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Fingerprinting of heavy metal and microbial contamination uncovers the unprecedented scale of water pollution and its implication on human health around transboundary Hudiara drain in South Asia

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ABSTRACT

Environment and health hazards posed by the contamination of existing underground water resources are amongst the leading anthropogenic challenges in South Asia. One such major environmental hazard is posed by the transnational wastewater drain – *Hudiara*, which is degrading the quality of underlying aquifers. Towards a spatiotemporal assessment of Hudiara's wastewater quality and drinking water from its adjoining areas, a total of 56 wastewater and 42 drinking water samples were obtained over seven months. Analysis of spatiotemporal data revealed a complex interplay of geogenic and anthropogenic factors underlying the HM burden leading to a high contamination factor(CF) (>6 for Mercury in wastewater and >150 for Arsenic in drinking water) and heavy metal pollution index(HPI) (>100 for 9 heavy metals). Moreover, high health risk assessment(HRA) scores (>1 for Arsenic, Mercury, Chromium, Lead, Cadmium, and Copper) indicated the unprecedented scale of drinking water contamination. A concomitant public health survey conducted in the surrounding population reported a high prevalence of gastrointestinal diseases (40%), chronic obstructive pulmonary diseases (COPD) (38.7%), dermatitis (34%), bone deformities (31%), and hepatic disorders (12%). Lastly, 16S rRNA sequences of Hudiara were submitted to the Comprehensive Antibiotic Resistance Database (CARD) and point mutations in the sequences were potentially found to confer antimicrobial resistance (AMR) against three classes of drugs including aminoglycosides, peptides, and tetracyclines.

Taken together, this pilot study attempts to uncover the unprecedented scale of water contamination along the transboundary Hudiara drain thus highlighting the critical need

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for international regulatory action towards ameliorating its catastrophic effects on the environment.

1. Introduction

Freshwater constitutes a mere 3% of the existing water resources on our planet (Avlonas and Nassos, 2013; Dudgeon, 2020). Most of this freshwater is trapped in ice caps and glaciers; of it, only 0.01% is available for human consumption in the form of surface and groundwater (Hinrichsen and Tacio, 2002). With the growing human population and industrialization, these paltry usable freshwater resources are under constant threat by natural and anthropogenic pollutants (World Health Organization, 2000). Worldwide, it is estimated that about 80% of the polluted water is discharged untreated into the water bodies (Connor, 2017); more so, the production of this wastewater is projected to reach 51% by 2050 (Qadir et al., 2020). In particular, Asia's contribution in terms of wastewater production stands at about 42% of global output (Qadri and Faiq, 2020) out of which only 32% is treated (Nations, 2013) thereby jeopardizing the sustainability of freshwater resources and associated habitat. In one of the largest anthropogenic disasters in Asia, industrial discharge containing untreated methyl mercury was released into Minimata Bay, between 1932 and 1968, which affected over 50,000 people with outcomes such as paralysis, brain damage, and delirium (Baxter, 1990; Okada and Peterson, 2000; World Health Organization, 2000). In another environmental disaster, mining activities in Bangkok have caused arsenic contamination of topsoil and groundwater, affecting thousands of local inhabitants (World Health Organization, 2001). In the Indian subcontinent, unrelenting anthropogenic contamination of the transboundary *Hudiara* stormwater channel has converted it into one of the largest contaminated wastewater drains in the region (Khattak et al., 2012). Originating from Batala District in Gurdaspur, India, Hudiara (also known as Tung Dhab in India) runs for 44.2 km before entering Pakistan at Laloo village. It then stretches for another 55 km in Lahore before emptying into River Ravi at Mohlanwal (Afzal et al., 2000; Kashif et al., 2009). With over 100 industrial sites on its banks in India (World Wide Fund For Nature, 2007) and another 600 in Pakistan (Ahmed, 2017), Hudiara receives untreated effluents from pharmaceuticals, textiles, tanneries, pesticides, and metallurgical industries (Afzal et al., 2000; Ejaz et al., 2011). Consequently, a multitude of toxic heavy metals, including arsenic, lead, cadmium, mercury, and chromium are introduced into the drain (Ahmad et al., 2019; Majeed et al., 2018). Additionally, agricultural activities including crops and livestock farming contribute to the discharge of a significant amount of nitrates, phosphates, and other heavy metals into Hudiara (Akhtar et al., 2014a). Besides industrial and agricultural pollutants, domestic run-offs also contribute a substantial amount of heavy metals and biological contaminants as indicated by total coliform, fecal coliform, *Escherichia coli*, and *Enterococci* counts (Anwar and Gaffar, 2018; Hamid et al., 2013; Haydar et al., 2014; Yasar et al., 2015). This renders Hudiara drain one of the largest carriers of untreated industrial, agricultural, and municipal discharge that is fed by a network of smaller drains within India and Pakistan (Akhtar and Nawaz, 2012; The Tribune, 2022, 2021) before emptying into River Ravi.

Research has already established that heavy metal exposure has undesirable repercussions on fundamental environmental elements including the atmosphere, lithosphere, hydrosphere, and biosphere (Mitra et al., 2022). Literature reports that cadmium, lead, nickel, and mercury are atmospheric pollutants and are involved in causing long-range transboundary air pollution with adverse impacts on the associated biota (World Health Organization, 2007). Moreso, heavy metals' persistent and non-biodegradable nature, specifically cadmium, chromium, copper, mercury, lead, and nickel, can enable their accumulation in the soil beside the consequent leaching and run-off into the aquatic environment. Several research studies on the seepage of contaminated wastewater channels have elucidated its adverse effects on underground aquifers as well as surface water (Akhtar et al., 2014b; Ly and Chui, 2011; McArthur et al., 2012). Consumption of contaminated water or agricultural products grown using it, lead to the introduction and accumulation of toxic heavy metals into the food chain, posing severe detrimental effects on crop yields, livestock, and human health thereby potentially ruining the ecosystem (Martin and Griswold, 2009; Naser et al., 2014; Yamin and Ahmad, 2007). Moreso, the discharge of metal-laden water into the nearby river bodies can lead to the bioaccumulation, trophic transfer, and biomagnification of hazardous metals along the aquatic food chain thereby imparting deleterious effects on local fish population besides increasing the production of reactive oxygen species (Ali and Khan, 2019).

The imminent surge of heavy metals into the ecosystems can also impose a myriad of consequences on the terrestrial and aquatic microbial communities by decreasing the diversity, population size, and overall activity of the microbes (Fan et al., 2016; Yao et al., 2003). Conversely, long-term exposure to persistently elevated levels of heavy metals from geogenic–anthropogenic sources and their complex interplay can impose selection pressures playing crucial roles in the establishment of contaminated ecosystems (de Mora et al., 2005). This is particularly true of microbial loads, which are established causal agents of water-borne infections broadly classified into diarrhoeal, parasitological, skin, and eye diseases (Anwar and Gaffar, 2018; Cesa et al., 2016). Microbes evolve via biochemically and genetically encoded mechanisms which affect their metabolic activity as well as diversity (Hoostal et al., 2008; Ndeddy Aka and Babalola, 2017; Sobolev and Begonia, 2008). Several studies have shown a positive correlation between multi-metal contaminated environments and the incorporation of metal resistance genes (MRG) (Besaury et al., 2013; Zhao et al., 2019). Research has further helped establish the co-selection of metal resistance and antibiotic resistance in heavy metal contaminated ecosystems, which could further potentiate the emergence of antimicrobial resistance (AMR) - a leading threat to public health (Hu et al., 2016; Zhu et al., 2013).

Here it is important to note that the adverse effects of heavy metals on human health vary by age group, gender, and genetics of the individual alongside the type of metal, and its exposure route, dosage, and frequency. Specifically, short-term exposure to arsenic, mercury, cadmium, and chromium has been documented to cause adverse health effects including, but not limited to gastrointestinal disorders, contact dermatitis, respiratory distress, and even death, in extreme cases (Jaishankar et al., 2014; Tchounwou et al., 2012). Chronic exposure can induce carcinogenesis (Chen et al., 2019; Tchounwou et al., 2012), muscular (Martin and Griswold, 2009), immunological (Lehmann et al., 2011), cardiovascular (Ötleş and Çağindi, 2010), and neurological disorders (Thomas et al., 2009). In particular, exposure to arsenic (Tofail et al., 2009), cadmium (Chandravanshi et al., 2021), and lead (Shannon, 2003) are established causes of cognition development disorders in utero and early childhood besides increasing the risk of spontaneous abortion and stillbirths. Furthermore, prolonged mercury exposure, lead, and cadmium have been reported to accelerate the age-related deterioration of vital organs including kidneys, liver, lungs, and bones (Bernard, 2008; Bui Huy et al., 2014; Järup, 2003). Hudiara being a prime carrier of such contaminants is hence a direct threat to the lives of millions of people residing in its vicinity (Anwar and Gaffar, 2018; Haydar et al., 2014; Kumar et al., 2015; Singh, 2015). Field surveys indicate that 79% of the water samples collected from different sites of the Hudiara drain are heavily metal-laden and, therefore, unfit even for irrigational purposes (Ahmad et al., 2020; Khattak et al., 2012); food crops irrigated with this contaminated water have a significant heavy metal load. Towards establishing the effect of heavy metal contamination in Hudiara drain and its adjoining areas, we designed a novel study on Hudiara drain and its adjoining areas.

