

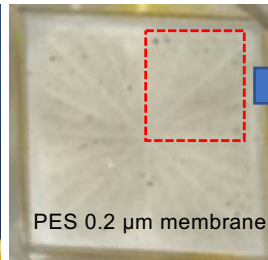
# Experimental procedure for metagenomic DNA analysis of WWTP effluent.

## Effluent from WWTP

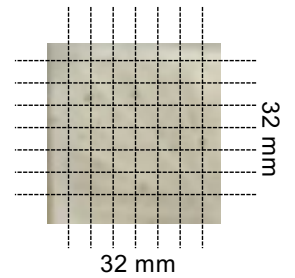


500 mL sampling  
Transport at room temperature (23°C)

Filtration within 6 hours after sample collection.



Cut to small pieces



TPP Rapid Filtermax Vacuum Filtration



- Add 1/4 membrane into Zymoresearch BasingBeads (0.1mm/0.5mm)
- Add 800 µl Roche Bacterial lysis buffer
- Freeze at -25°C
- Thaw room temperature



Bead-beating (1,500 rpm, 10 min)  
Geno/Grinder 2010



400 µL



DNA purification

Roche,  
MagNa Pure Compact Isolation

(Elution: 50 µL EB)

One microliter of DNA sol. corresponds to 1.25 mL effluent DNA, if 100% recovery rate.



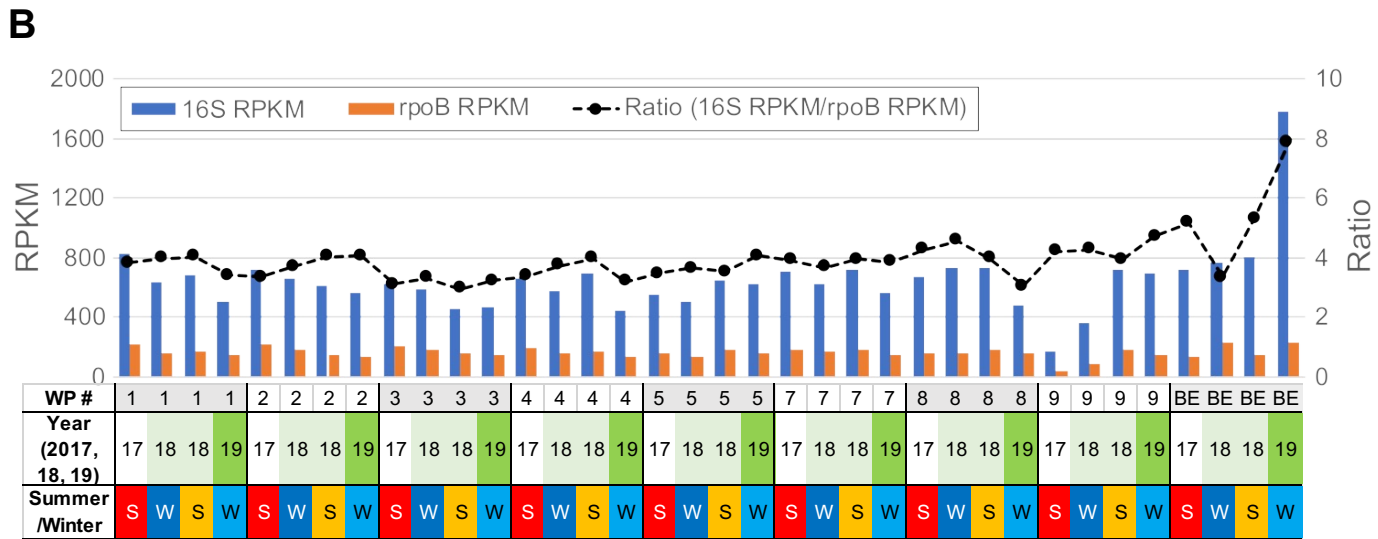
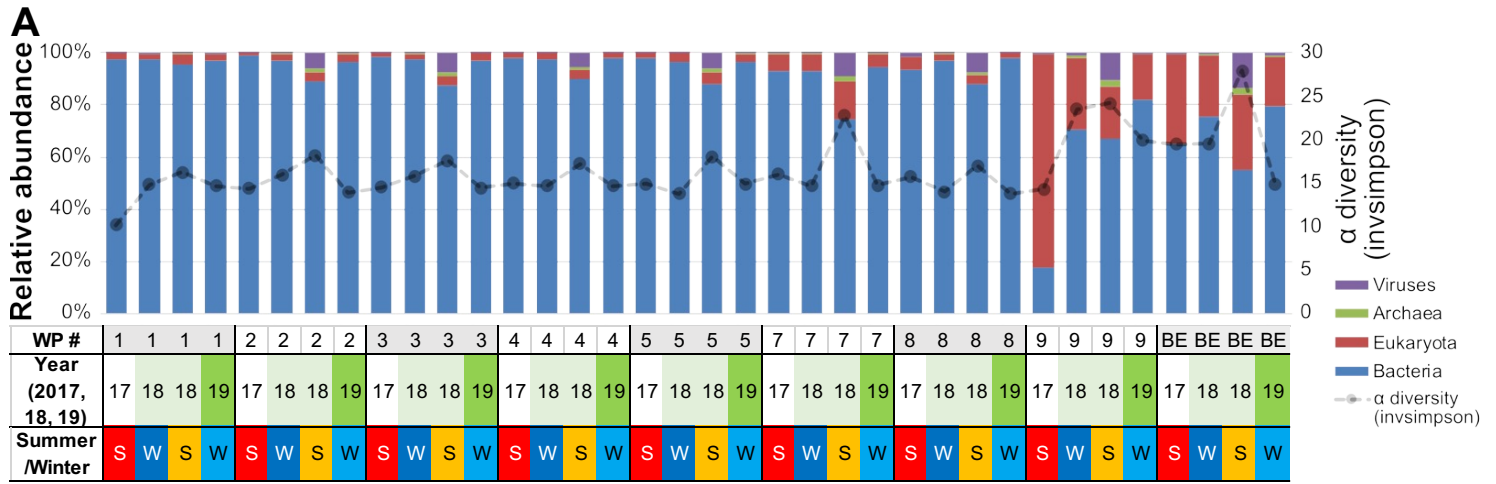
Metagenomic DNA-Seq library preparation (QIAGEN QIAseq FX DNA library kit)  
DNA sol. 2.5 µl, 4 cycle PCR enrichment.



