SHORT COMMUNICATION

Assessment of bacterial species present in Pasig River and Marikina River soil using *16S rDNA* phylogenetic analysis

Maria Constancia O. Carrillo*, Paul Kenny L. Ko, Arvin S. Marasigan, and Arlou Kristina J. Angeles

Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila, Padre Faura St., Ermita, Manila Philippines 1000

Abstract—The Pasig River system, which includes its major tributaries, the Marikina, Taguig-Pateros, and San Juan Rivers, is the most important river system in Metro Manila. It is known to be heavily polluted due to the dumping of domestic, industrial and solid wastes. Identification of microbial species present in the riverbed may be used to assess water and soil quality, and can help in assessing the river's capability of supporting other flora and fauna. In this study, *16S rRNA* gene or *16S rDNA* sequences obtained from community bacterial DNA extracted from riverbed soil of Napindan (an upstream site along the Pasig River) and Vargas (which is along the Marikina River) were used to obtain a snapshot of the types of bacteria populating these sites. The *16S rDNA* sequences of amplicons produced in PCR with total DNA extracted from soil samples as template were used to build clone libraries. Four positive clones were identified from each site and were sequenced. BLAST analysis revealed that none of the contiguous sequences obtained had complete sequence similarity to any known cultured bacterial species. Using the classification output of the Ribosomal Database Project (RDP) Classifier and DECIPHER programs, *16S rDNA* sequences of closely related species were collated and used to construct a neighbor-joining phylogenetic tree using MEGA6. Six out of the 8 cloned samples were found to belong to obligate anaerobe species, suggesting that these species live deep within the sediment layer and do not have access to dissolved oxygen. Three species were found to be associated with sulfate-reducing bacteria, which suggests an abundance of sulfur containing compounds in the riverbed. This is the first census report of the Pasig River microbial population using an approach that utilizes *16S rDNA* sequences without culture nor isolation of bacteria. Further studies employing multiple composite samples and larger sample sizes are recommended for more comprehensive bacterial taxonomic profiles as well as evaluation of interactions between community members and bacterial response to environmental perturbations.

Keywords—*16S rDNA*, metagenomics, Pasig River system, DNA sequence analysis, molecular phylogeny

INTRODUCTION

The Pasig River system (Figure 1) is the major river system in Metro Manila and is composed of the Pasig River, Marikina River, Taguig-Pateros River, and San Juan River. The principal river, the Pasig River, traverses the width of Metro Manila and connects Laguna de Bay and Manila Bay. Due to its location and reach, the river has traditionally been an important means of transport for city dwellers, as well as a water source for domestic and industrial use. However, the Pasig River and all three tributaries are heavily polluted due to the dumping of domestic waste, and industrial waste from tanneries, textile mills, food processing plants, and distilleries. The Department of Environment and Natural Resources classifies the rivers under class D status, which means that river water may be used only for agricultural applications such as irrigation and livestock watering, and secondary industrial applications like cooling. In recent years, the riverbed has become more silted with organic matter and non-biodegradable garbage (Gorme et al. 2010).

Water-saturated sediments make up the bulk of biomass in a river environment (Fischer and Pusch 2001), usually in the form of microbial biofilms. These microbes are responsible for the bulk of metabolic activity in river and stream ecosystems, and 76–96% of total respiration (Craft et al. 2002; Naegeli and Uehlinger 1997; Pusch 1997; Pusch et al. 1998; Vaque et al. 1992). Bacterial growth in this environment is dependent on physico-chemical traits, such as temperature, salinity, pH, as well as presence of pollutants. Fluctuations in these conditions may result to either an increase or decrease of bacterial growth and may also result in drastic changes in bacterial populations (Daniel 2005; Fierer et al. 2007; Hidayat et al. 2012). Identification of microbial species present in the riverbed may be used to assess water and soil quality, and may also help in assessing a river's capability of supporting other flora and fauna. Although soil microbial communities are known to possess incredible diversity with tens of thousands of species of bacteria and fungi in a gram of soil, they have remained poorly characterized (Kristiansson et al. 2011). This is due not only to often low

*Corresponding Author
Email Address: mcocarrillo@post.upm.edu.ph
Submitted: January 23, 2015
Revised: July 16, 2015
Accepted: September 23, 2015
Published: November 14, 2015

concentrations of each bacterial species present (Engel et al. 2010; Myrold et al. 2014), but also to the difficulty in isolating and culturing individual species.

