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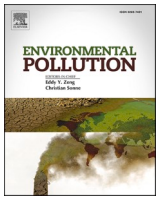

Discovery of sulfonamide resistance genes in deep groundwater below Patna, India☆

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ABSTRACT

Global usage of pharmaceuticals has led to the proliferation of bacteria that are resistant to antimicrobial treatments, creating a substantial public health challenge. Here, we investigate the emergence of sulfonamide resistance genes in groundwater and surface water in Patna, a rapidly developing city in Bihar, India. We report the first quantification of three sulfonamide resistance genes (*sul*I, *sul*II and *sul*III) in groundwater (12–107 m in depth) in India. The mean relative abundance of gene copies was found to be *sul*I (2.4×10^{-2} copies/16S rRNA gene) > *sul*II (5.4×10^{-3} copies/16S rRNA gene) > *sul*III (2.4×10^{-3} copies/16S rRNA gene) in groundwater ($n = 15$) and surface water ($n = 3$). A comparison between antimicrobial resistance (AMR) genes and wastewater indicators, particularly tryptophan:fulvic-like fluorescence, suggests that wastewater was associated with AMR gene prevalence. Urban drainage channels, containing hospital and domestic wastes, are likely a substantial source of antimicrobial resistance in groundwater and surface water, including the Ganges (Ganga) River. This study is a reference point for decision-makers in the fight against antimicrobial resistance because it quantifies and determines potential sources of AMR genes in Indian groundwater.

1. Introduction

The world is facing an imminent and significant threat from antimicrobial resistance (AMR) (Ashbolt et al., 2013; Grenni et al., 2018; Hendriksen et al., 2019; Munk et al., 2022; WHO, 2014; Yang et al., 2018; Zainab et al., 2020). Since the mid-20th century, the ever-increasing and indiscriminate usage of antibiotics to treat pathogenic microbial diseases (Hutchings et al., 2019), coupled with insufficient wastewater treatment (Michael et al., 2013; Pazda et al., 2019), has led to the input of unmetabolized antibiotics in water sources (Michael et al., 2013; Pazda et al., 2019). Antibiotics, along with antimicrobial agents and co-selectors, have provided a selection pressure for the proliferation of AMR genes in the environment (Lin et al., 2016; Maillard, 2018; Martinez and Baquero, 2000; Munk et al., 2022; Vats et al., 2022; Zainab et al., 2020).

Many of the previous studies on antibiotic residues and AMR genes have reported on surface water, often geographically limited to the USA (Martens and Demain, 2017), Europe (Hilton and Thomas, 2003) and China (Hilton and Thomas, 2003; Liu and Wong, 2013). Yet, few studies have attempted to survey antimicrobial resistance in India (Das et al., 2017; Walia et al., 2019) – one of the largest manufacturers (Greene, 2007), consumers (Klein et al., 2018) and polluters (Mutiyar and Mittal, 2014; Wilkinson et al., 2022) of antibiotics in the world. Patna, Bihar, is of particular interest to study in the context of antimicrobial resistance as an exemplar rapidly developing city with inadequate wastewater infrastructure (Alakshendra, 2019). The city is situated along the iconic Ganges (Ganga) River, which has been reported to be impacted by emerging organic contaminants (Lapworth et al., 2018; Richards et al., 2023). Here, reliance on antibiotic-contaminated groundwater (Fick et al., 2009; Richards et al., 2021) to fulfil water demands, including for

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drinking, highlights the need for understanding processes such as surface-derived ingress and associated controls on the development of antimicrobial resistance in groundwater sources used for drinking (Burch et al., 2022).

Among all the antibiotic groups found in water, sulfonamides are one of the oldest (Aminov, 2010) and least degradable (Liu et al., 2019a). Sulfonamides are effective against both gram-positive and gram-negative bacteria, along with some fungi and protozoa (Capasso and Supuran, 2014). The sulfonamide antimicrobial class is commonly used to treat both human and animal bacterial infections (Supuran et al., 2003; Upmanyu and Malviya, 2020). As recognition of their medicinal significance, sulfonamides are listed as *Highly Important Antimicrobials* in human medicine by the World Health Organisation (WHO, 2018) and *Critically Important Antimicrobials* in veterinary use by the World Organisation for Animal Health (WOAH, 2007).

The emergence of drug-resistant microbes presents a global health challenge (Larsson and Flach, 2021). Resistance to sulfonamides is mediated by the acquisition of the *folP* gene and/or sulfa-insensitive dihydropteroate synthetase (DHPS) enzymes, which are encoded by *sul* genes in gram-negative enteric bacteria (Sköld, 2000; Venkatesan et al., 2022). The *sul*I gene is often found on large conjugative plasmids and is linked to other resistance genes of the Tn21 transposon, in particular the class 1 integron-integrase gene (*intI*1; Chaturvedi et al., 2021; Sköld, 2000), whilst *sul*II is often located on small non-conjugative plasmids or large transmissible multi-resistance plasmids (Sköld, 2000). The *sul*III gene is similar to *sul*I and *sul*II genes and was first detected on conjugative plasmids in *Escherichia coli*, though the gene has been less extensively studied (Alcock et al., 2020; Perreten and Boerlin, 2003). In the context of India, *sul* genes have previously been detected in wastewater (Saxena et al., 2021; Talat et al., 2023), rivers (Chaturvedi et al., 2021; Kumar et al., 2020) and groundwater to a limited extent (Shinde et al., 2020), though quantification data is severely lacking.

The aim of this study was thus to quantify and explain the prevalence of three sulfonamide resistance genes (*sul*I, *sul*II and *sul*III) in groundwater and surface water around the rapidly developing city of Patna, Bihar, where sulfonamide-class antibiotics were detected previously (Richards et al., 2023, 2021). To build new understanding of the development of sulfonamide resistance genes in Indian groundwater, we quantify and investigate the AMR genes below Patna as a function of potential antibiotic inputs.

