Overexpression of antibiotic resistance genes in hospital effluents over time

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Received 20 October 2016; returned 21 December 2016; revised 6 January 2017; accepted 9 January 2017

Objectives: Effluents contain a diverse abundance of antibiotic resistance genes that augment the resistome of receiving aquatic environments. However, uncertainty remains regarding their temporal persistence, transcription and response to anthropogenic factors, such as antibiotic usage. We present a spatiotemporal study within a river catchment (River Cam, UK) that aims to determine the contribution of antibiotic resistance gene-containing effluents originating from sites of varying antibiotic usage to the receiving environment.

Methods: Gene abundance in effluents (municipal hospital and dairy farm) was compared against background samples of the receiving aquatic environment (i.e. the catchment source) to determine the resistome contribution of effluents. We used metagenomics and metatranscriptomics to correlate DNA and RNA abundance and identified differentially regulated gene transcripts.

Results: We found that mean antibiotic resistance gene and transcript abundances were correlated for both hospital (q = 0.9, two-tailed P < 0.0001) and farm (q = 0.5, two-tailed P < 0.0001) effluents and that two β-lactam resistance genes (blaGES and blaOXA) were overexpressed in all hospital effluent samples. High β-lactam resistance gene transcript abundance was related to hospital antibiotic usage over time and hospital effluents contained antibiotic residues.

Conclusions: We conclude that effluents contribute high levels of antibiotic resistance genes to the aquatic environment; these genes are expressed at significant levels and are possibly related to the level of antibiotic usage at the effluent source.

Introduction

The rise of antibiotic resistance in clinical pathogens is occurring at an alarming rate, severely jeopardizing the sustainability of antibiotic use in human and veterinary medicine.1 The antibiotic resistance genes (ARGs) found in these pathogenic bacteria are thought to derive from the long-term evolution of resistance mechanisms in non-pathogenic bacteria.2 The resistome, comprising the resistance determinants within environmental microbial communities, offers an explanation for the diversity of ARGs that is observed in clinical situations.3 Indeed, there is a large body of evidence that the resistome serves as a reservoir for ARGs that can be acquired by clinically significant pathogens through transfer of mobile genetic elements (MGEs).4

The impact that anthropogenic activities are having on the resistome has fuelled a great deal of research and debate concerning the augmentation of the resistome and the emergence of antibiotic-resistant pathogens.5–8 Of particular interest is the role that effluents may have in the dissemination of ARGs and the expansion of the resistome.9 Using a comparative metagenomic approach, we have recently shown that effluents entering a river catchment contain ARGs and that the abundance of these ARGs is greater than that of the receiving environment, suggesting that effluents are contributing ARGs to the resistome.10

Metagenomics, combined with the Search Engine for Antimicrobial Resistance (SEAR),11 allows the resolution of full-length ARGs from environmental samples of unknown composition, thus providing an excellent method for investigating the resistome.7 However, it is important when assessing the impact of anthropogenic activities on the resistome that one technique is not used in isolation but rather that several techniques should be
used to determine the ARG sample load. With this in mind, the identification of selection pressures and expressed ARGs within ARG-containing samples is vital information when determining the impact of anthropogenic activities on the resistome. For example, it has been found that pharmaceutical antibiotic residues present in effluents are selective pressures that can directly impact ARGs in the environment. However, linking selective pressures to ARG and ARG transcript abundance is yet a feature of environmental monitoring.

In this study, we examined ARG abundance across a series of monthly effluent samples that originated from two sites of varying antibiotic usage (a municipal hospital and a dairy farm). ARG abundance was compared against background samples of the receiving aquatic environment (i.e. the source of the river catchment) to determine the relative contribution of effluents to the resistome. In addition, we employed metatranscriptomics to detect ARG transcripts and correlated them to the corresponding ARGs in each sample, facilitating the identification of differentially expressed transcripts over the sampling period. This study also assessed the selective pressures acting on the sampled microbial communities through use of LC–MS to detect antibiotic residues in the samples, as well as examining antibiotic usage data for the site of high antibiotic consumption. The combined data from this study are used to assess the impact of anthropogenic activities on the resistome.