To assess the spatiotemporal variance in physicochemical parameters and heavy metal burden of Hudiara drain and its adjoining areas, we (i) profiled the temperature, pH, and heavy metal content in waste and drinking water samples, (ii) apportioned the complex interplay of these parameters into anthropogenic and geogenic sources, (iii) calculated pollution indices for water samples towards gauging the hazards posed by the consumption of metal-laden food and water, (iv) computed health risk assessment scores of drinking water followed by the mapping of water-associated communicable and non-communicable disease burden in the vicinity of Hudiara, and (v) characterized the Hudiara specific microbes to elucidate the impact of heavy metal contamination on the flora and fauna and their potential for antimicrobial resistance.

Our results show that mercury is the leading contaminant in wastewater (∼80 folds higher as compared to National Environmental Quality Standards, NEQS), and arsenic in drinking water (∼200 times as compared to World Health Organization (WHO) levels), at both sampling sites. Apportioning of heavy metal load and pH via principal component analysis (PCA) indicates a composite interplay of nine heavy metals and pH. Additionally, higher agglomerative clustering analysis (HACA) established that the leaching of arsenic, chromium, and iron is potentially occurring at alkaline pH. Next, an evaluation of water quality indices indicated abnormally high contamination factor (CF) values for mercury in wastewater (mean CF value ∼ 80) and arsenic in drinking water (mean CF value ∼ 200). Moreover, the heavy metal pollution index (HPI) of drinking water samples was found to be well over the permissible limits (index limit of 100). Health risk assessment (HRA) scores revealed drinking water to be unfit for human consumption due to heavy metal contamination. A follow-up health survey confirmed the impact of contaminated drinking water leading to respiratory, gastrointestinal, skin, hepatic and bone-related disorders in the local population. Besides leading to the onset of communicable and non-communicable diseases, heavy metal contamination also conferred antimicrobial resistance (AMR) against aminoglycosides, peptides, and tetracyclines classes of drugs to Hudiara-specific microbiota from both waste and drinking water samples. Taken together, our study is a crucial step towards mapping the environmental effects of water contamination in one of the most populous regions of the Indian subcontinent. Findings from the study highlight the gaps in the development and implementation of stringent international environment protection laws and industrial effluent discharge guidelines.

2. Materials and methods

2.1. Sampling methodology

Wastewater samples were obtained from pre- and post-Satukatla sites on the Hudiara drain (Fig. 1). Pre-Satukatla, which carries wastewater load from India, is located at Dullu Kalan (31°24′44N, 74°22′17E) near Gajju Matta, just before the confluence of Satukatla and Hudiara drains. Post-Satukatla, which carries wastewater load from Lahore, is situated at Descon (31°24′1N, 74°10′39E), downstream to the confluence of Satukatla and Hudiara drains. Since the cross-sectional width of Hudiara is approximately 25 m, samples were drawn from 4 different positions i.e. the right bank (site ‘A’), center-top of mainstream (site ‘B’), center-bottom of mainstream (site ‘C’), and left bank (site ‘D’), at both sites. Drinking water samples were collected from underground water pumps which were located at a distance of 100 m (site ‘E’), 300 m (site ‘F’), and 500 m (site ‘G’) from the banks of Hudiara, for both pre- and post-Satukatla sites. Sampling was carried out at site A–G for seven months (October 2018 until April 2019) in triplicates at each location in 500 ml pre-sterilized and labeled plastic bottles containing 1 ml of 50 mM Phenylmethylsulfonyl fluoride (PMSF) (Thermo Fisher Scientific, Waltham, United States) inhibitor. Physicochemical parameters e.g. color, temperature, and pH were measured for each sample on the site. The bottles were then immediately shifted to the ice for further processing. Note that the period between May and September was omitted due to seasonal rains and monsoons.

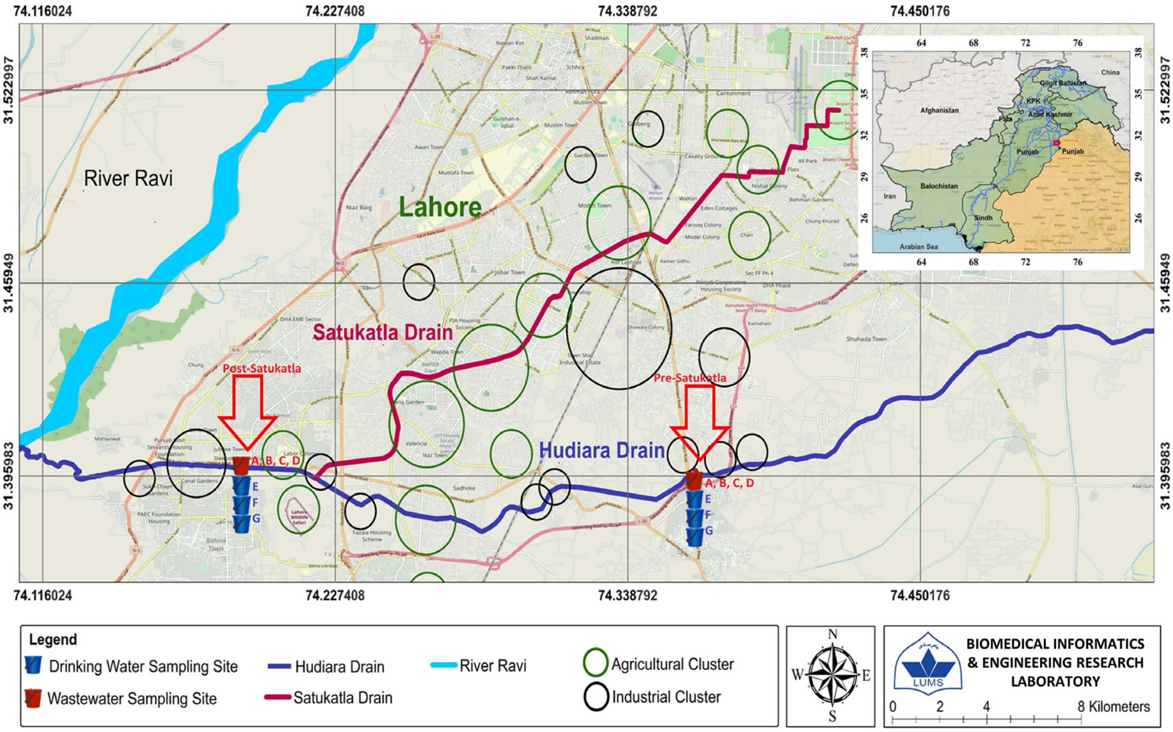

Fig. 1. Geographic location of sampling sites on Hudiara Drain. Google® map of the confluence between Hudiara drain (dark blue) and Satukatla drain (dark red) as it flows through Lahore carrying wastewater from agricultural (green circles) and industrial hubs (black circles) before entering River Ravi (light blue). The size of the circles is indicative of the spread of agricultural and industrial zones. Samples were collected from two sites (marked with red arrows): Pre-Satukatla, before the confluence of Satukatla drain, and Post-Satukatla, just after Satukatla drain empties into Hudiara drain. Wastewater samples were obtained from 4 points on Hudiara: sites 'A' (right bank), 'B' (center-top), 'C' (center-bottom), and 'D' (left bank). Drinking water samples were collected from 3 points: sites 'E' (100 m), 'F' (300 m), and 'G' (500 m) away from the drain. . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Heavy metals content analysis in water samples

Samples were profiled for the presence of nine heavy metals including (i) arsenic, (ii) mercury, (iii) chromium, (iv) lead, (v) cadmium, (vi) iron, (vii) copper, (viii) nickel and, (ix) manganese using atomic absorption spectrophotometer (AAS) (Thermo Electron Corporation MKII-6 series). Specifically, arsenic content in the acidified sample was determined through the hydride generation-atomic absorption spectrophotometer technique (HG-AAS) (Delgado-Andrade et al., 2003).

2.3. Pollution indices

To determine the pollution indices of metal load, we computed the contamination factor for both wastewater and drinking water samples and the heavy metal pollution index for drinking water samples only. The contamination factor (CF) was computed as follows:

$$C_f^i = \frac{C_i}{C_{ri}} \tag{1}$$

where C_i is the mean concentration of metal i and C_{ri} is recommended safe level of metal i. Samples can be classified into 4 classes based on contamination factor including low pollution ($CF < 1$), moderate pollution ($1 \leq CF < 3$), considerable pollution ($3 \leq CF < 6$), and high pollution ($CF \geq 6$) (Díez et al., 2005; Gashi et al., 2017)

The heavy metal pollution index (HPI) was computed as follows (Usero et al., 2005):

$$HPI = \frac{\sum_{i=1}^{n} W_i Q_i}{\sum_{i=1}^{n} W_i} \tag{2}$$

where Q_i is the sub-index of ith parameter, Wi is the unit weightage of ith parameter, and n is the number of parameters. The sub-index Q_i is computed as follows:

$$Q_i = \frac{M_i}{S_i} * 100 \tag{3}$$

where M_i is the monitored value of heavy metal of the i*th* parameter and S_i is the standard recommended levels i.e., World Health Organization (WHO) defined values of heavy metals in drinking water (Hussain et al., 2019; Prasad and Sangita, 2008).

