

Article

Antibiotic Resistance Genes and Faecal Sterols in Marine Sediments: An Evidence of Their Presence away from Point Sources—Kuwait's Example

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Abstract: Coastal areas are vital ecosystems, supporting diverse marine life and providing resources essential to human well-being. However, sewage pollution poses a significant threat to these delicate environments, impacting water quality, biodiversity, and public health. Antimicrobial resistance (AMR) has gained importance. We believe the presence of faecal sterols in coastal and marine sediments is a reliable indicator of sewage contamination. At most sites, the faecal contamination was observed with ranges from <dl to 18713 ng g⁻¹ dw. The coprostanol/cholesterol ratio was >0.2 at 68% of the sampling sites, suggesting a large spatial distribution of sewage contamination beyond the discharge points. The positive correlation of faecal sterols with AMR provides an insight that antibiotic resistance genes (ARGs) can spread to areas away from point sources. We propose that sterol ratios be considered as a screening tool for selecting the sites for AMR assessment. The analyses of sterols will be less time and cost-intensive compared to pharmaceutical analyses and can be a reliable indicator for AMR studies in areas where prior knowledge and experience are lacking.

Keywords: sterols; faecal contamination; coprostanol; cholesterol; pharmaceuticals



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1. Introduction

Almost 40% of the world's population resides in the coastal areas [1], making the coastal zone an economic [2–4], recreational [5], and ecological hub, hosting a multitude of organisms and providing various ecosystem services [6]. Multiple stressors affect the coastal and marine environment of the Gulf, including heavy metals [6,7], persistent organic pollutants [8], hydrocarbon pollution [9–11], and pharmaceutical residues [11] to name a few. The wastewater treatment plants (WWTPs) are, generally, not very efficient in removing pharmaceutical compounds resulting in measurable levels of these compounds in the aquatic environment where the effluent is discharged, likely to affect the biota [12–15]. The urban build-up in the coastal areas is linked to sewage contamination and pharmaceutical discharge [16] into the coastal waters, which often results in the development of antimicrobial resistance (AMR). However, since most of these chemicals are hydrophobic, their likelihood of settling and remaining in the sediments is high. The accentuation of drug resistance by aquatic environments has garnered a great deal of attention recently [17]. Antimicrobial compounds, resistance genes, and bacteria are introduced into marine and aquatic habitats mostly by agricultural run-off and faecal source discharges into the water through wastewater treatment plants or illicit discharges. Furthermore, a wide range of anthropogenic contaminants that have been demonstrated to co-select for AMR are known to be present in both treated and untreated sewage discharges.

The most widely used indicator of sewage contamination in coastal areas is Coliform bacteria [18], but their biodegradation at higher salinities and temporal fluctuations are well known [19], which can compromise their usefulness as a biomarker of sewage contamination and AMR. Faecal sterols, on the other hand, are quite stable under anoxic conditions. Coprostanol (predominant sterol in human faeces) is produced in the gut of mammals by enzymatic reduction of cholesterol and is a reliable indicator of human faecal contamination and, being hydrophobic, assimilates into the bottom sediments [20]. The persistent nature of coprostanol and other sterols (such as cholesterol, epicoprostanol, and cholestanol) makes them a reliable indicator for faecal contamination and useful in tracking sediment transport from sources to sink [20–24]. In this study, the ratio of coprostanol and cholesterol was used to ascertain the degree of pollution. The faecal sterols as biomarkers can differentiate between human and animal sources [25]. However, individual sterols cannot pinpoint sources of faecal pollution, as they are not exclusive to one organism. The ratios of cholesterol, cholestanol, coprostanol, and epicoprostanol [26–34] are a useful indicator for distinguishing between human and non-human faecal contamination.

The coprostanol/cholesterol ratio has been suggested as a valuable indicator of sewage due to its normalisation for total lipid content, as cholesterol is ubiquitous in most organisms. This ratio examines various biogenic sources of sterols and confirms the presence of urban sewage, displaying a positive correlation with other sewage pollution markers in oceans and estuarine sediments. To detect contamination originating from human waste, the ratio of coprostanol to other sterols has been suggested. Another ratio proposed by Grimalt et al. [21] suggested differential hydrogenation of cholesterol to cholestanol versus coprostanol, distinguishing urban sewage pollution from natural sources.

Although all the wastewater is treated in Kuwait, the expectations were that there would be less prevalence of AMR in coastal sediments. Unfortunately, the observations were contrary; resistance genes and faecal sterols were detected even away from point sources, which is likely to call for additional explanations and research on the process of transmission.

In addition, the samples were subjected to high throughput shotgun metagenomics sequencing to capture the presence of antibiotic resistant genes (ARGs). Since then, some studies have indicated the presence of AMR in *Escherichia coli* in Kuwait's coastal waters from non-polluted sites [35,36], raising a concern if this characterisation of non-polluted sites based on coliform presence is reliable, or whether these ARGs are associated with the redistribution of sewage-contaminated sediments which is substantiated by the presence of sterols.

2. Material and Methods

The sediment samples were collected from twenty coastal sites that were selected based on prior knowledge of the area approximately 3–4 kilometres away from stormwater and emergency outlets. Some remote and pristine sites were also selected. The details of the site and its coordinates are provided in Table 1, and spatial disposition is plotted in Figure 1 for a synoptic overview. The samples from the coastal area were collected in January–February 2023, while the samples from the Jahra Pool Reserve were collected in May 2023.

Table 1. Sampling locations and physico-chemical parameters.