Materials and methods

Sample collection and DNA and RNA sequencing

Samples were collected from three sources within the River Cam catchment, Cambridge, UK, on 2 May 2013 and five further samples over a 5 month period between August 2014 and December 2014 an average of 5 weeks apart (Table 1). Collections were made from the combined wastewater effluent of the main wards of Cambridge University Hospitals, Cambridge, UK, via a combined sewage pit (latitude 52.174343, longitude 0.1497) prior to the effluent entering the municipal sewers. Collections were also made from the effluent lagoon of the University of Cambridge dairy farm (latitude 52.22259, longitude 0.02603) and the River Cam source water (Ashwell Spring, Hertfordshire, latitude 52.0421, longitude 0.1497). The river source water served as a background sample for the environment both effluents were entering.

Samples for antibiotic residue testing were collected in 1 L sterile glass containers and transported at 4 °C to the laboratory. Samples for metagenome and metatranscriptome preparation were collected in 1 L sterile polypropylene containers and transported at 4 °C to the laboratory, and prokaryotic cells were isolated as described by Rowe et al. (2016). Each sample of prokaryotic cells was split in two, for separate DNA and RNA extractions to generate a metagenome and metatranscriptome for each sample. Metagenomes were prepared as described by Rowe et al. (2016). For metatranscriptome preparation, prokaryotic cells were washed in PBS solution before being treated with Max Bacterial Enhancement reagent (ThermoFisher Scientific, UK) to denature bacterial proteins and deactivate RNases. Bacterial cell lysis and RNA extraction were then performed using TRIzol reagent (ThermoFisher Scientific, UK). For each metatranscriptome, 2 μg of RNA was subjected to ribosomal RNA depletion (Ribo-Zero Gold, Epicentre, UK), quality checked using a BioAnalyzer (Agilent Technologies, US) and used to generate Illumina TruSeq RNA libraries (100 bp). All metagenome and metatranscriptome libraries were sequenced using an Illumina HiSeq2500 (Exeter Sequencing Service, UK). All sequencing data and metadata are available under the European Nucleotide Archive study with accession numbers PRJEB12083 (metagenomes) and PRJEB12284 (metatranscriptomes) (Table 1).

Identification of ARGs, MGEs and abundance analysis

ARGs were identified in metagenomes and metatranscriptomes using the SEAR with default parameters. MGE detection was carried out on all metagenomes in as in Rowe et al. (2016). Briefly, reads were mapped to a custom MGE database using BWA-MEM (default options). MGEs were annotated and binned by MGE type if mapping coverage was >90%. The abundances of ARGs and MGEs were normalized to the number of 16S sequences in each metagenomic dataset, as discussed in Bengtsson-Palme et al. (2014). Taxonomic profiling was performed on all metagenomes using MetaPhAn.

Antibiotic residue testing

Antibiotic residues were quantified in effluent samples using LC–MS (RPS Mountainheath, UK). Quantification standards were created for the three most used compounds in each class of antibiotics prescribed at Cambridge University Hospitals in 2013 as described below. No standards could be generated for aminoglycosides or trimethoprim.

Antibiotic usage and statistical analysis

Monthly antibiotic usage data for periods overlapping sample collection were obtained for Cambridge University Hospitals. Hospital antibiotic usage was calculated as DDDs (per 1000 bed days), which is the assumed average maintenance dose per day for a given drug and is used as a statistical measure of drug consumption.

Differentially regulated transcripts were identified as in Franzosa et al. (2014). Briefly, log DNA/RNA abundance ratios were calculated for each ARG and one-sample t-tests were used to determine significant deviation of abundance ratios from zero. The resulting two-tailed P values then underwent false discovery rate (FDR) correction using the Benjamini–Hochberg method (P = 0.05) and were used to identify differentially regulated transcripts.

Results

Detection of ARGs in effluents

In order to compare the relative ARG abundance in each sample type, metagenomic DNA from three sites was sequenced on multiple occasions. This study generated 102 giga bp (Gbp) of sequencing data across all samples (Table 1). Metagenomes from three samples of the river source water failed sequencing library quality checking and were removed from the study. A total of 15 metagenomes were successfully sequenced, passed quality checking and were found to contain ARGs (Table 1). The percentage of reads matching ARGs was an average 10-fold greater in the hospital effluent samples when compared with the farm effluent samples, and ~70-fold greater compared with the background samples of river source water (Table 1). The percentage of reads matching ARGs was an average 8-fold greater in the farm effluent compared with the background samples of river source water; however, one metagenome from a background sample was found to have a greater percentage of ARG reads than a metagenome from the farm effluent (see AS:M:2 and DF:M:1 in Table 1). After reconstructing ARGs from sequence reads using SEAR and then normalizing the ARG abundance in each sample to the 16S sequence abundance, the mean normalized abundance of ARGs in the hospital effluent samples was found to be significantly greater than in the farm effluent and background samples (9- and 34-fold greater respectively) (Figure 1a). In addition to a higher mean...
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Table 1. Summary of samples used in this study