2.4. Health risk assessment

To assess the risk to human health caused by the consumption of contaminated drinking water obtained from the vicinity of Hudiara drain, chronic daily intake (CDI) was computed as follows:

$$CDI = C * (\frac{DI}{BW}) \tag{4}$$

where C is the concentration of heavy metal in µg/L for the drinking water samples collected, DI is the daily intake of water (assumed to be 2L), and BW is body weight (assumed to be 70 kg). The health risk index (HRI) of drinking water was calculated as follows:

$$HRI = \frac{CDI}{RfD} \tag{5}$$

where RfD is the reference dose for each metal as defined by Environment Protection Agency (EPA) (Hussain et al., 2019; United States Environmental Protection Agency, 2022).

2.5. Health survey layout

A cross-sectional questionnaire-based health survey was designed to estimate the prevalence of various diseases in the vicinity of sampling sites and included bone deformities, dermatitis, hepatitis, gastrointestinal, and chronic obstructive pulmonary diseases (COPD) irrespective of age, sex, and ethnicity (**Table S1**). The survey was conducted on 60 adults residing in areas located at 100 m (site E), 300 m (site F), and 500 m (site G) away from pre- and post-Satukatla sites of the Hudiara drain during the sampling period. Additionally, 26 people were sampled from the Askari-XI neighborhood — an area without any major wastewater drain in its vicinity, to be used as control.

2.6. Bacterial isolation

To investigate the culture-dependent bacterial load in the samples obtained from pre- and post-Satukatla sites, we performed bacterial isolation. Serial dilutions of the wastewater and drinking water samples were prepared at 1:100, 1:500, and 1:1000. 100 µl of each prepared water dilution was spread on the Luria–Bertani (LB) agar (Merck KGaA, Darmstadt, Germany) plates using the spread plate method and incubated at 37 °C for 24 h. The preliminary characterization of bacterial colonies was conducted based on their morphological differences. Colonies were then purified by subculturing twice using the streak plate method.

2.7. Sequencing and phylogenetic analysis

The genomic DNA of bacterial strains was isolated using the boiling and snap chilling method. 16S rRNA gene was amplified using Taq polymerase enzyme (Thermo Scientific, Waltham, Massachusetts) through polymerase chain reaction (PCR) using the universal primer set: 8F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′). The PCR was performed at the following conditions: Initial denaturation at 95 °C for 3 min; 30 amplification cycles at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 75 s; final extension was carried out at 72 °C for 5 min. The PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sent for sequencing (Macrogen, Seoul, South Korea). Raw reads obtained were processed using Geneious Prime (Geneious Prime Software 2020.1) and used as a query sequence to identify their homologs (i.e. greater than 97% homology) through the Basic Local Alignment Search Tool (BLAST) from NCBI (National Centre for Biotechnology Information) database. Next, the query and homologous sequences obtained were globally aligned through the close-neighbor joining statistical method (Tamura and Nei, 1993) to generate a phylogenetic tree in Geneious Prime.

2.8. Prediction of AMR potential

Bacterial FASTA sequences were queried in the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020) and analyzed for the antimicrobial-resistant (AMR) genes using the built-in rRNA gene variant model of CARD. AMR is detected in light of the clinically proven mutations in the ribosomal RNA gene which confers resistance to known antibiotic(s) relative to the wild-type rRNA sequence. The AMR detection model then generates a reference rRNA sequence, a BLASTN bit-score cut-off, and a set of resistance variants. The resulting data was annotated with antibiotic-resistant genes and antibiotic targets.

2.9. Statistical data analysis

Data normality was assessed through the Shapiro–Wilk test (Shapiro and Wilk, 1965) and means were compared using the Mann–Whitney U test ($p < 0.05$) (Mann and Whitney, 1947), Kruskal–Wallis H test ($p < 0.05$) (Kruskal and Wallis, 1952), and one-sample t-test ($p < 0.05$) (Pearson, 1894). The suitability of the data was assessed using the Kaiser–Meyer–Olkin (KMO) value (>0.5) (Kaiser, 1970) and the significance level of Bartlett's test ($p < 0.05$) (Bartlett, 1937) before applying multivariate analysis. Principal component analysis (PCA) (Hotelling, 1933) was employed to interpret the potential sources of heavy metals in wastewater and drinking water sources. PCA was used to extract significant factors of variability towards distinguishing the geological and anthropogenic contribution of metal by applying VARIMAX rotation. Data was further subjected to hierarchical agglomerative cluster analysis (HACA) using Ward's method of clustering (Ward, 1963) to recognize the relationship between heavy metals and pH. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 25.0.

3. Results and discussion

3.1. Temporal evaluation of physicochemical properties of wastewater from pre- and post-Satukatla sites of Hudiara drain

The color of wastewater obtained from both pre- and post-Satukatla sites of Hudiara drain remained gray over the sampling period, for both sites (Fig. 1). The temperature, however, exhibited significant fluctuations between October and April at both sites, 16 °C to 30 °C (Kruskal–Wallis H test, $p < 0.05$) **(Fig. S1, Table S2-S3)**. A significantly higher mean temperature was observed at post-Satukatla sites during December (20 °C at post-Satukatla in comparison with 18 °C at pre-Satukatla) and February (22.6 °C at post-Satukatla against 20.9 °C at pre-Satukatla). In March, however, pre-Satukatla exhibited a higher mean temperature of 26.9 °C as compared to 22.6 °C at post-Satukatla (Mann–Whitney U test, $p < 0.05$) **(Table S4).** These variations could be attributed to the seasonal changes and the type of industrial and domestic effluents brought in by Satukatla into Hudiara. For the case of pH, a significant variation was observed within each site i.e., from 6.6 to 9.68 and 6.72 to 9.25 at pre- and post-Satukatla, respectively (Kruskal–Wallis H test, $p < 0.05$) **(Fig. S2, Table S5–S6).** Our results indicate a higher pH value range (between 6.6 to 9.68) than what was reported in earlier studies (between 7.25 to 7.92) (Haydar et al., 2014) **i**n the Hudiara drain. The alkalinity of wastewater (measured through pH) can be attributed to the unregulated anthropogenic activities taking place along the banks of the Hudiara drain in India and Pakistan. This includes dumping of wastewater from the adjoining tanning, pharmaceuticals, dyeing and printing, textile, electroplating, and agrochemical industry along with domestic discharge containing detergents and batteries which is indicative of the unprecedented scale of water pollution (Ahmed, 2017; Kaur and Dua, 2016; Majeed et al., 2018; Qureshi and Sayed, 2014). Moreover, no significant difference was found between the pH values of pre- and post-Satukatla sites. The pH values, however, at pre-Satukatla were found to be significantly higher in October (average pH: 9.25), November (average pH: 8.27), and January (average pH: 8.08) and significantly lower in February (average pH: 6.87) and March (average pH: 6.83) in comparison with the mean National Environmental Quality Standards (NEQS, Environment Protection Department, 1999) of 7.5 (one-sample t-test, $p < 0.05$). While at post-Satukatla, the pH was significantly higher in October (average pH: 8.96) and November (average pH: 8.01), and significantly lower in February (average pH: 7.04) and March (average pH: 6.84) as compared to the NEQS recommendation (one-sample t-test, $p < 0.05$) **(see Table S7 for more details).** Seasonal variations in pH could be attributed to the fluctuations in temperature (Romanescu and Stoleriu, 2014) and the consequent microbial activity i.e. a rise in temperature from 5 °C to 15 °C can result in an accelerated decomposition rate of organic compounds (0.0041 day $^{-1}$ to 0.01 day^{-1}) thereby lowering the pH value of water (Ferreira and Chauvet, 2011; Khan et al., 2003; Lim et al., 2001). Thus, the evaluation of physicochemical properties furnishes evidence of the unrelenting pollution of wastewater in Hudiara.

3.2. Heavy metal analysis of wastewater in Hudiara drain

Results from the temporal comparison of wastewater samples obtained from pre- and post-Satukatla sites of Hudiara drain showed consistently higher levels of arsenic, mercury, and cadmium whereas iron, copper, and manganese remained within the safe NEQS limits — confirming the unprecedented scale of water pollution in Hudiara (Table 1; **See details in Table S8**). Pre-Satukatla site reported the highest mean concentration of arsenic over 7 months followed by mercury, iron, lead, chromium, nickel, manganese, copper, and cadmium. At post-Satukatla, arsenic remained the leading contaminant followed by lead, nickel, mercury, copper, chromium, iron, manganese, and cadmium Multiple studies have already established a high burden of heavy metals in Lahore, explicitly in Hudiara and its adjoining areas (Hamid et al., 2013; Kashif et al., 2009; Muhammad et al., 2013; Noreen et al., 2022). Moreso, arsenic, in particular, is the predominant contaminant of Hudiara both due to geological and anthropological factors as confirmed by previous studies (Muhammad et al., 2016); the deep fingerprint of arsenic in wastewater of Hudiara drain.

