Impact of trimethoprim on the river microbiome and antimicrobial resistance.

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Introduction

Antimicrobial resistance (AMR) is a global health issue, with some forecasts predicting a significant and substantial increase in the levels of untreatable infections within the next 8 years (Li and Webster, 2018; Aslam et al, 2018). The struggle against the development of AMR has conventionally been viewed as a medical problem (Bengtsson-Palme et al, 2018). However, the environment is now being

acknowledged as a substantial contributor to the global spread of antibiotic resistance (Wellington et al, 2013 & Singer et al, 2016;2019). Rivers and waterways are prominent examples of environmental settings that have been been identified as a major repository and route for dissemination of both low levels of antibiotics and antibiotic resistance genes (Pärnänen et al, 2019; Amos et al, 2015).

A recent study quantified drug concentrations in freshwater samples taken at a range of sites located on the Thames catchment; the highest levels of antibiotics and other drugs were recorded downstream of waste water treatment plants (WWTPs) (Holden et al, 2020). While the origin and fate of these antibiotics could be strongly linked to community and clinical use, the impact of these drugs on the emergence of antimicrobial resistance in the environment remains elusive (Aslam et al, 2018). It is widely recognised that the evolutionary arms race, or red queen hypothesis, plays an important role in shaping the environmental resistome (Papot et al, 2017). However, (Märtinez et al, 2012) proposed that antimicrobial resistance genes (ARGs) had also emerged within environmental bacteria to provide a protective effect against antibiotic producing organisms and possibly polluting drugs in the environment. This is of concern to human health, as the genes under selection may be clinically relevant and have the potential to be transferred between environmental and clinical bacteria via horizontal gene transfer (HGT) (Baquero et al, 2008).

We have previously shown that the prevalence of an integron borne ARG, *sul1,* correlates with the concentration of several antibiotic residues across river sites on the Thames catchment (Holden et al, 2020). *sul1* provides resistance to sulfonamides, a bacteriostatic class of antibiotics that were originally synthesised in the 1930s (Drugbank, 2020). Sulfonamides bind to dihydropteroate synthase (DHPS) in bacteria and hinder dihydrofolic acid formation which is vital for vitamin B formation and bacterial growth (Sköld, 2000). One particular sulfonamide; Sulfamethoxazole (SMX) can be prescribed with Trimethoprim (TMP) as co-trimoxazole to treat chronic bronchitis, shigellosis

and *Pneumocystis carinii* pneumonitis (Alsaad et al, 2013). TMP and SMX target successive steps of the folate synthesis pathway. SMX inhibits dihydropteroate synthase (DHPS) FolP, which catalyses the addition of dihydropterin diphosphate to *p*-aminobenzoic acid. The product of FolP, 7,8-dihydropteroate, will then react with glutamate to form dihydrofolate. This is then reduced to tetrahydrofolate by the dihydrofolate reductase (*Dhfr*), the TMP target (Vilchéze and Jacobs, 2012).

Despite the combined effects of SMX and TMP, recent antibiotic prescription surveillance data suggests that TMP is more commonly prescribed as a single-dose antibiotic for clinical purposes (Ashiru-Oredope et al, 2014). Furthermore, the recent veterinary medicines directorates report on antibiotics showed that a combined volume of 66.2mg/kg of sulfonamides/TMP was used to treat infections in pigs in 2015, which accounted for 24% of the total antibiotics prescribed for pigs (Bos et al, 2019). Studies have revealed several different ARGs relevant to TMP (*DfrE*, *DfrB2/3, DfrB1/6)* (Šeputienė et al, 2010) and sulfonamides (*sul1*, *sul2, sul3, sul4*) (Razavi et al, 2017). Due to the prominence of sulfonamide resistance in the environment, the *sul1* gene has become incorporated into the structure of class 1 integrons (CL1s) (Na et al, 2014). Several studies have noted the persistence of sulfonamide and TMP resistance genes in the same environmental settings, often located on the same plasmids (Muziasari et al, 2014; Kiiru et al, 2013; Phuong Hoa et al, 2008).

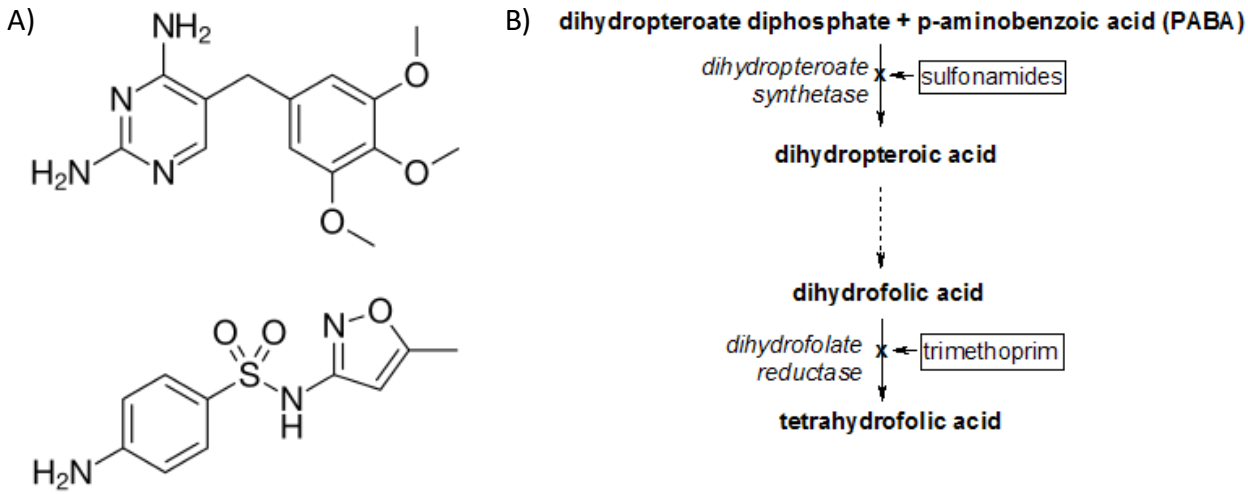

Figure 1- A) Chemical structure of SMX/TMP (PubChem, 2020) B) Sulfonamide and TMP combined mechanism of action (PubChem, 2020).