| Sample | Sample type | Date collected | Latitude | Longitude | Data type | ENA accession | Total reads | Total ARG reads | % ARG reads |
|--------|-------------|----------------|----------|-----------|-----------|---------------|--------------|----------------|-------------|
| AH:M:1 | hospital effluent | 02.05.2013 | 52.174343 | 0.139346 | metagenome | ERS1019923 | 64,659,230 | 97,698 | 0.1511 |
| AH:M:2 | hospital effluent | 04.08.2013 | 52.174343 | 0.139346 | metagenome | ERS1019924 | 52,355,416 | 122,164 | 0.2333 |
| AH:M:3 | hospital effluent | 15.09.2014 | 52.174343 | 0.139346 | metagenome | ERS1019925 | 109,795,652 | 207,767 | 0.1892 |
| AH:M:4 | hospital effluent | 29.09.2014 | 52.174343 | 0.139346 | metagenome | ERS1019926 | 61,573,380 | 125,019 | 0.203 |
| AH:M:5 | hospital effluent | 27.10.2014 | 52.174343 | 0.139346 | metagenome | ERS1019927 | 50,845,128 | 25,987 | 0.0511 |
| AH:M:6 | hospital effluent | 24.11.2014 | 52.174343 | 0.139346 | metagenome | ERS1019928 | 53,928,494 | 28,629 | 0.0531 |
| DF:M:1 | farm effluent | 02.05.2013 | 52.22259 | 0.02603 | metagenome | ERS1019955 | 66,120,642 | 2317 | 0.0035 |
| DF:M:2 | farm effluent | 06.08.2013 | 52.22259 | 0.02603 | metagenome | ERS1019956 | 184,149,408 | 13,094 | 0.0071 |
| DF:M:3 | farm effluent | 15.09.2014 | 52.22259 | 0.02603 | metagenome | ERS1019957 | 262,283,622 | 29,006 | 0.011 |
| DF:M:4 | farm effluent | 29.09.2014 | 52.22259 | 0.02603 | metagenome | ERS1019958 | 58,179,398 | 31,518 | 0.0542 |
| DF:M:5 | farm effluent | 27.10.2014 | 52.22259 | 0.02603 | metagenome | ERS1019959 | 53,192,154 | 6999 | 0.0132 |
| DF:M:6 | farm effluent | 24.11.2014 | 52.22259 | 0.02603 | metagenome | ERS1020022 | 495,16,248 | 4072 | 0.0082 |
| AS:M:1 | river source water | 02.05.2013 | 52.0421 | 0.1497 | failed sequencing | - | - | - | - |
| AS:M:2 | river source water | 04.08.2013 | 52.0421 | 0.1497 | failed sequencing | - | - | - | - |
| AS:M:3 | river source water | 15.09.2014 | 52.0421 | 0.1497 | failed sequencing | - | - | - | - |
| AS:M:4 | river source water | 29.09.2014 | 52.0421 | 0.1497 | failed sequencing | - | - | - | - |
| AS:M:5 | river source water | 27.10.2014 | 52.0421 | 0.1497 | failed sequencing | - | - | - | - |
| AS:M:6 | river source water | 24.11.2014 | 52.0421 | 0.1497 | failed sequencing | - | - | - | - |
| AH:T:1 | hospital effluent | 02.05.2013 | 52.174343 | 0.139346 | metatranscriptome | ERS1027345 | 152,298,536 | 308,848 | 0.2028 |
| AH:T:2 | hospital effluent | 04.08.2013 | 52.174343 | 0.139346 | failed sequencing | - | - | - | - |
| AH:T:3 | hospital effluent | 15.09.2014 | 52.174343 | 0.139346 | failed sequencing | - | - | - | - |
| AH:T:4 | hospital effluent | 29.09.2014 | 52.174343 | 0.139346 | failed sequencing | - | - | - | - |
| AH:T:5 | hospital effluent | 27.10.2014 | 52.174343 | 0.139346 | failed sequencing | - | - | - | - |
| AH:T:6 | hospital effluent | 24.11.2014 | 52.174343 | 0.139346 | failed sequencing | - | - | - | - |
| DF:T:1 | farm effluent | 02.05.2013 | 52.22259 | 0.02603 | metatranscriptome | ERS1027346 | 74,411,930 | 948,890 | 1.752 |
| DF:T:2 | farm effluent | 06.08.2013 | 52.22259 | 0.02603 | failed sequencing | - | - | - | - |
| DF:T:3 | farm effluent | 15.09.2014 | 52.22259 | 0.02603 | failed sequencing | - | - | - | - |
| DF:T:4 | farm effluent | 29.09.2014 | 52.22259 | 0.02603 | failed sequencing | - | - | - | - |
| DF:T:5 | farm effluent | 27.10.2014 | 52.22259 | 0.02603 | failed sequencing | - | - | - | - |
| DF:T:6 | farm effluent | 24.11.2014 | 52.22259 | 0.02603 | failed sequencing | - | - | - | - |