To evaluate the significance of temporal variations in mean heavy metal load within each sampling site, Kruskal–Wallis H test was employed. The results showed a statistically significant difference ($p < 0.05$) for the mean concentrations of cadmium at the post-Satukatla site that corresponds to seasonal industrial manufacturing and agricultural activities (**Table S9**). Next, to evaluate the significance of variation in the mean concentration of heavy metals in wastewater

Table 1
Variation in the mean concentration of arsenic (As), mercury (Hg), chromium (Cr), lead (Pb), cadmium (Cd), iron (Fe), copper (Cu), nickel (Ni), and manganese (Mn) in wastewater from pre-Satukatla and post-Satukatla sites of Hudiara Drain over seven months (October–April).

Heavy metals[a]	Satukatla site	Months							NEQS levels[b]
		October	November	December	January	February	March	April	
As	Pre-	1.55 ± 0.2	1.41 ± 0.2	1.43 ± 0.5	2.20 ± 0.2	1.66 ± 0.2	2.42 ± 0.2	2.30 ± 0.5	1
	Post-	2.20 ± 0.4	2.05 ± 0.4	1.89 ± 0.5	2.04 ± 0.4	1.50 ± 0.3	1.69 ± 0.4	2.49 ± 0.3	
Hg	Pre-	0.24 ± 0.03	0.63 ± 0.2	1.01 ± 0.3	0.74 ± 0.2	0.34 ± 0.1	0.69 ± 0.1	0.57 ± 0.2	0.01
	Post-	0.40 ± 0.08	0.61 ± 0.2	1.49 ± 0.6	0.51 ± 0.2	1.12 ± 0.3	1.50 ± 0.3	1.73 ± 0.3	
Cr	Pre-	0.039 ± 0.008	0.52 ± 0.1	0.53 ± 0.2	0.51 ± 0.1	0.63 ± 0.1	0.67 ± 0.09	0.65 ± 0.1	1
	Post-	0.22 ± 0.07	0.70 ± 0.3	1.04 ± 0.4	0.64 ± 0.2	0.74 ± 0.4	0.54 ± 0.2	1.20 ± 0.2	
Pb	Pre-	0.78 ± 0.2	0.46 ± 0.4	0.81 ± 0.4	0.35 ± 0.2	0.39 ± 0.3	0.40 ± 0.3	0.45 ± 0.3	0.5
	Post-	0.89 ± 0.3	1.06 ± 0.3	1.28 ± 0.5	1.31 ± 0.4	1.34 ± 0.5	1.66 ± 0.4	1.59 ± 0.3	
Cd	Pre-	0.003 ± 0.002	0.24 ± 0.2	0.27 ± 0.256	0.17 ± 0.16	0.24 ± 0.21	0.27 ± 0.23	0.21 ± 0.16	0.1
	Post-	0.01 ± 0.003	0.052 ± 0.007	0.42 ± 0.20	0.27 ± 0.21	0.23 ± 0.18	0.34 ± 0.17	0.27 ± 0.13	
Fe	Pre-	0.52 ± 0.1	0.46 ± 0.18	0.69 ± 0.17	0.32 ± 0.15	0.73 ± 0.24	0.68 ± 0.22	0.43 ± 0.17	8
	Post-	0.63 ± 0.13	0.57 ± 0.13	0.89 ± 0.33	0.70 ± 0.21	0.61 ± 0.21	0.65 ± 0.23	0.75 ± 0.15	
Cu	Pre-	0.07 ± 0.002	0.33 ± 0.18	0.60 ± 0.14	0.53 ± 0.23	0.24 ± 0.14	0.25 ± 0.14	0.14 ± 0.04	1
	Post-	0.49 ± 0.21	0.84 ± 0.07	0.92 ± 0.24	0.94 ± 0.14	0.63 ± 0.20	0.99 ± 0.10	0.96 ± 0.12	
Ni	Pre-	0.11 ± 0.09	0.50 ± 0.23	0.59 ± 0.24	0.41 ± 0.20	0.24 ± 0.14	0.62 ± 0.14	0.56 ± 0.17	1
	Post-	0.85 ± 0.34	0.91 ± 0.33	1.19 ± 0.41	1.11 ± 0.30	1.32 ± 0.34	1.03 ± 0.24	1.03 ± 0.19	
Mn	Pre-	0.11 ± 0.05	0.47 ± 0.21	0.53 ± 0.16	0.24 ± 0.17	0.26 ± 0.13	0.33 ± 0.15	0.33 ± 0.12	1.5
	Post-	0.13 ± 0.01	0.24 ± 0.13	0.54 ± 0.13	0.43 ± 0.21	0.35 ± 0.15	0.37 ± 0.18	0.41 ± 0.20	

[a] All units are in mg/L.
[b] National Environmental Quality Standards (NEQS) for metal are provided in mg/L (Environment Protection Department, 1999).

from pre- and post-Satukatla sites, Mann–Whitney U test was performed. Results showed significant differences in the mean concentration of mercury and chromium for April, and copper for October, March, and April ($p < 0.05$) **(Table S10 for details).** Such seasonal variations in the concentration of heavy metals can be associated with the compositional alterations of industrial discharge — indicative of the unrelenting pollution of Hudiara. Next, the mean monthly concentration of each heavy metal was compared with the respective NEQS reference value to determine the significance of variance for both sites. A significant difference was found for almost all metals at pre- Satukatla (except lead) and post-Satukatla (except copper and nickel) (one-sample t-test, $p < 0.05$) **(Table S11 for details).** This is indicative of an unregulated/unprecedented industrial discharge from adjoining pharmaceutical, agrochemicals, packaging, textile, dyeing, and printing units as well as agrochemical run-offs. Overall, the results show that the Hudiara channel carries an abnormally high anthropogenic heavy metal load which may be underpinning the elevated pH levels in its wastewater besides the alkaline soil of Indus River plain **(Table S12)**. Earlier research has also uncovered the correlation between alkaline pH and increased heavy metal concentration, which results from wastewater seepage accelerated heavy metal leaching from natural geogenic sources (Komonweeraket et al., 2015; Li et al., 2013). The consequent heavy metal exchange between sediments and underground aquifers is further known to have devastating long-term effects on drinking water as well as human health. In conclusion, the substantial imprint of heavy metals underscores the unprecedented scale of Hudiara's contamination.

3.3. Temporal evaluation of physicochemical properties of drinking water from areas adjacent to pre- and post-Satukatla sites of Hudiara drain

The color of drinking water samples was observed to vary from transparent to mild brown, manifesting the high content of contaminants, at both locations. In terms of temperature, the samples exhibited significant fluctuations from October until February within each site, for both sites, 22 °C to 28 °C (Kruskal–Wallis H test, $p < 0.05$) which can be attributed to seasonal changes **(Fig. S3, Table S13–S14).** No significant difference was observed between the mean temperatures of pre- and post-Satukatla sites. For the case of pH, a substantial temporal fluctuation was observed within each site i.e., from 7.02 to 9.76 and 7.17 to 10.34 at pre- and post- Satukatla sites, respectively (Kruskal–Wallis H test, $p < 0.05$) **(Fig. S4, Table S15–S16).** The range of pH values for groundwater samples (7.02 to 10.34) was higher as compared to those reported in the previous case studies (between 6.86 to 8.50) (Haydar et al., 2014). Here, it is important to note that the pH of groundwater sources is consistently elevated throughout the Indus plains which have led to accelerated leaching of heavy metals from geogenic sources into groundwater; this may lead to the elevated scale of metal pollution shortly (Podgorski et al., 2017). Furthermore, the seepage of alkaline wastewater of Hudiara, under the influence of anthropogenic insults, can potentially exacerbate the quality of drinking water. No significant difference was found between the pH values of pre- and post-Satukatla sites. However, at pre-Satukatla, the average pH was found to be significantly higher in October

(9.56), November (7.72), January (9.03), and April (8.23) than the mean World Health Organization (WHO) standard value of 7.5 using a one-sample *t*-test, $p < 0.05$ (WHO, 2007). At post-Satukatla, pH remained significantly higher in October (9.83), January (8.96), and April (8.11) than the WHO permissible levels **(Table S17).** Lastly, no relationship could be established between the pH values and the distance (i.e. 100 m (E), 300 m (F), and 500 m (G)) of groundwater sources located orthogonally to Hudiara. Absence of any relationship between the pH and distance (both orthogonal and vertical) has also been established from the previous literature findings (Haydar et al., 2014).