During our previous work, sites on the Thames catchment with elevated levels of TMP were identified (Holden et al, 2020). Despite this, there is still no evidence for a causative relationship between the residue of antibiotics such as TMP and the prevalence of antibiotic resistance genes such as *sul1* (Holden et al, 2020). It is possible that the correspondence between ARGs and drugs downstream of WWTPs is purely correlative, as both are being released from the treatment process at the same time. Studies have suggested that WWTPs themselves, as opposed to river sites are 'hotspots' of AMR genes and therefore most of the selection for resistance occurs before antibiotic residue enters the environment (Lood et al, 2017; Pazda et al, 2019; Fouz et al, 2020).

The aim of the current study was to conduct an in-depth analysis using a combination of large metagenomic, geospatial and chemical datasets in order to conduct a comparison between those sites with the highest TMP levels and lowest TMP levels on the Thames catchment. We aimed to establish the role that factors such as proximity to WWTPs, population equivalence (PE) and land coverage play in the accumulation of TMP at river sites. A secondary aim was to investigate seasonal variation in TMP and ARGs. Exploring these factors may also help to decipher the clinical relevance of ARG accumulation at river sites. Lastly, as TMP can be prescribed alongside SMX as co-trimoxazole, we aimed to establish if the levels of TMP identified at these specific sites show particularly high correlations with the prevalence of the *sul1* gene.

Methods

Sampling regime

Please see methods described in (Holden et al, 2020)

Sample processing

Please see methods described in (Holden et al, 2020)

Metagenome assembly and annotation

Metagenome assembly was performed using megahit v1.1.3 with option -k--min 29 (Dinghua et al, 2015). Three assembly schemes were implemented, assemblies per sample, co-assemblies of samples from the same tributary and a unique co-assembly built from all samples. Comparison of the 3 schemes lead to the global co-assembly being chosen for the rest of the analysis. Contigs smaller than 500 nucleotides were discarded. Gene prediction was carried out using prodigal v2.6.3, with option -p meta (Hyatt et al, 2015). A selection of 36 Single copy Core Genes (SCG) were annotated through rpsblast v2.9.0 using pssm formatted COG database made available by the CDD (Tatusoy et al 2000; Lu et al, 2020). Best hits were filtered so that hit span at least 50% of the query with an e-value smaller than 1e-5. Additionally, ARG and *intI1* annotation was completed using diamond v0.9.9 (Buchfink et al, 2015). Databases for both were sourced from (Lee et al, 2020), clustered respectively from of CARD (Jia et al, 2017) and NCBI sequence search. Best hits were filtered using 80% query coverage, 80% percentage identity and 20% subject coverage. Taxonomic annotation was carried out using diamond blastp on a NR database reduced to prokaryotes (Pruitt et al, 2005).

Assembly was converted to contig graph using megahit toolkit, it was then translated to orf graph using result from gene prediction in custom python script. Visualisation of annotations on orf graph is done through bandage v0.8.1 (Wick et al, 2015).

Samples were mapped to contigs using bwa-mem v0.7.17-r1188 (Li, 2013). Contig and genes mean coverage were computed through both samtools v1.5 (Li et al, 2009) and bedtools v2.28.0 (Quinlan et al, 2013). Coverage of functional feature, such as SCG, AMR or *intI1* were obtained by summation of coverage of genes sharing the same annotation. All coverages are normalised by sample, using the median of the 36 SCG coverages.

Chemical Analysis

For each site 100 mL of each water sample was acidified to pH 3, internal standards were added and the sample was extracted with a solid phase extraction (SPE) vacuum manifold system using Oasis HLB (200mg) cartridges. Thereafter each sample was evaporated to <10 µL, reconstituted to a final volume of 0.1 mL 5% acetonitrile with 0.1% formic acid and 10 µL of each water sample was analysed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). A detailed description of the two analytical methods used to analyse most of the pharmaceuticals, and the validation of each method is shown in (Holden et al, 2020). However, at the time of sample analysis, contamination in the LC-MS/MS system interfered with the analysis of the six biocides included in the study. Instead, an analytical method developed by (Östman et al, 2017) specifically for biocides, on the LC-MS/MS system, was used to analyse the six biocides.

Geospatial analyses and meteorological data

All geospatial analyses were performed using ArcGIS v10.4 software (ESRI, Aylesbury, UK). To analyse possible drivers of AMR for each sampling site, an upstream catchment of that location was delineated using the hydrology tools to create a flow accumulation and flow direction layer per site,

and subsequently a watershed (upstream catchment), using a 50m grid interval hydrological digital terrain model (the Centre for Ecology and Hydrology Integrated Hydrological Digital Terrain Model (IHDTM)) (Morris & Flavin, 1994). A Network Dataset was created from the Thames river data, using the Ordnance Survey MasterMap Water Network 2016 (Ordnance Survey, 2016) and comprised all layers needed for distance measurements, including WWTPs. Data on WWTPs was obtained from the environment agency (Environment Agency, 2012; Environment Agency, 2013). To analyse upstream land use, and more specifically to calculate the percentage land cover of each sample site watershed, the Centre for Ecology and Hydrology Land Cover Map 2015 (Rowland et al, 2017) and Land Cover Plus: Crops Map 2016 were used (NERC CEH, 2016).