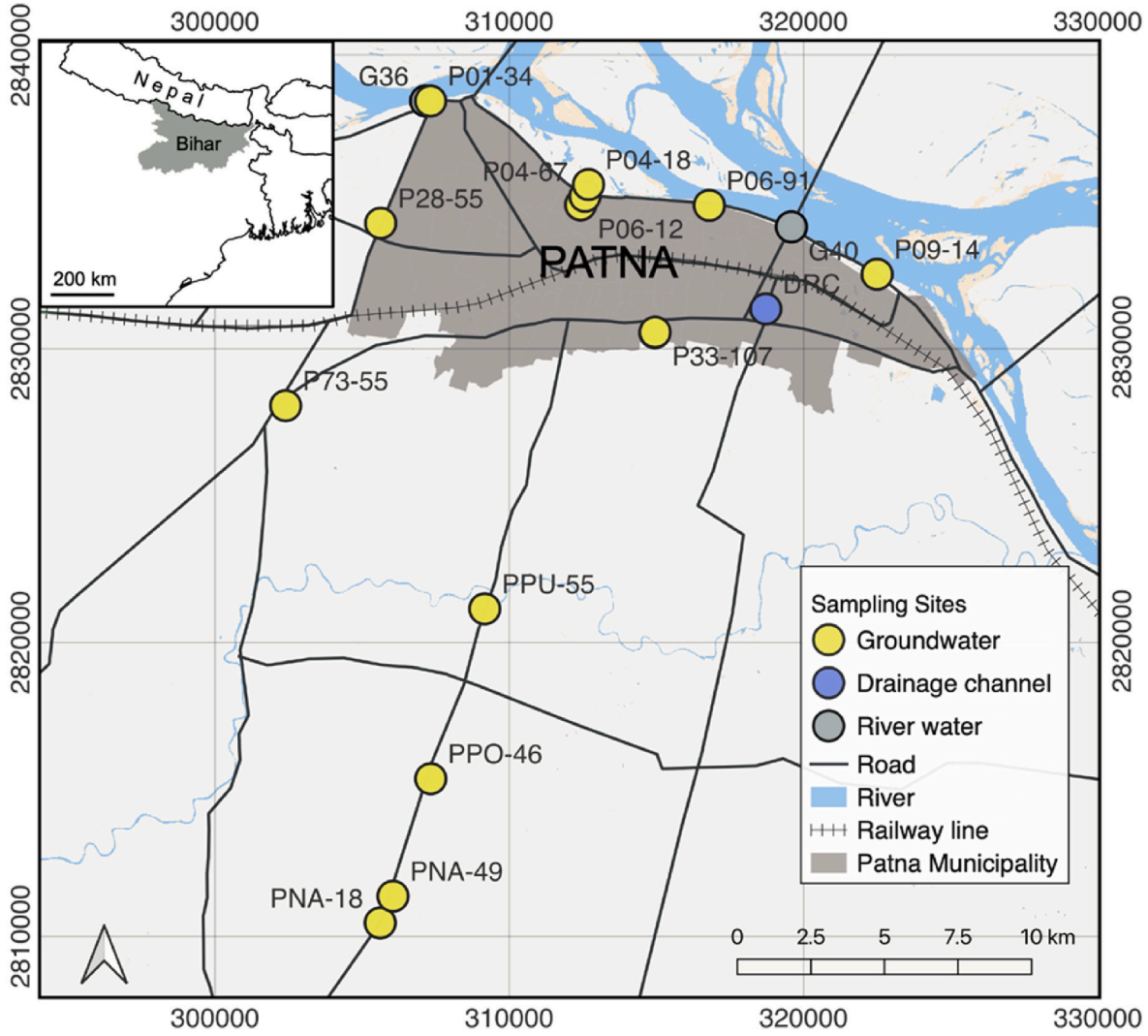

Fig. 1. Location of Patna sampling area and groundwater and surface water sites sampled for antimicrobial resistance in this study. Site selection was based on previous characterisation of antibiotics in the Patna area, with a preference for sites where detectable concentrations of sulfonamides were found (Richards et al., 2021, 2023). DRC = drainage channel, G = river water sampling (consistent with Richards et al. (2023) samples), first P = Patna groundwater sampling (consistent with Richards et al. (2021) samples), PO = Pothahi, NA = Nadwan and PU = Punpun. The reported depth (m) is given as -XX (groundwater sites only).

2. Methods

2.1. Study area

Patna, the largest and capital city of Bihar, is situated on the south side of the Ganges River in the Middle Gangetic Plain, India. Patna is one of the fastest growing cities in the world by population, which has grown approximately 47% from 2000 to 2020 to 2.5 million people (UN, 2018).

Sampling sites were selected from or nearby those previously surveyed for waterborne antibiotics by Richards et al. (2023, 2021), with a preference for sites where detectable concentrations of sulfonamides had previously been found (Fig. 1). Groundwater samples in this study were taken from identical boreholes to Richards et al. (2021), or the nearest borehole of similar depth, in cases where the borehole was no longer functional ($n = 4$). The surface sites were comprised of two near-bank samples from the Ganges River from Richards et al. (2023), one upstream and one downstream of the Patna urban area and a ~5 m-wide drainage channel close to several hospitals. More context on the field area is reported by Richards et al. (2022b). This manuscript extends preliminary data initially presented by Wilson et al., 2023a.

2.2. Sample collection

Samples were collected from groundwater ($n = 15$) and surface water ($n = 3$) sites in Patna during late-August (monsoon season) 2022. Groundwater was collected from private and government handpumps (12–107 m depth), which were flushed for approximately 90 s before sample collection. Approximately 2 L of water was collected in sterile Nalgene bottles from both groundwater and surface water and cooled at 4 °C in a portable refrigerator until the 'sample preparation' step. Two sampling repeats were collected at a groundwater ($n = 1$; P04-91) and surface water ($n = 1$; G36) site.

2.3. Quantification of sulfonamide resistance genes

2.3.1. Sample preparation

Following sample collection, the samples were transported to Mahavir Cancer Sansthan (MCS). Here, the samples were concentrated by filtering surface water (250 mL) or groundwater (1.5–2.1 L) through 0.45 μm sterile membrane filters (Sartorius) under laminar airflow and sterile conditions. Ultrapure water (2 L; provided by MCS) was filtered in the same manner as the sample (Procedural Control 1), after the samples had been filtered. The filter papers were placed in sterile foil envelopes (furnaced to 400 °C) using sterile forceps (SteriWare) and frozen (−20 °C). After each sample was filtered, the filtration apparatus was cleaned with ethanol wipes (70%), sodium hypochlorite (10%), UV light exposure (10 min) and was rinsed with the new sample. Filter papers were transported from MCS to KTH Royal Institute of Technology, Sweden in a vacuum-insulated flask (Milton Thermosteel; ~24 h in transit) containing an ice pack, chilled to −20 °C. Samples were stored at −20 °C for ~1 month before DNA extraction took place.

2.3.2. Genomic DNA extraction

Genomic DNA was extracted using the NucleoSpin® Soil DNA kit (Macherey-Nagel, Germany) according to the manufacturer's protocol, with a modified lysing step to smash the filter papers: filter papers were ripped into small pieces and placed into lysing tubes containing ceramic beads. Samples were pulverised at 10,000 rpm for 60 s using a Precelleys Evolution homogenizer. A sterile filter paper was also lysed in the same manner (Procedural Control 2).

2.3.3. Quantification of sulfonamide resistance genes with real-time qPCR

Quantification of sulfonamide resistance genes (*sul*I, *sul*II and *sul*III) in the water samples was achieved through qPCR (in duplicate). Specific primers and probes were used and designed for qPCR – these are reported in Table 1. Gene sequences were obtained from the National Center for Biotechnology Information (NCBI) webpage. The primers and probes were tested in silico using SnapGene software. The PCR reaction volume (20 μL) was comprised of 5 μL TaqMan Reliance One-Step Multiplex Supermix (BioRad), 1 μL of 20 mg/mL Bovine Serum Albumin, 5 μL of DNA template, 7 μL nuclease-free water and 2 μL of forward, reverse and probe primers mixture. The qPCR was conducted using an Applied Biosystems QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific) with amplification conditions of initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s with a final elongation step at 61 °C for 30 s. Standard curves were created using constructed plasmids containing the appropriate target for *sul*I, *sul*II and *sul*III (Integrated DNA Technologies, Custom MiniGene 25–500 bp), which were also used as positive controls in each qPCR reaction. Nuclease free water was used as a negative control.

2.3.4. Detection of 16S rRNA genes

As an estimation of *sul* gene relative abundance within the bacterial community, *sul* genes were quantified relative to 16S ribosomal RNA (rRNA) copy number (Yin et al., 2023). Quantification of the 16S rRNA gene was undertaken with a qPCR reaction medium (20 μL) composed of 5 μL PowerUp SYBR Green Master Mix, 1 μL forward primer, 1 μL reverse primer, 12 μL nuclease-free water and 1 μL of DNA template. The qPCR amplification of the 16S rRNA gene was carried out using primers 515 F (GTGYCAGCMGCCGCGGTAA) and 806 R (GGACTACNVGGGTWTCTAAT; Walters et al., 2015). The PCR conditions comprised of thermal cycling (50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 min, 95 °C for 10 min, 60 °C for 1 min).