3.4. Heavy metal analysis in the drinking water of areas adjoining Hudiara drain

Month-wise profiling of drinking water samples obtained from pre- and post-Satukatla sites showed consistently higher levels of arsenic, mercury, chromium, lead, cadmium, iron, and nickel as compared to WHO recommended levels (World Health Organization, 2011) which is reflective of the magnitude of metal pollution in drinking water. The average concentration of copper and manganese, however, remained within the safe limits (Table 2; **See details in Table S18**). Pre-Satukatla reported the highest mean concentration of arsenic over 7 months followed by nickel, iron, manganese, mercury, copper, chromium, lead, and cadmium. At post-Satukatla, arsenic remained the leading contaminant followed by nickel, lead, copper, cadmium, manganese, chromium, iron, and mercury. Such abnormally high levels of heavy metals in groundwater have also been reported in the earlier studies on the housing schemes around Hudiara (Akhtar et al., 2014b; Haydar et al., 2014; Khattak et al., 2012).

To evaluate the significance of temporal variations in the mean concentration of heavy metals within each sampling site, the Kruskal–Wallis H test was employed for both sites. The results showed significant differences ($p < 0.05$) in the mean concentrations of cadmium at both pre- and post-Satukatla sites (**Table S19-S20).** Next, to evaluate the statistical significance of variation in the mean concentration of heavy metals in drinking water from pre- and post-Satukatla sites, Mann–Whitney U test was performed. No significant differences were found in the mean concentration of heavy metal loads. Finally, the mean monthly concentration of each heavy metal was compared against the respective WHO reference value to determine the variance at both sites using a one-sample *t*-test. At pre-Satukatla, this difference was found to be significant ($p < 0.05$) for arsenic, mercury, chromium, lead, copper, and manganese. At post-Satukatla, levels of arsenic, chromium, lead, cadmium, copper, and nickel were found to be significantly different from the WHO recommended levels (**Table S21).** High heavy metal concentrations over the permissible limits are indicative of the magnitude of heavy metal pollution in drinking water.

Moreso, our results indicate a substantial heavy metal load at pre-Satukatla sites E, F, and G located adjacent to Hudiara drain with the highest mean metal load reported at site E (0.604 mg/L), followed by G (0.346 mg/L) and F (0.397 mg/L). At post-Satukatla, however, site F reported the highest mean metal content (0.0.921 mg/L) followed by site E (0.868 mg/L) and G (0.790 mg/L) **(Table S22).** This gives credence to the hypothesis that the percolation of metal-laden wastewater reaches the aquifer and contaminates the groundwater sources — indicating the fingerprint of heavy metal pollution (Javaid et al., 2020; Mohankumar et al., 2016). Furthermore, these results also highlight the pressing need to map the aquitards beneath transboundary Hudiara to elucidate the irregularity of effluent seepage patterns.

3.5. Compositional analysis and apportioning of heavy metal load in Hudiara and its surroundings

Compositional evaluation of pH and heavy metals concentration obtained from both waste and drinking water samples recovered multiple rotated principal components (PC) with eigenvalues >1.0 (**Table S23**). Analysis of wastewater data from pre-Satukatla output 3 components: PC1 (positive loading for arsenic, lead, and chromium), PC2 (positive loading for cadmium, copper, and manganese), and PC3 (positive loading for nickel and mercury) with a cumulative variance of 76.74% after removing data for iron and pH. Wastewater data from post-Satukatla was classified into 3 components: PC1 (positive loading for chromium, lead, and nickel), PC2 (positive loading for arsenic, cadmium, iron, and mercury), and PC3 (positive loading for arsenic and pH) accounting for a cumulative variance of 76.12%, after removing the data for copper. Analysis of drinking water data (heavy metal concentration and pH) from pre- and post-Satukatla site using Kaiser–Meyer–Olkin (KMO) value (<0.5) and Bartlett's test ($p > 0.05$) rendered it non-significant, and therefore removed from PCA-based data apportioning.

To correlate the pH and heavy metal load in wastewater and drinking water from pre- and post-Satukatla, hierarchical agglomerative cluster analysis (HACA) using the Wards method was employed. The outcomes indicated a close relationship of pH with iron, chromium, and arsenic at pre-Satukatla in wastewater. For the post-Satukatla site, however, arsenic and copper were clustered closely with variations in pH. The strongest temporal association of pH was observed with arsenic and iron in drinking water at pre-Satukatla, while for post-Satukatla, arsenic and manganese were clustered with variations in pH (**See Table S24 for more details**). The PCA and HACA enable the determination of potential sources which are in charge of the differences in water quality. Our PCA results reveal that the abundance of heavy metals in water sources is not controlled by a single factor but rather by a combination of a wider anthropogenic landscape and its complex interactions with geological formations. In particular, HACA indicates that leaching of arsenic, chromium, and iron occurs at alkaline pH which is also in concert with earlier literature reports (Komonweeraket et al., 2015; Smedley and Kinniburgh, 2002).

Table 2
Variation in the mean concentration of arsenic (As), mercury (Hg), chromium (Cr), lead (Pb), cadmium (Cd), iron (Fe), copper (Cu), nickel (Ni), and manganese (Mn) in drinking water from pre-Satukatla and post-Satukatla sites of Hudiara Drain over seven months (October–April).

Heavy metals[a]	Satukatla site	Months							WHO levels[b]
		October	November	December	January	February	March	April	
As	Pre-	2.05 ± 0.26	1.44 ± 0.64	0.85 ± 0.59	1.42 ± 0.67	1.58 ± 0.76	1.82 ± 0.24	2.39 ± 0.24	0.01
	Post-	2.79 ± 0.27	3.00 ± 0.11	2.34 ± 0.32	2.70 ± 0.33	2.30 ± 0.43	2.17 ± 0.45	2.64 ± 0.65	
Hg	Pre-	0.002 ± 0.0008	0.05 ± 0.025	0.10 ± 0.04	0.22 ± 0.17	0.16 ± 0.13	0.61 ± 0.26	0.59 ± 0.54	0.006
	Post-	0.03 ± 0.018	0.04 ± 0.025	0.22 ± 0.14	0.27 ± 0.19	0.24 ± 0.18	0.67 ± 0.29	0.62 ± 0.52	
Cr	Pre-	0.02 ± 0.005	0.05 ± 0.02	0.10 ± 0.02	0.29 ± 0.2	0.25 ± 0.2	0.36 ± 0.29	0.40 ± 0.20	0.05
	Post-	0.16 ± 0.08	0.22 ± 0.05	0.38 ± 0.03	0.28 ± 0.07	1.39 ± 0.99	0.26 ± 0.04	0.61 ± 0.33	
Pb	Pre-	0.35 ± 0.2	0.33 ± 0.19	0.22 ± 0.08	0.09 ± 0.008	0.09 ± 0.04	0.04 ± 0.015	0.36 ± 0.26	0.01
	Post-	0.92 ± 0.17	0.77 ± 0.43	1.20 ± 0.39	1.80 ± 1.27	0.65 ± 0.16	0.61 ± 0.06	1.04 ± 0.31	
Cd	Pre-	0.00 ± 0	0.00 ± 0	0.002 ± 0.002	0.002 ± 0.002	0.39 ± 0.28	0.33 ± 0.22	0.47 ± 0.28	0.003
	Post-	0.02 ± 0.006	0.07 ± 0.03	0.83 ± 0.39	0.40 ± 0.14	0.77 ± 0.31	0.67 ± 0.13	1.01 ± 0.33	
Fe	Pre-	0.57 ± 0.08	0.36 ± 0.16	0.57 ± 0.28	0.25 ± 0.19	0.47 ± 0.22	0.51 ± 0.17	0.40 ± 0.16	0.3
	Post-	0.34 ± 0.01	0.54 ± 0.2	0.71 ± 0.21	0.52 ± 0.11	0.30 ± 0.12	0.33 ± 0.14	0.42 ± 0.28	
Cu	Pre-	0.09 ± 0.02	0.51 ± 0.23	0.27 ± 0.18	0.37 ± 0.29	0.09 ± 0.05	0.10 ± 0.03	0.12 ± 0.04	2
	Post-	0.63 ± 0.1	0.64 ± 0.24	1.42 ± 0.13	0.64 ± 0.01	0.50 ± 0.09	0.51 ± 0.14	0.71 ± 0.09	
Ni	Pre-	0.36 ± 0.34	0.38 ± 0.33	0.67 ± 0.29	0.59 ± 0.29	0.69 ± 0.31	0.65 ± 0.28	0.38 ± 0.32	0.07
	Post-	0.77 ± 0.35	0.89 ± 0.16	1.19 ± 0.14	1.26 ± 0.45	1.26 ± 0.53	1.65 ± 0.39	1.45 ± 0.43	
Mn	Pre-	0.22 ± 0.02	0.22 ± 0.05	0.65 ± 0.28	0.42 ± 0.26	0.21 ± 0.11	0.17 ± 0.10	0.60 ± 0.21	0.4
	Post-	0.50 ± 0.06	0.32 ± 0.16	0.54 ± 0.19	0.47 ± 0.18	0.48 ± 0.11	0.57 ± 0.23	0.50 ± 0.13	

[a] All units are in mg/L.
[b] World Health Organization permissible limits for metal are provided in mg/L (World Health Organization, 2011).