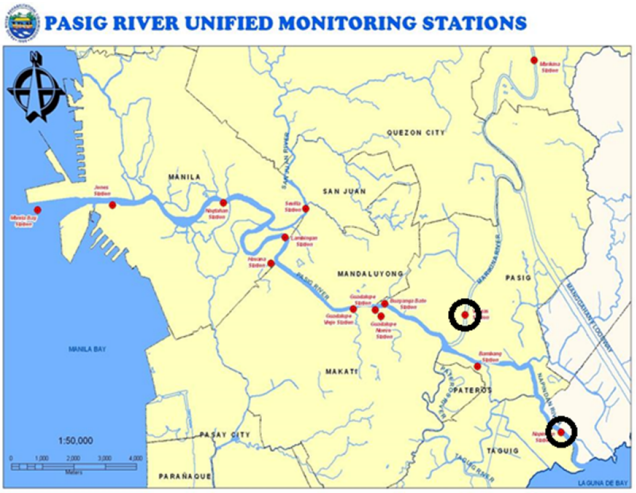

Figure 1. Map of Pasig River and its tributaries. The two sampling sites, Napindan/C6, which is along Pasig river, and Vargas, which is along Marikinina River, are indicated by black circles (Map courtesy of the Pasig River Rehabilitation Commission).

A metagenomic approach that employs culture-independent analysis of genomes from total microbial DNA extracted from an environmental sample (such as oil, water, fluids and fecal matter) has made possible the rapid identification of multiple bacterial species present. This approach allows the identification of previously uncultured (and therefore unknown) bacterial species. Previous studies have demonstrated that this approach provides a better description of the taxonomic composition of soil microbial communities or soil microbiome (Myrold et al. 2014). Various culture-independent studies investigating sediment microbial populations and their phylogenetic structure have used techniques such as denaturing gradient gel electrophoresis (DGGE) and clone libraries. Data from these studies have demonstrated that microbial communities are extremely sensitive to changes in the physicochemical state of freshwater sediments (Feris et al. 2004; Feris et al. 2003; Ramsey et al. 2005; Zeglin et al. 2011), and can be used as indicators of ecological degradation. Metagenomics has also been employed to assess direct or indirect impact of microbes on health and biogeochemical cycles, and has even led to the discovery of new genes (Gomez-Alvarez et al. 2009; Huson et al. 2009; Kröber et al. 2009; Petrosino et al. 2009; Shah et al. 2011; Thomas et al. 2012). For instance, in a study by Ferrer et al. (2011) on the microbial community of the anoxic sediments of Laguna de Carrizo in Madrid, Spain, annotated genes from species found in this site were predominantly related to sulfur oxidation, sulfur metabolism, thiosulfate reduction, iron-oxidation and denitrification. Another useful application of metagenomics is the use of phylogenetics to look at the distribution of bacteria populating an environment (Devereux and Mundfrom 1994).

In this study, a metagenomics approach that makes use of sequence comparison of the *16S rDNA* from total bacterial genomic DNA extracted from riverbed soil was employed to obtain a snapshot of the types of bacteria present in two riverbed sampling sites – Napindan, an upstream site along the Pasig River, and Vargas, which is along the Marikina River, a tributary to the Pasig. Phylogenetic tree construction based on sequence comparison, which can help reveal the evolutionary relationships of the unknown bacteria, was also performed. The types of bacterial species present in the sampling sites were correlated with the types or status of the environment in order to obtain necessary information to assess the state of health of the Pasig River.

METHODOLOGY

Soil sample collection and pH determination

Riverbed soil samples were obtained from the Pasig River Rehabilitation Commission (PRRC). These were collected by divers from the Philippine Coast Guard contracted by PRRC, who used shovels and rice sacks to collect soil sediment samples from the target collection sites. A portion of the samples collected were transferred to resealable non – sterile plastic bags and immediately frozen. The sites of the Pasig River Unified Monitoring Stations (Figure 1) used in this study were Napindan (14.5351° N, 121.1022° E) and Vargas (14.5663° N, 121.0735° E). Soil pH values were determined by mixing sediment and deionized water at a 1:5 mass to volume ratio.

Soil genomic DNA extraction and construction of 16S rDNA clone library

Total genomic DNA from each soil sample was extracted using the Mo Bio Powersoil® DNA isolation kit (California, USA) which was used according to the manufacturer's protocol. DNA extract quality was assessed using 1% agarose gel electrophoresis stained with ethidium bromide and visualized using BIO-RAD UV transilluminator 2000 (Milan, Italy).

PCR primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify targeted *16S rDNA* of the genomic DNA extracted from soil samples. The PCR reaction mixture contained 1 µL of genomic DNA and both primers, and 2X Taq Master Mix (Vivantis). Amplification conditions were as follows: initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of denaturation (94ºC, 1 minute), annealing (54ºC, 1 minute), and extension (72ºC, 1 minute 45 seconds), ending with a final extension at 72ºC for 5 minutes. Amplicon size and quality were assessed by 1% agarose gel electrophoresis.

Amplicons were ligated into pCR™ 2.1-TOPO® vectors using Invitrogen TOPO® TA Cloning® kit (California, USA) and the ligation mixtures were used to transform competent E. coli cells using heat shock process, following the manufacturer's protocol. Randomly picked transformants were screened for the presence of cloned *16S rDNA* using colony-PCR with M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers. Amplification followed a similar temperature profile as *16S rDNA* amplification from the previous step except for the initial denaturation being set to 10 minutes and the annealing temperature at 55ºC. The presence of cloned *16S rDNA* amplicons was assessed using 1% agarose gel electrophoresis.