For more detailed information, please see (Holden et al, 2020).

Statistical analysis and model development

Initial analysis of chemical data used a mixed-effects model with Tukey's multiple comparison tests to assess differences in trimethoprim (TMP) and sulfamethoxazole (SMX) concentrations between seasons (summer & winter) and between twenty sample sites (Coln1, Coln2, Coln3, Coln4, Leach1, Leach2, Key1, Ray1, Ray6, Ray4, Ray3, Thames1, Thames3, Thame7, Thame2, Cut1, Cut2, Cut4, Cut6, Thames6).

Analysis of geospatial data relating to the distance (metres) of the twenty sample sites downstream from waste-water treatment plants (WWTPs) and the concentration of TMP was carried out using Spearman's rank correlation coefficient due to the non-parametric nature of the data. Both variables were log-transformed prior to analysis.

Analysis of metagenomic data used multiple t-tests with Bonferri-Dunn correction to assess differences in ARG (*sul1*, *sul2*, *dfrB1/6*, *dfrB2/3*, *dfrE*) and *intI1* prevalence between seasons (summer and winter). Two-way ANOVA was also applied to assess differences in the prevalence of aforementioned ARGs & *intI1* between seasons and sites with the four highest (Cut4, Ray4, Cut6, Ray6) and four lowest (Coln1, Coln2, Coln3, Leach1) TMP concentrations. All analysis was performed using GraphPad® Prism version 8 and R® software. For all tests, p values of statistical significance are signified by * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ & **** $p<0.0001$.

Maps

Maps shown in Figure 4 were created using ArcGIS v10.4 software. Geographic coordinates of sites were taken from (Ordnance Survey, 2016).

Results

Analysis of TMP and SMX levels

The original study of the Thames catchment sampled 69 sites for all data sets including qPCR, but only 20 of these were selected from metagenomics (Holden et al, 2020). The levels of antibiotics at these latter sites illustrated significant seasonal variation in levels of TMP and SMX (Figure 2).

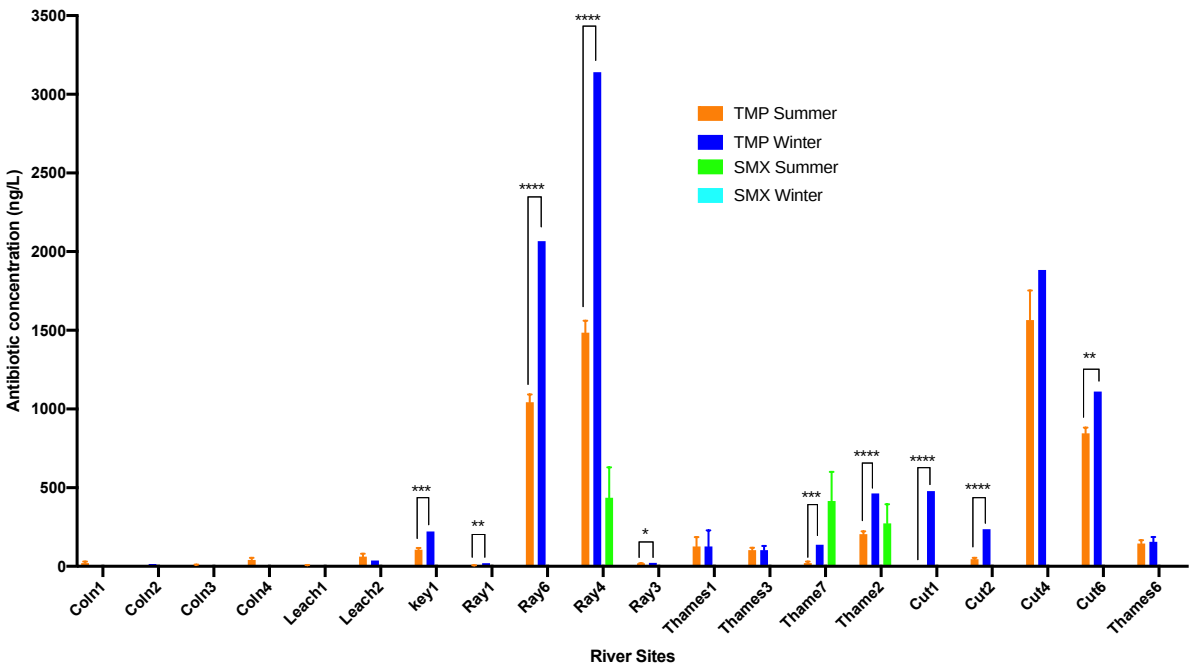

Figure 2- The concentrations of TMP and SMX at twenty sites across the Thames catchment in summer (n=6) and winter (n=3) (SEM). The sites were ordered by geographical location across the catchment, with Coln1 being furthest East and Thames6 being furthest West.

TMP concentrations were higher than SMX concentrations at all sites other than Thame7. Ten of the twenty sites had significantly higher TMP in winter compared to summer. Four sites had elevated levels of TMP (Ray4, Ray6, Cut4 and Cut6), with Ray4 providing a TMP concentration of 3000ng/L. Aside from Cut6, the level of TMP was significantly higher in winter compared to summer for these sites. For three sites (Ray4, Thame7 and Thame2), the concentration of SMX was higher in summer compared to winter, but these differences were not significant.