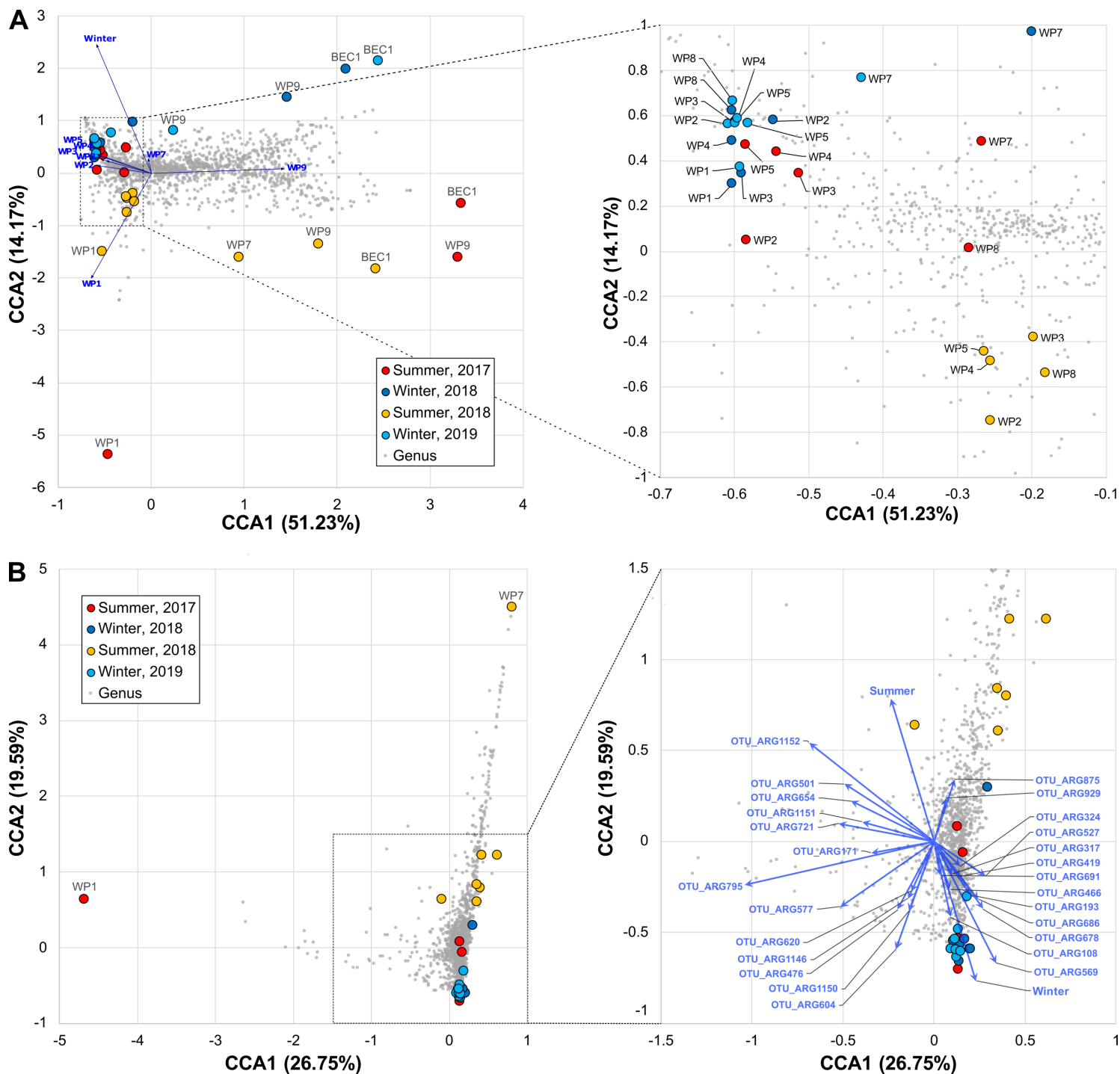
Next-Generation Sequencing  
NextSeq 500  
150-mer paired-end