3.6. Evaluation of heavy metal pollution indices in Hudiara and its surroundings

The contamination factor (CF) index was computed for waste and drinking water samples while the heavy metal pollution index (HPI) was computed using a mean heavy metal concentration in drinking water. Our results exhibit elevated CF values of $\sim$60 and $\sim$100 for mercury in wastewater samples obtained from pre- and post-Satukatla sites of Hudiara drain, respectively (high pollution index: CF ≥ 6) thereby establishing Satukatla drain as a mercury-laden effluent carrier. Moreover, high values of CF were also observed in drinking water samples obtained from the pre-Satukatla site for arsenic (CF value $\sim$150), mercury ($\sim$41), lead ($\sim$21), and cadmium ($\sim$57) (high pollution index: CF ≥ 6). A high CF was also observed for arsenic (CF value $\sim$250), mercury ($\sim$50), chromium ($\sim$10), lead ($\sim$100), cadmium ($\sim$180), and nickel ($\sim$17) (high pollution index: CF ≥ 6) in drinking water samples at the post-Satukatla site **(Table S25)**. Extensive discharge from point and non-point pollution sources and its subsequent seepage across aquitards can result in higher CF values for groundwater sources as compared to wastewater obtained from Hudiara drain. Such aberrant CF values indicate the unparallel scale of pollution in wastewater and drinking water samples obtained from Hudiara and its vicinity, respectively.

Next, the composite influence of 9 heavy metals on the quality of groundwater was determined by calculating the HPI for each month (October-April). The outcome reveals abnormally high HPI values (HPI of 6033.854 and 14101.01 at pre and post-Satukatla, respectively) in comparison with the index limit of 100 — an indicator of the poor quality of drinking water in areas adjacent to both pre- and post-Satukatla sites of Hudiara **(Table S26).**

Aberrant HPI values are also indicative of the possibility that groundwater is contaminated by both geogenic and anthropogenic sources including urbanization, industrialization, and agricultural activities. High HPI confirms the unprecedented scale of heavy metal pollution around Hudiara. These alarming findings indicate that the groundwater sources around Hudiara are not only marginally suitable for irrigation but also strictly unsuitable for drinking purposes (Javaid et al., 2020; Khattak et al., 2012) due to an unprecedented scale of pollution. Such findings necessitate an evaluation of the potential health risks resulting from oral consumption of heavy metal contaminated water.

3.7. Health risk assessment of heavy metals in drinking water collected from the vicinity of Hudiara drain

Health risk assessment (HRA) scores, for drinking water obtained from the surroundings of Hudiara, were found to be >1.0 for all metals except iron, nickel, and manganese at the pre-Satukatla site. For post-Satukatla, all metals except iron and manganese (<1) exhibited an HRA index >1.0. Overall, arsenic showed the highest mean index (HRA $\sim$188) followed by mercury ($\sim$100), lead ($\sim$87), cadmium ($\sim$13), chromium ($\sim$7), copper ($\sim$6), nickel ($\sim$1.5), manganese ($\sim$0.4), and iron ($\sim$0.03) **(Table S27).** HRA scores establish the strong fingerprint of heavy metal seepage into the groundwater sources.

Exposure to consistently high levels of heavy metals, as established by the spatiotemporal profiling of groundwater resources and critically high levels of pollution indices, may elicit serious health consequences. We, therefore, set out to establish the prevalence of water-borne diseases in the individuals residing along the banks of Hudiara to measure the outcomes of unprecedented scale of pollution.

3.8. Measuring disease burden in the population residing along the banks of Hudiara drain

Preliminary descriptive analysis of the health survey data of the population dwelling along the drain showed that 12% of the participants suffered from hepatitis, 38.7% suffered from chronic obstructive pulmonary diseases (COPD), and 40% had diarrhea. Moreover, 34% and 31% of participants reported dermatitis and bone-related deformities, respectively. Note that the earlier heavy metal analyses from three drinking water collection sites (E, F, and G) at pre- and post-Satukatla had shown significantly higher concentrations ($p < 0.05$) of arsenic (average value — pre-site: 1.65 mg/L; post-site: 2.56 mg/L), mercury (pre-site: 0.25 mg/L; post-site: 0.3 mg/L), chromium (pre-site: 0.21 mg/L; post-site: 0.47 mg/L), lead (pre-site: 0.17 mg/L; post-site: 0.54 mg/L), cadmium (pre-site: 0.45 mg/L; post-site: 0.45 mg/L), iron (pre-site: 0.22 mg/L; post-site: 0.72 mg/L), and nickel (pre-site: 0.36 mg/L; post-site: 0.48 mg/L). Copper, on the other hand, had, however, exhibited significantly lower levels, 0.22 mg/L and 0.72 mg/L at pre- and post-Satukatla site, respectively, as compared to WHO recommended levels of 2 mg/L **(Table S28).** Such alarming levels of heavy metal pollution in drinking water subsequently affect the health of people residing in the locales of Hudiara.

To factor in the health risks posed by heavy metal contaminated groundwater to public health, we undertook an extensive literature review. Foremost, research reports a positive correlation between respiratory symptoms and arsenic, mercury, cadmium (Naidoo et al., 2019), and chromium (Jamal et al., 2017; Shekhawat et al., 2015). Our findings from the health survey conform with this literature-reported correlation between communicable respiratory diseases including COPD (38.7%) under the effect of significantly high concentrations of arsenic (2105 ± 138.7 μg/L), mercury (274.7 ± 65.7 μg/L), chromium (340.5 ± 84.7 μg/L), and cadmium (355.6 ± 70.5 μg/L). Survey findings that 40% of the local population suffered from diarrhea are also positively associated with considerably higher levels of arsenic (Franzblau and Lilis, 1989), nickel (Sunderman et al., 1988), and chromium (Sharma et al., 2012) in groundwater ($p < 0.05$). A high incidence of gastrointestinal symptoms in the vicinity of Hudiara may also be induced by the elevated heavy metal load as indicated by literature on arsenic (in 79.8% of the subjects) (Xu et al., 2008), chromium (39.3% of the subjects) (Sharma et al., 2012), and nickel (Sunderman et al., 1988). Literature further correlates dermatitis (at 34%) and significantly high levels of nickel (869.8 ± 98.9 μg/L) and chromium (340.5 ± 84.7 μg/L) ($p <0.05$).

Industrial labor encountering occupational exposure to chromium report dermatitis (Bregnbak et al., 2014) as well as in cases of exposure to nickel (13% (Duarte et al., 2005), 39% (Warshaw et al., 2008)). Likewise, a prevalence rate of 31% bone-related deformities in the vicinity of Hudiara may be linked to metals including arsenic (Akbal et al., 2014), lead (Lim et al., 2016), and cadmium (Suhartono et al., 2019) which is indicated by the published data. Lastly, the incidence of hepatic disorders in the surroundings of Hudiara may also be associated with various heavy metals including lead (Kasperczyk et al., 2013) and cadmium (Goering and Barber, 2010). An overview of the linkages between nine heavy metals and five classes of heavy metal-induced diseases is given in Fig. 2 **(See Table S29 for further details).**

Summarily, findings from the health survey indicate that the high disease burden of communicable and non-communicable diseases in adjoining areas of Hudiara may be potentially linked to abnormally high heavy metal load in groundwater. Dumping of municipal and industrial discharge into Hudiara, and its subsequent seepage and mixing with the groundwater sources may be underpinning the catastrophic health outcomes in the vicinity of Hudiara as has also been established by previous studies (Jabbar, 2020).

3.9. Profiling and phylogenetic analysis of microbiota in wastewater and drinking water samples

Results from the profiling of water samples from both wastewater and drinking water samples revealed a multitude of bacterial strains throughout the sampling period. Based on the morphological differences, a total of 20 bacteria (17 from wastewater and 3 from drinking water) were selected from purified culture plates **(Fig. S5).** 16S rRNA sequencing followed by a homology search revealed the presence of genus *exiguobacterium, bacillus*, and *klebsiella* in the water samples. Sequences of individual bacteria were also deposited to NCBI **(Table S30)** and phylogenetic analysis was performed to elucidate the diversity of these microbes and their evolutionary lineages **(Fig. S6).**

The two bacterial species of the genus *exiguobacterium,* isolated from the wastewater in Hudiara drain, are known to tolerate high concentrations of arsenic, mercury, chromium, cadmium, copper, nickel, and manganese (Gupta et al., 2012; Pandey, 2020). In particular, *Exiguobacterium profundum* (MW485975.1) *and E. mexicanum* (MW486128.1) show a close phylogenetic relationship to extremophiles with a potential to thrive in high metal and salt concentration (Strahsburger et al., 2018). Bacillus species including *Bacillus. aerius* (MW486125.1), *B. pumilus* (MW486124.1), and *B. safensis* (MW486122.1) exhibited a common ancestor. Our findings are strengthened by literature reports that these species are also known to exhibit multi-metal resistance (De and Ramaiah, 2007; Elahi and Rehman, 2019; Raja and Omine, 2012). *B. licheniformis* (MW485985.1), *B. paralicheniformis* (MW486110.1), and *B. subtilis* (MW486129.1), among others, constitute the second major branch of our phylogenetic tree. These species also have a demonstrated resistance potential against heavy metals (Alotaibi et al., 2021; Guzmán-Moreno et al., 2022; Sunil et al., 2015). The microbial profiling exercise also revealed the presence of *Klebsiella pneumoniae* (MW486037.1) which is phylogenetically more divergent than other reported species. Literature shows a high tolerance potential of *K. pneumoniae* under elevated levels of arsenic, mercury, chromium, cadmium, nickel, lead, and copper (Choudhury and Kumar, 1998; Kumar et al., 2021; Zeroual et al., 2001).