Results from melting curve detection (95 °C for 15 s, 60 °C for 1 min, 60–95 °C with an increment of 0.15 °C/s and 95 °C for 1 s) of all target genes were compared to positive controls, to ensure specific amplification of PCR products (Fig. 2). Reactions were considered positive if the cycle threshold (C_t) was below 40 cycles with a single melting peak at an appropriate temperature.

2.4. Analysis of wastewater indicators

Inorganic and organic parameters were quantified to understand the prevalence of antibiotic resistance genes in the study area. Ammonium (NH_4^+) and nitrite (NO_2^-) were selected as established tracers of sewage and anthropogenic waste (Abdel-Shafy et al., 2008; Fick et al., 2009; Heng et al., 2004). Measurements of NH_4^+ and NO_2^- were made at the point of sample collection using a field spectrophotometer (Spectroquant Nova 60 A, Merck, Germany). An indophenol blue test (Spectroquant 114739) was used for the quantification of NH_4^+ (0.01–2.00 mg/L NH_4^+-N). A Griess reaction test (Spectroquant 114776) was used to quantify nitrite at low concentrations (0.002–1.000 mg/L NO_2–N),

Table 1
Primers and probes used in quantitative polymerase chain reaction (qPCR) to detect sulfonamide resistance genes in this study. The GenBank accession number of each sulfonamide gene is listed.

Gene	*sul*I	*sul*II	*sul*III
Forward	GCCGATGAGATCAGACGTATTG	AACCGCCTTGTCCTTGATC	GCAGTGTCACGGAAATCATTC
Reverse	GAAGCTGTCGATTGAAACACG	CAGCCGCAATTCATCGAAC	ATCATGGGTGCGGAGATAATC
Probe	CGCTCTTAGACGCCCTGTCCG	CTGCTCCCGAAACCTCGCTCTC	TGGTGCTAAACGAGATTTCACATCGGT
GenBank accession number	X12869	AY232670	KF240814

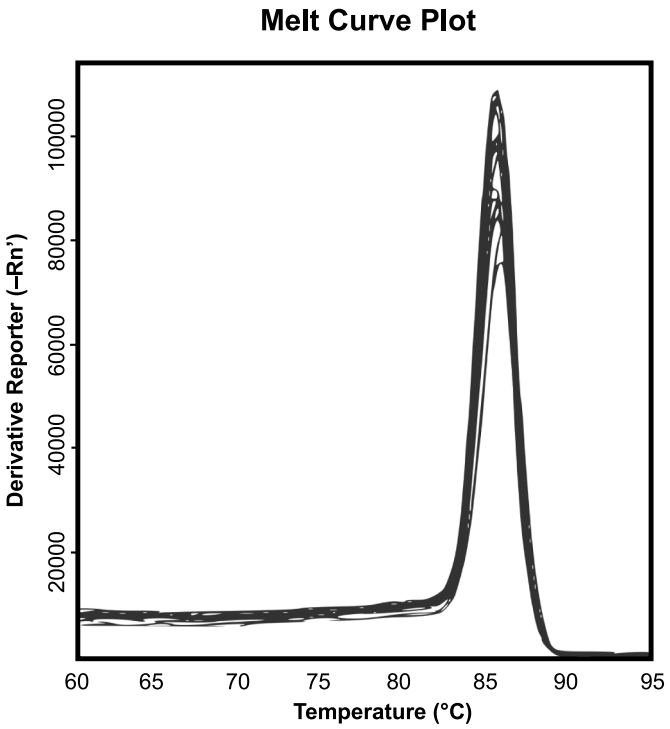

Fig. 2. Melting curve detection (95 °C for 15 s, 60 °C for 1 min, 60–95 °C with an increment of 0.15 °C/s and 95 °C for 1 s) analysis of target genes was compared to positive controls to confirm specific amplification of PCR products. Analysis was performed on the QuantStudio 3 Real-Time PCR System.

whilst an iron (II) ethylenediammonium sulfate test (Spectroquant 100, 609) was used for a higher range of concentrations (1.0–90.0 mg/L NO_2–N). The photometric accuracy and linearity of the photometer was checked daily using PhotoCheck solutions (PhotoCheck 14693, Merck, Germany).

Fluorescent dissolved organic matter (fDOM) was quantified using an Agilent Cary 60 UV–Vis and Cary Eclipse Fluorescence spectrophotometers at the British Geological Survey (Wallingford, UK). The excitation (Ex.) and emission (Em.) scanning ranges were 200–400 nm (5-nm bandwidth) and 250–500 nm (2-nm bandwidth), respectively. A 1-cm quartz cuvette was used for the spectrophotometer measurements; the cuvette was cleaned with ultrapure water between samples. After absorbance and blank correction, following methods of Lakowicz (1994) and Murphy (2011), the ratio of tryptophan-like to fulvic-like fluorescence (Tryp:FA) was quantified as a proxy for DOM bioavailability. This ratio has previously been used as a tracer for wastewater in groundwater (Baker, 2002; Kulkarni et al., 2017). Full fDOM quantification methods have previously been described in Richards et al. (2019).

2.5. Statistical analysis

Statistical analysis was undertaken using Microsoft Excel 16.71. Regression analysis was conducted through two-tailed Spearman's rank correlation in the format "*r(degrees of freedom) = r value; p = p value*". All statistics are reported at 95% confidence level. A Bonferroni correction, $\frac{\alpha}{n}$, where $\alpha = 0.05$ and $n =$ the number of tests performed, was applied to correct for biased false-positive result in instances where multiple tests were made simultaneously.

3. Results

3.1. Distribution of sulfonamide resistance genes in groundwater and surface water around Patna

AMR genes in Patna's water resources were measured using quantitative polymerase chain reaction (qPCR). Three sulfonamide resistance genes (*sul*I, *sul*II and *sul*III) were found to be prevalent in studied groundwater (12–107 m in depth) and surface water of Patna. This is the first study to quantify *sul* genes in groundwater in India; the detection and quantification of *sul* genes in deep groundwater provides crucial insight into the proliferation of AMR in the environment.

To estimate *sul* gene relative abundance in the bacterial community, *sul* genes were reported as gene copies per 16S ribosomal RNA (rRNA) copy number. Overall, the mean relative abundance of sulfonamide resistance genes was *sul*I (2.4×10^{-2} copies/16S rRNA gene) > *sul*II (5.4×10^{-3} copies/16S rRNA gene) > *sul*III (2.4×10^{-3} copies/16S rRNA gene; Table 2). The detection rate of *sul*I and *sul*II genes was 100% in the studied groundwater and surface water, whilst *sul*III was detected in 100% of surface water and 53% of groundwater. The abundance of all three quantified sulfonamide resistance gene (copies/mL) was significantly correlated to 16S rRNA genes ($r\ (16) = 0.99$; $p < 0.05$; *sul*I, *sul*II and *sul*III). The relative abundance of *sul* genes, normalised to the 16S rRNA gene, in groundwater, river water and a drainage channel is reported in Table 2.

A Ganges surface water sample taken downstream of Patna city (G40) was an order of magnitude higher in all three sulfonamide resistance genes compared to a sample taken upstream of the city (G36; Fig. 3), suggesting substantial urban inputs from Patna. The concentration of 16S rRNA gene sequences was the same order of magnitude between G40 and G36 (1.71×10^6 and 1.12×10^6 copies/mL, respectively). Although the number of samples was limited, this observation may indicate that drainage channels such as the one sampled from Patna city (containing abundant AMR genes at least two orders of magnitude higher than river samples; Table 2) and other urban inputs are likely a key source of antimicrobial resistance in the Ganges River.