Supplementary Figure 1. Experimental procedure for metagenomic DNA analysis of WWTP effluent.



**Supplementary Figure 2.** A) Taxonomic classification of the domain rank of effluents from fresh water areas (WP1 to 8) based on the metagenomic DNA-Seq. B) Bacteria population size using RPKM normalization for 16S-rRNA genes and *rpoB* RNA polymerase  $\beta$  subunit orthologs showed no significant difference with an average 4 copies of 16S-rRNA gene for *rpoB*, suggesting that RPKM estimation is an effective approach to characterize the ARG content.



**Supplementary Figure 3. Canonical correspondence analysis (CCA) based on genus taxonomic classification.** A) CCA exhibited the season-dependent manner among wastewater treatment plants (WPs). Colored circles indicate each WP site and sampling time (see detail in Table S1). Gray circles indicate the 1603 bacterial genera that were detected among all tested WP effluents.

B) Biplot corresponding to ARGs and seasonal features (summer and winter) was included in the CCA plot. The results showed that OUT\_ARG875 and OUT\_ARG929 were positively correlated to the summer samples, while OUT\_ARG108 was correlated to the winter samples. Variables were chosen based on significance calculated from individual CCA results.

**Supplementary Table 1. Metagenomic DNA-Seq information for Effluent of WWTP and environment water samples in this study**

Place ID	Sampling site (GPS)	Sampling date (mm/dd/yyyy)	Season (Summer/Winter)	Temperature (°C)	DNA conc. (ng/μl)	Metagenome DNA-Seq Total reads	Total RPKM of AMR genes	BioSample ID	SRA ID
<b>Wastewater treatment plant (WWTP)</b>									
WP1-S17	35°41'06.8"N 139°24'37.2"E	07/10/2017	Summer	32.1	4.0	17,471,507	97.2	SAMD00193144	DRR198489
WP1-W18	35°41'06.8"N 139°24'37.2"E	02/13/2018	Winter	9.7	12.0	5,022,485	93.7	SAMD00193145	DRR198490
WP1-S18	35°41'06.8"N 139°24'37.2"E	08/06/2018	Summer	34.0	9.7	3,786,852	100.8	SAMD00193146	DRR198491
WP1-W19	35°41'06.8"N 139°24'37.2"E	02/05/2019	Winter	8.1	11.7	15,624,087	90.1	SAMD00193147	DRR198492
WP2-S17	35°39'50.5"N 139°26'16.0"E	07/10/2017	Summer	32.1	6.4	12,108,247	39.7	SAMD00193148	DRR198493
WP2-W18	35°39'50.5"N 139°26'16.0"E	02/13/2018	Winter	9.7	2.9	1,461,838	48.1	SAMD00193149	DRR198494
WP2-S18	35°39'50.5"N 139°26'16.0"E	08/06/2018	Summer	34.0	1.9	931,653	71.8	SAMD00193150	DRR198495
WP2-W19	35°39'50.5"N 139°26'16.0"E	02/05/2019	Winter	8.1	7.3	9,309,728	148.0	SAMD00193151	DRR198496
WP3-S17	35°40'06.1"N 139°25'40.0"E	07/10/2017	Summer	32.1	9.4	13,370,239	50.8	SAMD00193152	DRR198497
WP3-W18	35°40'06.1"N 139°25'40.0"E	02/13/2018	Winter	9.7	7.0	2,187,631	84.1	SAMD00193153	DRR198498