AH, hospital effluent (Addenbrooke’s Hospital/Cambridge University Hospitals); DF, farm effluent (University of Cambridge dairy farm); AS, river source water (Ashwell Spring).

Table 1. Summary of samples used in this study

abundance of ARGs, the hospital effluent samples were frequently found to contain a higher abundance of MGEs and a greater number of distinct bacterial species in comparison with the farm effluent samples (Figure 1b).

**Relating ARGs in the environment at the DNA and RNA levels**

In order to determine whether the ARGs that were identified in hospital and farm effluent samples (at higher abundance than those found in the background samples of river source water) were being expressed, metatranscriptomics was used to interrogate effluent samples for the presence of ARG transcripts. Two RNA samples from both the hospital and the farm effluents failed sequencing library quality checking and were removed from the study (Table 1). A total of eight metatranscriptomes were successfully sequenced, passed quality checking and were found to contain ARG transcripts at varying abundance levels (Table 1).

To identify differentially regulated transcripts, particularly over-expressed ARGs, across our effluent samples, we applied the global model for metagenome versus metatranscriptome regulation described by Franzosa et al.20 (2014), predicting that the mean gene and transcript abundance would be correlated across the samples if the gene regulation and rate of transcription were constant. Overall, the mean ARG and ARG transcript abundances were highly correlated for both hospital effluents ($P < 0.0001$) and farm effluents ($P < 0.5$, two-tailed $P < 0.0001$) (Figure 2). The deviation of mean log ARG/ARG transcript ratios from zero (following FDR correction) identified two differentially regulated ARG transcripts in the hospital effluent samples; the relative abundance of each of these transcripts was greater than expected (based on the abundance of the corresponding ARGs) (Figure 2). The β-lactam resistance genes blaGES and blaOXA were consistently overexpressed in all hospital effluent samples; a significant 11-fold mean change in transcript abundance was observed for blaGES transcripts (two-tailed $P < 0.005$, $q < 0.05$) and a significant 2-fold mean change in transcript abundance was observed for blaOXA transcripts (two-tailed $P < 0.0001$, $q < 0.05$).
observed for \( \text{bla}_{\text{OXA}} \) transcripts (two-tailed \( P < 0.005, q < 0.05 \)) [data not shown and Table S1 (available as Supplementary data at JAC Online)]. The \( \beta \)-lactam genes \( \text{bla}_{\text{GES}} \) and \( \text{bla}_{\text{OXA}} \) were present but not overexpressed in the farm effluent samples.

**Determining the effect of \( \beta \)-lactam antibiotic usage on ARG expression**

Following the identification of two differentially regulated ARG transcripts \( \text{bla}_{\text{GES}} \) and \( \text{bla}_{\text{OXA}} \) in the hospital effluent samples, to investigate whether the genes were overexpressed as a result of anthropogenic activity we looked at the effect of hospital antibiotic usage on the abundance of \( \beta \)-lactam ARG transcripts. Antimicrobial drug usage data were obtained for the hospital and used to calculate the total volume of drugs used in the hospital each month (Table S2). Monthly drug usage for each antibiotic class was converted into a DDD (per 1000 bed days) value and the relative \( \beta \)-lactam usage was calculated for each month preceding metatranscriptome sample collection; monthly \( \beta \)-lactam usage (proportion of total usage) was found to change in line with relative ARG transcript abundance in the three sequential hospital effluent metatranscriptomes (Figure 3).