Two procedural controls were utilised to quantify potential contamination of the samples. Procedural Control 1 (Section 2.3.1) was a control for filtration-induced contamination. This control sample resulted in a small number of *sul*I (0.35 copies/mL), *sul*II (0.75 copies/mL) and no detectable *sul*III. The 16S rRNA gene was also detectable (8.88×10^2 copies/mL) in this control sample, albeit low, relative to the

Table 2
Concentration ranges of three sulfonamide resistance genes in groundwater ($n = 15$), river water ($n = 2$) and a drainage channel ($n = 1$) in Patna, Bihar, quantified by qPCR. As an estimation of the relative abundance of *sul* genes in the bacterial community, *sul* genes are reported as gene copies per 16S ribosomal RNA (rRNA) copy number. ND = not detected after 45 PCR cycles.

Category	*sul*I		*sul*II		*sul*III	
Copies-	*/mL*	*/16S rRNA*	*/mL*	*/16S rRNA*	*/mL*	*/16S rRNA*
Ground-water	1.4×10^0–6.2×10^3	1.3×10^{-4}–1.2×10^{-1}	7.2×10^{-1}–6.8×10^3	2.4×10^{-5}–1.7×10^{-2}	ND–1.7×10^1	ND–2.6×10^{-3}
River water	1.3×10^3–5.4×10^4	1.1×10^{-3}–3.1×10^{-2}	1.2×10^3–2.4×10^4	1.0×10^{-3}–1.4×10^{-2}	2.7×10^2–3.0×10^3	2.4×10^{-4}–1.8×10^{-3}
Drainage channel	5.1×10^6	1.6×10^{-1}	1.1×10^6	3.4×10^{-2}	1.3×10^6	3.8×10^{-2}
Mean	–	2.4×10^{-2}	–	5.4×10^{-3}	–	2.4×10^{-3}

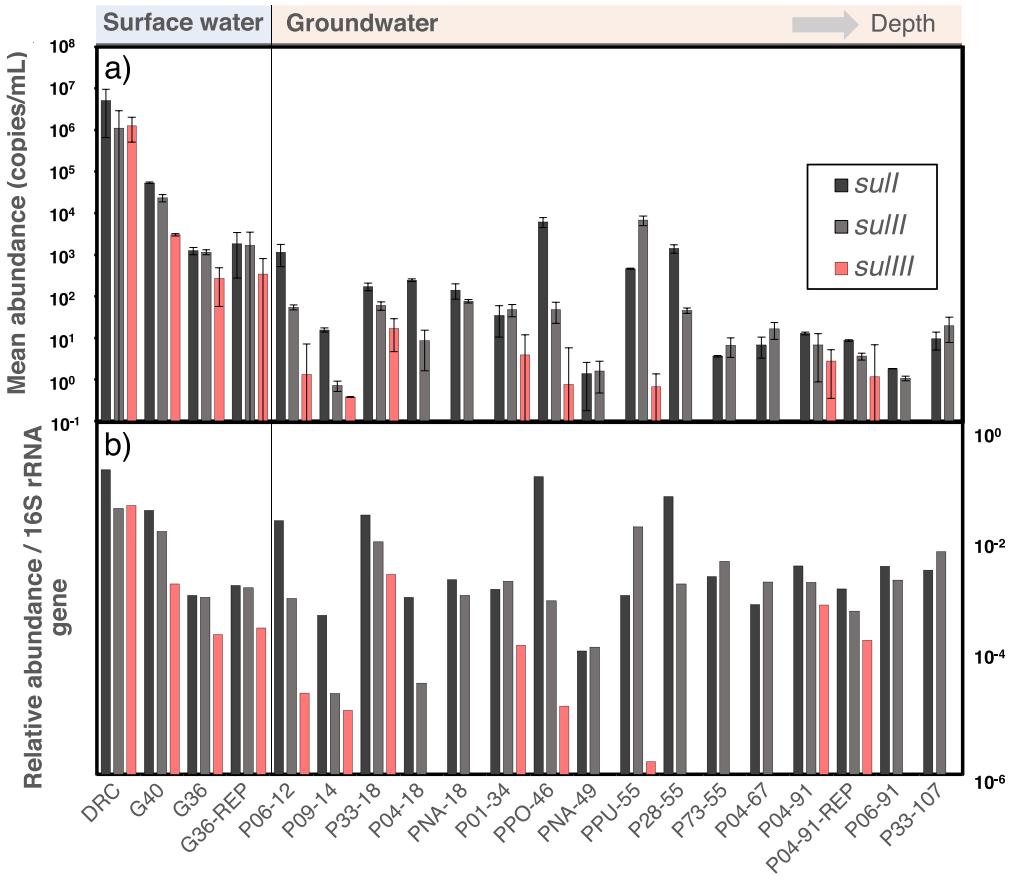

Fig. 3. Three quantified sulfonamide resistance genes (*sul*I, *sul*II and *sul*III) in groundwater ($n = 15$) and surface water ($n = 3$) were prevalent in studied water of Patna, Bihar. Groundwater samples are displayed in order of increasing depth, from left to right as a) mean *sul* abundance (copies/mL) and b) *sul* relative abundance (/16S rRNA). Confidence intervals (95%) relate to samples analysed in duplicate. DRC = drainage channel, G = river water sampling (consistent with Richards et al. (2023) samples), first P = Patna groundwater sampling (consistent with Richards et al. (2021) samples), PO = Pothahi, NA = Nadwan and PU = Punpun. The reported depth (m) is given as -XX (groundwater sites only). Sampling repeats ($n = 2$) are reported as '-REP'.

observed abundances of genes measured in samples in this study. Procedural Control 2 (Section 2.3.2) was the contamination control for the DNA extraction. There were no *sul* nor 16S rRNA genes detected in this control, implying that a there was a residual level of PCR targets present on the filtration equipment and/or the ultrapure water used for the blank, rather than contamination introduced during the DNA extraction process. Two sampling repeats were taken at sites G-36 and P04-91 to assess and quantify potential differences in sample measurements – both repeats exhibited a high level of consistency, as depicted in Fig. 3.

To investigate the spatial dynamics of AMR genes within the groundwater profile, sulfonamide resistance and 16S rRNA gene concentrations were determined as a function of depth. The abundance of the *sul*I gene (copies/mL) and 16S rRNA gene both showed a significant correlation with reported depth ($p < 0.05$; Fig. 4 and Table 3, respectively), whereas the abundancies of *sul*II and *sul*III did not. These findings may suggest that *sul*I has disseminated to a higher degree in bacterium present here, compared to *sul*II and *sul*III genes (Alcock et al., 2020). The prevalence of *sul*I–III, relative to 16S rRNA genes were not significantly correlated with reported depth ($p < 0.05$) (Table 3; Fig. 4c). Interestingly, *sul*I > *sul*II > *sul*III described the overall abundance of *sul* genes in this study, yet for the drainage channel the order of abundance was *sul*I > *sul*III > *sul*II. The *sul*III gene was not present in several ($n = 8$) groundwater samples > 20 m.