DNA sequencing and Data Analysis

PCR amplicons were sent to Macrogen (Seoul, South Korea) for sequencing using the Sanger method. Sequences were analyzed using CodonCode Aligner v4.2.7 (http://www.codoncode.com/aligner/). Contiguous sites of the forward and reverse sequences were assembled and edited using the same program. The 1400 - 1600 base pairs sequences were subjected to editing to produce contigs with around 450 – 1000 base pairs. Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) website.

The contig sequences were run through DECIPHER, an online tool used for deciphering and managing biological sequences, to weed out chimeric sequences (Wright et al. 2012). The sequences were also classified using Ribosomal Database Project (RDP) Classifier (Wang et al. 2007). Using the classification output of both programs, *16S rDNA* sequences of closely related species were collated and used to construct a neighbor-joining phylogenetic tree using MEGA6 (Tamura et al. 2013).

RESULTS AND DISCUSSION

The two sites included in this study are Napindan, which is located near the outlet of Laguna de Bay to the Pasig River, and Vargas, which is found along the Marikina River and near the Marikina-Pasig River junction. Being major upstream contributors of water in the Pasig River, these sites may be considered as benchmarks for water and soil quality throughout the stretch of the river. The metagenomics approach, which employs culture-independent sequence-based analysis of the collective microbial genomes contained in environmental samples, can detect known as well as unknown (because they have not been cultured, or are unculturable) bacterial species. The type of bacterial species detected can be useful in assessing the health of the river because a correlation between bacterial species present and the quality or health of a river sampling site can be made.

The total genomic DNA profiles of samples taken from two sampling sites exhibited smears in agarose gel. PCR amplification of *16S rDNA* using the genomic DNA as template, however, produced the expected size bands of about 1400 base pairs (data not shown). PCR using clones that were positive for *16S rDNA* insertion produced amplicons of about 1700 base pairs, suggesting the inclusion of amplified plasmid DNA. Four positives clones were detected out of 70 colonies picked from each clone library.

Bacterial enumeration and identification in this study was based on sequence analysis of *16S rDNA* amplicons (Table 1). Sequence analysis of the *16S rDNA* amplicons is considered the gold standard for bacterial identification and characterization studies (Mizrahi-Man et al. 2013). The *16S rDNA* from bacteria is universal and functionally conserved; thus, random sequence changes are considered to be useful evolutionary markers. Its 1500 base pair gene length, which includes 9 hypervariable regions, is also sufficiently long for bioinformatics – based sequence analysis.

Chimeric sequences, which may cause overestimation of species diversity that can account for most errors in metagenomic data analysis (Janda and Abbott 2007; Shah et al. 2011), were weeded out using DECIPHER prior to further analysis. BLAST search to find the closest *16S rDNA* sequences hits revealed that none of the closest matches for each clone (which include *16S rDNA* complete and partial sequences of bacterium from various phyla) had 100% sequence similarity with the contig sequences (Table 1). The sequences for each clone contig (designated NP-01 to NP-04 for the samples from the Napindan sampling site, and V-01 to V-04 for samples from the Vargas site) have been submitted to, and are currently accessible in, GenBank (Accession # KM107850 – KM107853 for NP-01 to NP-04; KM107856 – KM107859 for V-01 to V-04).

Contig sequences of microbial species from unique DNA sequences run through the RDP classifier (a Naïve Bayesian classifier) generated taxonomic classification and sequences of *16S rDNA* of related species were collated according to taxonomic classifications of both RDP and DECIPHER (Table 2). The sequences used for phylogenetic analysis were restricted to cultured representatives; therefore, the number of representatives that can be used for each sample was subject to availability in online databases.

Table 1. Top results of contig sequences when run through BLAST including the max score, total score, query coverage, E value, percentage identity, and the accession number and description of the closest sequence.

Sample	GenBank Accession No.	Description of closest sequence	Max score	Total score	Query cover	E value	Ident	GenBank Accession No.
NP-01	KM107850	*Syntrophus aciditrophicus* strain SB 16S ribosomal RNA gene, complete sequence	992	992	100%	0	91%	NR_102776.1
NP-02	KM107851	*Caldilinea aerophila* strain DSM 14535 16S ribosomal RNA gene, complete sequence	658	658	100%	0	86%	NR_074397.1
NP-03	KM107852	*Syntrophus gentianae* strain HQgoe1 16S ribosomal RNA gene, partial sequence	1461	1461	99%	0	93%	NR_029295.1
NP-04	KM107853	*Dethiobacter alkaliphilus* strain AHT 1 16S ribosomal RNA gene, partial sequence	688	688	100%	0	87%	NR_044205.1
V-01	KM107856	*Longilinea arvoryzae* strain KOME-1 16S ribosomal RNA gene, partial sequence	686	686	99%	0	88%	NR_041355.1
V-02	KM107857	*Bellilinea caldifistulae* strain GOMI-1 16S ribosomal RNA gene, partial sequence	673	673	99%	0	89%	NR_041354.1
V-03	KM107858	*Kozakia baliensis* strain NBRC 16664 16S ribosomal RNA gene, partial sequence	1454	1454	100%	0	95%	NR_113858.1
V-04	KM107859	*Desulfuromonas acetexigens* strain 2873 16S ribosomal RNA gene, partial sequence	1548	1548	100%	0	93%	NR_044770.1

