# Cultivatable bacterial community in water from Lai Nullah contaminated with household sewage and industrial waste is more diverse and populated compared with nonpolluted water

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ARTICLE

# Cultivatable bacterial community in water from Lai Nullah contaminated with household sewage and industrial waste is more diverse and populated compared with nonpolluted water

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**Abstract:** The effect of environmental pollutants on living organisms can be assessed by studying the changes in the indigenous microbial community. Therefore, in this study, cultivatable bacterial community in nonpolluted as well as household sewage and industrially polluted water of Lai Nullah flowing through Islamabad and Rawalpindi, Pakistan was analyzed. Bacterial community composition and population present in the polluted water were significantly different from the nonpolluted water ($P < 0.05$). Nonpolluted water had much fewer species and population of bacteria compared with polluted water. Sequence analysis of bacterial 16S rRNA gene revealed that *Citrobacter freundii, Klebsiella pneumoniae, Escherichia coli, Lactobacillus plantarum, Geobacillus stearothermophilus, Enterococcus faecalis, Acinetobacter guillouiae, Ralstonia* sp., *Comamonas* sp., and *Stenotrophomonas maltophilia* were specific to the polluted water. On the other hand, *Aeromonas veronii, Exiguobacterium* sp., and *Lysinibacillus macroides* were only found in the nonpolluted water. Among measured physicochemical parameters, higher colony count in the polluted water was best correlated with higher biological oxygen demand, phosphate, sodium, and chloride values (Spearman's rho = 0.85). Concentration of heavy metals such as cadmium, chromium, copper, nickel, and lead were below 0.03 $\mu g \cdot mL^{-1}$ at all the study sites. During plate assay, bacterial strains found at polluted sites showed resistance to selected heavy metals with highest minimum inhibitory concentration for lead (8 $mmol \cdot L^{-1}$) followed by copper (5 $mmol \cdot L^{-1}$), nickel (3 $mmol \cdot L^{-1}$), and cadmium (1 $mmol \cdot L^{-1}$). All the bacterial isolates also showed various levels of resistance against antibiotics ampicillin, tetracycline, ciprofloxacin, and vancomycin using broth microdilution method. Current research provides new insight into the effect of household sewage and the industrially polluted water of Lai Nullah on the indigenous bacteria.

*Key words:* water pollution, cultivatable bacterial community, bacterial metal and antibiotic resistance, minimum inhibitory concentration, physicochemical parameters, Lai Nullah.

**Résumé :** On peut évaluer l'effet des polluants environnementaux sur les organismes vivants en examinant les changements que subit la microflore indigène. Les auteurs ont analysé les bactéries cultivables dans les eaux de la Lai Nullah non polluées ou polluées par les ordures ménagères et les rejets industriels qui traversent Islamabad-Rawalpindi, au Pakistan. La composition et la densité de la microflore présente dans les eaux polluées diffèrent sensiblement de celles de la microflore retrouvée dans les eaux non polluées ($P < 0,05$). Dans ces dernières, les espèces sont beaucoup moins nombreuses et leur population est plus faible. Le séquençage de l'ARNr 16S bactérien révèle que *Citrobacter freundii, Klebsiella pneumoniae, Escherichia coli, Lactobacillus plantarum, Geobacillus stearothermophilus, Enterococcus faecalis, Acinetobacter guillouiae, Ralstonia* sp., *Comamonas* sp. et

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*Stenotrophomonas maltophilia* peuplent spécifiquement les eaux polluées. En revanche, on ne retrouve *Aeromonas veronii*, *Exiguobacterium* sp. et *Lysinibacillus macroides* que dans les eaux non polluées. Le nombre supérieur de colonies obtenu avec les eaux polluées est le paramètre physicochimique le mieux corrélé à la plus forte demande biologique d'oxygène ainsi qu'à la plus importante concentration de phosphate, de sodium et de chlore (facteur rho de Spearman = 0,85). La concentration de métaux lourds (cadmium, chrome, cuivre, nickel et plomb) était inférieure à 0,03 $\mu g \cdot mL^{-1}$ à tous les endroits examinés. Les souches bactériennes issues des sites pollués identifiées lors de la culture sur gélose résistent à certains métaux lourds, la plus haute concentration minimale inhibitrice étant celle du plomb (8 $mmol \cdot L^{-1}$), suivie par le cuivre (5 $mmol \cdot L^{-1}$), le nickel (3 $mmol \cdot L^{-1}$) et le cadmium (1 $mmol \cdot L^{-1}$). D'après la technique de la microdilution, tous les isolats bactériens résistent de façon variable aux antibiotiques (ampicilline, tétracycline, ciprofloxacine et vancomycine). Ces résultats nous en apprennent davantage au sujet de l'incidence que les ordures ménagères et les rejets industriels ont sur la microflore indigène dans les eaux polluées de la Lai Nullah. [Traduit par la Rédaction]

*Mots-clés :* pollution de l'eau, bactéries cultivables, résistance des bactéries aux métaux et aux antibiotiques, concentration minimale inhibitrice, paramètres physicochimiques, Lai Nullah.

## Introduction

Living organisms and their environment face permanent toxic effects due to water pollution caused by household sewage and industrial waste (Halder and Islam 2015; Haseena and Malik 2017). In addition to metals, organic and inorganic materials, microorganisms especially bacteria and fungi are the main constituents of wastewater (Stottmeister et al. 2003; Raja et al. 2009).

For efficient management and rehabilitation of polluted environments, we first need to assess the extent or level of pollution. Different types of physiochemical analyses are used to check water pollution. However, physicochemical analysis can give an idea about the concentration of pollutants in the environment but is unable to provide the information about the possible toxicity of pollutants and their integrated influence on the organisms and ecosystem. Actual impact of pollutants on organisms can only be measured through biological analysis (Smejkalova et al. 2003; Zhou et al. 2008). Among living organisms, microorganisms especially bacteria respond quickly to the environmental stress, as they have short generation time and have close interaction with their surroundings due to their high surface to volume ratio. Therefore, bacteria may be ideal candidates for studying the impact of pollutants on living organisms (Nielsen and Winding 2002; Pearl et al. 2003; García-Armisen et al. 2014)

Bacteria adopt various mechanisms in order to tolerate high concentrations of pollutants, and they are associated with particular contaminants. Thus, bacteria can be used as biosensors for environmental forensics, especially those that are specific to certain pollutants (Haq and Shakoori 2000; Sumampouw and Risjani 2014; De La Rosa-Acosta et al. 2015). Moreover, changes in bacterial community structure, biomass, and functional activity (organic matter decomposition, enzymatic activity, nitrogen mineralization, and respiration) serve as valuable indicators of alterations in physical and chemical properties of polluted environments (Khan et al. 2007; Romero et al. 1999; Guo et al. 2012; Chen et al. 2014; Pająk et al. 2016).