WP3-S18	35°40'06.1"N 139°25'40.0"E	08/06/2018	Summer	34.0	7.7	3,160,162	35.4	SAMD00193154	DRR198499
WP3-W19	35°40'06.1"N 139°25'40.0"E	02/05/2019	Winter	8.1	13.0	15,381,005	55.8	SAMD00193155	DRR198500
WP4-S17	35°39'04.9"N 139°28'52.8"E	07/10/2017	Summer	32.1	6.9	9,654,509	71.9	SAMD00193156	DRR198501
WP4-W18	35°39'04.9"N 139°28'52.8"E	02/13/2018	Winter	9.7	8.1	3,656,160	98.7	SAMD00193157	DRR198502
WP4-S18	35°39'04.9"N 139°28'52.8"E	08/06/2018	Summer	34.0	6.6	3,127,256	94.4	SAMD00193158	DRR198503
WP4-W19	35°39'04.9"N 139°28'52.8"E	02/05/2019	Winter	8.1	12.9	15,700,728	54.3	SAMD00193159	DRR198504
WP5-S17	35°39'13.8"N 139°30'45.2"E	07/10/2017	Summer	32.1	12.2	13,613,329	74.5	SAMD00193160	DRR198505
WP5-W18	35°39'13.8"N 139°30'45.2"E	02/13/2018	Winter	9.7	16.0	6,850,264	84.1	SAMD00193161	DRR198506
WP5-S18	35°39'13.8"N 139°30'45.2"E	08/06/2018	Summer	34.0	2.9	1,187,246	73.3	SAMD00193162	DRR198507
WP5-W19	35°39'13.8"N 139°30'45.2"E	02/05/2019	Winter	8.1	4.4	6,552,281	104.3	SAMD00193163	DRR198508
WP7-S17	35°33'42.9"N 139°45'11.0"E	07/10/2017	Summer	32.1	5.9	15,352,840	69.6	SAMD00193164	DRR198509
WP7-W18	35°33'42.9"N 139°45'11.0"E	02/13/2018	Winter	9.7	3.6	2,438,058	69.8	SAMD00193165	DRR198510
WP7-S18	35°33'42.9"N 139°45'11.0"E	08/06/2018	Summer	34.0	6.2	2,148,293	23.0	SAMD00193166	DRR198511
WP7-W19	35°33'42.9"N 139°45'11.0"E	02/05/2019	Winter	8.1	7.6	10,567,429	46.3	SAMD00193167	DRR198512
WP8-S17	35°37'53.8"N 139°44'45.6"E	08/23/2017	Summer	33.7	42.1	10,261,235	129.7	SAMD00193168	DRR198513
WP8-W18	35°37'53.8"N 139°44'45.6"E	02/13/2018	Winter	9.7	19.6	7,269,472	157.6	SAMD00193169	DRR198514
WP8-S18	35°37'53.8"N 139°44'45.6"E	08/06/2018	Summer	34.0	8.5	3,715,095	118.0	SAMD00193170	DRR198515
WP8-W19	35°37'53.8"N 139°44'45.6"E	02/05/2019	Winter	8.1	47.8	24,567,160	93.5	SAMD00193171	DRR198516
WP9-S17	35°37'49.9"N 139°47'02.9"E	07/10/2017	Summer	32.1	54.0	24,630,107	3.2	SAMD00193172	DRR198517
WP9-W18	35°37'49.9"N 139°47'02.9"E	02/13/2018	Winter	9.7	4.1	3,490,194	7.4	SAMD00193173	DRR198518
WP9-S18	35°37'49.9"N 139°47'02.9"E	08/06/2018	Summer	34.0	5.7	2,469,983	15.3	SAMD00193174	DRR198519
WP9-W19	35°37'49.9"N 139°47'02.9"E	02/05/2019	Winter	8.1	1.1	1,713,450	36.6	SAMD00193175	DRR198520
<b>Recreational Beach</b>									
BEC1-S17	35°37'50.0"N 139°46'31.3"E	07/10/2017	Summer	32.1	98.8	19,083,448	6.2	SAMD00193176	DRR198521
BEC1-W18	35°37'50.0"N 139°46'31.3"E	02/13/2018	Winter	9.7	2.8	2,353,547	11.9	SAMD00193177	DRR198522
BEC1-S18	35°37'50.0"N 139°46'31.3"E	08/06/2018	Summer	34.0	40.3	11,585,753	4.1	SAMD00193178	DRR198523
BEC1-W19	35°37'50.0"N 139°46'31.3"E	02/05/2019	Winter	8.1	1.8	1,514,954	4.2	SAMD00193179	DRR198524