A phylogenetic tree (Fig. 2) was generated using MEGA6 that employs the neighbor-joining method. This is a distance based method which joins pairs of sequences, or "neighbors", with the least number of differences or evolutionary distance (Saitou and Nei 1987). A phylogenetic tree using the maximum-likelihood method (that uses probability for production of optimal trees; Pagel 1999) yielded similar results, but with varying branch lengths and bootstrap values (data not shown). Results generated from both phylogenetic trees clearly suggest that the sequence comparison groups for each sample analyzed were closely related and in accordance to the classification output from DECIPHER and RDP (Table 2). Both results were consistent with the top results from BLAST analysis (Table 1), with the exception of NP-04, which was classified under *Acidobacteria* by both DECIPHER and RDP but was grouped under *Syntrophomonadaceae* according to the BLAST analysis.

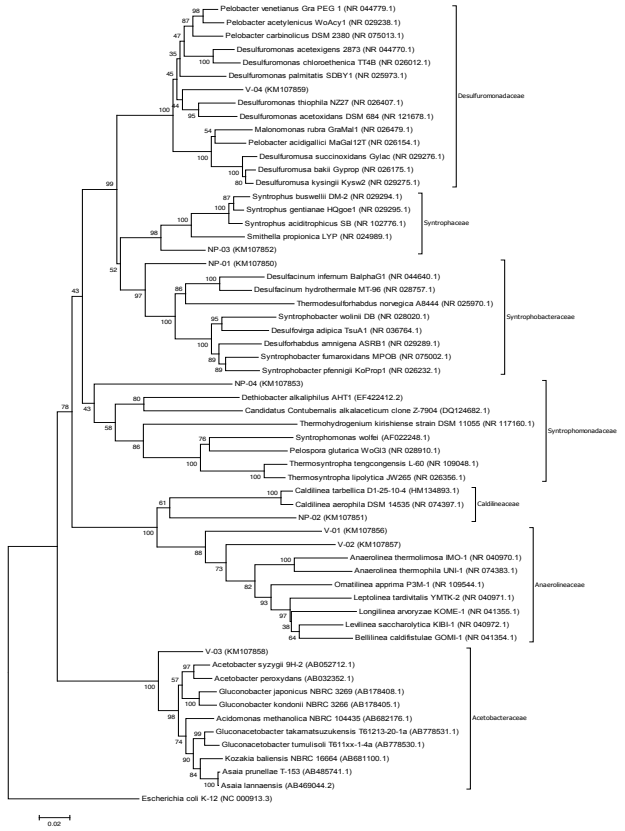

Figure 2. Phylogenetic tree of contig sequences and *16S rDNA* sequences of closely related species using the neighbor-Joining method with 1000 replicate bootstrap test generated using MEGA6. *Escherichia coli* was included as outgroup. Samples from the study are labeled NP-01 to NP-04 and V-01 to V-04.

Phylogenetic analysis showed that NP-01 and members of the family *Syntrophobacteraceae*, Class *Deltaproteobacteria*, (which include *Desulfacinum infernum*, *Desulfacinum hydrothermale*, and *Thermodesulforhabdus norvegica*) share a common ancestor. Members of the *Syntrophobacteraceae* are Gram-negative and are neutrophilic thermophiles. They are also strictly anaerobic and able to utilize sulfate and sulfite as electron acceptors (Beeder et al. 1995; Rees et al. 1995; Sievert and Kuever 2000). NP-02 share common ancestry with *Caldilinea aerophila* and *Caldilinea tarbellica* of the family *Caldilineaceae*, Phylum *Chloroflexi*. Both species are Gram-negative, thermophilic, neutrophilic, and facultative anaerobic (Gregoire et al. 2011; Sekiguchi 2003). NP-03 share a common ancestor with the members of the family *Syntrophaceae*, Class *Deltaproteobacteria*. Known representatives of this family, such as *Smithella propionica*, *Syntrophus buswellii*, *Syntrophus aciditrophicus*, and *Syntrophus gentianae*, are all Gram-negative, mesophilic, neutrophilic, and strictly anaerobic (Auburger and Winter 1995; Jackson et al. 1999; Liu et al. 1999; Mountfort et al. 1984; Wallrabenstein et al. 1995). NP-04 share common ancestry with *Dethiobacter alkaliphilus* and *Candidatus Contubernalis alkalaceticum* of the family *Syntrophomonadaceae*, Phylum *Firmicutes* and Class *Clostridia*. Both are Gram-positive, mesophilic, alkaliphilic, and obligate anaerobic. *Dethiobacter alkaliphilus* is able to utilize thiosulfate and elemental sulfur or polysulfide as an electron acceptor (Sorokin et al. 2008; Zhilina et al. 2005).