Bacterial activity also plays an important role in determining metal bioavailability. The impact of metals on aquatic health is dependent on microbial activities. For instance, over a small time period, the concentration of pollutants in an aquatic environment will not change, but the bioavailability may change. Therefore, the total content of chemicals in water is not a reliable indicator of their bioavailability and thereby of water quality. Instead, bioavailability has to be measured in relation to bioassays and specific bacterial processes (Nielsen and Winding 2002; Li and Wong 2010). In this regard, bacterial community structure has to be identified first to assess the bioavailability of metals.

In addition to the potential of biological methods in bioindication and bioavailability assessment, they can also be used to clean up metals from wastewater generated from industries and household sewage, a phenomenon known as bioremediation, which has many potential leads. Bioremediation is cost-effective, has high efficiency, and its end products are also nonhazardous. Metal-resistant bacteria can be used for bioremediation of contaminated waters (Lloyd and Lovley 2001; Rehman and Shakoori 2001; Valls and Víctor de Lorenzo 2002; Gao et al. 2018).

An example of wastewater contamination is Lai Nullah, which is the largest natural water channel of Islamabad and Rawalpindi, Pakistan (Iram et al. 2013), stretching from Margallah Hills in Islamabad at northwestern edge till Soan River at the southeastern edge in Rawalpindi. It has a total length of about 30 km and has a catchment area of 239.8 km², 161.2 km² of it is present in Islamabad and 73.6 km² is in Rawalpindi (Kamal 2004). Total sewage coverage by Lai Nullah from Rawalpindi is about 65% and wastewater flowing in Lai Nullah is also contributing to ground water contamination. Various industries such as oil refineries, textile mills, marble crushing units, flour mills, soap and detergent, steel and electroplating, hydrogenated oils, automobiles, and rubber industries are operating in Islamabad and Rawalpindi. Immense amounts of wastes from different industries, domestic sources, and

agricultural practices contribute to the overall pollution of Lai Nullah (Iram et al. 2013).

Lai Nullah is an ideal site in Islamabad and Rawalpindi to isolate metal-resistant bacteria and study the effect of pollutants on indigenous aquatic bacteria, as various types of wastewater enter it (Sheikh et al. 2008). Lai Nullah also serves as an important source of water for drinking and irrigation purposes for a variety of communities living near the banks and catchment area (Iram et al. 2013). This further makes it critical to study the effect of water pollutants on living organisms in Lai Nullah. Therefore, this research was carried out to study the diversity of cultivatable bacteria in Lai Nullah. As microbial community responds to environmental contamination (Paerl et al. 2003; García-Armisen et al. 2014), we hypothesized that bacterial community thriving in household sewage and industrially polluted water of Lai Nullah would be different from the community present in nonpolluted water. Moreover, as bacteria thriving at contaminated sites adopt various mechanisms to resist toxic metals (Issazadeh et al. 2013), we also hypothesized that bacteria present at polluted water of Lai Nullah would be resistant to heavy metals as well as antibiotics because metal resistance and antibiotic resistance are correlated (Spain and Alm 2003). To test these hypotheses, we correlated the cultivatable bacterial community isolated from different locations of Lai Nullah with the physicochemical parameters and determined minimum inhibitory concentration of selected heavy metals and antibiotics for isolated bacteria. The objective of the study was to study the effect of household sewage and industrial waste on bacterial community present in Lai Nullah, which may provide us an idea about how this contamination may affect higher organisms and human beings.

## Materials and Methods

### Survey and sampling

A survey of Lai Nullah was conducted to select the sampling sites. Lai Nullah starts from Margallah Hills, where it contains uncontaminated water before entering Islamabad, whereafter it receives domestic and industrial wastewater when passing through different areas of Islamabad and Rawalpindi such as Sector I-8, Kattarian, Gawalmandi, Murree Brewery, and High Court (Fig. 1). Four independent running water samples were collected in sterilized plastic bottles from each of the above mentioned locations of Islamabad and Rawalpindi. Collected samples were transported to laboratory on ice and were stored at 4 °C for bacterial and physicochemical analysis.

### Physiochemical analysis of water samples

Physicochemical analysis was carried out at Pakistan Institute of Nuclear Science and Technology (PINSTECH), Islamabad, Pakistan.

Metals including calcium (Ca), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), sodium (Na), nickel (Ni), lead (Pb), zinc (Zn), and sulfate ($SO_4^{2-}$) were checked through inductively coupled plasma optical emission spectrometry (ICP-OES). Electrical conductivity, total dissolved solids (TDS), pH, turbidity, chloride ($Cl^-$), phosphate ($PO_4$), and nitrate ($NO_3$) were detected through instrumental method. Dissolved oxygen (DO) of water samples was analyzed through portable meter. Water samples were analyzed through titration for alkalinity and total hardness as calcium carbonate $CaCO_3$.