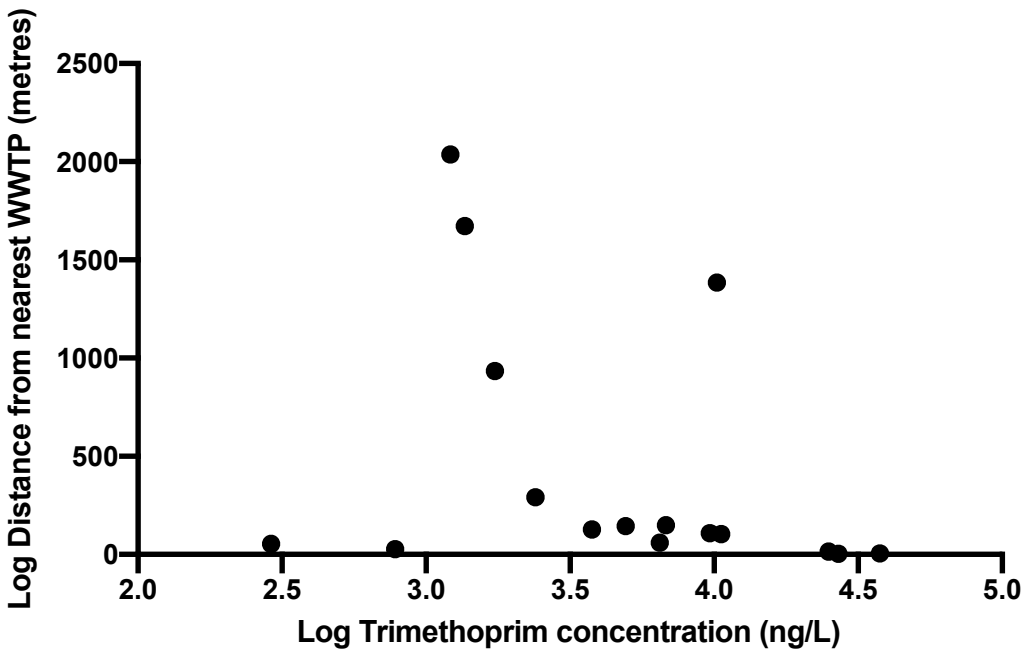

Figure 3- Relationship between distance of sample sites downstream from a WWTP and the concentration of TMP across 20 sites on the Thames catchment.

A significant negative correlation was observed between the distance of sites downstream from the nearest WWTP and the TMP concentration across 20 sites on the Thames catchment (Spearman r = -0.456 & $p<0.05$). This relationship was further explored to determine where the highest and lowest TMP levels were detected (Table 1).

Table 1- A sub-set of four sites that showed the highest and lowest TMP concentrations from the 20 sites. TMP concentrations shown were averaged across summer/winter seasons.

***Secondary:** 'Treatment methods include biological filtration (including conventional filtration, rotating biological contactors and root zone treatment (where used as a secondary treatment stage), alternating double filtration and high rate filtration'.

(Sewage Treatment in the UK, 2020).

***Tertiary:** 'Works with a secondary activated sludge process whose treatment methods also include rapid-gravity sand filters, moving bed filters, pressure filters, nutrient control using physico-chemical and biological methods, disinfection, hard COD and colour removal, where used as a tertiary treatment stage' (Sewage Treatment in the UK, 2020).

River Site	Nearest WWTP	Distance from WWTP (m)	WWTP Treatment Type*	Trimethoprim concentration (ng/L)
Cut4	Bracknell	1361	Tertiary	1671
Cut6	Maidenhead	1540	Tertiary	934
Ray4	Swindon	1213	Tertiary	2037
Ray6	Swindon	10208	Tertiary	1384
Coln1	Withington	16677	Secondary	14
Coln2	Withington	18688	Secondary	4
Coln3	Bibury	9479	Secondary	5
Leach1	Northleach	20270	Secondary	4

The four sites with the highest TMP concentrations were located closer to WWTPs (Table 1) as expected from the data in Figure 4. The four sites with the highest TMP concentrations were located closest to tertiary WWTPs, whereas the three of the four sites with the lowest TMP concentrations were located closest to secondary WWTPs. Two of the four sites with the highest TMP concentrations (Ray4 and Ray6) were located closest to the same Swindon WWTP.

Analysis of land use and population equivalence (PE)

Land use and PE data were assembled for the four sites with the highest and lowest TMP concentrations across the Thames catchment, as shown in Figures 4, 5 and 6.