Sample ID	Location	Latitude	Longitude	Site Description	Dates of Sample Collection
1	Khadmah Bay	29°23'10'' N	47°44'43'' E	A distal end of Kuwait Bay	15 January 2023
2	Near MPA	29°21'12'' N	47°53'15'' E	Near Doha Multi-stage flash Power and Desalination Plant outfall	15 January 2023
3	Shuwaikh Outfall	29°21'51'' N	47°56'59'' E	Distant to outfall receiving emergency waste near Shuwaikh Beach	15 January 2023
4	Sharq	29°23'49'' N	47°59'02'' E	Distant to outfall receiving emergency waste near Kuwait City	15 January 2023
5	Kuwait Tower	29°23'46'' N	48°00'12'' E	Distant to outfall receiving emergency waste	15 January 2023
6	Marina Food Court	29°20'55'' N	48°04'52'' E	Distant to outfall receiving emergency waste	15 January 2023
7	Al Bida	29°18'54'' N	48°05'41'' E	Distant to outfall receiving emergency waste	15 January 2023
8	Khairan	28°39'18'' N	48°23'50'' E	Wastes from recreational resorts	16 January 2023
9	Ras Zour	28°44'59'' N	48°24'17'' E	Proximity to Al-Zour refinery	16 January 2023
10	Bnaider	28°53'27'' N	48°16'45'' E	Recreational beach	16 January 2023
11	CFP Mina Abdulla	28°58'44'' N	48°10'53'' E	The area receiving refinery waste	16 January 2023
12	Fahaheel	29°05'09'' N	48°08'36'' E	Distant to outfall receiving emergency waste	16 January 2023
13	Mahboula	29°08'19'' N	48°08'12'' E	Distant to outfall receiving emergency waste	16 January 2023
14	Eagila	29°11'01'' N	48°07'24'' E	Distant to outfall receiving emergency waste	16 January 2023
15	Messila	29°15'29'' N	48°05'48'' E	Distant to outfall receiving sewage discharge	16 January 2023
16	Al Qaruh	28°48'52'' N	48°46'45'' E	A remote island with oil contamination	5 February 2023
17	Umm Ul Maradim	28°41'70'' N	48°39'30'' E	Remote island	5 February 2023
18	Subiya outfall	29°28'54'' N	48°07'10'' E	Waste of Subiya power plant	6 February 2023
19	Failaka	29°25'57'' N	48°15'54'' E	Wastes from resorts on the island	6 February 2023
20	Auha	29°23'10'' N	48°17'40'' E	Remote uninhabited island	6 February 2023
JR1	Jahra Reserve [†]	29°21'32'' N	47°41'37'' E	Lake in Jahra Pool Reserve	11 May 2023
JR2	Jahra Reserve [†]	29°21'41'' N	47°42'22'' E	Lake in Jahra Pool Reserve	11 May 2023
JR3	Jahra Reserve [†]	29°21'42'' N	47°41'25'' E	Outflow to Sea	11 May 2023
JR4	Jahra Reserve [†]	29°21'52'' N	47°41'33'' E	Lake in Jahra Pool Reserve	11 May 2023
JR5	Jahra Reserve [†]	29°21'32'' N	47°41'27'' E	Lake in Jahra Pool Reserve	11 May 2023

Average water temperature = 18.7 °C; average depth = 5–30 m; average salinity = 41.82‰; [†] average salinity (17‰) shown as open triangles in Figure 1, average temperature (24.6 °C), depth (0–40 cm); outfall receiving emergency waste are conduits that are usually a stormwater network that is sometimes used to discharge waste in emergencies.

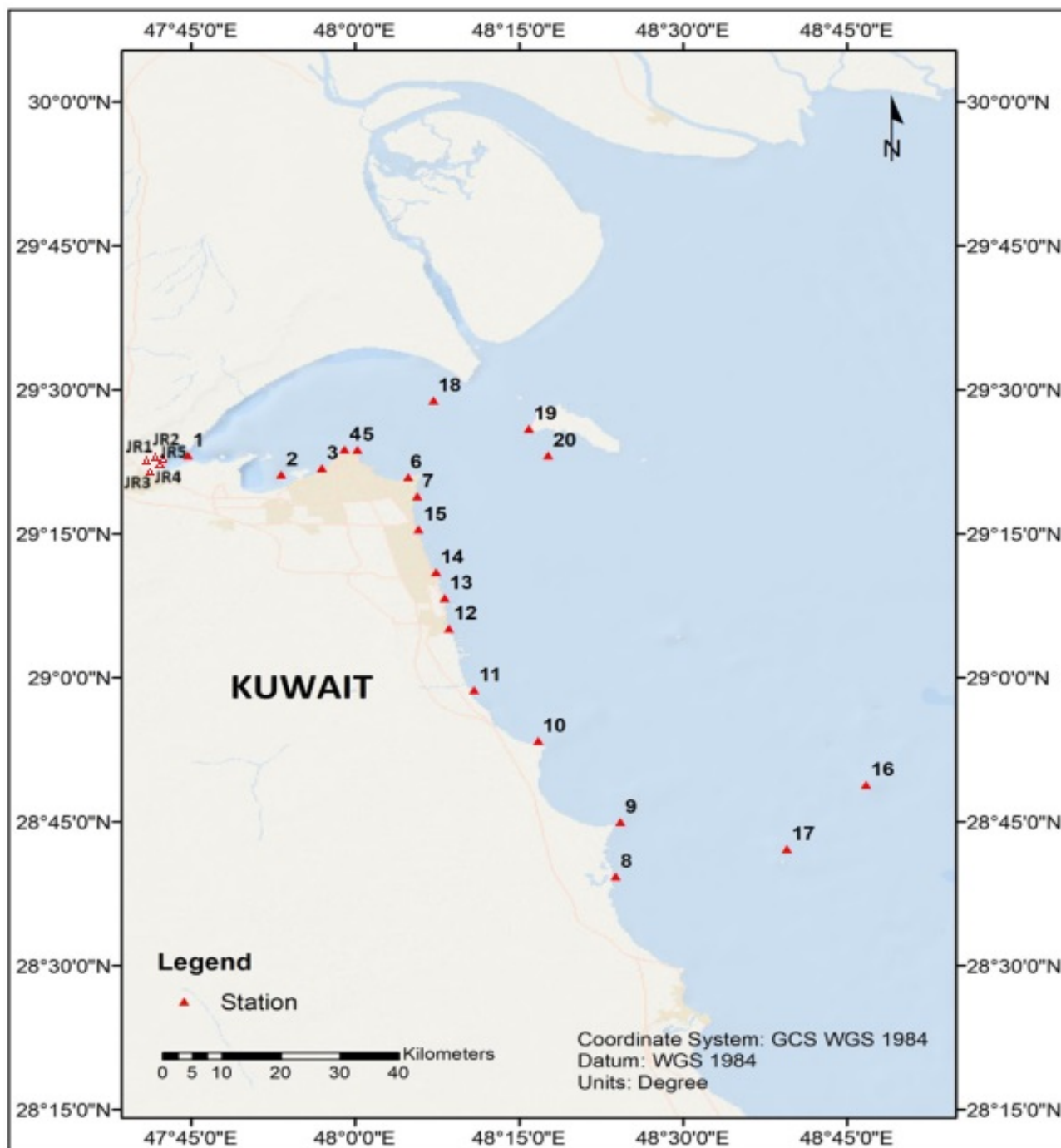


Figure 1. Kuwait map with marked points of sample collection.