In brief, sulfonamide resistance genes were found to be prevalent in studied groundwater and surface water of Patna. All three target *sul* genes were found in groundwater up to 91 m depth (Fig. 4). The mean abundance of sulfonamide resistance genes in groundwater and surface water was *sul*I > *sul*II > *sul*III (Table 2). The abundance of *sul*I and 16S rRNA genes showed a significant correlation with reported depth (Fig. 4a; Table 3). However, the relative abundance of *sul* genes, normalised to 16S rRNA genes, showed no significant relationship to increasing groundwater depth (Fig. 4c; Table 3). To investigate whether the depth distribution of *sul* genes was indicative of less sulfonamide antibiotics, a comparison of sulfonamide resistance genes to previously characterized antibiotic concentrations was undertaken.

3.2. Comparison to sulfonamide antibiotics

Antibiotic resistance gene concentrations were compared to published data on antibiotics concentrations (Richards et al., 2021, 2023) at the sampling sites. Although environmental persistence of sulfonamide resistance genes has been demonstrated (Archundia et al., 2017; Enne et al., 2001; Kaiser et al., 2023), none of the measured sulfonamide resistance genes were significantly correlated to the antibiotics detected previously in groundwater, nor to surface water at $p = 0.05$ level (Fig. 6a–d; sulfamethazine, sulfanilamide, sulfamethoxazole and sulfathiazole). In both groundwater and surface water, the low detection rates of sulfonamides plausibly impeded a relationship between antimicrobials and AMR gene abundance (Fig. 6). Particularly in surface water, this disparity could also be attributed to substantial temporal fluctuations in antibiotic concentrations (Richards et al., 2022a), given the difference in sampling time between AMR genes and antibiotics. However, groundwater sites, which were associated with a lower abundance of AMR genes (Table 2), also had lower concentrations of

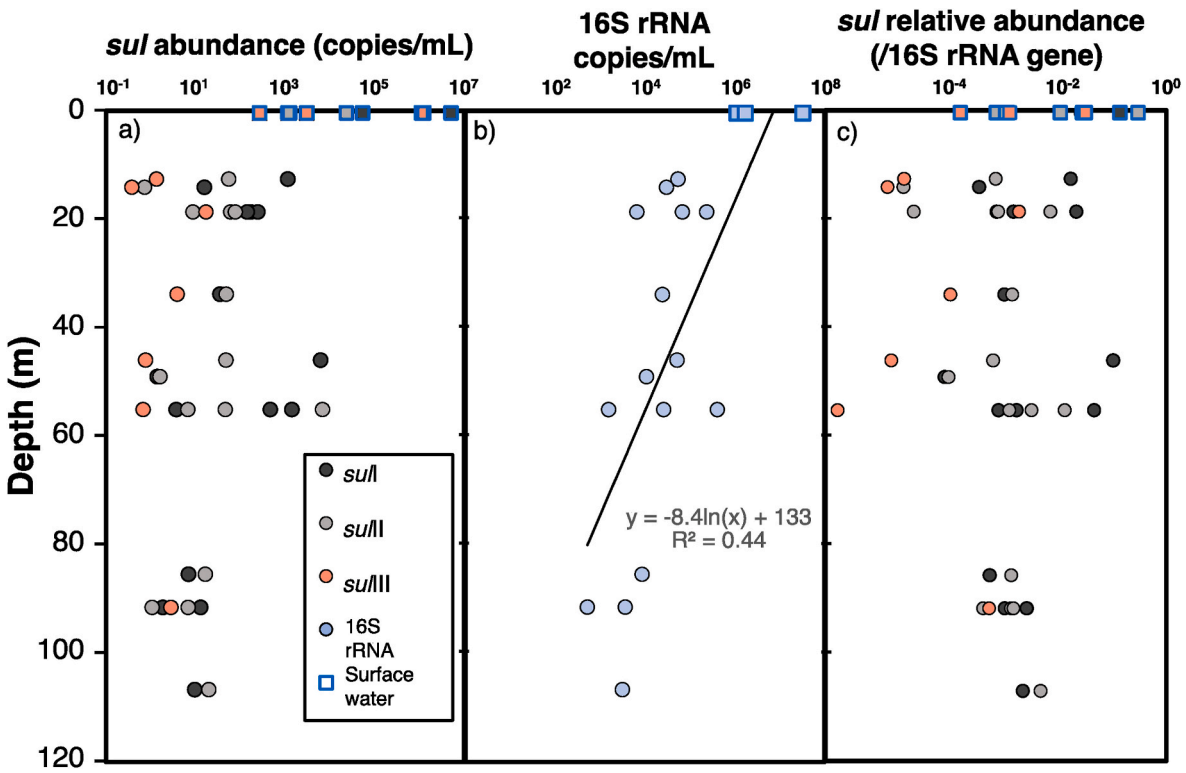

Fig. 4. Depth profiles of a) *sul* gene abundance, b) 16S rRNA gene copy number and c) *sul* gene relative abundance (/16S rRNA gene), detected in surface water (square with blue outline) and groundwater (circle with black outline) below Patna City, Bihar using qPCR. Spearman's rank correlation tests are reported in Table 3. Trendline in plot **b** shows a logarithmic correlation between depth and 16S rRNA gene concentration, excluding river water samples.

Table 3
Spearman's rank correlation revealed *sul*I and 16S rRNA genes were significantly correlated with depth at $p = 0.05$ level, though relative to 16S rRNA sequences, none of the sulfonamide resistance genes were correlated with depth at $p = 0.05$ level. For the purpose of calculating correlation coefficients: the drainage channel sample was used at depth = 0 m; river samples (G40 and G36) were not included in the correlation analysis and the concentration of genes below the detection limit was assumed to be 0 copies/mL. *Result significant at $p = 0.05$ level. [B]Result significant at Bonferroni corrected significance level.

	*sul*I (copies/mL)	*sul*II (copies/mL)	*sul*III (copies/mL)	16S rRNA (copies/mL)	*sul*I/16S rRNA	*sul*II/16S rRNA	*sul*III/16S rRNA
***r* value**	−0.57	−0.40	−0.47	−0.69	−0.10	0.26	−0.47
***p* value**	**0.02***	0.12	0.06	**0.00*[B]**	0.71	0.34	0.06

sulfamethoxazole (~10x; Fig. 6c), compared to surface water sites. Prominence of sulfamethoxazole in rivers in India has been reported elsewhere (Lapworth et al., 2018; Wilkinson et al., 2022). Sulfamethazine, sulfanilamide and sulfathiazole were not detected in the two surface water sampling points.

In groundwater, the *sul*III gene was only detected around the Patna urban centre where the highest concentrations of antibiotics were reported previously (Richards et al., 2021, Fig. 5a and b). The results may indicate that the abundance of the *sul*III gene in the environment may be related to a higher degree of selection pressure (*e.g.* wastewater-contaminated groundwater below a rapidly developing city) (Kampouris et al., 2021; Pruden, 2014; Uluseker et al., 2021). Indeed, higher concentrations of sulfonamides are present in groundwater around Patna centre, compared to less urban areas (Fig. 5a; Richards et al., 2021). In contrast to trends in total antimicrobial concentrations, this observation may indicate sulfonamides in Patna's groundwater are largely derived from human medicine, rather than veterinary use.

In summary, 10 antimicrobial drugs of medicinal/veterinary use were previously detected in post-monsoon groundwater and surface water in our study area (Richards et al., 2021, 2023). Notably, these include four sulfonamides: sulfamethazine, sulfanilamide, sulfamethoxazole, sulfathiazole (Fig. 5a). The distribution of sulfonamide antibiotics in groundwater below the Patna urban area coincided with the detection of the *sul*III gene (Fig. 5b). Though, none of the measured sulfonamide resistance genes were significantly correlated to the detection of antibiotics in groundwater, nor to surface water at $p = 0.05$ level (Fig. 6).