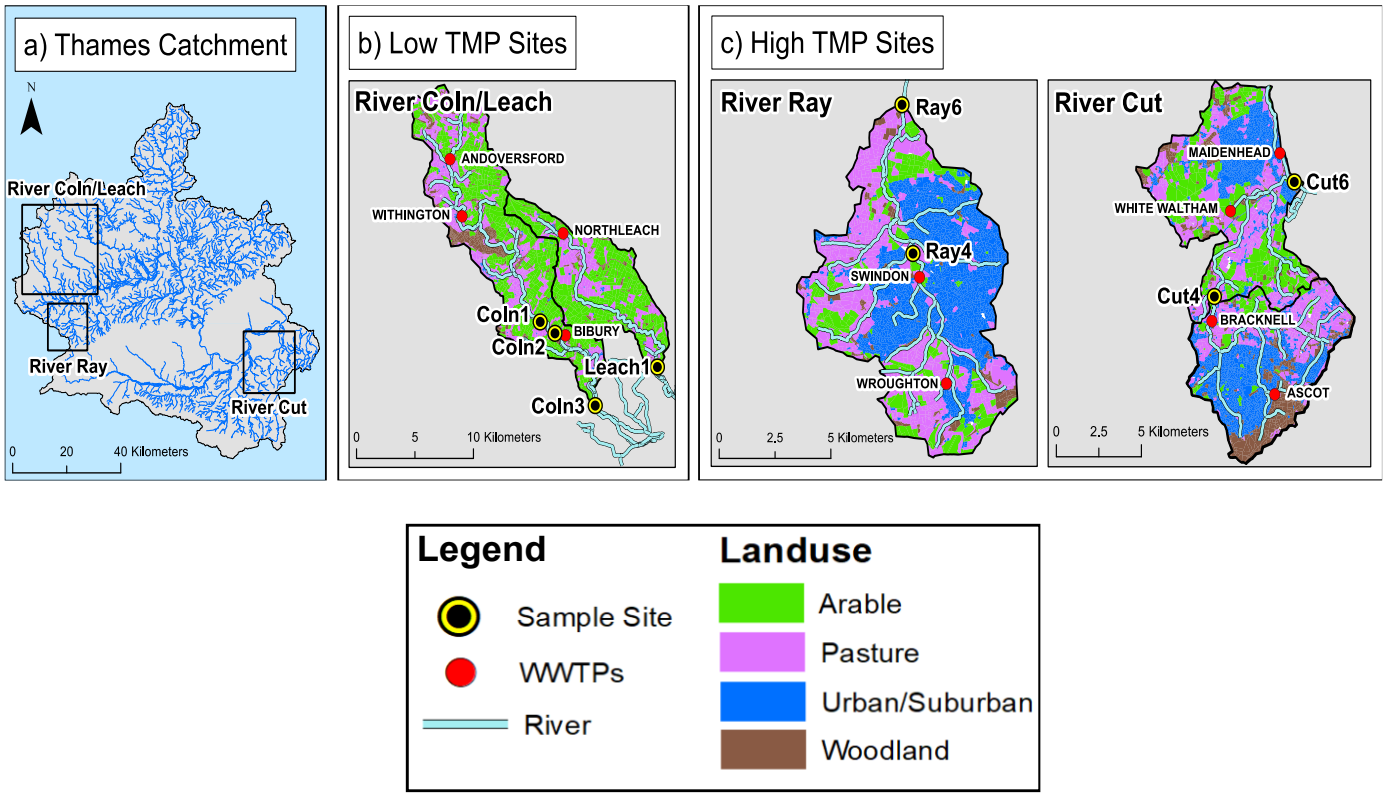

Figure 4

A) Locations of the Rivers Cut, Ray, Coln and Leach on the Thames catchment.

B) Locations and land-use corresponding to the four sites with the lowest TMP concentrations.

C) Locations and land-use corresponding to the four sites with the highest TMP concentrations.

Examination of the land-use data in proximity to sites with highest and lowest TMP levels indicate urbanization correlating with tertiary WWTPs and high TMP levels (Figure 4). The Coln1 and Coln2 were located closest downstream to Withington WWTP, which serves a population of approximately 230 people (ThamesWater, 2018). Coln3 was located closest to Bibury WWTP, which serves approximately 600 people (ThamesWater, 2018). Leach1 was located closest to Northleach WWTP, which serves a population of approximately 1,670 (ThamesWater, 2018).

The sites Ray4 and the Ray6 were both located closest to Swindon WWTP, which serves approximately 210,000 people (ThamesWater, 2018). Cut4 and Cut6 were situated closest to Bracknell and Maidenhead WWTPs, which serve populations of 90,000 and 87,400 respectively (ThamesWater, 2018).

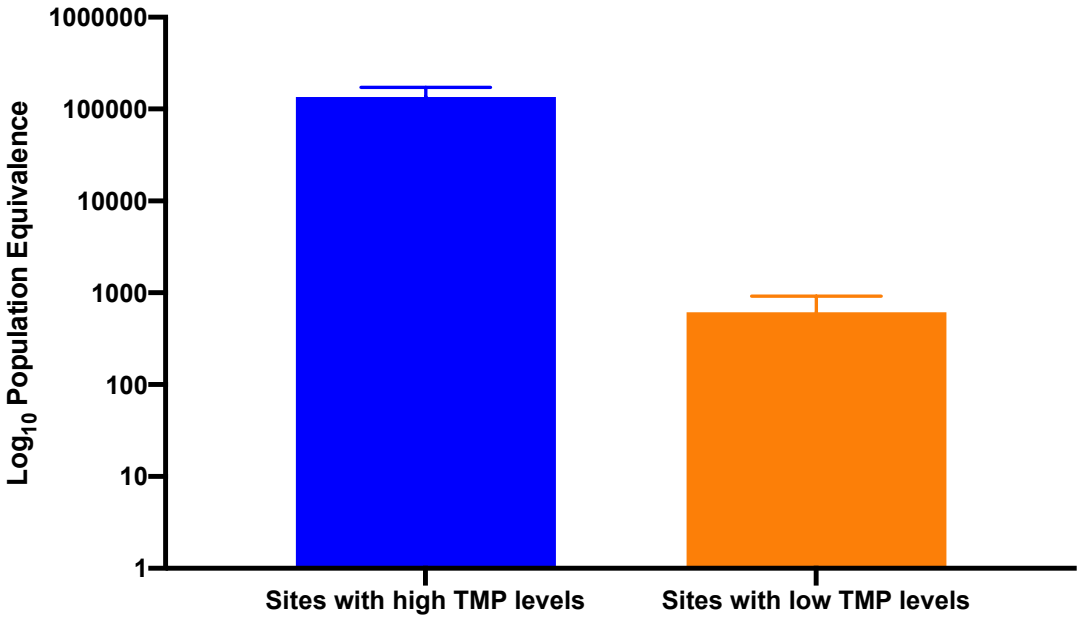

Figure 5- The average Log_{10} population equivalence (PE) for four sites with the highest TMP concentrations (left) and four sites with the lowest TMP concentrations (right) (n=4) (SEM).