To supplement the antibiotic usage data, LC–MS was used to detect specific antibiotic residues in the effluent samples (Table S3). In total, 23 antibiotic residues from the testing panel were detected in the hospital effluent across all the sampling dates. However, in the sequentially sampled monthly metatranscriptomes, \( \beta \)-lactam antibiotic residues (for fluocoxacinilin) were only detected in November 2014 (Figure 3 and Table S3).

**Discussion**

To our knowledge this work represents the first study to combine metagenomics, metatranscriptomics and an attempt to relate environmental ARG expression to antibiotic resistance selective pressures. Although there have been many good studies towards cataloguing ARG diversity in effluent-impacted waters,\(^ {21-24} \) previous work in this area has been limited in terms of demonstrating how anthropogenic factors may be influencing the resistome in receiving waters. In this study we correlate, for the first time, the ARG and ARG transcripts in hospital effluents and relate this to the hospital antibiotic usage.

As previously suggested,\(^ {8} \) to extend our understanding of the risks that ARGs pose to the augmentation of the resistome and the impact this may have on global health, we must go beyond the presence and absence of genes and gain a more comprehensive understanding of ARG dynamics. With this in mind we employed metatranscriptomics to identify ARG transcripts in effluents, allowing us to elucidate the expression of key ARGs in a given sample, identify consistent overexpression across samples and classify antibiotic or ARG types that are routinely being released into the environment via effluents. Despite the complexities of generating metatranscriptomes from environmental samples,\(^ {25} \) we were able to produce a sufficient number of paired metagenomes/metatranscriptomes to determine the
The correlation between ARG and ARG transcripts for hospital and farm effluents. Within a sample, the presence of transcripts was used as a proxy for gene expression and the ratio of ARG to ARG transcript abundance was used to determine differentially expressed transcripts. Although a commonly used method, metatranscriptomic analysis of gene expression in microbial communities may be biased by factors such as transcript half-life, causing over- or under-representation of particular genes. However, metatranscriptomics is a powerful tool as it provides a snapshot of ARG expression from a microbial community and can be linked with metagenomic data to give a taxonomic, genomic and functional overview of an environmental sample.

Using the metagenomic data, we found the mean normalized ARG abundance in effluents to be much greater than in the background samples of the river catchment, particularly in the case of effluents originating from a hospital. Our results complement...
similar findings of ARG carriage by effluents that originate from anthropogenic sources, and the work described here also complements the findings of a previous study of the same river catchment by identifying additional ARG-containing effluents and sampling over an extended time period.

Similar to our previous study, we encountered difficulty in obtaining sufficient biomass from several background samples, leading to failure to prepare metagenomic libraries. However, notwithstanding the reduced number of background samples, the metagenomic libraries revealed ARG abundance in hospital and farm effluents was always higher than in the corresponding background sample for the month of sampling (if available) and the mean abundance values for the effluents were significantly greater than the mean abundance value for the background river catchment.

This study also incorporated LC–MS and antibiotic usage data for the site of high antibiotic consumption, Cambridge University Hospitals. Although anecdotal, the similar trends observed between β-lactam transcript abundance and the hospital β-lactam usage over the same 3 month period, combined with the presence of antibiotics at low concentrations in the effluents, gives weight to the argument that anthropogenic activities may impact ARGs in the environment. It should be noted that the LC–MS data were for only a panel of antibiotics and the LC–MS standards used were designed for undecayed antibiotics, and consequently this LC–MS screen only provided a snapshot of selected antibiotic compounds that were present and will not have detected decayed compounds.

In terms of the overexpression of β-lactam resistance genes, the genes blatGES and blaGNOX were expressed in both hospital and farm effluents but they were only found to be overexpressed in hospital effluents. This significant overexpression of ARGs occurring in an environment that is heavily impacted by antibiotic use, compared with the much lower expression found in farm effluents (subject to a much lower use of antibiotics), may be an example of human activity augmenting the aquatic resistome. The combined data from this study were used to assess the impact of anthropogenic activities on the resistome. To date there have been limited temporal studies in this area and the use of background sampling is still to be routinely adopted. The powerful combination of metagenomics and metatranscriptomics provides further insight into a complex problem and we believe this to be the first report of ARGs being overexpressed in effluents. Although we try to link between ARG expression and antibiotic usage at the effluent source (Figure 3), it must be noted that there are many confounding factors (e.g. higher temperatures of effluents) that may be responsible for the observed overexpression of ARGs and further work is needed to support an association between ARG expression in effluents and antibiotic usage at the effluent source. Indeed, more must be done to investigate factors such as the genetic context of ARGs (e.g. promoter proximity) and the metabolic activity of the community being sampled, which could both impact gene expression in microbial communities. For instance, the expression of ARGs we have observed in this study could be attributed to the high temperatures of hospital effluents and the recent excretion of bacteria from human hosts. Untangling the various factors that could be involved in the observed overexpression of ARGs remains a significant challenge and determining the fate of these genes once the effluents are received by the wider aquatic environment will go towards facilitating our understanding of this complex interaction between ARGs and the environment.