3.3. Comparison between AMR genes and wastewater indicators

To investigate the source of AMR genes in Patna's water, wastewater indicators (NH_4^+, NO_2^- and tryptophan-like to fulvic-like [Tryp:FA] fluorescent dissolved organic matter) were compared to sulfonamide resistance genes. Though NH_4^+ and NO_2^- in groundwater seemed to have little association with *sul* and 16S rRNA genes, surface water sites were characterized by high concentrations of NH_4^+ and NO_2^- along with high AMR gene prevalence (Fig. 7a and c) and also high 16S rRNA gene counts (Fig. 7b and d). Interestingly, Tryp:FA showed no correlation to 16S rRNA genes (Fig. 7f), yet there was a plausible association to *sul* gene relative abundance, normalised to 16S rRNA genes (Fig. 7e; though not statistically significant at $p < 0.05$ level). This may suggest that wastewater has contributed to an increased prevalence of antimicrobial resistance in organisms in groundwater and surface water.

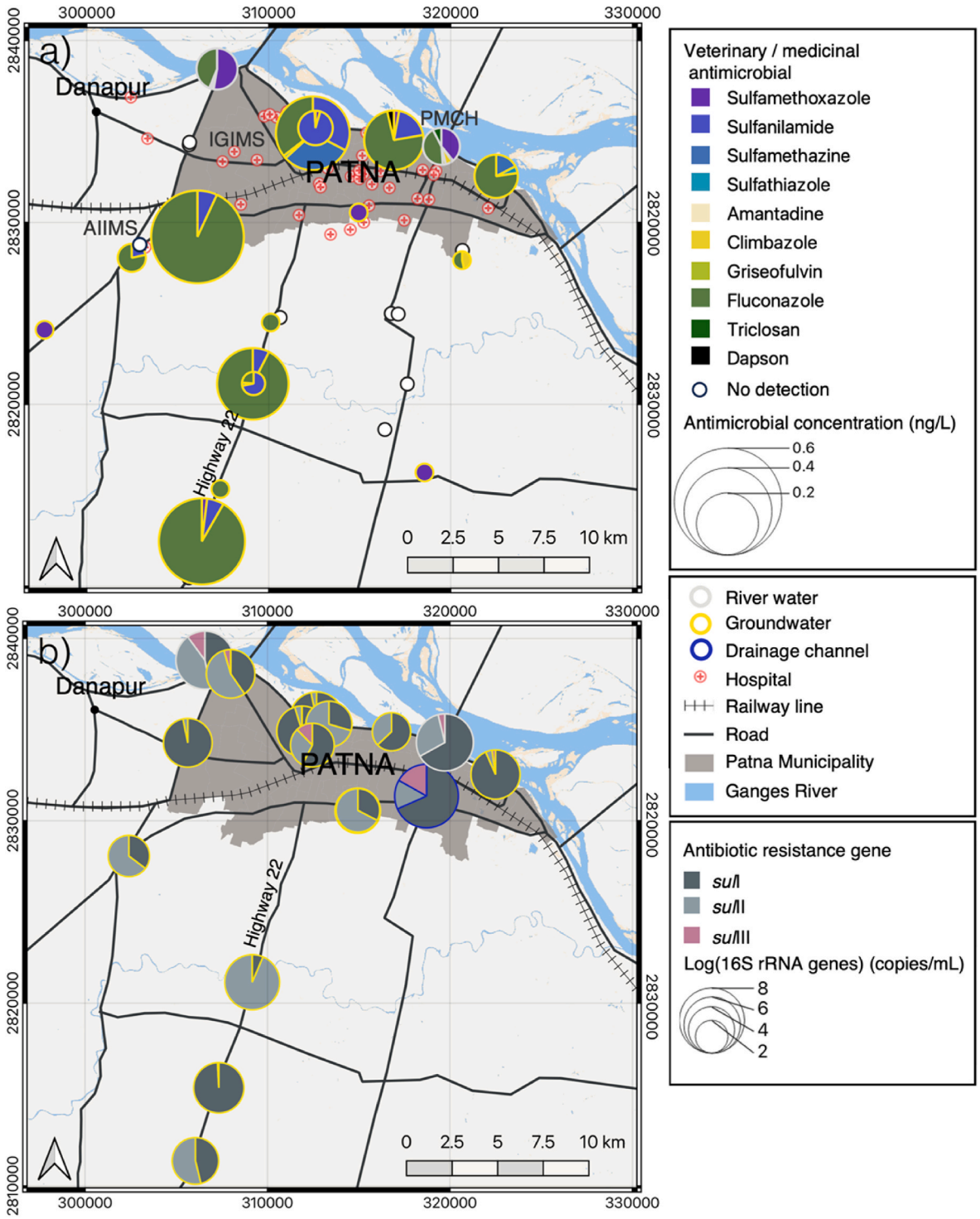

Fig. 5. Sulfonamide resistance genes were prevalent in studied groundwater and surface water around Patna, but the *sul*III gene was restricted to groundwater below the urban area, which was associated with a greater number of antimicrobials. a) Detected inputs of medicinal/veterinary antimicrobials in post-monsoon river and groundwater from Richards et al. (2023, 2021; respectively). Locations of hospitals are shown as a red cross (not exhaustive). The size of the circles represents the total medicinal/veterinary antimicrobial concentration. Major medical institutions (private and government) are shown by a red cross. AIIMS = All India Institute of Medical Sciences, IGIMS, Indira Gandhi Institute of Medical Science, PMCH = Patna Medical College Hospital. b) Sulfonamide resistance genes (*sul*I, *sul*II and *sul*III) in groundwater and surface water samples from Patna. The size of the circle represents the $\log_{10}$-converted concentration of 16S ribosomal RNA. White, yellow and blue outlines represent river water, groundwater and a drainage channel, respectively (both plots).

4. Discussion

Three sulfonamide resistance genes (*sul*I, *sul*II, *sul*III) were found to be prevalent in studied groundwater (12–107 m depth) and surface water of Patna, the first known quantification of these genes in groundwater in India. Although the sample size in this study was relatively low (n = 18), the results highlight a novel insight into the dissemination of AMR genes in groundwater used for drinking in India. The detection rate of the *sul*II gene in groundwater in our study (53–100%) was more frequent than in 20–40 m depth groundwater in