Samples were collected from coastal areas using a 45 cm Van Veen grab sampler that was deployed from Research Vessel Bahith 5 of the Kuwait Institute for Scientific Research (KISR). The sediment samples were extracted from the grab using 50 mL falcon tubes by pressing them straight down and retrieving 10 cm of the top layer. From each grab, 6 tubes were extracted. The samples were transported on ice and stored in a freezer at -20°C until analysed.

2.1. Sterol Analysis

The sediment samples collected were freeze-dried using an Operon freeze dryer (Operon, South Korea) and sieved through a $250\ \mu\text{m}$ sieve on a Retsch Sieve Shaker (Retsch, Germany). For analysis, 2–5 g of freeze-dried sample was spiked with $5\ \mu\text{L}$ of $2\ \text{mg L}^{-1}$ of internal standard (5α -androstan- 3β -ol) and extracted using hexane-dichloromethane 1:1 (*v:v*) three times by ultrason-

ication. Activated copper filings were added for sulphur removal. The dried extract was saponified with 0.5 M methanolic potassium hydroxide at 70 °C for 30 min. Neutral lipids (containing sterols) were separated with hexane, drying over sodium sulfate and evaporating to dryness under a nitrogen stream. The dried residue was reacted with *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) reagent to prepare silyl derivatives of sterols. The dried residue was taken up in 1 mL iso-octane. The derivatised sterols were analysed using a Shimadzu GC2030 coupled to a Shimadzu QP-2020 NX mass spectrometer. A Shimadzu AOC-20i plus autosampler was used for introducing the samples to the GC-MS. The temperatures of the injection port, interface, and ion source were 280 °C, 270 °C, and 240 °C, respectively. Helium was used as a carrier gas and was flowing through the column (coated with SPB-5, 0.25 mm ID and 30 m length) at 1 mL min⁻¹. The initial temperature of the GC was 60 °C and was raised to 100 °C at the rate of 10 °C min⁻¹. From 100–270 °C, the rate of temperature increase was kept at 4 °C min⁻¹ and finally held for 20 min. The data were acquired and processed using Shimadzu GCMS Solution software. To identify and quantify the compounds of interest, the selective ion monitoring (SIM) mode was used. The SIM mode identified androstanol: 333; cholesterol: 368 and 458; cholestanol: 445 and 460, coprostanol: 370, 355; and epicoprostanol: 370 and 355. The quantification was carried out using internal standard androstanol (5 α -anthrostan-3 β -ol) in addition to its usage as a recovery standard. The recoveries were between 79 and 103%. Recoveries less than 75% were repeated until an acceptable recovery was obtained. A total of 20% of the samples were repeated for quantity assurance and quality control. As part of the QA/QC, a standard reference material IAEA-408 was concurrently analysed with the sample batch. In addition, reagent blanks were also processed. The detection limit for the method was 5 ng g⁻¹ dw.

2.2. DNA Isolation

DNeasy® Power Soil Pro® Kit (QIAGEN, Germantown, MD, USA) was used to extract DNA from each sample (0.25 g). The DNA was extracted multiple times ($n = 6$) from each site and pooled to obtain a homogenous sample. Each sample was weighed and placed in a sterile 1.5 mL microcentrifuge tube (Eppendorf, Darmstadt, Germany) containing lysis solution C1. The lysate was then transferred to the power bead tube and vortexed for 10 min at room temperature. The manufacturer's protocol was followed to precipitate, bind, wash, and elute the DNA. The quantity of isolated DNA was estimated on a fluorometer (Qubit, Thermo Fisher Scientific, Waltham, MA, USA) employing the BR dsDNA kit (Qubit, Thermo Fisher Scientific, USA). The quality was assessed on a 0.8% agarose gel run for 45 min at 100 V (Bio-Rad, Darmstadt, Germany) [36]. DNA was stored at –80 °C until further processing.

2.3. Bacterial Counts by qPCR

Quantitative polymerase chain reaction (qPCR) was used to estimate the 16S rRNA gene copy numbers employing degenerate primers [37]. The polymerase chain reaction (PCR) was assembled in a volume of 20 μ L and consisted of 2 $\times$ PCR master mix (iQTM SYBR® Green supermix (BioRad), 300 nM primers (Forward 5'-CCTACGGGNBGCASCAG-3' and Reverse 5'-GACTACNVGGGTATCTAATCC-3), template DNA (2 ng), and nuclease-free water. PCR was run on a CFX96™ Deep Well thermal cycler (BioRad, Hamburg, Germany). The cycling conditions for the PCR are described in our previous studies [38]. A melt curve analysis (60 °C) was performed to rule out non-specific amplification and primer-dimers. Standard *Escherichia coli* DNA was used as a positive control. A non-template control (NTC) sample was run along with the PCR reaction as a negative control. The C_T was set at 30, and values above this threshold were excluded. Six technical replicates were used per site. Mean C_T was used to calculate the gene numbers at each location. A PCR efficiency of 100.0% was achieved. The melt curve analysis was carried out at 60 °C to check for non-specific amplification [13]. For each sample, six replicates were analysed. A box and whisker plot was drawn for the counts obtained at each sampling location in the GraphPad Prism v10 software. The data were distributed in the box encompassing the 25 to 75% interquartile range, whereas the upper and lower whiskers represented the 10 and 90% of data, respectively. The median values were marked as a black line in the centre.

2.4. Shotgun Metagenomic Sequencing

The isolated DNA was also subjected to whole genome metagenomic sequencing. The steps of analysis for this method have been described elsewhere [36]. Shortly, 1 µg of DNA was sonicated, end-repaired, A-tailed, indexed, and sequenced on a NovaSeq 6000 (Illumina, San Diego, CA, USA) following the 2×150 bp paired-end chemistry. The raw sequences were processed through standard bioinformatics pipelines. Firstly, the raw sequences were checked for quality statistics (FASTQC); thereafter, the low-quality bases were removed, adapters were trimmed (Trimmomatic), and clean reads assembled through MEGAHIT v 1.04 into ≥ 500 bp scaffigs [39]. The scaffigs were used for gene prediction using the MetaGeneMark v 2.10. that were aligned against the Comprehensive Antibiotic Research Database (CARD) (BLASTP e value $\leq 1 \times 10$). The visualisation of genes was performed in the R package, 2D track CIRCOS for the circus plots [40]. gplots in R was used to create the heat maps [41].