Combining genomic technologies and environmental metad ata to inform antibiotic stewardship is an exciting prospect and one that is being actively pursued. Future studies should consider including metatranscriptomics to identify ARG transcripts in environmental samples, developing the methodology further or using a single-cell approach to gain greater resolution of the species involved in the expression of genes of interest.

While similar approaches that combine metagenomics and metatranscriptomics have been used to study the impact of anthropogenic activities on microbial communities, our study combined metagenomics, metatranscriptomics and antibiotic usage data to identify hospital effluents containing consistently overexpressed ARGs. With the suggestion of incorporating gene weight- ing or scoring systems into environmental antibiotic resistance risk assessments, the findings presented here suggest that it would be worthwhile incorporating ARG expression data when designing ARG scoring matrices in future risk assessments.

To conclude, our results indicate that effluents originating from sites of antibiotic usage and entering a river catchment are regularly contributing ARGs to the resistome; these ARGs are expressed and are more abundant than in background samples of the river catchment. The clinical impact of these ARGs re-entering the human population after release into the natural environment is clearly an area of future work, and consideration must also be given to the role of effluents in effective antibiotic stewardship.

Acknowledgements
Metagenomic and metatranscriptomic sequencing was performed by the Sequencing Service and Wellcome Trust Biomedical Informatics Hub, Exeter (UK). LC–MS was performed by RPS Mountainheath, Hertfordshire (UK). Antimicrobial usage data were provided by Cambridge University Hospitals, Cambridge (UK).

Funding
This research was funded by the Biotechnology and Biological Sciences Research Council, GlaxoSmithKline and the Centre for Environment, Fisheries and Aquaculture Science.

Transparency declarations
C. B.-A. and D. W. V.-J. are employed by the Centre for Environment, Fisheries and Aquaculture Science. J. J. R. is employed by GlaxoSmithKline. All other authors: none to declare.

Supplementary data
Tables S1 to S3 are available as Supplementary data at JAC Online.

References
1 Holmes AH, Moore LSP, Sundsfjord A et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet 2016; 387: 176–87.
2 Gaze WH, Krone SM, Larsson DG et al. Influence of humans on evolution and mobilization of environmental antibiotic resistome. Emerg Infect Dis 2013; doi:10.3201/eid1907.120871.
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3 D’Costa VM, McGrann KM, Hughes DW et al. Sampling the antibiotic resistance gene pool in hospital effluents: A metagenome-assembled contig approach. *Nature* 2015; 519: 47–52.

4 Forsberg KJ, Reyes A, Wang B et al. The shared antibiotic resistance of soil bacteria and human pathogens. *Science* 2012; 337: 1107–11.

5 Martinez JL, Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* 2009; 157: 2893–902.

6 Wellington EMH, Boxall ABA, Cross P et al. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect Dis* 2013; 13: 155–65.

7 Port JA, Cullen AC, Wallace JC et al. Metagenomic frameworks for monitoring antibiotic resistance in aquatic environments. *Environ Health Perspect* 2014; 122: 222–8.

8 Berendonk TU, Mania CM, Merlin C et al. Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol* 2015; 13: 310–7.

9 Scott GI, Porter DE, Norman RS et al. Antibiotics as CECs: an overview of the hazards posed by antibiotics and antibiotic resistance. *Front Mar Sci* 2016; doi: 10.3389/fmars.2016.00024.

10 Rowe W, Verner-Jeffreys DW, Baker-Austin C et al. Comparative metagenomics reveals a diverse range of antimicrobial resistance genes in effluents entering a river catchment. *Water Sci Technol* 2016; 73: 1541–9.