RPKM, per kilobase of exon per million mapped reads.

SRA, short read archive.

Supplementary Table 2. Summary of RPKM value for operational taxonomic unit (OTU) of antimicrobial resistance

ARG cluster ID	class	subclass	gene list	length	Total RPKM in all 28 samples																																						
					WP1-170	WP1-170	WP1-185	WP1-190	WP2-170	WP2-170	WP2-185	WP2-190	WP3-170	WP3-170	WP3-185	WP3-190	WP4-170	WP4-170	WP4-185	WP4-190	WP5-170	WP5-170	WP5-185	WP5-190	WP7-170	WP7-170	WP7-185	WP7-190	WP8-170	WP8-170	WP8-185	WP8-190	WP9-170	WP9-170	WP9-185	WP9-190	REC1-170	REC1-170	REC1-185	REC1-190			
OTU_ARG57	AMINOGLYCOSIDE	STREPTOMYCIN	STR1Faa44aa, aadA11, aph37-1b	909	88.15	4.62	1.98	2.65	1.04	1.37	2.27	2.40	2.41	2.40	4.54	2.82	2.44	3.67	3.93	4.96	1.84	2.69	2.74	1.89	3.23	3.25	3.63	0.52	2.64	3.79	4.11	3.89	3.76	0.00	0.32	0.91	1.32	0.00	0.47	0.19	0.00		
OTU_ARG64	AMINOGLYCOSIDE	STREPTOMYCIN	aadA2, aadA2b, aadA3, ai	792	51.40	3.78	0.51	2.03	1.03	0.63	1.73	2.75	2.96	2.19	0.00	0.00	0.42	2.76	1.73	0.81	2.77	0.74	4.33	1.95	2.90	1.04	1.20	0.85	1.37	5.24	3.44	3.25	0.00	0.36	1.04	1.32	0.00	0.00	0.22	0.00			
OTU_ARG71	AMINOGLYCOSIDE	STREPTOMYCIN	aadA, aadA5	789	50.46	3.87	1.27	1.70	1.88	0.84	0.87	1.52	2.07	1.14	0.88	0.81	0.99	1.32	0.70	0.82	1.06	1.78	2.79	3.26	0.78	1.66	0.52	0.00	1.46	2.87	1.53	1.72	3.76	0.32	0.00	1.05	0.00	0.34	1.09	0.00	0.00		
OTU_ARG80	AMINOGLYCOSIDE	STREPTOMYCIN	aph37-1b	804	39.028	1.65	1.49	1.67	0.89	1.03	0.85	1.36	1.22	1.03	2.28	1.59	2.22	3.24	2.39	0.00	1.20	1.29	0.18	3.20	0.38	0.90	1.03	0.00	0.00	1.84	2.92	1.89	1.18	0.00	0.36	0.00	0.00	0.00	0.00	0.00			
OTU_ARG82	AMINOGLYCOSIDE	STREPTOMYCIN	aadA	465	31.91	0.62	1.19	0.61	0.76	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61	0.76	0.61
OTU_ARG34	AMINOGLYCOSIDE	STREPTOMYCIN	aadA1, aadA1b, aadA11	786	35.81	0.88	0.76	0.00	0.17	0.53	3.50	5.55	2.88	0.48	1.17	1.22	0.84	0.80	2.10	1.64	0.16	0.00	1.97	2.42	0.00	4.52	0.83	0.15	0.00	0.49	1.38	1.41	0.00	1.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
OTU_ARG64	AMINOGLYCOSIDE	STREPTOMYCIN	aph(6)-Ia	837	31.91	1.38	0.72	1.28	0.78	1.29	0.82	0.00	0.65	1.08	1.64	1.15	2.12	2.95	0.77	0.92	1.68	0.53	1.02	1.66	0.63	0.00	0.00	0.11	0.71	2.48	1.30	1.13	0.00	0.00	0.99	0.00	0.00	0.00	0.00	0.00	0.00		
OTU_ARG29	AMINOGLYCOSIDE	AMIKACIN/KANAMYCIN	ant(4)-Ib, aac(6)-Ib, aac(6)-Ib-cr, ai	555	21.01	0.41	0.72	3.86	0.47	0.75	0.00	0.00	0.20	0.68	0.00	1.15	0.48	0.75	0.99	1.16	0.00	1.07	0.00	3.09	0.00	0.59	0.74	0.00	0.00	0.89	1.25	1.47	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
OTU_ARG92	AMINOGLYCOSIDE	AMINOGLYCOSIDE	aac(6)-I1, aac(6)-I1a	555	8.07	0.21	0.90	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.27	0.83	0.00	0.48	0.00	0.49	0.00	0.12	0.80	0.79	0.00	0.56	0.00	0.00	0.89	0.00	0.48	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
OTU_ARG108	AMINOGLYCOSIDE	STREPTOMYCIN	aadA	798	7.89	0.25	0.00	1.15	0.76	0.00	0.00	0.23	0.32	0.99	0.00	0.14	0.22	0.68	0.00	0.14	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.64	0.89	0.00	0.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