The average PE for the four sites with the highest TMP concentrations was higher than the PE for the four sites with the lowest TMP concentrations.

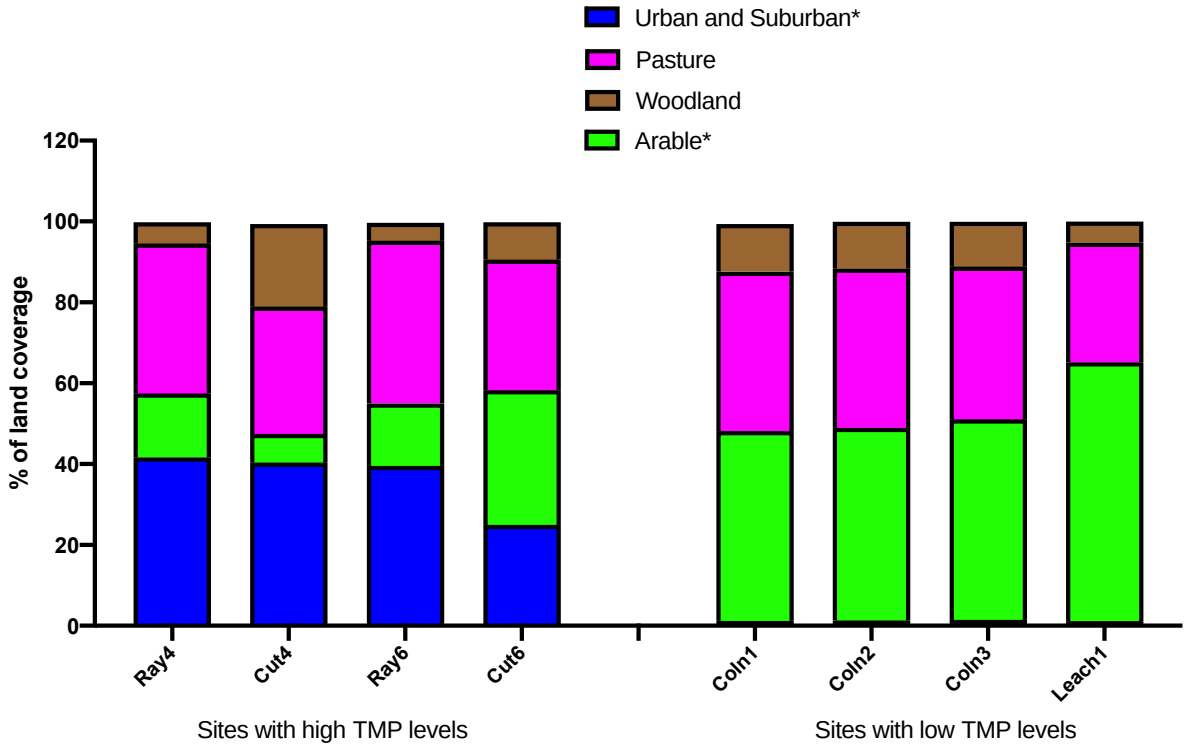

Figure 6- Percentage of land coverage at four sites with the highest TMP levels (left) and the lowest TMP levels (right).

The proportion of urban/suburban land coverage was found to be higher across the four sites with the highest TMP concentrations compared to the four with the lowest TMP concentrations. By contrast, the proportion of arable land coverage was higher across the sites with the lowest TMP concentrations compared to sites with the highest TMP concentrations.

Prevalence of ARGs

Only two SMX ARGs and three TMP ARGs were at detectable levels in the metagenomes analysed (Figure 7) and of these *sul1* was detected at significantly higher levels in the winter. These were compared with *intI1* (CL1 marker gene) prevalence.

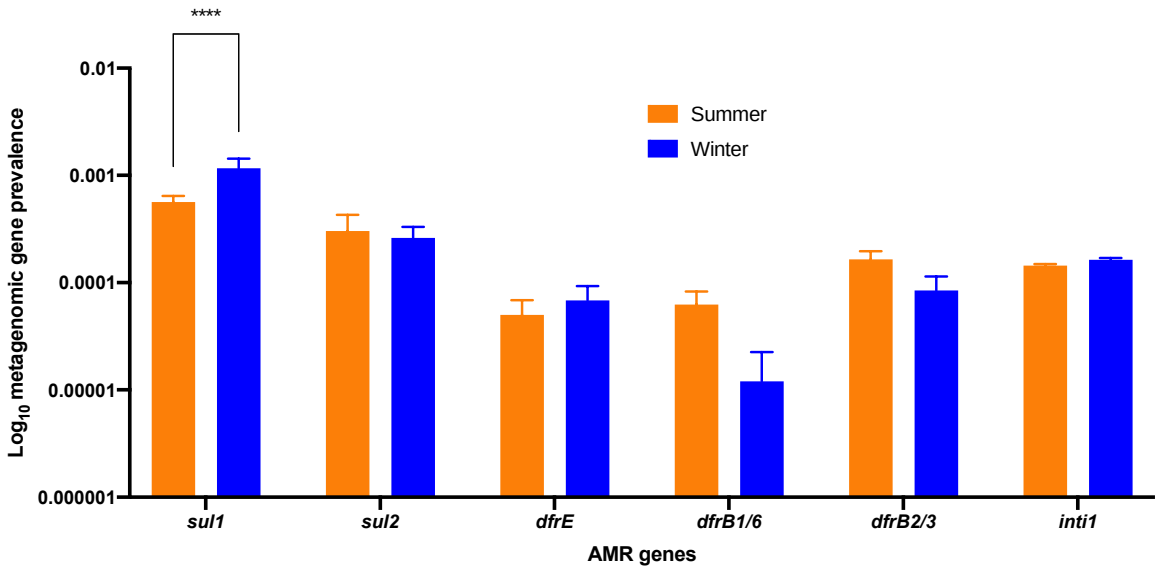

Figure 7- Prevalence of ARGs relevant to TMP/SMX resistance across 20 sites on the Thames catchment.

