \times 10^2$ CFU ml$^{-1}$ standard set by U.S Environmental Protection Agency [17]. Although the upstream is generally receiving relatively less input from urban sewage, heterotrophic bacteria concentrations are near at the threshold of the limit indicative of risk. Nevertheless, based to the mean TC data, the upstream portion is classified as class A (for primary contact recreation such as bathing, swimming, skin diving, etc. particularly those designated for tourism purposes), albeit the midstream and downstream are both under class D (for agriculture, irrigation, and livestock watering) [18]. On the other hand, bacterial concentrations were analysed for temporal variations to determine the extent of bacterial concentrations on river within the two pronounced tropical seasons: wet and dry. Significant mean differences on bacterial concentrations were presented on figure 3.

![Figure 3](image_url)

**Figure 3.** Mean values (SE = error bar) and differences of bacterial concentrations during wet and dry seasons. Different superscripts represent significant differences at p < 0.05 within bacterial groups. Bacterial concentrations were quantified for total heterotrophic counts (THC), total Gram-negative counts (TGNC), and total fecal coliform counts (TC).

Present findings revealed a significant seasonal variation on THC and TGNC. Occurrence of high bacterial concentrations during wet season can be attributed by surface runoffs and increased drainage inputs from increased frequency of atmospheric precipitations. Further, wet season is characterized with increased nutrient levels, river flow, and aeration which enhance aerobic decomposition of organic matter and hence bacterial population counts [19]. Pooled data on TC exhibited an insignificant seasonal
difference. However, it can be observed that dilution with water inputs during wet season from surface runoffs and rain drainage rendered high TC unabated. This suggests a consistent flow of contamination (e.g. fecal wastes) in the river as attributed to surface runoffs during rainy months. Observed TC can be linked to contamination due to animal wastes (e.g., wild birds and reptiles). This can possibly occur since coliforms could resist for a long period of time in the environment.

3.2. Similarities in bacterial concentrations

Analysis of spatiotemporal similarity produced conspicuous clusters in microbial loads. The dendrogram for THC showed high similarity (93%) in the midstream group, which deviated from the downstream during dry season (~88%) and wet season (~66%) (figure 4A). A similar pattern was observed also for TGNC (figure 4B). On the other hand, TC dendrogram shows lower dissimilarity of midstream and downstream groups to the upstream group at ~61%. Similarity within midstream group is high at ~79% and ~85% within downstream group (figure 4C).

Figure 4. A dendogram for Bray-Curtis similarity of unweight pair group average method showing the clustering of sites per season derived from bacterial load data. 1D = upstream in dry season; 1W = upstream in wet season; 2D = midstream in dry season; 2W = midstream in wet season; 3D = downstream in dry season; 3W = downstream in wet season. Total heterotrophic counts (THC), total Gram-negative counts (TGNC), and total fecal coliform counts (TC).
Both of the midstream and downstream groups tend to be deviated from upstream group, signifying the capacity of the river in self-purification process or the removal of pollutants. It was also observed that there were consistent lower bacterial levels in the upstream that are usually within prescribed ranges [12] [17], which suggests that most of the source of contamination and anthropogenic pollution is in the midstream and downstream.

3.3. Multivariate analyses

Based on component scores for sampling sites, all of the principal components were above the Jolliffe’s cut-off value (0.7) for correlation matrix except pH, salinity and NO$_3$. Scores on loadings above 0.7 give indication that a principal component is more significant than those with scores below the number. Loadings for PC2 were found to be all below significant levels. The PCA biplot (figure 5) shows the correlation among study parameters as well as their relationships to component scores for sampling sites at the first two dimensions (accounting for 81.64% of the total variation). The first dimension of variation accounts for 58.18% the second-dimension accounts for 23.46% of the total variation.

In PC1, variations can be observed between midstream and downstream against upstream. Positive correlation with DO in the upstream was observed. The riverscape in the upstream is characterized with high altitude, river rapids and a rocky bottom (boulders) allowing for vigorous aeration. Other parameters related to biological productivity and pollution are located on the positive x-axis indicating for higher values in the midstream and downstream portions (e.g., TC was highly correlated with nitrogen-containing compounds, whilst TVC, and TGNC was associated to high NH$_3$ level.) In PC2, significant variations can be observed between midstream and downstream portions and within upstream seasons. The positive axis was associated with the midstream and upstream during dry season. Maximal correlation of bacterial data with environmental parameters can be used to predict the changes in microbial integrity within the stream longitudinal gradient.
3.4. Water quality indices

The computed scores for CCME-WQI and (HI)c are shown in Figure 6. Both values for wet and dry season falls within “marginal” class with CCME-WQI values of 54.77 for wet season and slightly higher 57.67 for dry season. The (HI)c obtained scores of 47.97 for wet season falling between the “suitable with high restriction” (30<(HI)c<55) class and 62.24 for dry season in the “suitable with medium restriction” (55<(HI)c<75) class. Both indices show higher values for dry season. This supports the observed better bacterial load and water quality during dry season compared to wet season.

![Figure 6](image)

Figure 6. Application of the Canadian water quality index (CCME-WQI) and shrimp hydrological index (HI)c to evaluate river water during wet, and dry seasons. Multiple vertical axes present references of classes for general quality: CCME-WQI (left side); and shrimp culture suitability: (HI)c (right side).

The present findings divulged the spatial and seasonal variability on microbial and ecological data within the river system. Increase in microbial growth showed an ascending order following the sequence — upstream < downstream < midstream, with higher microbial build-up during wet months. Regardless of seasons, midstream and downstream microbial concentrations exceeded water quality standards (consumption point). Anthropogenically-induced dynamics in microbial condition and water quality indices were observed, which can be attributed to reduced self-purification capabilities of river due to continuous waste discharge from urbanized areas even during rainy months. PCA further revealed this scenario as high microbial loads were detected in water samples with high nitrogen-containing compounds (mainly from decomposition process). Further, river water quality indices indicate better values during dry season for shrimp culture and for water quality in general. The routine determination of key microbial communities is vital for the refinement of management regimes necessary for specific conditions [5]. For instance, intensified pond management is necessary during wet season to mitigate the observed higher bacterial load, and poorer riverine water quality. Further, establishment of sewage treatment plants can circumvent the negative impacts of anthropogenic wastes being released into the rivers. Although this study is limited to Orani river, complementary studies can be done to validate the results and initiate further investigations in other adjacent streams. The baseline dataset is hoped for the impetus of proper management protocols for both the river and river-fed brakishwater ponds [5].

4. References

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