3. Results

There are many sources of sterols in the aquatic environment, e.g., fungi, planktons, protozoan, vascular plants, zooplanktons, and sewage. The sterols found can have in situ origin or can be distributed by oceanic processes i.e., currents and tides. The spatial distribution of sterols is presented in Table 2. Detectable levels of coprostanol have been measured in most samples ranging between <dl to 18,713 ng g⁻¹ dw. Coprostanol constitutes about 60% of sterols in human faeces. The sample results have been colour-coded for ease of representation, with values in 10–100 ng g⁻¹ indicating uncontaminated samples [21] (indicated as green). The coprostanol concentration of >100 ng g⁻¹ indicates sewage contamination (colour-coded yellow), and values >500 ng g⁻¹ indicating severe sewage contamination are indicated as red in Table 2. Epicoprostanol is present only in trace amounts in human faeces; however, it is considered an indicator of sewage treatment of faecal material, as it originates in the digested sludge of the wastewater treatment plants [42,43]. The concentration of epicoprostanol in the study area was variable between <dl to 16,401 ng g⁻¹ dw.

Table 2. Faecal sterols in sediment samples (ng g⁻¹, dry wt.).

Samples ID	Coprostanol	Epicoprostanol	Cholesterol	Cholestanol	Cop/Cholesterol	Epicop/Cop	Cop/(Cop + Cholestanol)
1	778.39	565.10	2889.20	2227.15	0.269	0.726	0.259
2	<dl	<dl	<dl	<dl	-	-	-
3	<dl	<dl	<dl	<dl	-	-	-
4	279.14	257.67	604.29	388.04	0.462	0.923	0.418
5	303.80	230.98	1315.22	480.71	0.231	0.760	0.387
6	403.85	399.04	873.08	658.65	0.463	0.988	0.380
7	261.28	237.33	795.54	428.97	0.328	0.908	0.379
8	194.17	203.88	427.18	257.28	0.455	1.050	0.430
9	196.32	202.94	963.24	311.27	0.204	1.034	0.387
10	268.37	274.76	2635.78	472.84	0.102	1.024	0.362
11	238.23	227.15	5531.58	396.12	0.043	0.953	0.376
12	206.11	206.11	531.81	282.44	0.388	1.000	0.422
13	265.85	200.00	485.37	275.61	0.548	0.752	0.491
14	253.59	207.69	425.64	292.31	0.596	0.819	0.465
15	215.54	0.00	729.32	300.75	0.296	0.000	0.417
16	243.77	227.15	1016.62	324.10	0.240	0.932	0.429
17	<dl	215.43	436.17	281.91	-	-	-
18	459.77	244.25	681.03	445.40	0.675	0.531	0.508
19	290.78	297.87	645.39	586.88	0.451	1.024	0.331
20	<dl	207.69	384.62	256.41	-	-	-
JR1	428.92	16,401.96	5017.16	1345.59	0.085	38.240	0.242
JR2	5021.46	4727.47	3849.79	2493.56	1.304	0.941	0.668
JR3	888.13	271.69	2093.61	863.01	0.424	0.306	0.507
JR4	18,713.13	4885.45	9858.59	405.66	1.898	0.261	0.979
JR5	2800.00	5828.57	29,800.00	22,971.43	0.094	2.082	0.109

Note: red is severely contaminated; yellow indicates faecal contamination; green is uncontaminated sites.

Several authors have used the coprostanol/coprostanol + cholesterol ratio to assess faecal contamination [44,45], with a consensus that <0.3 represents uncontaminated areas and >0.7 confirms sewage contamination. The ratio in the samples was >0.7 , except for the four stations 2, 3, 17, and 20.

4. Bacterial Gene Counts

The bacterial genes (16S rRNA) were present in all 20 marine sediment samples (Figure 2). The counts ranged between 10^4 and 10^9 16S rRNA copies per gram of sediment. Maximum gene copies were found at Station 17 (5.6×10^9 gene copies per gram), followed by Station 18 (2.6×10^9 gene copies per gram) and Station 19 (1.3×10^9 gene copies per gram). There were 0.0 gene copies per gram at JR5, while, in the coastal sediments, Station 10 had the minimum load of 3.7×10^4 gene copies per gram. Within the Jahra Reserve, the highest count of 3.3×10^8 gene copies per gram was at JR2 (Figure 2).