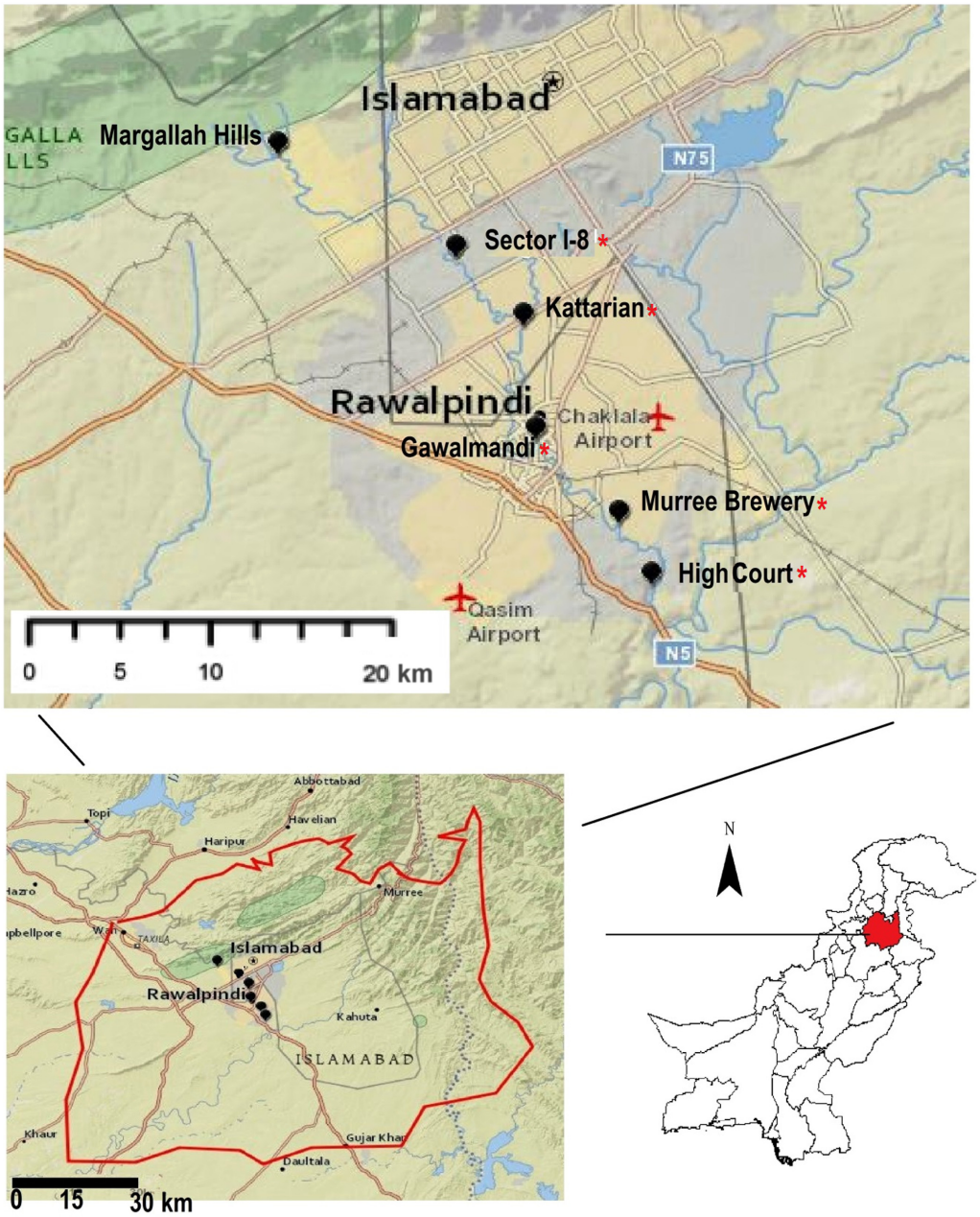
### Isolation of bacteria

Bacteria were isolated by serial dilution method (Maltseva and Oriel 1997). Briefly, 1 mL of each water sample was added to 9 mL of sterilized saline solution and serial dilutions were made up to $10^{-9}$ dilution. Hundred µL of each dilution was plated on MacConkey and Nutrient agar petri plates and incubated at 37 °C overnight to grow bacterial colonies. Individual colonies were subcultured on Luria Bertani (LB) agar plates to get pure colonies. Morphological data of each colony was recorded.

### Molecular identification of bacterial isolates

Genomic DNA was extracted using the method described by Cao et al. (2003). Amplification of bacterial 16S rRNA was done using universal primers P1 (5′ – CGGGATCCAGAGTTTGATCCTGGTCAGAACGAACGCT – 3′) and P6 (5′ – CGGGATCCTACGGCTACCTTGTTACGACTTCACCCC – 3′) (Tan et al. 1997). A total of 50 µl PCR reaction mixture contained 1 µl of DNA template (10–20 ng), 2 µl of each primer (5 mmol·$L^{-1}$), 6 µl of $MgCl_2$ (25 mmol·$L^{-1}$), 1 µl dNTPs (2 mmol·$L^{-1}$), 5 µl buffer, and 0.5 µl of Taq polymerase (1.5 U). Amplifications were performed using a Labnet Multigene Thermal Cycler (Labnet International, Inc. Edison, NJ, USA) and included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 92 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplified PCR products were separated on 1% (w/v) agarose gel through agarose gel electrophoresis in 1x TAE buffer and ethidium bromide (0.5 µg·$mL^{-1}$). PCR products were then purified using the protocol recommended by GeneJET PCR Purification Kit (Fermentas, EU). Purified PCR amplicons were sequenced commercially in both forward and reverse directions by Macrogen Inc., Korea. Sequences were trimmed, and forward and reverse sequences were then edited and reconciled using MacVector, version 15.5 (MacVector, Inc., Cary, NC, USA) to obtain the consensus sequence for each PCR amplicon. Phylogenetic affiliation of the sequences was checked using the BLAST search program (Altschul et al. 1997) at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.-nih.gov/).

**Fig. 1.** Study sites at Lai Nullah. An asterisk (*) represents contaminated sites. Maps were created using basic version of Scribble Maps (an online tool for geographic information system and annotation). [Colour online.]




**Statistical analysis of the cultivatable bacterial community and the physicochemical parameters**

PRIMER (Plymouth Routines in Multivariate Ecological Research, version 6.1.12, Primer-E Ltd, United Kingdom) (Clarke and Warwick 2001) was used to analyze physicochemistry and bacterial data. The mean of physicochemical data was normalized, log (square root) transformed, and resemblance matrix was calculated using Euclidian distance. The mean of bacterial data was standardized to account for variation in total bacterial colony counts among different samples and were then square root transformed to reduce the effect of abundant bacterial strains. A resemblance matrix was calculated using Bray Curtis similarity. The RELATE (Spearman rank correlation method) was used on bacterial and physicochemical resemblance matrices to measure the correlation between bacterial and physicochemical data. The resemblance matrices were also used to generate Non-metric multidimensional scaling (MDS) plots, principal coordinate analysis (PCA), and to calculate permutational analysis of variance using PERMANOVA+ in PRIMER. BEST (BIOENV) was used to determine which physicochemical parameters were correlated best with changes in the bacterial community.