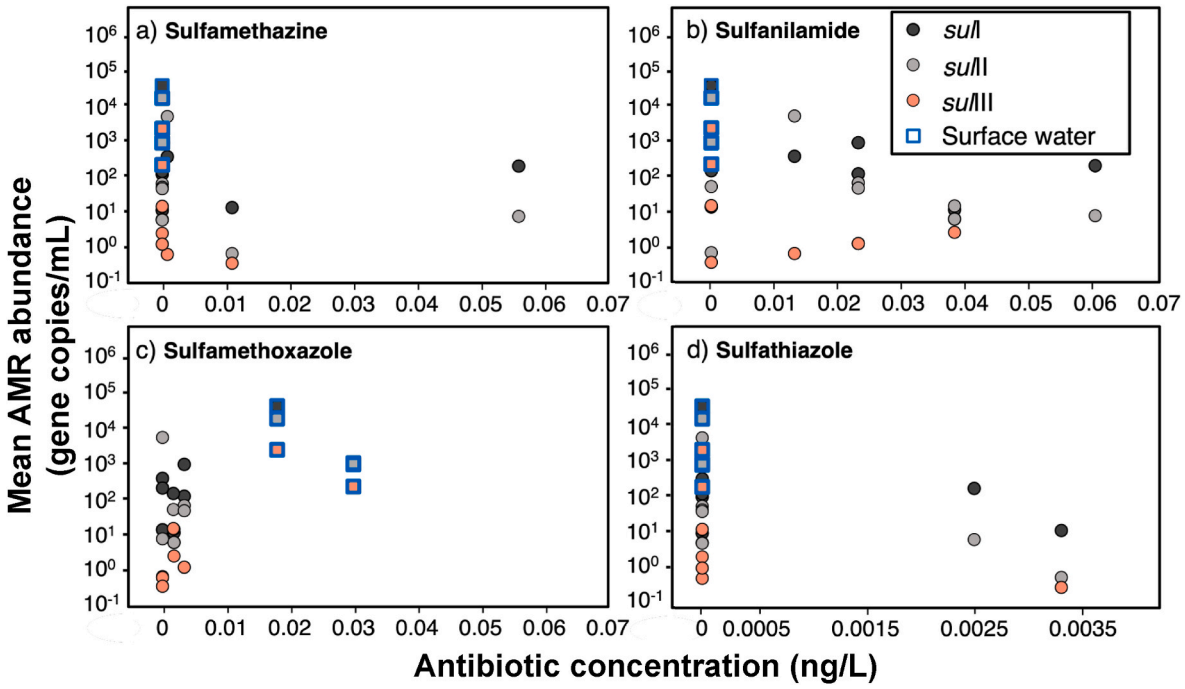

Fig. 6. Comparison between sulfonamide resistance gene (*sul*I, *sul*II and *sul*III) abundance in this study with previously reported (Richards et al., 2021, 2023) antibiotic concentrations shows a disparity, though higher sulfamethoxazole is found in association with a higher abundance of *sul* genes. Antibiotics a) sulfamethazine, b) sulfanilamide, c) sulfamethoxazole and d) sulfathiazole are from the post-monsoon season in river (blue outline) and groundwater (black outline). Groundwater and surface water sites in this study were taken from identical locations/boreholes to Richards et al. (2021, 2023; respectively), or the nearest borehole of similar depth, in cases where a borehole was no longer functional ($n = 4$). Antibiotic concentrations below detection limits are plotted as 0 ng/L.

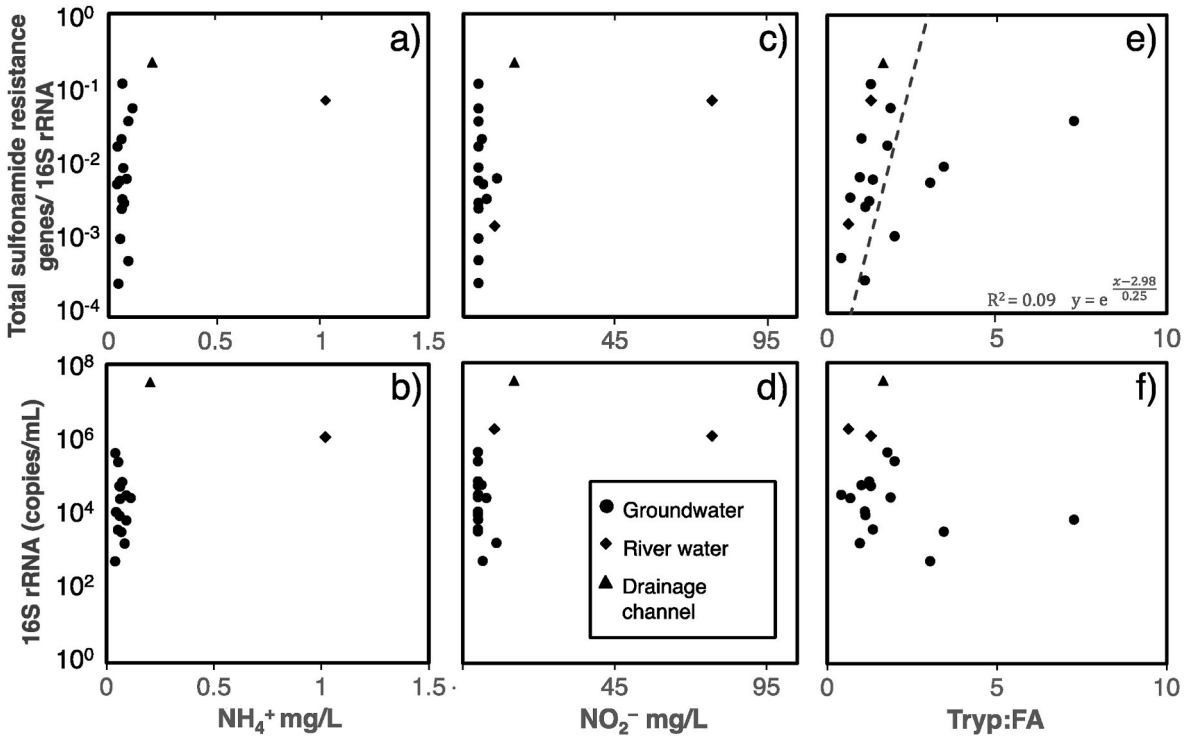

Fig. 7. The prevalence of three sulfonamide resistance genes (*sul*I + *sul*II + *sul*III), relative to 16S rRNA genes, and 16S rRNA (copies/mL) were found to correspond to wastewater tracers. Target genes were compared to a) and b) Ammonium – NH_4^+, c) and d) Nitrite – NO_2^- and e) and f) Tryptophan-like to fulvic-like fluorescent dissolved organic matter (Tryp:FA; Section 2.4) in groundwater and surface water of Patna. An exponential trendline (dashed line; $p = 0.07$) is plotted for **e** and the equation of the line is displayed in the bottom right.

Gujurat (10%; Shinde et al., 2020). Interestingly, all three *sul* genes in our study were detected in deep groundwater (*sul*I–III up to 91 m and *sul*I–II up to 107 m), which is increasingly targeted for drinking supplies in Bihar and elsewhere in India (Lapworth et al., 2018). Groundwater *sul* abundance in this study was of similar magnitude to reported *sul* abundance in shallow groundwater near Shenzhen, China (Table 4; Wu et al., 2020). In the river samples, the more *sul*-contaminated Ganges sample (G40) was one and two orders of magnitude lower in *sul*I and *sul*II genes/mL than samples from the Haihe River, China, respectively (Luo et al., 2010, Table 4).

The contrast in *sul* gene detection suggests differing gene transfer methods and rates in bacteria. A collation of whole-genome shotgun assemblies available in the Comprehensive Antibiotic Resistance Database (CARD) suggests *sul*I is located more on plasmid DNA, than *sul*II and sulIII (12%, 6% and 1% of pathogens, respectively (Alcock et al., 2020). As a result, gene transmission through horizontal gene transfer is plausibly highest for *sul*I, compared to *sul*II and *sul*III. As found in this study, others have reported significantly less *sul*III relative to *sul*I and *sul*II in soils in Jiangsu Province, China (Wang et al., 2014), in farm waste from California, USA (Wang et al., 2021) and an absence of *sul*III was also reported in an upstream control site of the *sul*-abundant Katari catchment (Archundia et al., 2017). The *sul*III gene in our study was more abundant, relative to *sul*I and *sul*II, in sites where exposure to antibiotics would be expected to be more significant (*i.e.* an urban drainage channel or shallow groundwater; Fig. 3). However, the *sul*III gene is also of more recent genetic origin than *sul*I and *sul*II and reduced propagation of this gene has been reported by CARD (Alcock et al., 2020; Perreten and Boerlin, 2003). Dissemination of the *sul*III gene across many species of pathogens (*e.g. Escherichia coli)* is reported as much lower than for *sul*I and *sul*II; though for a small number of species (*e.g. Shigella dysenteriae*), the *sul*III gene has a higher propagation rate (Alcock et al., 2020). Because it is highly likely that the drainage channel contains a different diversity of microorganisms than the groundwater and river water sites, the prevalence of *sul*III likely varies according to the bacterial community.