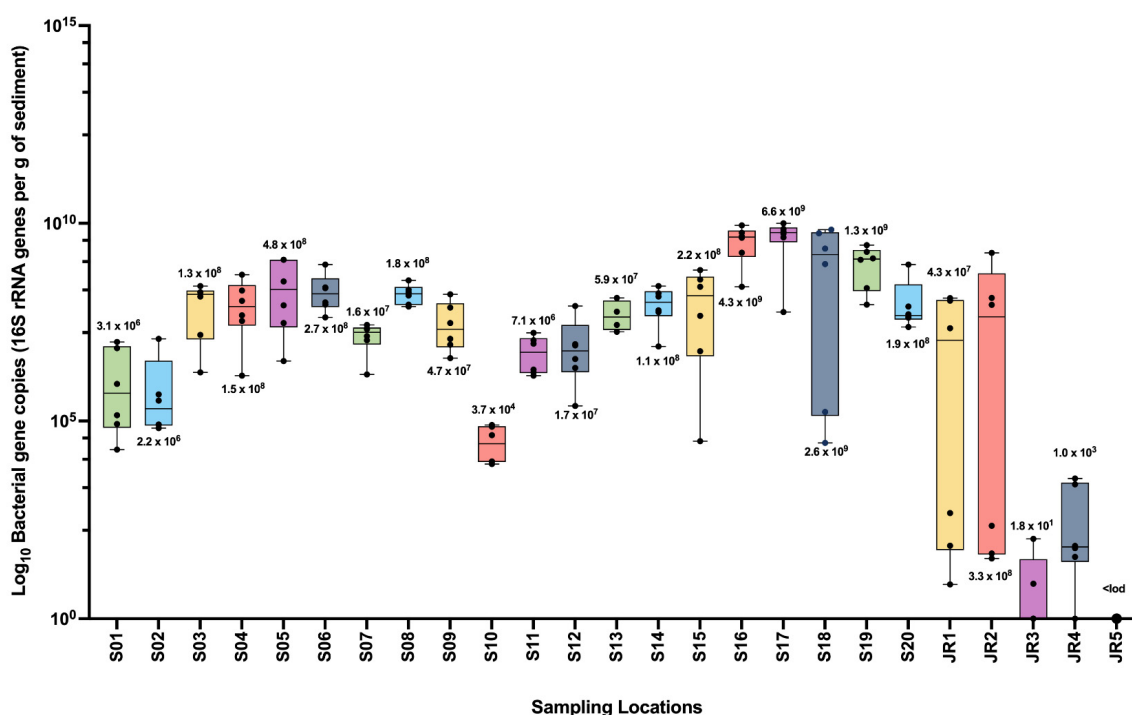


Figure 2. Bacterial gene (16S rRNA) copies detected within the marine sediments collected from Kuwait. The sampling locations are located on the x-axis. The respective gene counts as estimated by the quantitative polymerase chain reaction (qPCR) have been plotted on the y-axis. Each box represents an interquartile range of 25–75%. The upper and lower whiskers point to 10 and 90%, and the black line indicates mean copy numbers. The box plots were created in GraphPad Prism version 10.

5. Sequencing Statistics

Sequencing of twenty marine sediment samples (1–20) with an insert size of 350 bp yielded 11,959.96 to 13,535.25 raw reads (Table S1). The Jahra sediments (JR1–JR5) generated sequences between 6037.02 and 6280.11. With post-quality filtering, almost 99% of the data passed for downstream processing. On average, 97% and 93% of data had a Phred score $> Q20$ and $> Q30$, respectively. The GC% of the clean data ranged between 55 and 61.7% (Table S1). Roughly, 130,551,113 bp to 403,266,246 bp were assembled into contigs (185,838 to 485,156) with an average length of 784.57 bp (Max—58,542.58 bp). The average N50 and N90 were 746.55 bp and 530.60 bp, respectively (Table S2). The effective size $> 95\%$ is regarded as good quality usable data for downstream analysis.

6. Prevalence and Abundance of ARGs

Shotgun metagenomics provided a qualitative estimation of the genes based on the number of sequences aligned against the CARD database. Each site recorded variable abundances of hundreds of antibiotic resistance genes (ARGs) (Tables S3 and S4). Genes with a sample prevalence above 5% and relative abundance > 0.01 are shown on the heat maps (Figure 3a,b). ARGs such as *rpsL*, *patA*, *ImrD*, *adeF*, and PEDO-3 were omnipresent (100% prevalence) in all the samples (Stations 1–20). The relative abundances (RA%) of these genes were 4.83 ± 3.5 (Max—14.08, Min—0.42), 3.15 ± 1.3 (Max—6.16, Min—1.2), 1.7 ± 1.2 (Max—5.7, Min—0.41), 2.8 ± 1.16 (Max—5.4, Min—1.3), and 0.99 ± 1.0 (Max—0.99, Min—0.07), respectively. Among other dominant genes ($>80\%$ sample prevalence) were *tetB46*, *APH4-lb*, *arnA*, *TLA-2*, *ileS*, *emrB*, *ceoB*, and *rgt1438*. The RA% of all the genes are presented in Table S4.