**Table 1.** Physicochemical parameters of Lai Nullah water samples.

| Parameters | Location | | | | | |
|---|---|---|---|---|---|---|
| | Margallah Hills | Sector I-8 | Gawalmandi | Murree Brewery | Kattarian | High Court |
| Calcium ($\mu g \cdot mL^{-1}$) | 154 ± 3 | 105.44 ± 1.22 | 104.52 ± 3 | 109.17 ± 1 | 101.71 ± 2 | 88.87 ± 0.72 |
| Iron ($\mu g \cdot mL^{-1}$) | 0.01 ± 0.001 | 0.08 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0 | 0.03 ± 0.004 | 0.11 ± 0.01 |
| Manganese ($\mu g \cdot mL^{-1}$) | BDL | 0.1 ± 0.01 | 0.08 ± 0 | 0.11 ± 0.01 | 0.06 ± 0.003 | 0.14 ± 0.02 |
| Sodium ($\mu g \cdot mL^{-1}$) | 15.42 ± 0.6 | 108.34 ± 1.96 | 84.93 ± 0.68 | 90.42 ± 0.91 | 67.03 ± 1.73 | 87.68 ± 0.79 |
| Sulfate ($\mu g \cdot mL^{-1}$) | 166 ± 2 | 18.52 ± 0.57 | 17.83 ± 0.25 | 25.28 ± 0.62 | 23.96 ± 0.62 | 50.27 ± 0.90 |
| Zinc ($\mu g \cdot mL^{-1}$) | BDL | BDL | 0.02 ± 0 | 0.02 ± 0 | BDL | BDL |
| Calcium carbonate ($\mu g \cdot mL^{-1}$) | 552 ± 5 | 369.79 ± 1.51 | 380.05 ± 1.54 | 396.15 ± 5.45 | 361.56 ± 2.08 | 345.78 ± 2.50 |
| Alkalinity ($\mu g \cdot mL^{-1}$) | 350 ± 3 | 652.91 ± 2.74 | 823.77 ± 2.6 | 713.93 ± 1.71 | 720.03 ± 1 | 579.69 ± 0.88 |
| Conductivity ($\mu S \cdot cm^{-1}$) | 948 ± 7 | 1149 ± 3.22 | 1438 ± 4.05 | 1326 ± 4.64 | 1115 ± 2 | 1181 ± 6.07 |
| TDS ($\mu g \cdot mL^{-1}$) | 520 ± 5 | 636 ± 2.56 | 790 ± 5.51 | 734 ± 2.41 | 615 ± 3 | 650 ± 4.49 |
| pH | 7.1 ± 0.1 | 7.05 ± 0.01 | 6.9 ± 0.05 | 6.9 ± 0.06 | 7.01 ± 0.01 | 7.3 ± 0.03 |
| Turbidity ($\mu g \cdot mL^{-1}$) | 1.8 ± 0.05 | 55 ± 1.2 | 138 ± 1.2 | 123 ± 1.33 | 177 ± 2 | 34.4 ± 1.04 |
| Chloride ($\mu g \cdot mL^{-1}$) | 1.76 ± 0.01 | 28.87 ± 0.65 | 49.28 ± 1.17 | 54.51 ± 1.01 | 34.40 ± 1 | 36.91 ± 1.51 |
| Dissolved oxygen ($\mu g \cdot mL^{-1}$) | 4.86 ± 0.01 | 2.23 ± 0.01 | 1.58 ± 0.01 | 2.87 ± 0.01 | 1.98 ± 0.01 | 4.71 ± 0.08 |
| BOD ($\mu g \cdot mL^{-1}$) | 2 ± 0.05 | 19 ± 0.58 | 20 ± 1 | 22 ± 0.88 | 23 ± 0.88 | 26 ± 1.16 |
| Phosphate ($\mu g \cdot mL^{-1}$) | 0 | 5 ± 0.33 | 5 ± 0 | 5 ± 0.33 | 5 ± 0 | 6 ± 0.33 |

**Note:** Values are mean of four replicates from each of the six sampling sites ± standard deviation. TDS, total dissolved solids; BOD, biological oxygen demand; BDL, below detection limit. Cadmium, chromium, copper, nickel, lead, and zinc were BDL. The detection limits are as follows: cadmium = 0.01 $\mu g \cdot mL^{-1}$; chromium = 0.02 $\mu g \cdot mL^{-1}$; copper = 0.03 $\mu g \cdot mL^{-1}$; nickel = 0.01 $\mu g \cdot mL^{-1}$; lead = 0.03 $\mu g \cdot mL^{-1}$; zinc = 0.01 $\mu g \cdot mL^{-1}$.

#### Heavy metal resistance of isolated bacteria

The bacterial isolates were exposed to different concentrations of Cd (0.5 $mmol \cdot L^{-1}$, 1 $mmol \cdot L^{-1}$, 3 $mmol \cdot L^{-1}$, 5 $mmol \cdot L^{-1}$, 7 $mmol \cdot L^{-1}$), Ni (2.5 $mmol \cdot L^{-1}$, 3 $mmol \cdot L^{-1}$, 4.5 $mmol \cdot L^{-1}$, 6.5 $mmol \cdot L^{-1}$, 8.5 $mmol \cdot L^{-1}$), Pb (6 $mmol \cdot L^{-1}$, 8 $mmol \cdot L^{-1}$, 8.5 $mmol \cdot L^{-1}$, 9 $mmol \cdot L^{-1}$, 10 $mmol \cdot L^{-1}$), and Cu (5 $mmol \cdot L^{-1}$, 5.5 $mmol \cdot L^{-1}$, 6 $mmol \cdot L^{-1}$, 6.5 $mmol \cdot L^{-1}$, 7 $mmol \cdot L^{-1}$) added to LB agar plates in the form of Copper Sulfate ($CuSO_4$), Cadmium Chloride ($CdCl_2$), Nickel Sulfate ($NiSO_4$), and Lead Nitrate [$Pb(NO_3)_2$] until no growth of bacteria was observed. Metal concentration at which bacteria were unable to grow was recorded as minimum inhibitory concentration (MIC) of each metal (Haroun et al. 2017).

#### Antibiotic resistance of isolated bacteria

Bacterial strains were also tested for their resistance against selected antibiotics and MIC of the antibiotics were determined using broth microdilution method (Wiegand et al. 2008). Four different antibiotics, that is, ampicillin, tetracycline, vancomycin, and ciprofloxacin were used. Bacteria were exposed to 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, and 0.03 $\mu g \cdot mL^{-1}$ of each antibiotic. Minimum inhibitory concentration was recorded as the minimum concentration of an antimicrobial agent that could inhibit the growth of microorganisms after overnight incubation.

## Results

#### Physicochemistry of sampling sites

Physicochemical analysis revealed that heavy metals such as Cd and Ni were below the detection limit of 0.01 $\mu g \cdot mL^{-1}$, Cr was below the detection limit of 0.02 $\mu g \cdot mL^{-1}$ and Cu and Pb were below their detection limit of 0.03 $\mu g \cdot mL^{-1}$ at all the locations while Zn was below the detection limit of 0.01 $\mu g \cdot mL^{-1}$ at Sector I-8, Kattarian and High Court (Table 1). Multi-dimensional scaling plot, PCA and PERMANOVA+ analysis ($P < 0.05$) based on physicochemical matrix showed that Margallah Hills site was significantly different in terms of the physicochemical parameters from other sites i.e., Sector I-8, Kattarian, Gawalmandi, Murree Brewery and High Court of Lai Nullah as Margallah Hills site scattered away from rest of the sites while some variation was also observed among Sector I-8, Kattarian, Gawalmani, Murree Brewery and High Court of Lai Nullah (Figs. 2 and 3).

Based on physicochemistry, Margallah Hills site was rich in $SO_4^{2-}$, Ca and $CaCO_3$, Sector I-8, Gawalmandi, Kattarian and Murree Brewery had higher level of Zn, TDS, conductivity, turbidity and alkalinity while High Court had higher concentration of $Cl^-$, Mn, Na, DO, BOD, Fe, $PO_4$, and pH (Table 1, Fig. 3).