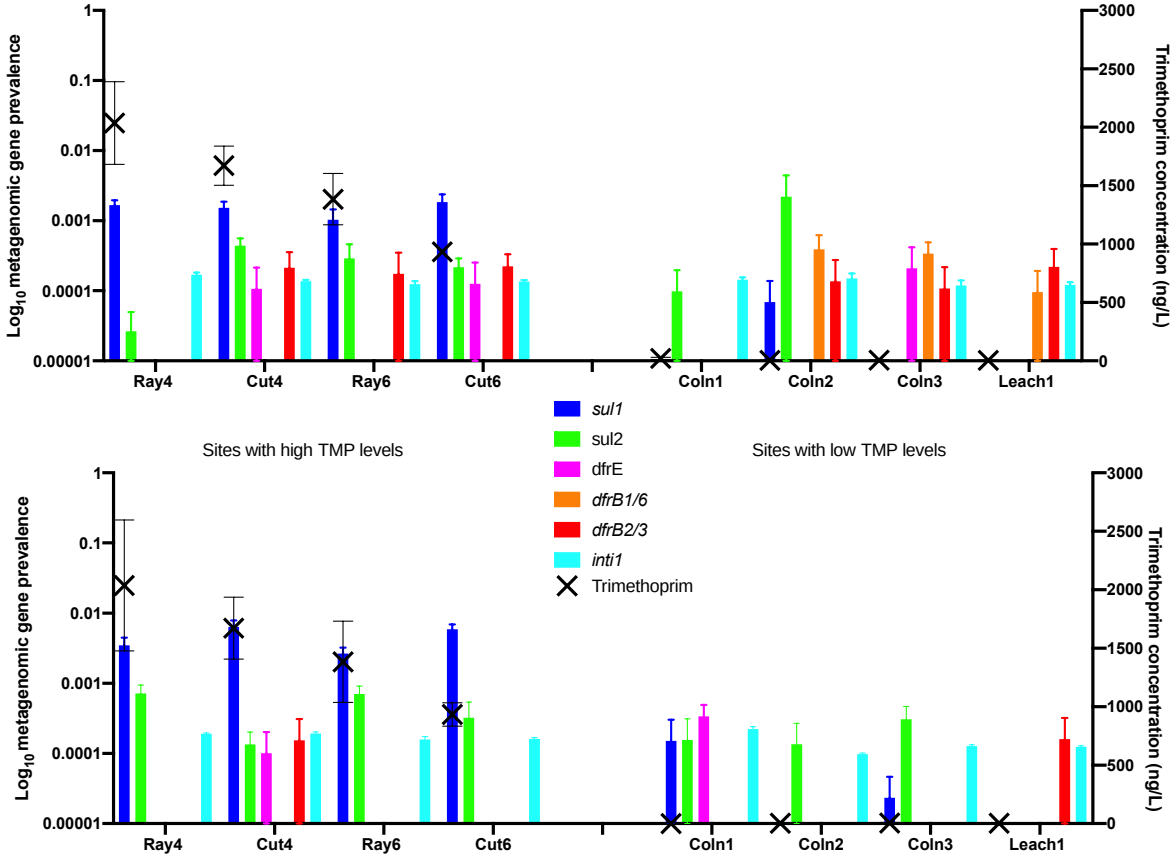

Figure 8- Prevalence of ARGs and *intI1* at sites with the four highest and four lowest TMP levels in summer (n=6) (above) compared to winter (n=3). Two-way Analysis of Variance (ANOVA) and unpaired t-tests was used to compare the prevalence of ARGs in Summer (n=6) vs Winter (n=3).

The prevalence of *sul1* was higher at sites Ray4, Ray6 and Cut4 in winter compared to summer. At these four sites, the prevalence of *sul1* was higher than any of the prevalence of any other genes in both winter and summer. Coln2 and Coln3 showed significantly higher prevalence's of *dfrB1/6* in summer compared to winter. The prevalence of *sul2* at Coln2 was significantly higher in summer compared to winter.

Discussion

This study highlighted river sites with significantly higher concentrations of TMP than SMX. Antibiotic surveillance data shows that TMP is more commonly prescribed as a single dose antibiotic than co-trimoxazole for clinical purposes (Ashiru-Oredope et al, 2014). By contrast, veterinary surveillance data suggests that TMP is co-prescribed with 'sulfonamides' in high levels in agricultural settings (Bos et al, 2019). As a result, it is possible that TMP is higher than SMX at these sites because the two drugs are more often being used for different purposes and therefore more likely to be found at different locations. The fact that the sites with higher TMP than SMX have a large proportion of urban/suburban land coverage and high PE values further suggests that the TMP originates from clinically relevant settings. By contrast, the one site that had higher SMX than TMP; Thame7 had a low proportion of urban/suburban land coverage, but a high proportion of pasture-based land coverage. This suggests that the SMX is more likely to be arising from agricultural settings.