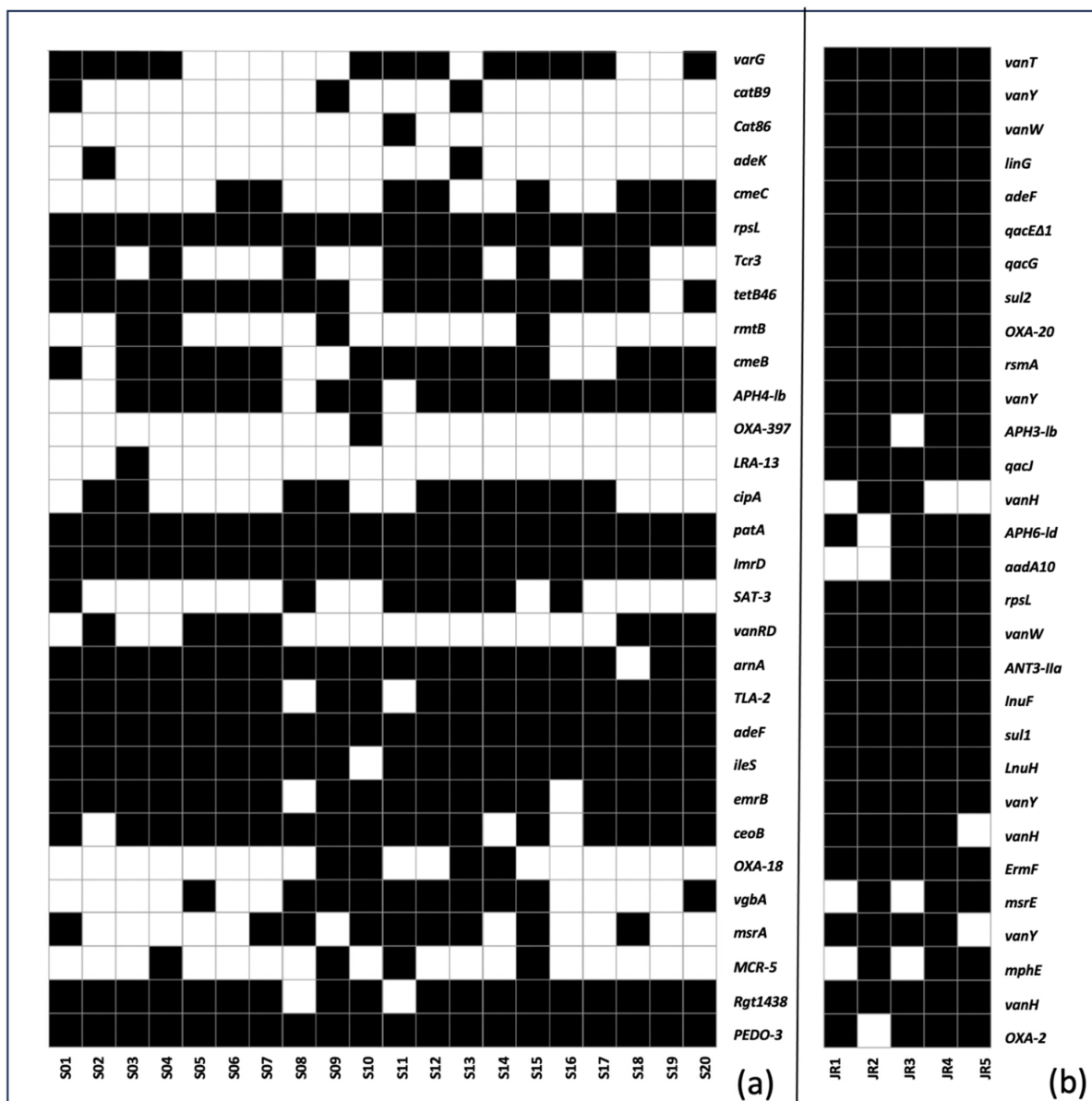


Figure 3. Detection of ARGs in (a) marine sediments of Kuwait and (b) sediments from Jahra reserve. The heat maps were created in Rpackage (gplots). The x-axis denotes the sampling locations, and the y-axis shows the genes identified. Genes were filtered through the Comprehensive Antibiotic Resistance Genes Database (CARD) (blastp e value $\leq 1 \times 10$). Black colour signifies the presence, whereas white represents the absence of the gene at the respective location.

The sediments from the Jahra Reserve (JR1–JR5) were richer in ARG abundance. About 21 genes exhibited a prevalence of 100%. Intriguingly, these samples exhibited a richness of *van* cluster genes.

7. Mechanism of Action and Microbial Hosts

The mechanism of antibiotic action was investigated. The most dominant action of ARGs was antibiotic efflux, followed by antibiotic inactivation in marine sediments. The other mechanisms were antibiotic target alteration, antibiotic target replacement, and antibiotic target protection (Figure 4a). Some genes synergistically acted through two mechanisms of action (Table S5). The phyla *Proteobacteria* possessed maximum genes involved in the antibiotic efflux mode of action. Bacterial phyla such as *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobiae*, and ARGs also originated from some archaeal phyla such as *Candidatus*, *Tectomicrobia*, *Thaumarchaeota*, and *Euarchaeota*.

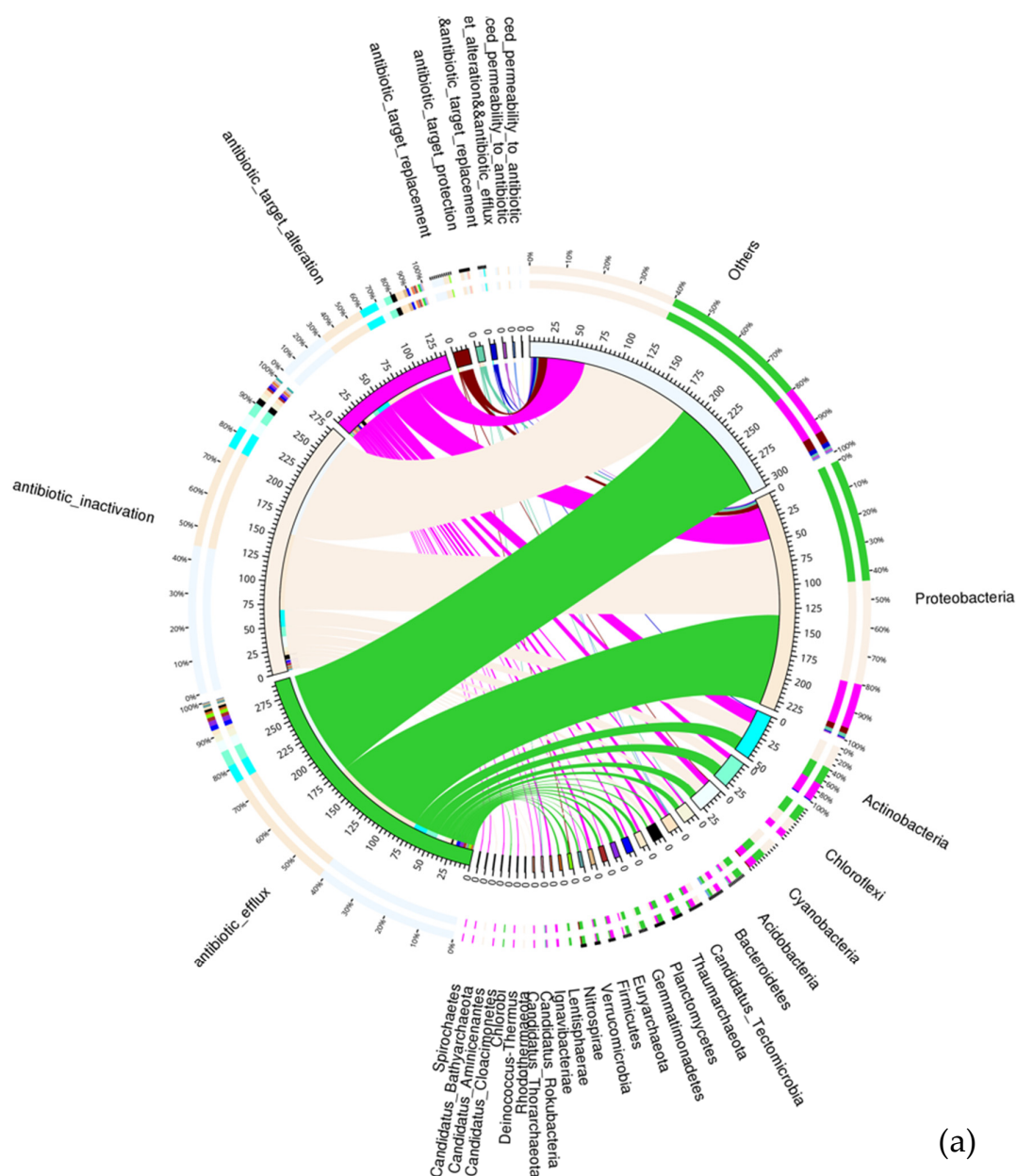


Figure 4. Cont.