#### Bacterial community at Lai Nullah sampling sites

Diverse cultivatable bacterial community was identified at Lai Nullah study sites. Among these group of bacteria, *Bacillus,* comprising *Bacillus subtilis, Bacillus atrophaeus, Bacillus licheniformis* was the most abundant genus retrieved from polluted sites (Sector I-8, Kattarian, Gawalmandi, Murree Brewery and High Court) of Lai Nullah followed by *Staphylococcus,* which was represented by *Staphylococcus epidermidis* and

**Fig. 2.** Multi-dimensional scaling (MDS) plot based on physicochemistry at different locations of Lai Nullah. The plot was generated using mean values of four replicates from each of the six sampling sites. [Colour online.]

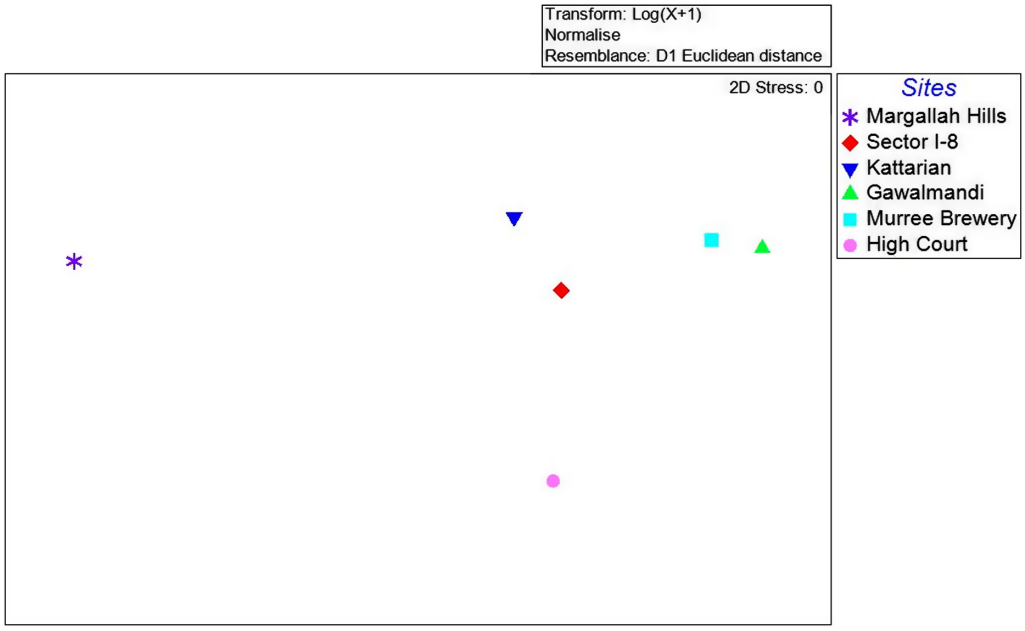
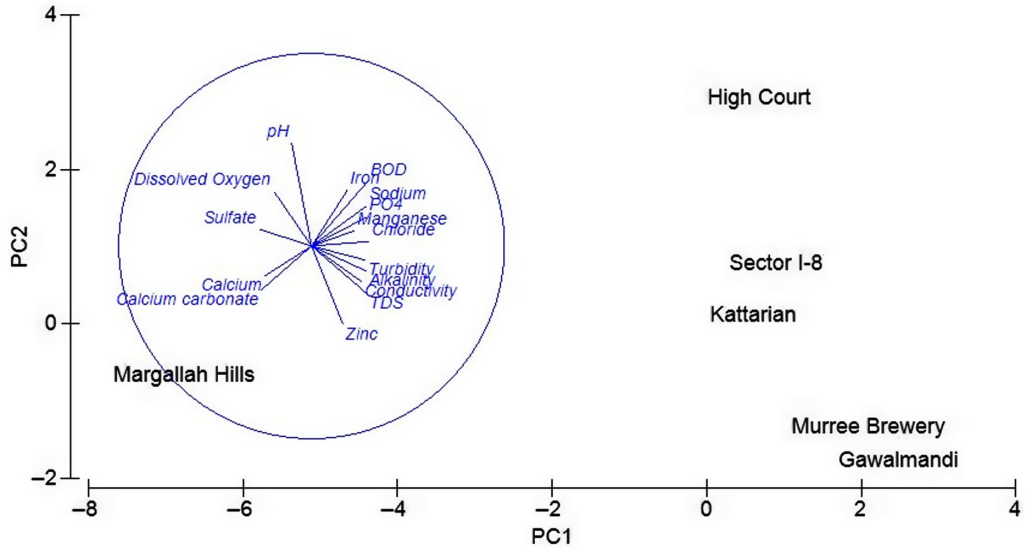



**Fig. 3.** Principal coordinate analysis (PCA) plot generated from physicochemistry indicating the distribution of measured physicochemical parameters among different locations of Lai Nullah. The plot was generated using mean values of four replicates from each of the six sampling sites. [Colour online.]




*Staphylococcus caprae*. At the species level, *Staphylococcus epidermidis* was the most abundant followed by *Bacillus subtilis, Staphylococcus caprae, Citrobacter freundii, Klebsiella pneumoniae, Bacillus atrophaeus, Bacillus licheniformis, Escherichia coli, Lactobacillus plantarum, Enterobacter cloacae, Geobacillus stearothermophilus, Enterococcus faecalis, Acinetobacter guillouiae, Ralstonia* sp., *Comamonas* sp., and *Stenotrophomonas maltophilia* (Table 2). Among polluted sites, maximum colony count was recorded for High Court followed by Kattarian, Murree Brewery, Gawalmandi, and Sector I-8 (Table 2). Nonpolluted site of Margallah Hills showed least colony count with *Enterobacter cloacae, Staphylococcus epidermidis, Staphylococcus caprae, Aeromonas veronii, Exiguobacterium* sp., and *Lysinibacillus macroides* representing the bacterial community at the site. So, *Bacillus* spp., *Citrobacter freundii, Klebsiella pneumoniae, Escherichia coli, Lactobacillus plantarum, Geobacillus stearothermophilus, Enterococcus faecalis,*