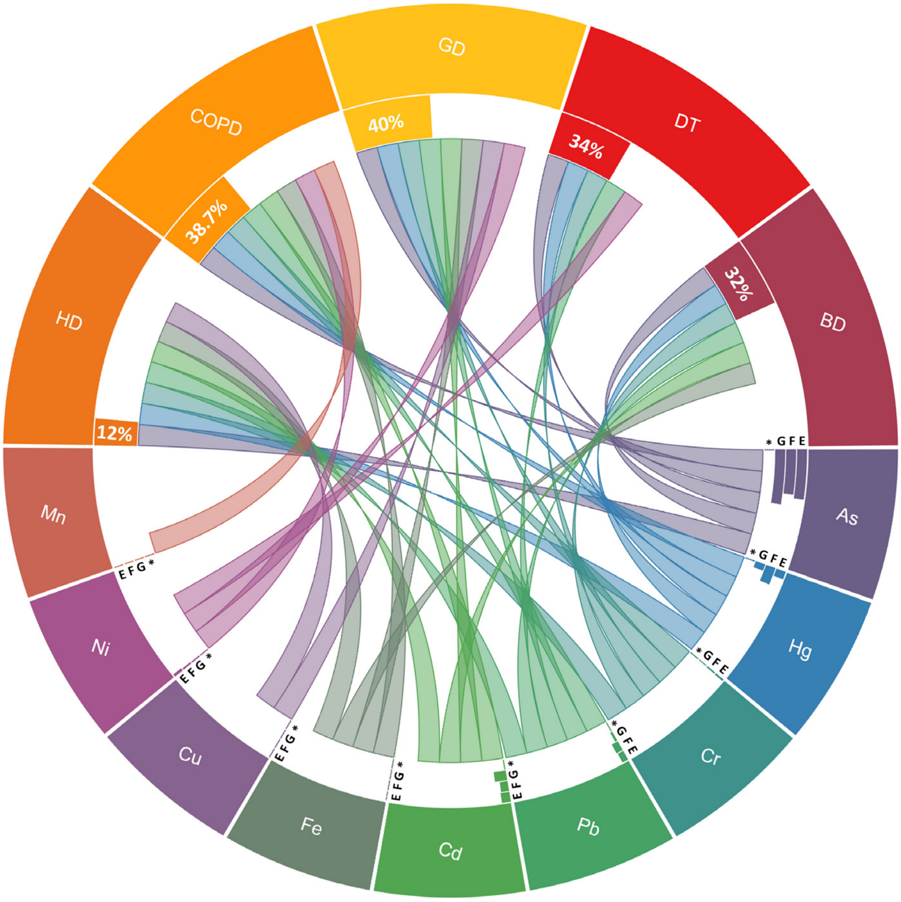

Fig. 2. CIRCOS representation of the heavy metal contamination in Hudiara and disease prevalence in the surrounding population along with their association. (Outer to inner) *Circle 1*: Names of the heavy metals: arsenic (As), mercury (Hg), chromium (Cr), lead (Pb), cadmium (Cd), iron (Fe), copper (Cu), nickel (Ni), and manganese (Mn) and associated diseases: hepatic diseases (HD), chronic obstructive pulmonary diseases (COPD), gastrointestinal diseases (GD), dermatitis (DT), and bone deformities (BD) are mentioned (circle 1). *Circle 2*: Bars represent the respective levels of each heavy metal at sites 'E', 'F', and 'G'. * represent the WHO permissible levels of heavy metal in drinking water. Percentage prevalence of each disease is also represented: HD (12%), COPD (38.7%), GD (40%), DT (34%), and BD (32%). *Circle 3*: The inner ribbons represent the association between heavy metals and diseases confirmed through literature-based analysis.

For the case of drinking water samples, microbial species belonged to the genus bacillus (MW486015.1; MW486121.1; MW486127.1); interestingly these bacteria were phylogenetically linked closely to *B. velezensis* (MW486119.1), which is proven to be sensitive to elevated levels of metals (Vörös et al., 2019), and *B. subtilis* (MW486109.1), which exhibits multi-metal resistance (Alotaibi et al., 2021). Moreso, the *B. zanthoxyli* strain was shown to be related to *exiguobacterium* and *klebsiella*, thereby establishing the high scale and magnitude of microbial pollution around Hudiara.

Microbial survival under unremittingly high levels of heavy metals is known to alter their genetic and metabolic machinery thereby inducing anti-microbial resistance — an indication of prolonged anthropocentric intervention into natural environments (Hobman and Crossman, 2015; Ndeddy Aka and Babalola, 2017). Therefore, towards monitoring the antimicrobial resistance potential of these multi-metal resistant microbes, we proceeded to perform an in-silico screening and evaluation of 16S rRNA sequences.

3.10. Evaluation of antimicrobial resistance potential in Hudiara's microbiota

Evaluation of antimicrobial resistance (AMR) potential using the Comprehensive Antibiotic Resistance Database (CARD) showed that mutations present in Hudiara-specific bacteria exhibited resistance against three classes of drugs: aminoglycosides (tobramycin, kanamycin, gentamicin C, amikacin, neomycin, kanamycin A, capreomycin, streptomycin, hygromycin B, spectinomycin, kasugamicin, paromomycin, G418, and gentamicin), peptides (edeine and viomycin) and tetracyclines (tetracycline). BLASTN bit-score cut-off value for each bacterial sequence was computed against each type

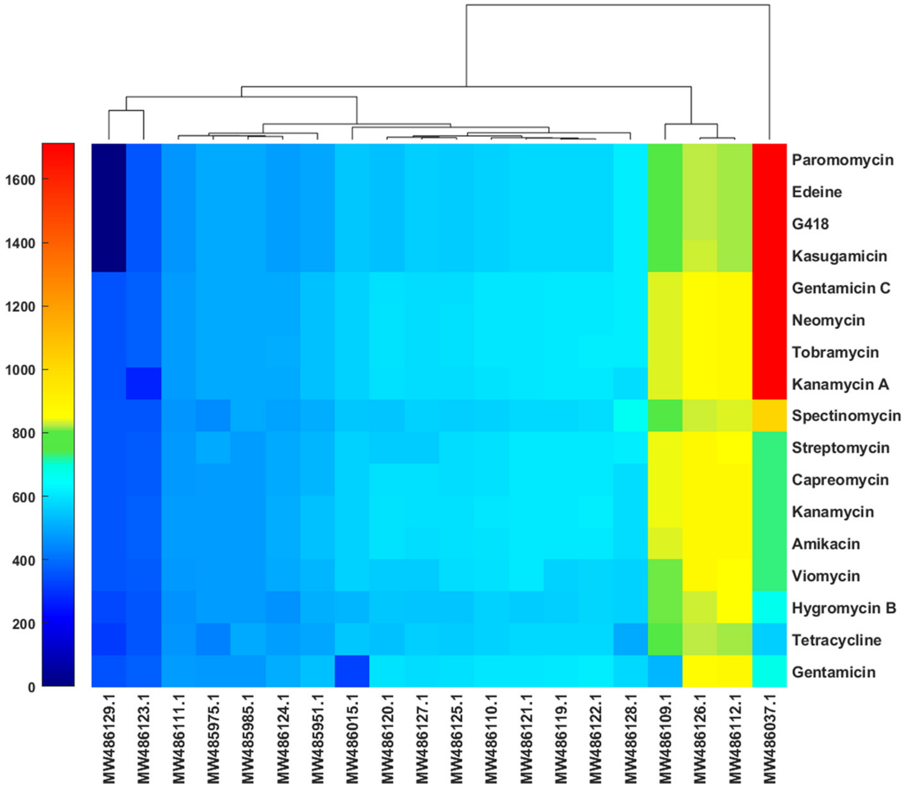

Fig. 3. Heatmap of relative BLASTN bit-score cutoff value for 20 bacterial species against different types of antibiotics. A bit-score value of zero can be abstracted to show the absence of a particular mutation to confer resistance to a particular antibiotic.

of antibiotic and a heat map of resistance was developed (Fig. 3, **Table S31**) (Alcock et al., 2020). Bit-scores of zero can be interpreted as minimum homology of the query sequence with the reported sequences in CARD and indicate an absence of resistance-conferring mutations for particular antibiotic(s). Our results showed that bacterial species reported from drinking water that included *Bacillus zanthoxyli* (MW486015.1), *B. subtilis* (MW486121.1), and *B. safensis* (MW486127.1) exhibited low antimicrobial resistance ($300 < \text{bit-score} < 600$) (Fig. 3). On the other hand, the microbes reported from wastewater, including *Klebsiella pneumoniae* (MW486037.1) (Li et al., 2014), *B. subtilis* (MW486109.1) (Tsonis et al., 2018), *B. licheniformis* (MW486112.1) (Salkinoja-Salonen et al., 1999), and *B. sonorensis* (MW486126.1), exhibited strong AMR potential and are established causal agents of water-borne diseases.