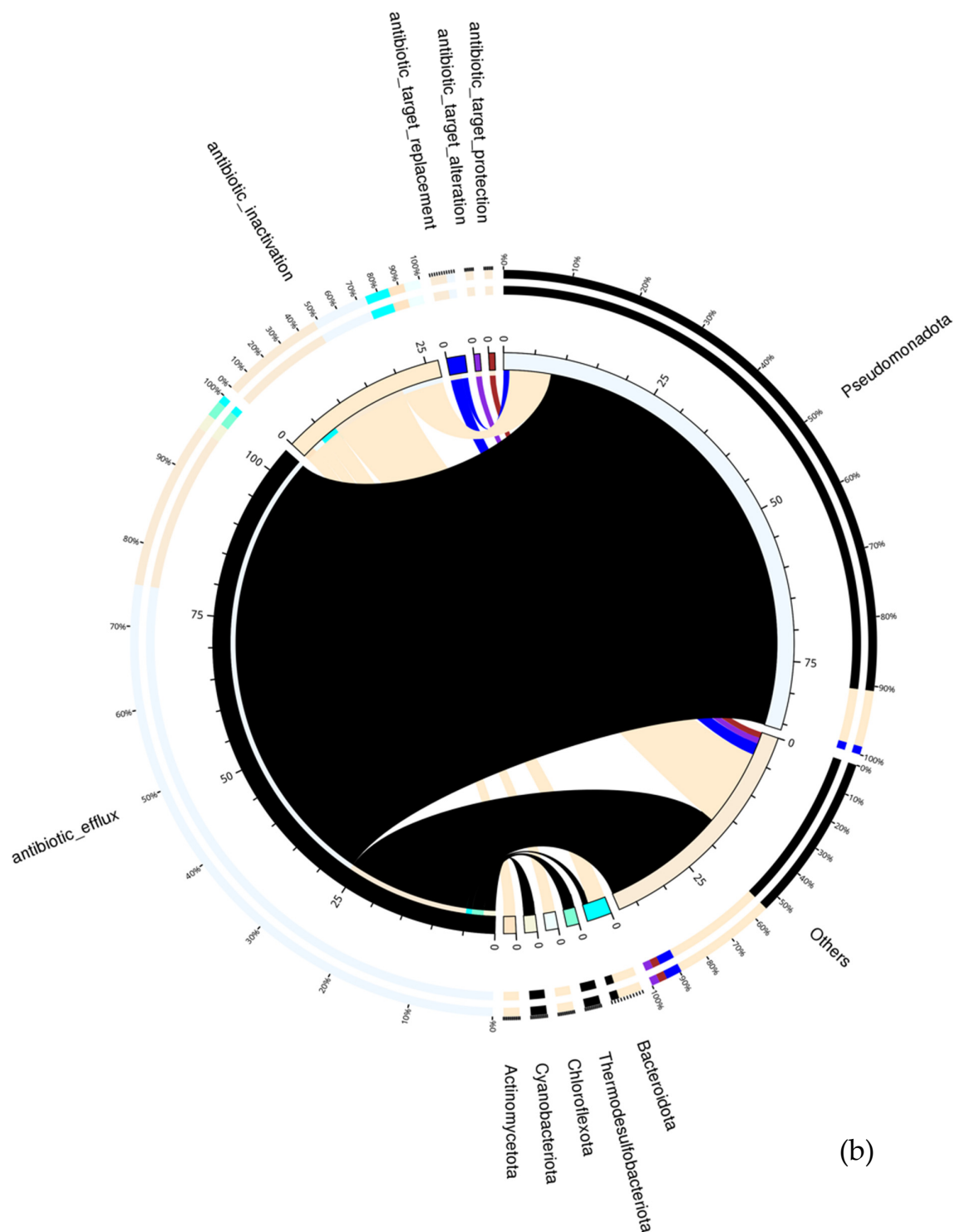


Figure 4. Mechanism of action and microbial hosts of the ARGs found in (a) marine sediments and in (b) Jahra reserve sediments. The predominant 10 bacterial taxa and their mechanism of action were laid on the circus plots employing Rcircos.

In the case of Jahra reserve samples, the microbial hosts were *Pseudomonadota*, *Bacteroidota*, *Thermodesulfobacteriota*, *Chloroflexota*, *Cyanobacteriota*, *Actinomycetota*, and others. The mechanism of antibiotic action was antibiotic efflux > antibiotic inactivation > antibiotic target replacement > antibiotic target alteration and antibiotic target protection (Figure 4b; Table S6).

8. Discussions

The data show that out of the 25 sampled locations, the coprostanol concentration was <dl at only four locations, and in 21 locations it was significantly higher, suggesting the ubiquitous presence of faecal contamination in the study area despite the enormous wastewater treatment facilities and the claims that all the wastewater generated is treated. This is not the first time such exceedances in coprostanol have been observed; in a previous investigation, Lyons et al. [46] reported 3 locations where coprostanol levels were $>500 \text{ ng g}^{-1}$, 6 locations where the levels were between 100 and 500 ng g^{-1} , and 18 coastal and 2 open water locations where the concentration was $<100 \text{ ng g}^{-1}$. Saeed et al. [47] reported $>500 \text{ ng g}^{-1}$ coprostanol from 13 locations in Kuwait Bay and 5 locations in the southern waters. Correlating the data generated in this study with the previous reports suggests sewage contamination is a chronic problem in the region, even though most of the wastewater is treated. The presence of pharmaceuticals in the wastewater effluent and the coastal waters of Kuwait was linked to the emergence of antimicrobial resistance. In addition to the presence of sterols, pharmaceuticals are reported in measurable concentrations in coastal and marine sediments [48–54] indicating the spatial distribution of sewage much beyond point source discharges. The presence of sterols, especially coprostanol, is a reliable indicator of faecal contamination. The ratios of coprostanol/cholesterol and coprostanol/(coprostanol + cholesterol) are robust indicators of faecal contamination. The coprostanol/cholesterol ratio >1 indicates severe sewage contamination; however, in this study, most sites have a coprostanol/cholesterol ratio of >0.2 , which is indicative of faecal contamination. This lower ratio can be a result of the large spatial distribution of sewage, and the lowering of the ratio can also be due to the in situ production of cholesterol. A more robust coprostanol/(coprostanol + cholesterol) ratio indicates a similar trend, where most sites have a ratio >3 indicating sewage contamination.