**Table 2.** Bacterial species colony count found at different locations of Lai Nullah.

| | | Study sites | | | | | |
|---|---|---|---|---|---|---|---|
| Closest homologue | Homology | Margallah Hills | Sector I-8 | Gawalmandi | Murree Brewery | Kattarian | High Court |
| *Staphylococcus epidermidis* | 99% | 7 ± 1 | 63 ± 3 | 44 ± 3 | 225 ± 5 | 284 ± 6 | 100 ± 5 |
| *Bacillus Subtilis* | 98% | 0 | 80 ± 3 | 26 ± 3 | 44 ± 2 | 201 ± 3 | 281 ± 6 |
| *Staphylococcus caprae* | 99% | 8 ± 2 | 32 ± 1 | 12 ± 1 | 80 ± 2 | 90 ± 5 | 40 ± 1 |
| *Citrobacter freundii* | 97% | 0 | 21 ± 2 | 54 ± 3 | 94 ± 4 | 23 ± 2 | 59 ± 4 |
| *Klebsiella pneumoniae* | 99% | 0 | 10 ± 1 | 20 ± 2 | 100 ± 4 | 71 ± 2 | 34 ± 2 |
| *Bacillus atrophaeus* | 99% | 0 | 5 ± 1 | 15 ± 1 | 6 ± 1 | 0 | 155 ± 6 |
| *Bacillus licheniformis* | 99% | 0 | 11 ± 1 | 50 ± 2 | 18 ± 2 | 18 ± 1 | 52 ± 2 |
| *Escherichia coli* | 99% | 0 | 41 ± 2 | 7 ± 1 | 61 ± 2 | 16 ± 1 | 1 ± 0 |
| *Lactobacillus plantarum* | 99% | 0 | 7 ± 1 | 20 ± 2 | 17 ± 1 | 45 ± 3 | 34 ± 2 |
| *Enterobacter cloacae* | 98% | 6 ± 1 | 7 ± 1 | 16 ± 1 | 44 ± 2 | 22 ± 1 | 19 ± 1 |
| *Geobacillus stearothermophilus* | 99% | 0 | 8 ± 1 | 22 ± 1 | 14 ± 0 | 12 ± 1 | 50 ± 2 |
| *Enterococcus faecalis* | 97% | 5 ± 1 | 12 ± 1 | 22 ± 2 | 19 ± 1 | 13 ± 1 | 34 ± 2 |
| *Acinetobacter guillouiae* | 99% | 0 | 0 | 50 ± 2 | 5 ± 0 | 0 | 0 |
| *Ralstonia* sp. | 99% | 0 | 11 ± 1 | 5 ± 1 | 3 ± 0 | 2 ± 0 | 7 ± 1 |
| *Comamonas* sp. | 99% | 0 | 1 ± 0 | 3 ± 0 | 2 ± 0 | 1 ± 1 | 2 ± 0 |
| *Aeromonas veronii* | 99% | 8 ± 1 | 0 | 0 | 0 | 0 | 0 |
| *Exiguobacterium* sp. | 98% | 7 ± 0 | 0 | 0 | 0 | 0 | 0 |
| *Lysinibacillus macroides* | 96% | 7 ± 0 | 0 | 0 | 0 | 0 | 0 |
| *Stenotrophomonas maltophilia* | 99% | 0 | 0 | 0 | 3 ± 0 | 0 | 0 |
| Total | | 48 | 309 | 366 | 735 | 798 | 868 |

**Note:** Values are mean of four replicates from each of the six sampling sites ± standard deviation.

*Acinetobacter guillouiae, Ralstonia* sp., *Comamonas* sp., and *Stenotrophomonas maltophilia* were specific to polluted water of Lai Nullah and were not found at nonpolluted Margallah Hills site. On the other hand, *Aeromonas veronii, Exiguobacterium* sp., and *Lysinibacillus macroides* were limited to nonpolluted site of Margallah Hills only (Table 2).

### Comparison of bacterial community present at different locations of Lai Nullah

MDS plot based on bacterial resemblance matrix also showed that all the studied locations were different in terms of the bacterial community as shown in Fig. 4. However, greatest difference was found among Margallah Hills and rest of the sites of Lai Nullah. PERMANOVA+ analysis also indicated that sampling sites were significantly different in terms of the bacterial community ($P < 0.05$).

### Correlation between bacterial community and physicochemical parameters of Lai Nullah

The bacterial community was positively correlated with the physicochemical parameters at Lai Nullah (Spearman's rho = 0.61). Among physicochemical parameters measured, BOD, $PO_4$ $Cl^-$, and Na were best correlated with the changes in the bacterial community (Spearman's rho = 0.85). Among bacterial species isolated in this study, *Bacillus* species best responded to the changes in the physicochemical parameters as number of *Bacillus* colonies increased with the increase in the concentration of BOD, PO4, $Cl^-$, and Na.

### Heavy metal minimum inhibitory concentration for bacterial isolates

Minimum inhibitory concentration of Cu, Ni, Pb, and Cd for bacterial isolates is given in Table 3. Minimum inhibitory concentration of Pb ranged from 4.0 to 8.5 mmol·$L^{-1}$, for Cu MIC ranged from 2.0 mmol·$L^{-1}$ to 7.0 mmol·$L^{-1}$, MIC of Ni ranged from 2.0 mmol·$L^{-1}$ to 4.0 mmol·$L^{-1}$, whereas MIC of Cd ranged from 1.0 mmol·$L^{-1}$ to 1.5 mmol·$L^{-1}$. For Pb, maximum MIC of 8.5 mmol·$L^{-1}$ was shown by several isolates, that is, *Bacillus atrophaeus*, *Bacillus licheniformis, Bacillus subtilis, Citrobacter freundii, Klebsiella pneumoniae, Lactobacillus plantarum,* and *Enterobacter cloacae*. Maximum MIC for Cu (7.0 mmol·$L^{-1}$) was shown by *Klebsiella pneumoniae*. For Ni, *Klebsiella pneumoniae* and *Escherichia coli* both showed maximum MIC of 4.0 mmol·$L^{-1}$. *Comamonas* sp., *Klebsiella pneumoniae,* and *Escherichia coli* also showed maximum MIC (1.5 mmol·$L^{-1}$) for Cd. Hence, *Klebsiella pneumoniae* showed maximum resistance toward all the tested metals.

### Antibiotic minimum inhibitory concentration for bacterial isolates

All the bacterial isolates showed some level of resistance against the tested antibiotics. However, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST), *Bacillus subtilis, Citrobacter freundii,*

**Fig. 4.** Multi-dimensional scaling (MDS) plot based on bacterial community at different locations of Lai Nullah. The plot was generated using mean values of four replicates from each of the six sampling sites. [Colour online.]