Elevated *sul* gene prevalence in the drainage channel and a comparison between AMR genes to wastewater indicators alludes to an association between wastewater and the increase of AMR gene prevalence in groundwater (Fig. 7). Previous studies have suggested that nutrients may expedite AMR proliferation by promoting horizontal gene transfer (Blau et al., 2018; Lima et al., 2020). Improper management of wastewater likely facilitates the entry of antibiotic residues and nutrients into soils and groundwater, providing favourable conditions for AMR genes to concentrate (Jindal et al., 2021; Kunhikannan et al., 2021). Residence time indicators suggest most groundwater in the study area is < 70 years old (Richards et al., 2022b), though the infiltration of antimicrobials and affiliated AMR gene abundance may be exacerbated by extensive groundwater pumping below Patna (Lu et al., 2022; Saha et al., 2014; Wilson et al., 2023b).

Previously, a total of 10 antimicrobial drugs of medicinal/veterinary use were detected in groundwater and surface water in our study area (Richards et al., 2021, 2023). These were, listed in order of detection frequency: fluconazole (41%), sulfanilamide (25%), sulfamethoxazole (23%), climbazole (9%), sulfamethazine (7%), amantadine (5%), griseofulvin (5%), sulfathiazole (5%), dapson (2%), triclosan (2%) (Fig. 5a; detection frequency of all sites in Richards et al. 2021 and the two river sites used in this study, from Richards et al., 2023). The relative abundance of sulfonamides in groundwater in India is likely a result of their frequent usage against human diseases (Kumar et al., 2008) and veterinary application (Jindal et al., 2021) combined with their physical properties: low biodegradability and weak sorption to soils, which results in a high degree of leaching (Sarmah et al., 2006). Since the concentration of antimicrobial drugs in groundwater does not appear to directly correspond to the distribution of major hospitals in Patna (Fig. 5a; *e.g.* at All India Institute of Medical Sciences) nor the urban area, it is plausible that ingress of veterinary-derived antibiotics contribute substantially to observed antimicrobial distribution (Jindal et al., 2021), and/or that sewage ingress still contributes to groundwater composition even in less densely populated peri-urban or rural areas where wastewater infrastructure remains very limited. We note this study was designed spatially to capture urban to rural transitions in the context of a rapidly developing city and on the basis of previous antimicrobial inputs detected in groundwater, rather than the detection of AMR genes specifically around major hospitals; the latter of which is a topic for further investigation.

The presence of antimicrobial compounds in groundwater and subsequent resistance is plausibly mediated by numerous factors. These factors may be related to, though not limited to, solute transport parameters (*e.g.* diffusion and dispersion), biodegradation, hydrogeological factors (*e.g.* surface-groundwater interactions, groundwater age and pumping rate), co-selection of AMR genes, hydrochemical conditions (*e.g.* pH and redox conditions) and physiochemical parameters of antimicrobials (*e.g.* concentration) (Zainab et al., 2020). In our study, the disparity between antibiotic concentrations and sulfonamide resistance genes could be, in part, a result of different sampling periods between the two datasets (Fig. 6; Wilson et al., 2023b), whilst noting dynamic temporal changes occurring across various scales (*e.g.* diurnal and seasonal differences) are likely to be more substantial in surface waters as compared to groundwater; Richards et al., 2022a). Additionally, the potential for co-selection, plausibly driven by a numerous environmental selection variables (Lin et al., 2016; Vats et al., 2022) and the presence of a diverse collection of resistance genes and gene cassettes within integrons (Adelowo et al., 2018; Chaturvedi et al., 2021; Luo et al., 2010) may play a substantial role in influencing the prevalence of *sul* genes. To further elucidate this relationship, future investigations could employ PCR analysis of integrons in conjunction with DNA sequencing.

This study helps to characterise the problem of antimicrobial

Table 4
The abundance (copies/mL) and prevalence (normalised to 16S rRNA sequences) of *sul* genes (I–III) in a range of environmental matrices from a selection of published studies.

Gene	Location	Copies/mL	Copies/16S rRNA	Reference
***sul*I**	Kathmandu Valley, Nepal; shallow–deep groundwater		2.7×10^{-3}–2.2×10^{0}	Thakali et al. (2022)
	Wendeburg, Germany; topsoil pore-water		1×10^{-3}–1×10^{-2}	Kampouris et al. (2021)
	Shenzhen, China; groundwater	ND–8.0×10^{5}		Wu et al., (2020)
	Wenyu River, China		1.8×10^{-3}–1.2×10^{-1}	Liu et al. (2019b)
	Haihe River/Tributary, China	9.3×10^{4}–2.2×10^{6}		Luo et al. (2010)
	Durban, South Africa; urban river		1×10^{-2}–1 x10^{-1}	Suzuki et al. (2015)
	Diluvio River, Brazil	3×10^{0}–7×10^{4}		
***sul*II**	Wenyu River, China		6.0×10^{-5}–7.1×10^{-2}	Liu et al. (2019b)
	Haihe River/Tributary, China	3.9×10^{5}–3.6×10^{7} copies/mL		Luo et al. (2010)
	Delhi and Nagpur, India; wastewater influent	2×10^{2}–1×10^{6}		Saxena et al. (2021)
	Haihe River, Poyang Lake and Qingdao Beach, China	3×10^{5}–5×10^{7}		Su et al. (2020)
***sul*III**	Durban, South Africa; sewage		ND–1×10^{-4}	Suzuki et al. (2015)
	Manitoba, Canada; sewage lagoon		2×10^{-4}	Anderson et al. (2013)

resistance, in the context of a rapidly developing city. Although with potential global implications, this issue is particularly pertinent in Northeast India, where access to sanitation is very low in comparison to other regions (IIPS, 2022) and the ever-stressed reliance on groundwater (Goldin, 2016; Saha et al., 2014) highlights the crucial need to survey these resources. Our findings here warrant a more extensive and systematic study, to gain a comprehensive understanding of the origins and abundance of AMR genes in Indian waterbodies.

The pervasiveness of antibiotic residues and resulting resistance emergence in groundwater and major rivers raises concerns globally. Adequate waste management and potential remediation of antibiotic residues in water supplies is a critical task that requires policymakers, practitioners, scientists and other relevant stakeholders to develop, implement and manage appropriate and effective interventions to adequately mitigate pathological risks in impacted water supplies. Given the risk posed by AMR genes in the environment, we conclude by highlighting the need for further monitoring and research on antibiotics and AMR gene distribution in aqueous water sources, particularly in groundwater used as drinking supplies in rapidly developing cities.

CRediT authorship contribution statement

George J.L. Wilson: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Mariel Perez-Zabaleta:** Investigation, Methodology, Writing – review & editing. **Isaac Owusu-Agyeman:** Investigation, Methodology, Writing – review & editing. **Arun Kumar:** Conceptualization, Investigation, Project administration, Writing – review & editing. **Ashok Ghosh:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **David A. Polya:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. **Daren C. Gooddy:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. **Zeynep Cetecioglu:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Laura A. Richards:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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

Data will be made available on request.

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