The ARGs in Kuwait marine sediments originated from 38 antibiotic families (Table S5), whereas the Jahra reserve samples belonged to 12 drug classes (Table S6). The drug classes with higher abundances were aminoglycoside, beta-lactams, fluoroquinolones, macrolide, MLSB (macrolide-lincosamide-streptogramin B), peptide, tetracycline, and vancomycin (Figure 5a). Several drug classes with <10 ARGs (nucleoside, acridine dye, antibacterial free fatty acids, nitroimidazole, bicyclomycin, elfamycin, fusidic acid, tetracenomycin, rhodamine, benzalkonium chloride, oxazolidinone, nitrofurantoin) were merged as others. In the Jahra reserve, the majority of the genes originated from the drug class of vancomycin, followed by disinfecting reagents (Figure 5b). Genes conferring resistance against fluoroquinolone, phenicol, and diaminopyrimidine were grouped as multiple drugs. All the detected drug categories in the present study are used to treat bacterial infections of humans as well as animals. More intriguing is the presence of genes resistant against multiple antibiotics and the broad range of beta-lactams (cephalosporins, cephamycin, carbapenem, penam, penem, monobactam). Beta-lactams are the last choice of drugs used to treat chronic infections [55]. Multiple drug resistance is rising alarmingly and risking the lives of immunocompromised patients [56,57]. Resistance against aminoglycoside, beta-lactams, and macrolides was also observed elsewhere, i.e., deep ocean sediments of the northern South China Sea [58]. The efflux pumps were recorded in Kuwait's marine sediments similar to other regions like the marine sediments collected from the polluted sites of Tolo Harbour [59]. In contrast, antibiotic inactivation was more prevalent in contaminated sites of the South China Sea [58]. ARGs also originated from archaeal phyla such as Candidatus Tectomicrobia, Thaumarchaeota, and Euarchaeota. In addition to these archaeal families, viral contigs also hosted ARGs in Hadal oceanic environments [60].

The 20 stations sampled were strategically considered to cover areas that are 3–4 km away from the emergency outfall, while the 5 remote stations were considerably far (20–48 km) from any emergency outfall. The presence of ARGs at two near-coast stations, namely 2 and 3, and the offshore stations 17 and 20 where the coprostanol was <dl suggests potential confounding factors like horizontal gene transfer and heavy metals [61] can be responsible for the spread of ARGs. The presence of antibiotic resistant microbes at all other sites are suggestive of a positive correlation between the sterols and AMR. A study conducted in Oko Nigeria reported the co-occurrence of faecal coliforms ($\sim 10^5 \text{ CFU/mL}$) and multidrug resistant bacteria in shallow

hand-dug drinking water wells. These sites were in the vicinity of pit latrines in the region. The concentrations of coprostanol ranged between 1.6 and 2.6 abs, indicating the occurrence of faecal contamination at these sites [62]. Faecal indicator microorganisms and sterols were cofound in sediments from Admiralty Bay, Antarctica [63]. In addition to this, both phenotypically and genotypically resistant *E. coli* were reported earlier in the marine environment of Kuwait [64]. The present study endorses the applicability of a multiparamter approach in assessments of faecal contamination and antimicrobial resistance, as advisories based on Enterococci alone are misleading at times [65].

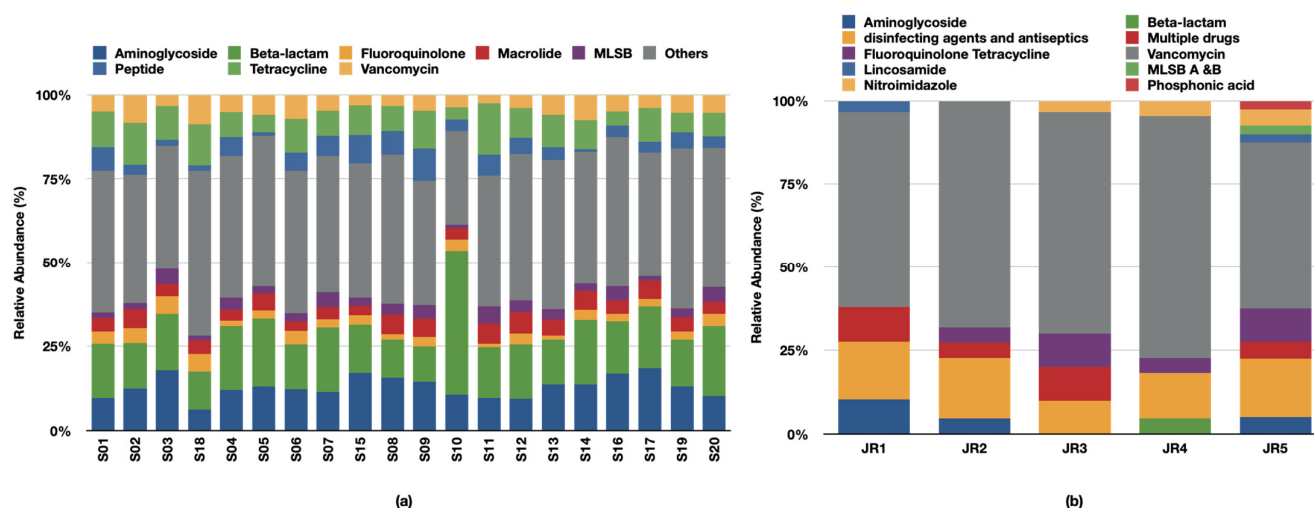


Figure 5. Bar charts showing the most abundant drug classes in (a) marine sediments and (b) Jahra reserve. The x-axis shows the sampling locations, and the relative abundances (RA%) are shown on the y-axis.

ARGs were recorded in the deepest abyssal–hadal transition zones of the Mariana Trench as well [66]. Approximately 40.0% ($n = 267$) of ARGs detected presently were also found in the near-shore surface sediments of Kuwait [36]. There were 133 and 400 genes unique to coastal and off-shore sediments, respectively.

9. Conclusions

The data indicate that sewage contamination in the coastal waters is on a large spatial scale much beyond the point inputs. The presence of coprostanol at most sites indicates faecal contamination. The ratios above the uncontaminated threshold indicate the likely dispersal of sewage contamination with the currents and suspended particulate matter. The presence of antimicrobial resistance across the spatial domain correlates positively with faecal sterols. The lower coprostanol/cholesterol ratio can be attributed to the dispersion of faecal contamination, more specifically coprostanol and the in situ contribution of cholesterol. The AMR reporting can be supported by sterol data to support observations in the absence of pharmaceutical data.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su16104320/s1>, Table S1: Raw Sequence statistics; Table S2: Assembly statistics; Table S3: ARGs found in 20 sediment samples collected away from Kuwait’s shore; Table S4: ARGs filtered from Jahra reserve sediment samples; Table S5: Drug Classes and Resistance Mechanism sediment samples of Kuwait; Table S6: Drug Classes and Resistance Mechanism sediment samples Jahra reserve.

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