Table 2. Taxonomic classification of contig sequences from the results of DECIPHER and RDP.

Name	DECIPHER	RDP
NP-01	Syntrophobacterales	Root[100%] Bacteria[100%] "Proteobacteria"[98%] Deltaproteobacteria[97%] Syntrophobacterales[88%] Syntrophobacteraceae[64%] Desulfovirga[39%]
NP-02	Caldilineae	Root[100%] Bacteria[100%] "Chloroflexi"[100%] Caldilineae[57%] Caldilineales[57%] Caldilineaceae[57%] Caldilinea[57%]
NP-03	Syntrophobacterales	Root[100%] Bacteria[100%] "Proteobacteria"[100%] Deltaproteobacteria[100%] Syntrophobacterales[100%] Syntrophaceae[100%] Smithella[60%]
NP-04	Acidobacteria_Gp22	Root[100%] Bacteria[100%] "Acidobacteria"[100%] Acidobacteria_Gp18[100%] Gp18[100%]
V-01	Anaerolineaceae	Root[100%] Bacteria[100%] "Chloroflexi"[99%] Anaerolineae[97%] Anaerolineales[97%] Anaerolineaceae[97%] Longilinea[87%]
V-02	Anaerolineaceae	Root[100%] Bacteria[100%] "Chloroflexi"[100%] Anaerolineae[100%] Anaerolineales[100%] Anaerolineaceae[100%] Longilinea[43%]
V-03	Acetobacteraceae	Root[100%] Bacteria[100%] "Proteobacteria"[100%] Alphaproteobacteria[100%] Rhodospirillales[100%] Acetobacteraceae[100%] Granulibacter[54%]
V-04	Desulfuromonadales	Root[100%] Bacteria[100%] "Proteobacteria"[100%] Deltaproteobacteria[100%] Desulfuromonadales[100%] Desulfuromonadaceae[100%] Desulfuromonas[100%]

V-01 and V-02 are closely related to the family *Anaerolineaceae*, Phylum *Chloroflexi*. Members of *Anaerolinea thermophile*, *Anaerolinea thermolimosa*, and *Ornatilinea apprima* are Gram-negative, thermophilic, neutrophilic to slightly *alkaliphilic*, and obligate anaerobic (Podosokorskaya et al. 2013; Sekiguchi 2003; Yamada 2006). V-03 share common ancestry with the members of the family *Acetobacteraceae*. Representative species *Acetobacter syzygii*, *Gluconobacter japonicas*, and *Gluconobacter kondonii* are known to be Gram-negative, mesophilic, acidophilic, and obligate aerobic (Lisdiyanti et al. 2001; Malimas et al. 2009; Malimas et al. 2007). V-04 share common ancestry with *Desulfuromonas thiophila* and *Desulfuromonas acetoxidans* of the family *Desulfuromonadaceae*, Class *Deltaproteobacteria*. *Desulfuromonas thiophila*, *Desulfuromonas acetoxidans*, *Desulfuromonas palmitatis*, *Desulfuromonas acetexigens*, and *Desulfuromonas chloroethenica* are Gram-negative, mesophilic, neutrophilic and obligate anaerobic. They can utilize different forms of sulfur as electron acceptor (Coates et al. 1995; Finster et al. 1997; Finster et al. 1994; Krumholz 1997; Pfennig and Biebl 1976).

Most of the cloned samples belong to families that are obligate anaerobes, except for NP-02 and V-03, which are grouped with facultative anaerobes and obligate aerobe families, respectively. These data suggest that the bulk of the cloned species most likely live deep within the sediment layer and do not have interaction or access to dissolved oxygen, whereas the facultative anaerobe and obligate aerobe live near or at the water-sediment boundary. A study by Qian, et al. (2000) reported that dissolved oxygen (DO) of waters from the surface in the Pasig River does not necessarily reflect the DO from waters right above the river bed. However, unlike mid- to downstream sites, upstream sites of the Pasig have dissolved oxygen near the riverbed.

The temperature determined by the Pasig River Rehabilitation Commission for the year 2013 was between 26 to 32°C. The pH of the sediments of two sites (Napindan and Vargas) were 7.90 and 8.66, respectively. These conditions are able to support the growth of neutrophiles and alkaliphiles, obligate and facultative anaerobes, and mesophiles and thermophiles. Some of the samples, such as NP-01, NP-04, and V-04, were also associated with sulfate-reducing bacteria or with bacteria able to use sulfur compounds as electron acceptor, possibly from the families *Syntrophobacteraceae*, *Syntrophomonadaceae*, and *Desulfuromonadaceae*, respectively. This suggests a high abundance of sulfur or sulfur containing compounds in the riverbed. Sulfate-reducing bacteria are known to help reduce heavy metal contamination in water by producing sulfides that easily react with divalent metals, such as cadmium and copper. The heavy metal sulfides produced have very low solubility, so the compounds can easily precipitate out (Muyzer and Stams 2008).