Next, we set out to correlate the results of phylogenetic analysis and the *in-silico* predictions of AMR potential. Multi-metal resistant bacteria, belonging to the genus *klebsiella* yielded the highest bit-score value (1714) which also conformed to literature reports. Compared to this, bacteria belonging to the genus *bacillus* exhibited AMR potential bit-score values ranging from 0 to 874. Such a variance in AMR might be due to the variable metal-resistance potential of the genus bacillus, as documented by earlier studies, i.e. metal-resistant and metal-susceptible bacteria. We concluded that metal-type exposure can directly select metal-resistant bacteria while co-selecting antibiotic-resistant bacteria.

The discovery of a potential AMR gene pool in the native microbial life of Hudiara, its transfer to other species residing in wastewater and consequently to the underground water sources can culminate in the dissemination of AMR through mechanisms such as co-resistance and cross-resistance (Singer et al., 2016). Previous studies have established heavy metal resistance (HMR) to be simultaneously associated and selected with antibiotic-resistant genes (ARG) (Knapp et al., 2017), with undesired consequences for human health. Taken together, the current study links the heavy metal contamination and potential antimicrobial resistance with the unprecedented scale of pollution in Hudiara's wastewater and drinking water from its adjoining areas.

3.11. Discussion on fingerprinting of heavy metal and microbial contamination

Spatial fingerprinting of heavy metals in Hudiara drain over 7 months has established mercury as the leading contaminant, with levels almost 80 times higher than recommended standards. Subsequent seepage of this metal-laden water into the underground water reservoirs deteriorates its quality thus raising the level of metals well above the permissible limits. Consequently, arsenic is the leading contaminant in drinking water with levels ∼200 times higher than the permissible limits. Towards the source apportionment of these heavy metals, multivariate statistical analysis was applied which shows the combinatorial nature of geogenic and anthropogenic fingerprints on the contamination of water sources around Hudiara. Next, to quantitatively gauge the degree of water contamination, pollution indices such as contamination factor and heavy metal pollution index were computed. The resultant outcomes helped elucidate the unprecedented scale of water pollution. Next, health risk assessment (HRA) scores were calculated to scale the fingerprint of metal-laden water consumption on the health of people residing around the drain. HRA scores were found to be high for all metals (except iron and manganese) representing the consistent health risk posed to the dwellers of Hudiara. A general health assessment of inhabitants residing around Hudiara was then carried out for communicable and non-communicable disease burdens. Particularly for chronic obstructive pulmonary diseases (COPD) and dermatitis, 38.7% and 34% of the participants, respectively, reported the disease around Hudiara compared to a prevalence rate of 2.1% for COPD (Khan et al., 2019) and 21.4% for eczema (Ahmed et al., 2019) in Pakistan. Furthermore, the profiling of the water samples revealed the presence of *klebsiella, exiguobacterium,* and *bacillus* which are the established causative agents of broad-spectrum infectious diseases (Baron, 1996; Chen et al., 2017; Keynan and Rubinstein, 2007). Presence of the exceptionally high levels of heavy metals can potentially induce antimicrobial resistance in these Hudiara-specific pathogenic microbes. To confirm our hypothesis, an *in-silico* screening of 16S rRNA sequences was directed which exhibited the multi-drug resistance potential against aminoglycosides, peptides, and tetracyclines in the Hudiara-specific microbes. Such ubiquitous fingerprints of heavy metals in water sources around Hudiara can further strengthen the antimicrobial resistance potential of these microbes leading to their dissemination consequently resulting in severe and inconceivable implications on human health.

4. Practical applications and prospects

This work highlights the potential of interdisciplinary and data-integrative approaches to assess waste and drinking water quality including physical, chemical, and biological parameters and their impact on human health. The work can be extended through the development of a longitudinal framework that helps elucidate linkages between spatiotemporal variation in Hudiara's anthropogenic landscape with communicable and non-communicable disease burdens around the drain. Given the established linkages between heavy metal contamination and antimicrobial resistance, follow-up metagenomic investigations can help establish causal agents of antimicrobial resistance in Hudaira-specific microbiota. Another impactful extension of this work can come in the form of a fungi and algae sorption study towards developing novel bioremediation strategies for the removal of heavy metals from soil and water.

Moreso, the study underlines the dire need for the establishment, implementation, and periodic monitoring of strict environmental practices towards the development and preservation of sustainable water resources along with the protection from slow poisoning of Hudiara in the Indian Subcontinent. Such efforts will also cope with the exponentially high disease burden of communicable and non-communicable diseases besides augmenting efforts in curbing AMR. Summarily, our study provides evidence to warrant a full-scale assessment of groundwater quality in areas adjoining the drains to pre-empt potential health hazards and the initiation of bioremediation projects towards the creation of wetlands.

5. Recommendations

Towards mitigating the unprecedented scale of water pollution and developing stewardship to this effect, the following recommendations are being made to the government and policymakers.

1. **Pollutant type-specific low-cost water treatment** programs need to be designed such that contaminants emanating from sewage, industrial discharge, and agricultural runoffs, are optimally treated before being discharged into wastewater channels.
2. **Wastewater volumes** emerging from anthropogenic sources need to be monitored and quantified towards devising cost-effective and scalable remediation strategies.
3. **Soil characterization of wastewater stream beds and geospatial mapping of aquitards** should be promulgated to check the diffusion of anthropogenic heavy metals into groundwater resources.
4. **Temporal monitoring and profiling of contaminant discharges**, in particular industrial effluents, should be mandated to inform and adjust the remediation strategies.
5. **Mandatory industrial sponsorship of wetlands** on banks of industrial wastewater channels.
6. **Mobile water quality assessment units should be created** to democratize heavy metal content testing in water and soil before their onward employment in agriculture.
7. **Bioavailability assessment framework for heavy metals in agricultural produce** should be established in wholesale markets.
8. **Domestic water quality** monitoring should be incorporated into the land development and house construction regulations.
9. **Sewage discharge from septic tanks in households** should be brought under the umbrella of environment protection agencies (EPA).

10. **Research and development of bioremediation strategies**, in this case, employing Hudiara-specific microbes – *exiguobacterium, bacillus* (including *B.tequilensis*, *B. velezensis*, *B. safensis*, *B. subtilis*, *B. licheniformis*) and *klebsiella* should be encouraged.
11. **Training, capacity building, and engagement of key stakeholders** including the communities, government bodies, and employees towards the risk assessment, long-term planning, and implementation of water sustainability laws.

6. Conclusion

Spatiotemporal assessment of the physicochemical parameters of Hudiara drain has established arsenic and mercury as the leading contaminants in wastewater and their subsequent seepage in groundwater reservoirs. Multivariate statistical analysis of heavy metals has shown the combinatorial impact of geogenic and anthropogenic factors on the contamination of water sources around Hudiara. Onwards, the evaluation of pollution indices and health risk assessment scores present the consistent health risk posed to the dwellers of Hudiara, besides foregrounding a high burden of diseases and the emergence of antimicrobial resistance (AMR). In conclusion, the in-depth investigation and analysis helped uncover the complex geogenic and anthropogenic landscape of Hudiara drain and elucidated its effects on underground water resources as well as human health.

CRediT authorship contribution statement

Zainab Nasir: Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas, Processed the bacterial samples obtained from wastewater and drinking water, Performed the data analysis, wrote the manuscript. **Ambreen Sabir:** Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas, Wrote the manuscript. **Hafiz Muhammad Salman:** Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas, Processed the bacterial samples obtained from wastewater and drinking water, Performed the data analysis. **Muhammad Usman Ashraf:** Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas, Processed the bacterial samples obtained from wastewater and drinking water. **Muhammad Farhan Khalid:** Performed the geographical mapping of sampling sites on Hudiara drain. **Muhammad Burhan Khalid:** Performed the geographical mapping of sampling sites on Hudiara drain. **Zonaira Khalid:** Devised the experimental protocols for the study, Wrote the manuscript. **Amna Tahir:** Performed the data analysis. **Fatima Arshad:** Devised the experimental protocols for the study. **Hafiz Gohar Ejaz:** Performed the sampling of water samples from Hudiara drain and its adjoining areas. **Saneela Ashraf:** Performed the sampling of water samples from Hudiara drain and its adjoining areas. **Sheikha Hina Liaqat:** Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas. **Huma Khawar:** Performed the sampling of water samples from Hudiara drain and its adjoining areas. **Risham Hussain:** Performed the sampling of water samples from Hudiara drain and its adjoining areas. **Muhammad Umer Sultan:** Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas, Processed the bacterial samples obtained from wastewater and drinking water. **Imran Afzal:** Devised the experimental protocols for the study. **Sadia Hamera:** Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas. **Numrah Nisar:** Performed the heavy metal analysis in water samples. **Shomaila Sikandar:** Devised the experimental protocols for the study. **Safee Ullah Chaudhary:** Conceived the idea and designed the study, Devised the experimental protocols for the study, Performed the sampling of water samples from Hudiara drain and its adjoining areas, Wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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