11 Rowe W, Baker KS, Verner-Jeffreys D et al. Search Engine for Antimicrobial Resistance: a cloud compatible pipeline and web interface for rapidly detecting antimicrobial resistance genes directly from sequence data. *PLoS One* 2015; 10: e0133492.

12 Baquero F, Tedim AP, Coque TM. Antibiotic resistance shaping multi-level population biology of bacteria. *Front Microbiol* 2013; 4: 15.

13 McEniff G, Barron L, Kelleher B et al. A year-long study of the spatial occurrence and relative distribution of pharmaceutical residues in sewage effluent, receiving marine waters and marine bivalves. *Sci Total Environ* 2014; 476–477: 317–26.

14 Bruchmann J, Kirchen S, Schwartz T. Sub-inhibitory concentrations of antibiotics and wastewater influencing biofilm formation and gene expression of multi-resistant *Pseudomonas aeruginosa* wastewater isolates. *Environ Sci Pollut Res* 2013; 20: 3539–49.

15 Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv 2013; https://arxiv.org/abs/1303.3997.

16 Segata N, Waldron L, Ballarini A et al. Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat Methods* 2012; 9: 811–4.

17 Hutchinson JM, Patrick DM, Marra F et al. Measurement of antibiotic consumption: a practical guide to the use of the Anatomical Therapeutic Chemical classification and Defined Daily Dose system methodology in Canada. *Can J Infect Dis* 2004; 15: 29–35.

18 WHO Collaborating Centre for Drug Statistics Methodology. *Defined Daily Dose*. http://www.whocc.no/ddd/definition_and_general_considerations/2016.

19 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Front Mar Sci* 1995; 57: 289–300.

20 Franzosa EA, Morgan XC, Segata N et al. Relating the metatranscriptome and metagenome of the human gut. *Proc Natl Acad Sci USA* 2014; 111: E2329–38.

21 Bengtsson-Palme J, Boulund F, Fick J et al. Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. *Front Microbiol* 2014; 5: 648.

22 Chen B, Yang Y, Liang X et al. Metagenomic profiles of antibiotic resistance genes (ARGs) between human impacted estuary and deep ocean sediments. *Environ Sci Technol* 2013; 47: 12753–60.

23 Pitta DW, Dou Z, Kumar S et al. Metagenomic evidence of the prevalence and distribution patterns of antimicrobial resistance genes in dairy agroecosystems. *Foodborne Pathog Dis* 2016; 13: 296–302.

24 Yang Y, Li B, Zou S et al. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res* 2014; 62: 97–106.

25 Poretsky RS, Gifford S, Rinta-Kanto J et al. Analyzing gene expression from marine microbial communities using environmental transcriptomics. *J Vis Exp* 2009; doi:10.3791/1086.

26 Zarraonaindia I, Smith DP, Gilbert JA. Beyond the genome: community-level analysis of the microbial world. *Biol Philos* 2013; 28: 261–82.

27 Aguilar-Pulido V, Huang W, Suarez-Ulloa V et al. Metagenomics, metatranscriptomics, and metabolomics approaches for microbiome analysis. *Evol Bioinform Online* 2016; 12 Suppl 1: 5–16.

28 Nayes NR, Yang X, Linke LM et al. Characterization of the resistome in marine, soil and wastewater from dairy and beef production systems. *Sci Rep* 2016; 6: 24645.

29 Karkman A, Johnson TA, Lyra C et al. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. *FEMS Microbiol Ecol* 2016; doi:10.1093/femsec/fiw014.

30 Li J, Cheng W, Xu L et al. Occurrence and removal of antibiotics and the corresponding resistance genes in wastewater treatment plants: effluents’ influence to downstream water environment. *Environ Sci Pollut Res* 2016; 23: 6826–35.

31 Bondczuk K, Markowicz A, Plotrowska-SEG et al. The urgent need for risk assessment on the antibiotic resistance spread via sewage sludge land application. *Environ Int* 2016; 87: 49–55.

32 Radziwiłł A, Gojobori T. Single-cell technologies in environmental omics. *Gene* 2016; 576: 701–7.

33 Mason OU, Hazen TC, Borglin S et al. Metagenome, metatranscriptome and single-cell sequencing reveal microbial responses to Deepwater Horizon oil spill. *ISME J* 2012; 6: 1715–27.

34 Martinez JL, Coque TM, Baquero F. What is a resistance gene? Ranking risk in resistomes. *Nat Rev Microbiol* 2015; 13: 116–23.