This study employs *16S rDNA*-targeted sequence analysis to conduct a phylogenetic analysis of bacterial population in two sampling sites in Pasig River. The low sensitivity of the clone screening method limited the number of unique sequences identified. Nevertheless, the study demonstrates the utility of the culture-independent metagenomics approach in characterizing microbial species in

the Pasig River system. Phylogenetic analysis, which was based on the comparison of individual clone contigs with gene sequences archived in annotated databases, produced insights on the types of bacteria present in the riverbed soil samples. Previous organismic studies of the Pasig River have been limited to rotifers (Lazo et al. 2010), fish (Chavez et al. 2006), fungi (Yap and Halos 2009), and cultured bacteria (Dela Cruz and Halos 1997). This study reports for the first time, an approach of utilizing community *16S rDNA* to generate a census of the microbial population in two sites of the Pasig River system. Future studies using multiple composite samples, larger sample sizes, and use of other techniques like pyrotagged sequencing (Hamady et al. 2008 and Muller et al. 2013) are recommended.

ACKNOWLEDGEMENTS

The authors would like to thank the Pasig River Rehabilitation Commission for providing the Pasig River soil samples and accompanying data, and the Office of the Vice President for Academic Affairs, University of the Philippines, for the Creative Work and Research Grant (awarded to MCC) used to fund the study.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: PKK AKA MCC. Performed the experiments: PKK AM AKA. Analyzed the data: PKK. Wrote the paper: PKK MCC. Read and edited the final version of the manuscript: PKK MCC

REFERENCES

Auburger G, Winter J. Isolation and physiological characterization of *Syntrophus buswellii* strain GA from a syntrophic benzoate-degrading, strictly anaerobic coculture. Appl Microbiol Biotechnol. 1995; 44(1-2):241–8.

Beeder J, Torsvik T, Lien T. *Thermodesulforhabdus norvegicus* gen. nov., sp. nov., a novel thermophilic sulfate-reducing bacterium from oil field water. Arch Microbiol. 1995; 164(5):331–6.

Chavez JM, de la Paz RM, Manohar SK, Pagulayan RC, Carandang JR. New Philippine record of south american sailfin catfishes (Pisces: *Loricariidae*). Zootaxa. 2006; 1109:57–68.

Coates JD, Lonergan DJ, Philips EJP, Jenter H, Lovley DR. *Desulfuromonas palmitatis* sp. nov., a marine dissimilatory Fe(III) reducer that can oxidize long-chain fatty acids. Arch Microbiol. 1995; 164(6):406–13.

Craft JA, Stanford JA, Pusch M. Microbial respiration within a floodplain aquifer of a large gravel-bed river. Freshw Biol. 2002; 47(2):251–61.

Daniel R. The metagenomics of soil. Nat Rev Microbiol. 2005; 3(6):470–8.

Dela Cruz J, Halos PM. Isolation, identification and bioremediation potential of oil-degrading bacteria from Manila Bay and Pasig River [Philippines]. 1997;

Devereux R, Mundfrom GW. A phylogenetic tree of 16S rRNA sequences from sulfate-reducing bacteria in a sandy marine sediment. Appl Environ Microbiol. 1994; 60(9):3437–9.

Engel AS, Meisinger DB, Porter ML, Payn RA, Schmid M, Stern LA, et al. Linking phylogenetic and functional diversity to nutrient spiraling in microbial mats from Lower Kane Cave (USA). ISME J. 2010; 4(1):98–110.

Feris KP, Ramsey PW, Frazar C, Rillig M, Moore JN, Gannon JE, et al. Seasonal Dynamics of Shallow-Hyporheic-Zone Microbial Community Structure along a Heavy-Metal Contamination Gradient. Appl Environ Microbiol. 2004; 70(4):2323–31.

Feris KP, Ramsey PW, Frazar C, Rillig MC, Gannon JE, Holben WE. Structure and seasonal dynamics of hyporheic zone microbial communities in free-stone rivers of the estern United States. Microb Ecol. 2003; 46(2):200–15.

Ferrer M, Guazzaroni M-E, Richter M, García-Salamanca A, Yarza P, Suárez-Suárez A, et al. Taxonomic and Functional Metagenomic Profiling of the Microbial Community in the Anoxic Sediment of a Sub-saline Shallow Lake (Laguna de Carrizo, Central Spain). Microb Ecol. 2011; 62(4):824–37.

Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, et al. Metagenomic and Small-Subunit rRNA Analyses Reveal the Genetic Diversity of Bacteria, Archaea, Fungi, and Viruses in Soil. Appl Environ Microbiol. 2007; 73(21):7059–66.

Finster K, Bak F, Pfennig N. *Desulfuromonas acetexigens* sp. nov., a dissimilatory sulfur-reducing eubacterium from anoxic freshwater sediments. Arch Microbiol. 1994; 161(4):328–32.