OTU_ARG89	AMINOGLYCOSIDE	STREPTOMYCIN	aadA13, aph37-1a	798	5.82	0.00	0.75	0.00	0.24	0.00	0.00	0.00	0.09	0.00	0.00	0.33	0.00	0.00	0.00	0.32	0.46	0.55	0.00	0.58	0.00	0.00	0.00	0.96	0.12	0.52	0.00	0.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
OTU_ARG39	AMINOGLYCOSIDE	GENTAMICIN/KANAMYCIN	Cfaac(6)-Ic	582	5.88	0.10	0.00	2.30	0.11	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.63	0.00	0.47	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG16	AMINOGLYCOSIDE	Gentamicin, Streptomycin, aph37-1b, aac(6)-Ic	1392	4.32	0.12	0.00	0.77	0.05	0.12	0.49	0.00	0.00	0.22	0.66	0.00	0.09	0.15	0.20	0.00	0.09	0.05	0.21	0.00	0.11	0.14	0.00	0.00	0.50	0.20	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
OTU_ARG104	AMINOGLYCOSIDE	GENTAMICIN/KANAMYCIN	Cfaac(6)-Ic	534	4.11	0.22	0.00	0.36	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.37	0.25	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.18	1.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG58	AMINOGLYCOSIDE	STREPTOMYCIN	aadA6	846	3.75	0.00	0.00	0.00	0.20	0.00	0.00	0.26	0.00	0.00	0.76	0.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.73	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG56	AMINOGLYCOSIDE	KANAMYCIN	aph(3)-Ia	816	3.60	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.38	0.34	0.00	0.34	0.00	0.24	0.18	0.00	0.00	0.00	0.00	0.00	0.24	0.34	0.00	0.91	0.00	0.00	0.00	0.00	0.53	0.00					
OTU_ARG122	AMINOGLYCOSIDE	AMINOGLYCOSIDE	aac(6)-29, aac(6)-29a, ai	404	3.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.37	0.00	0.00	0.33	0.26	0.68	0.00	0.32	0.18	0.73	0.00	0.00	0.00	0.00	0.24	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG58	AMINOGLYCOSIDE	STREPTOMYCIN	aadA	807	2.38	0.00	0.23	0.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.32	0.00	0.24	0.32	0.00	0.15	0.09	0.00	0.18	0.00	0.00	0.23	0.32	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG73	AMINOGLYCOSIDE	STREPTOMYCIN	ant(4)-Ib, aac(6)-Ic	798	2.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
OTU_ARG76	AMINOGLYCOSIDE	STREPTOMYCIN	aadA10	834	2.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
OTU_ARG65	AMINOGLYCOSIDE	GENTAMICIN/KANAMYCIN	Cfaac(6)-Ib	555	2.31	0.41	0.36	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
OTU_ARG70	AMINOGLYCOSIDE	AMIKACIN/KANAMYCIN	aph(3)-Ib	795	2.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.19	0.00	0.00	0.00	0.00	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG76	AMINOGLYCOSIDE	AMIKACIN/KANAMYCIN	aph(3)-IV	795	1.32	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG34	AMINOGLYCOSIDE	STREPTOMYCIN	ant(6)-Ia	909	1.22	0.00	0.00	0.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00						
OTU_ARG70	AMINOGLYCOSIDE	KANAMYCIN	aph(3)-Ia	795	1.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG59	AMINOGLYCOSIDE	AMINOGLYCOSIDE	aadA	516	1.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG19	AMINOGLYCOSIDE	STREPTOMYCIN	ant(4)-Ib, aac(6)-Ic	789	0.96	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.35	0.10	0.00	0.00	0.00	0.00	0.00						
OTU_ARG72	AMINOGLYCOSIDE	STREPTOMYCIN	ant(4)-Ib, aac(6)-Ic	789	0.93	0.36	0.00	0.33	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
OTU_ARG101	AMINOGLYCOSIDE	AMIKACIN/KANAMYCIN																																									