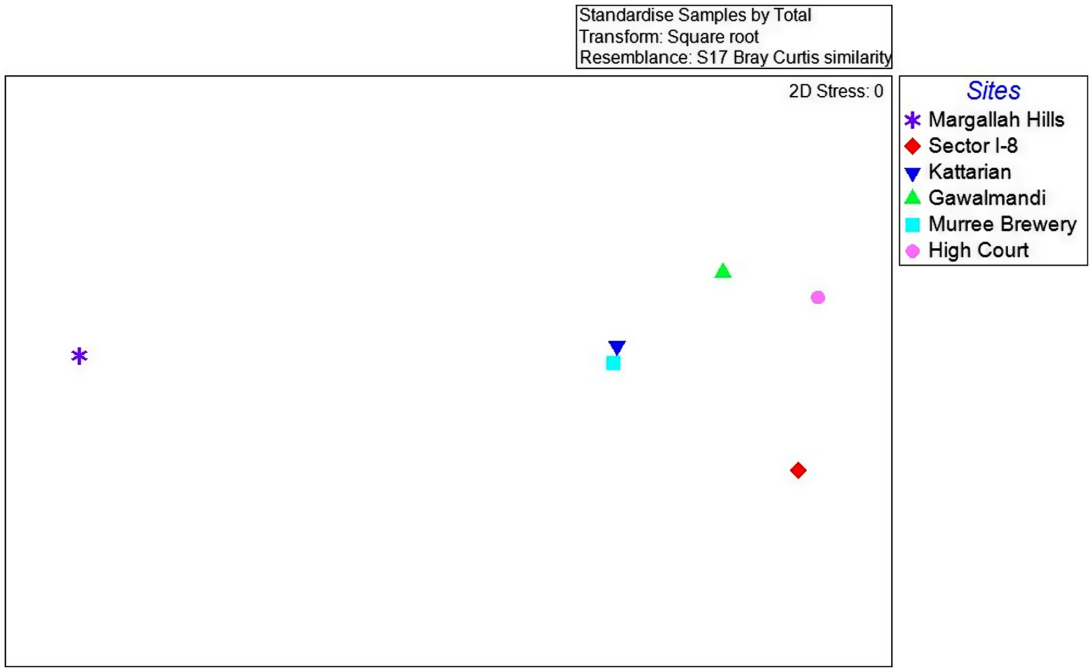



**Table 3.** Minimum inhibitory concentration (MIC) of lead, copper, nickel, and cadmium for bacterial species isolated from different locations of Lai Nullah.

| | MIC ($mmol \cdot L^{-1}$) | | | |
|---|---|---|---|---|
| Bacterial species | Lead | Copper | Nickel | Cadmium |
| *Staphylococcus epidermidis* | 5.5 | 5.0 | 3.0 | 1.0 |
| *Bacillus subtilis* | 8.5 | 2.0 | 2.5 | 1.0 |
| *Staphylococcus caprae* | 5.5 | 5.0 | 3.0 | 1.0 |
| *Citrobacter freundii* | 8.5 | 5.5 | 3.0 | 1.0 |
| *Klebsiella pneumoniae* | 8.5 | 7.0 | 4.0 | 1.0 |
| *Bacillus atrophaeus* | 8.5 | 5.5 | 2.5 | 1.0 |
| *Bacillus licheniformis* | 8.5 | 5.0 | 3.0 | 1.0 |
| *Escherichia coli* | 4.5 | 5.5 | 4.0 | 1.5 |
| *Lactobacillus plantarum* | 8.5 | 2.0 | 2.5 | 1.0 |
| *Enterobacter cloacae* | 8.5 | 2.0 | 3.0 | 1.0 |
| *Geobacillus stearothermophilus* | 4.0 | 5.5 | 2.0 | 1.0 |
| *Enterococcus faecalis* | 4.0 | 2.0 | 2.5 | 1.0 |
| *Acinetobacter guillouiae* | 4.0 | 2.0 | 2.5 | 1.0 |
| *Ralstonia* sp. | 5.5 | 5.0 | 3.0 | 1.0 |
| *Comamonas sp.* | 5.0 | 5.5 | 2.5 | 1.5 |
| *Aeromonas veronii* | 4.0 | 2.0 | 2.0 | 1.0 |
| *Exiguobacterium* sp. | 4.0 | 2.0 | 2.0 | 1.0 |
| *Lysinibacillus macroides* | 4.0 | 2.0 | 2.0 | 1.0 |
| *Stenotrophomonas maltophilia* | 5.0 | 5.0 | 2.5 | 1.0 |

*Lactobacillus plantarum, Enterobacter cloacae, Geobacillus stearothermophilus, and Enterococcus faecalis* were resistant to all the antibiotics tested while rest of the bacteria were susceptible to at least one of the antibiotics (Table 4).

## Discussion

We hypothesized that cultivatable bacterial community of Lai Nullah water polluted with household sewage and industrial waste would be different from nonpolluted water. In fact, our findings supported this

**Table 4.** Minimum inhibitory concentration (MIC) of ampicillin, tetracycline, ciprofloxacin, and vancomycin for bacterial species isolated from different locations of Lai Nullah.

| | MIC ($\mu g \cdot mL^{-1}$) | | | |
|---|---|---|---|---|
| Bacterial species | Ampicillin | Tetracycline | Ciprofloxacin | Vancomycin |
| *Staphylococcus epidermidis* | <0.5 | <0.5 | <0.25* | <1* |
| *Bacillus subtilis* | >4 | <4 | >2 | >8 |
| *Staphylococcus caprae* | <1* | >.5* | <0.06* | <8 |
| *Citrobacter freundii* | 8.5 | 5.5 | 3.0 | 1 |
| *Klebsiella pneumoniae* | >16 | >1* | >2 | *<0.125** |
| *Bacillus atrophaeus* | >4 | <4 | <0.5 | <2* |
| *Bacillus licheniformis* | >4 | <4 | <0.5 | <2* |
| *Escherichia coli* | <4 | <1* | <0.25 | <0.25* |
| *Lactobacillus plantarum* | <0.5 | >8 | <1 | <1 |
| *Enterobacter cloacae* | <4 | <4 | >1 | >4 |
| *Geobacillus stearothermophilus* | >16 | <4 | <2 | <4 |
| *Enterococcus faecalis* | >2 | >0.125 | <1 | >1 |
| *Acinetobacter guillouiae* | >4 | <1* | >1 | <2* |
| *Ralstonia* sp. | >8 | <4 | <0.06* | <4 |
| *Comamonas sp.* | >16 | <.5 | <1 | >4 |
| *Aeromonas veronii* | >1* | <4 | >1 | <1* |
| *Exiguobacterium* sp. | >1 | >2 | >0.5 | <2* |
| *Lysinibacillus macroides* | <0.5* | <0.125* | >0.5 | <1* |
| *Stenotrophomonas maltophilia* | >4 | <2 | <0.06* | <2 |

*Bacteria are susceptible to given antibiotic according to European Committee on Antimicrobial Susceptibility Testing (EUCAST).

hypothesis as the bacterial community in polluted water was significantly different from the nonpolluted water. Moreover, the bacterial community at polluted water was even more diverse and had a higher density than the nonpolluted water. *Bacillus* spp., *Citrobacter freundii, Klebsiella pneumoniae, Escherichia coli, Lactobacillus plantarum, Geobacillus stearothermophilus, Enterococcus faecalis, Acinetobacter guillouiae, Ralstonia* sp., *Comamonas* sp., and *Stenotrophomonas maltophilia* were specific to polluted water of Lai Nullah and were not found in nonpolluted sites. In fact, the genera *Escherichia, Klebsiella, Enterobacter,* and *Citrobacter* (collectively known as coliform bacilli) are used to assess the water quality (Guentzel 1996). Their occurrence indicates water contamination as in the case of Lai Nullah. Some of the bacterial species such as *Bacillus, Citrobacter, Acinetobacter, Klebsiella,* and *E. coli* have been previously isolated from industrial and household sewage water (Silva-Bedoya et al. 2016; Li et al. 2017).