Finster K, Coates JD, Liesack W, Pfennig N. *Desulfuromonas thiophila* sp. nov., a new obligately sulfur-reducing bacterium from anoxic freshwater sediment. Int J Syst Bacteriol. 1997; 47(3):754–8.

Fischer H, Pusch M. Comparison of bacterial production in sediments, epiphyton and the pelagic zone of a lowland river. Freshw Biol. 2001; 46(10):1335–48.

Gomez-Alvarez V, Teal TK, Schmidt TM. Systematic artifacts in metagenomes from complex microbial communities. ISME J. 2009; 3(11):1314–7.

Gorme JB, Maniquiz MC, Song P, Kim L-H. The Water Quality of the Pasig River in the City of Manila, Philippines: Current Status, Management and Future Recovery. Environ Eng Res. 2010; 15(3):173–9.

Gregoire P, Bohli M, Cayol J-L, Joseph M, Guasco S, Dubourg K, et al. *Caldilinea tarbellica* sp. nov., a filamentous, thermophilic, anaerobic bacterium isolated from a deep hot aquifer in the Aquitaine Basin. Int J Syst Evol Microbiol. 2011; 61(6):1436–41.

Hamada M, Walker JJ, Harris JK, Gold NJ, Knight R. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat Methods. 2008; 5(3):235–7.

Hidayat T, Abdul Sama MA, bin Elias MA, Hadibarata T. Metagenomic Analysis of 16S rRNA Sequences from Selected Rivers in Johor Malaysia. J Appl Sci. 2012; 12(4):354–61.

Huson DH, Richter DC, Mitra S, Auch AF, Schuster SC. Methods for comparative metagenomics. BMC Bioinformatics. 2009; 10(Suppl 1):S12.

Jackson BE, Bhupathiraju VK, Tanner RS, Woese CR, McInerney MJ. *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. Arch Microbiol. 1999; 171(2):107–14.

Janda JM, Abbott SL. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. J Clin Microbiol. 2007; 45(9):2761–4.

Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegård B, et al. Pyrosequencing of Antibiotic-Contaminated River Sediments Reveals High Levels of Resistance and Gene Transfer Elements. Rodriguez-Valera F, editor. PLoS ONE. 2011; 6(2):e17038.

Kröber M, Bekel T, Diaz NN, Goesmann A, Jaenicke S, Krause L, et al. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J Biotechnol. 2009; 142(1):38–49.

Krumholz LR. *Desulfuromonas chloroethenica* sp. nov. Uses Tetrachloroethylene and Trichloroethylene as Electron Acceptors. Int J Syst Bacteriol. 1997; 47(4):1262–3.

Lazo MAVA, Nieto KKP, Rayel MFS, Sto Domingo DM, Vergara MAM, Papa RDS. Composition, Abundance and Distribution of Rotifers in the Pasig River, Philippines. Philipp Sci. 2010; 46:47–64.

Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K. Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov., and *Acetobacter orientalis* sp. nov. J Gen Appl Microbiol. 2001; 47(3):119–31.

Liu Y, Balkwill DL, Aldrich HC, Drake GR, Boone DR. Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. Int J Syst Bacteriol. 1999; 49 Pt 2:545–56.

Malimas T, Yukphan P, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, et al. *Gluconobacter kondonii* sp. nov., an acetic acid bacterium in the alpha-Proteobacteria. J Gen Appl Microbiol. 2007; 53(5):301–7.

Malimas T, Yukphan P, Takahashi M, Muramatsu Y, Kaneyasu M, Potacharoen W, et al. *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the Alphaproteobacteria. Int J Syst Evol Microbiol. 2009; 59(3):466–71.

Mizrahi-Man O, Davenport ER, Gilad Y. Taxonomic Classification of Bacterial 16S rRNA Genes Using Short Sequencing Reads: Evaluation of Effective Study Designs. White BA, editor. PLoS ONE. 2013; 8(1):e53608.

Mountfort DO, Brulla WJ, Krumholz LR, Bryant MP. Syntrophus buswellii gen. nov., sp. nov.: a Benzoate Catabolizer from Methanogenic Ecosystems. Int J Syst Bacteriol. 1984; 34(2):216–7.

Muller EEL, Glaab E, May P, Vlassis N, Wilmes P. Condensing the omics fog of microbial communities. Trends Microbiol. 2013; 21(7):325–33.

Muyzer G, Stams AJM. The ecology and biotechnology of sulphate-reducing bacteria. Nat Rev Microbiol. 2008; 6:441-54.

Myrold DD, Zeglin LH, Jansson JK. The Potential of Metagenomic Approaches for Understanding Soil Microbial Processes. Soil Sci Soc Am J. 2014; 78(1): 3.

Naegeli MW, Uehlinger U. Contribution of the Hyporheic Zone to Ecosystem Metabolism in a Prealpine Gravel-Bed-River. J North Am Benthol Soc. 1997; 16(4):794.

Pagel M. The Maximum Likelihood Approach to Reconstructing Ancestral Character States of Discrete Characters on Phylogenies. Syst Biol. 1999; 48(3):612–22.