Statistical analysis revealed a positive correlation between the bacterial community and the physicochemical parameters at different locations of Lai Nullah. Higher BOD was one of the contributing factors responsible for higher bacterial population at polluted sites of Lai Nullah. As seen in this present study, the discharge of effluent adds nutrients into the water bodies resulting in an increase in the growth of bacterial isolates (Prabha et al. 2017). Higher BOD at our study sites indicates an excessive amount of biodegradable organic matter that serves as a source of energy for bacteria, which ultimately increased the growth and population of the bacterial community. Similarly, $PO_4$, Na, and $Cl^-$ were also positively correlated with the bacterial community. Phosphate is an important nutrient for bacterial growth (Vadstein 1989; Juhna et al. 2007) and Roessler et al. (2003) have shown that $Cl^-$ is essential for the growth of various bacteria living in high salinity ($Na^+$) conditions like Lai Nullah. Similar correlation between bacterial density and the concentrations of BOD, $PO_4$, Na, and $Cl^-$ was observed by Olutiola et al. (2010), Prabha et al. (2017), Vadstein (1989) and Juhna et al. (2007). Thus, physicochemistry directly affected the bacterial community and the bacterial population of Lai Nullah responded to increase in the physicochemical parameters. This reinforces earlier studies that bacterial community can serve as a bioindicator of environmental pollution (Haq and Shakoori 2000; Zhou et al. 2008).

It was interesting to note that a different colony number of isolated bacterial species was present at Margallah Hills, Sector I-8, Gawalmandi, Kattarian, Murree Brewery, and High Court of Lai Nullah. In general, highest colony number of bacterial species was present at High Court area possibly due to higher BOD and $PO_4$ at this site both of which indicates the presence of energy source for bacterial growth.

Higher concentration of heavy metals in contaminated waters adversely affects the growth of bacteria. However, in Lai Nullah, tested heavy metals were either not detected or their concentrations were well below the minimum inhibitory concentrations of the isolated

bacteria. Therefore, these heavy metals may not have negatively affected the bacterial community.

Among isolated bacteria, *Bacillus* species were the most abundantly present followed by *Staphylococcus*. *Bacillus* spp. have been shown to survive in contaminated environments through acquisition of specific resistance mechanisms to selectively accumulate and reversibly bind metals including Pb, Ni, and Cd from polluted environments (Mumtaz et al. 2013; Selenska-Pobell et al. 1999). Heavy metals are mainly uptaken by carboxyl groups present in the bacterial cell wall (Beveridge 1989; Yilmaz 2003). Other than this, *Bacillus* spp. have also been shown to produce endospores under stress conditions. These characteristics of *Bacillus* spp. may explain their high occurrence in polluted environment of Lai Nullah. Due to their ability to bind the substantial amount of heavy metals, *Bacillus* spp. can be used for heavy metal removal and bioremediation of pollutants from different sources (Selenska-Pobell et al. 1999; Augusto da Costa and Duta 2001; Wierzba 2015; García et al. 2016). Similarly, species belonging to *Staphylococcus* are normal wastewater contaminants and the presence of various drug resistance and virulence genes in wastewater inhabiting *staphylococci* can be of public health concern (Gómez et al. 2016; Ben Said et al. 2017).

We also predicted that bacteria thriving at household sewage and industrially polluted water of Lai Nullah would be resistant to heavy metals as bacteria adopt themselves to polluted environments through various resistance mechanisms (Issazadeh et al. 2013). As expected, the isolated bacteria exhibited resistance to studied heavy metals such as Pb, Cu, Cd, and Ni. Bacterial isolates showed greater resistance toward Pb and Cu compared with Cd and Ni. This shows that Cd and Ni are more toxic. Our results match with that of Hassen et al. (1998), Hussein and Joo (2013) and Marzan et al. (2017) who also observed higher MIC of Pb and Cu for studied bacteria.

Heavy metals resistance of bacteria present at contaminated environments has some ecological significance. There is a correlation between bacterial resistance to heavy metals and antibiotics resistance. Antibiotic resistance is acquired by the changes in the genetic makeup of bacterial genome either by mutation or by the transfer of antibiotic resistance gene between bacterial species in environment. Bacteria showing resistance to heavy metals can also be antibiotic resistant because both resistant genes are present close together on the same plasmid and can also be transferred into the environment simultaneously (Lazăr et al. 2002; Spain and Alm 2003; Safari and Younessi 2017). Thus, in our study, all the isolated bacterial species showed some level of resistance against tested antibiotics, which supported our hypothesis that bacteria resistant to heavy metals would also be able to resist antibiotics. Co-occurrence of heavy metals resistance and antibiotic resistance is well reported (Yamina et al. 2012; Jiang et al. 2020; Thomas et al. 2020). The microorganisms that have developed both heavy metals and antibiotic resistance in contaminated environments can act as pool for the resistance genes that can ultimately be acquired by the pathogenic species. These resistant bacteria can contaminate the water bodies and transfer these resistance genes to other pathogenic and nonpathogenic bacteria. Therefore, microbial resistance to heavy metals plays an important role in ecology especially those bacteria, which are resistant to antibiotics as well (Spain and Alm 2003).

The knowledge about the potential bioavailability of the physicochemical parameters provided by the bacterial community assessment may have not been possible by physicochemical analysis alone. The work provides important information of bacterial community present at Lai Nullah for further studies of water-microbe-metal interactions, which may help in developing sustainable management and rehabilitation strategies at polluted environment of Lai Nullah.

## Conclusion

This study revealed that household sewage and industrially polluted water of Lai Nullah, a water channel flowing through Islamabad and Rawalpindi, Pakistan, have changed the bacterial community structure and membership compared with the nonpolluted water. This indicates the effect of water pollution on microorganisms, which may be applicable to higher organisms and humans. Moreover, bacteria isolated from polluted water showed resistance against selected heavy metals and antibiotics. These bacteria can transfer the resistance genes to pathogenic bacterial strains. So, environmental contamination not only has a harmful effect on living organisms, but may also evolve antibiotic resistant pathogenic bacterial strains, which may be difficult to treat and could cause severe health issues.

## Conflict of Interest

Authors declare no conflict of interest.

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