Petrosino JF, Highlander S, Luna RA, Gibbs RA, Versalovic J. Metagenomic Pyrosequencing and Microbial Identification. Clin Chem. 2009; 55(5):856–66.

Pfennig N, Biebl H. Desulfuromonas acetoxidans gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch Microbiol. 1976; 110(1):3–12.

Podosokorskaya OA, Bonch-Osmolovskaya EA, Novikov AA, Kolganova TV, Kublanov IV. *Ornatilinea apprima* gen. nov., sp. nov., a cellulolytic representative of the class *Anaerolineae*. Int J Syst Evol Microbiol. 2013; 63(Pt 1):86–92.

Pusch M, Fiebig D, Brettar I, Eisenmann H, Ellis BK, Kaplan LA, et al. The role of micro-organisms in the ecological connectivity of running waters. Freshw Biol. 1998; 40(3):453–95.

Pusch M. Community respiration in the hyporheic zone of a riffle-pool sequence. In: Gilbert J, Mathieu J, Fournier F, eds. Groundwater/Surface Water Ecotones: Biological and Hydrological Interactions and Management Options. USA: Cambridge University Press, 1997: 51–6.

Qian X, Capistrano ET, Lee W, Ishikawa T, Yokoyama K, Shoji H. Field Survey on the Flow Structure and Water Quality of Pasig River in Metro Manila. PROCEEDINGS OF HYDRAULIC ENGINEERING. 2000; 44:1101–6.

Ramsey PW, Rillig MC, Feris KP, Gordon NS, Moore JN, Holben WE, et al. Relationship between communities and processes; new insights from a field study of a contaminated ecosystem: Contaminated systems; communities, processes. Ecol Lett. 2005; 8(11):1201–10.

Rees GN, Grassia GS, Sheehy AJ, Dwivedi PP, Patel BKC. *Desulfacinum infernum* gen. nov., sp. nov., a Thermophilic Sulfate-Reducing Bacterium from a Petroleum Reservoir. Int J Syst Bacteriol. 1995; 45(1):85–9.

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4(4):406–25.

Sekiguchi Y. *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila*

gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. Int J Syst Evol Microbiol. 2003; 53(6):1843–51.

Shah N, Tang H, Doak TG, Ye Y. Comparing bacterial communities inferred from 16S rRNA gene sequencing and shotgun metagenomics. Pac Symp Biocomput Pac Symp Biocomput. 2011; 165–76.

Sievert SM, Kuever J. *Desulfacinum hydrothermale* sp. nov., a thermophilic, sulfate-reducing bacterium from geothermally heated sediments near Milos Island (Greece). Int J Syst Evol Microbiol. 2000; 50(3):1239–46.

Sorokin DY, Tourova TP, Mußmann M, Muyzer G. *Dethiobacter alkaliphilus* gen. nov. sp. nov., and *Desulfurivibrio alkaliphilus* gen. nov. sp. nov.: two novel representatives of reductive sulfur cycle from soda lakes. Extremophiles. 2008; 12(3):431–9.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013; 30(12): 2725–9.

Thomas T, Gilbert J, Meyer F. Metagenomics - a guide from sampling to data analysis. Microb Inform Exp. 2012; 2(1):3.

Vaque D, Pace ML, Findlay S, Lints D. Fate of bacterial production in a heterotrophic ecosystem: Grazing by protists and metazoans in the Hudson Estuary. Mar Ecol Prog Ser Oldendorf. 1992; 89(2):155–63.

Wallrabenstein C, Gorny N, Springer N, Ludwig W, Schink B. Pure Culture of *Syntrophus buswellii*, Definition of its Phylogenetic Status, and Description of *Syntrophus gentianae* sp. nov. Syst Appl Microbiol. 1995; 18(1):62–6.

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 2007; 73(16):5261–7.

Wright ES, Yilmaz LS, Noguera DR. DECIPHER, a Search-Based Approach to Chimera Identification for 16S rRNA Sequences. Appl Environ Microbiol. 2012; 78(3):717–25.

Yamada T. *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*. Int J Syst Evol Microbiol. 2006; 56(6):1331–40.

Yap RA, Halos PS. Isolation, Enumeration And Identification Of Oil-Degrading Fungi In Pasig River. Philipp J Biotechnol. 2009; 6(1).

Zeglin LH, Dahm CN, Barrett JE, Gooseff MN, Fitpatrick SK, Takacs-Vesbach CD. Bacterial Community Structure Along Moisture Gradients in the Parafluvial Sediments of Two Ephemeral Desert Streams. Microb Ecol. 2011; 61(3):543–56.

Zhilina TN, Zavarzina DG, Kolganova TV, Tourova TP, Zavarzin GA. "*Candidatus* Contubernalis alkalaceticum," an Obligately Syntrophic Alkaliphilic Bacterium Capable of Anaerobic Acetate Oxidation in a Coculture with *Desulfonatronum cooperativum*. Microbiology. 2005; 74(6):695–703.