Another possible reason why TMP was significantly higher than SMX at certain sites could be the differing biodegradation profiles that the drugs display in the environment (Thiebault, 2020). One

particular study assessed the concentration profiles of TMP and SMX in large batch reactors containing different wastewater matrices (Perez et al, 2005). When they were exposed to aerobic treatments in order to simulate the degradation process of a WWTPs, the biodegradation rate of SMX was significantly higher than TMP. In addition, when sewage sludge was amended with 24µg/L of ^{14}C radiolabelled TMP in a batch reactor, no degradation of TMP was seen over a 21-day period (Perez et al, 2005). Most WWTPs are designed to extract nutrients and easily degradable carbon compounds. However, it has been shown that they have limited capacities to remove pharmaceuticals such as TMP, making them sources of TMP in rivers and streams (Metcalfe et al, 2010; Sengupta et al, 2014). The presence of antibiotics in the treatment process can inhibit the biological activity of activated sludge, meaning that the antibiotics themselves are not degraded effectively (Al-Riyami et al, 2018). As a result, it is possible that SMX is more readily degraded than TMP as part of the WWTP process and therefore was found in higher concentrations at certain sites.

Studies estimate that between 75% and 90% of antibiotics are incompletely absorbed by human or animal hosts and are excreted, unaltered, in urine and faeces (Uyaguari-Diaz et al, 2018). As a result, the spillage of antibiotics and their metabolites into the environment is widespread (Pruden et al, 2013). In areas of high human population, it has been shown that excretion by humans contributes to most of the pharmaceutical waste that ends up in domestic sewers and is eventually transported to WWTPs (Tehrani and Gilbrade, 2018). As a result of the incapacity of conventional systems to remove emerging contaminants such as antibiotics during treatment, studies have indicated that urban-associated river sites often have the highest concentrations of antibiotics (Chen et al, 2018; Lundborg et al, 2017). The fact that the PE and the proportion of urban/suburban land coverage were significantly higher in the four sites with the highest TMP concentrations compared to those with the lowest TMP concentrations in this study further emphasises this.

Data from this study showed seasonal variation in the prevalence of individual ARGs and the concentrations of TMP/SMX antibiotics at river sites. For example, the prevalence of *sul1* over the 20 sites was significantly higher in winter compared to summer. Also, the concentration of TMP was significantly higher in winter compared to summer for ten of the twenty sites analysed. There are a number of different factors that could be causing this.

Firstly, the increased prescription/use of antibiotics in the population during the winter months could increase the concentrations detected at river sites and potentially the prevalence of ARGs. A recent study looked at antibiotic prescription rates by 3635 General Practitioners (GPs) serving a population of 5.8 million patients in the West Midlands UK (Ironmonger et al, 2018). The data consistently showed increases in the prescription of antibiotics (which included TMP) by individual GPs and >1 GP in winter compared to three other seasons across a four-year period (Ironmonger et al, 2018). An increase in antibiotic consumption can increase the antibiotic concentration found in sewage, which will eventually be treated by WWTPs (Pärnänen et al, 2019). If antibiotics are not successfully removed during sewage treatment processes, then the higher concentrations of TMP shown in winter compared to summer could be due to increased antibiotic usage/consumption. However, with regards the chemical analysis of TMP and SMX, it must be acknowledged that the freshwater samples used were collected over two summer seasons (summer 2015 and summer 2016) and only one winter season (winter 2016). Also, the concentrations of chemicals determined at each site are only reflective of the concentration of that chemical at one particular time, with no corrections made for the flow rate of the individual river site or data on sewage spills from WWTPs. It is possible that winter samples show elevated TMP concentrations at some sites because there was a particularly large discharge from a CSO before the day of sampling or example. As a result, the chemical data shown is potentially not reflective of the whole seasons analysed and the differences between TMP concentrations in summer and winter is potentially more of a temporal effect as opposed to a seasonal one. Despite this, the fact that ten of the twenty sites analysed showed significantly higher

TMP concentrations in winter than summer does reduce the chances of the elevated TMP being coincidental. In order to resolve this, more information needs to be obtained on the flow rates of individual tributaries and the discharge process that WWTP companies undertake to release effluent into rivers.

A second factor that could influence the prevalence of *sul1* in winter is an element of the wastewater infrastructure in the UK. Systems known as combined sewers are used to simultaneously carry municipal sewage and storm water to WWTPs (SOAF, 2018). These sewers are often designed to carry several times the average dry-weather load, but in cases of high rainfall, the capacity can be overloaded. In these cases, combined sewer overflow (CSO) events occur to prevent the back-flooding of homes and streets. The storm flow naturally becomes mixed with untreated sewage, and is released into a nearby water body (Munro et al, 2019). In London for example, it was shown that approximately 39 million tonnes of untreated sewage are discharged into the Thames river every year (DEFRA, 2015). CSOs have been shown to have a significant impact upon the short-term concentrations of pharmaceutical drugs in the Thames catchment (Munro et al, 2019). This could also explain the higher prevalence of *sul1* genes at river sites and the higher concentrations of TMP in winter compared to summer.

A third factor that could explain increased prevalence of *sul1* in winter compared to summer was the record rainfall across the UK and Ireland in the Winter of 2015/2016. In fact, in England and Wales, it was the eighth wettest winter on record since 1766 (McCarthy et al, 2016). Previous studies have indicated that increased rainfall can increase the prevalence of antibiotic resistance genes in rivers (Pallares-Vega et al, 2019 and Di Cesare et al, 2017). HGT can be enhanced under altered conditions and when altering bacterial populations come into contact in spatially limited environments (Di Cesare et al, 2017). Soils are generally inhabited by larger and more diverse microbial communities

466 than aquatic environments and their chemical composition provides bacteria with substrates that
467 enhance biofilm formation. Multispecies biofilms can confer antibiotic resistance in these
468 environments. As a result of increased rainfall, soil biofilms can be dislodged, fractioned into
469 aggregates and become suspended in water systems (Stoodley et al, 2002). This would explain the
470 increase in *sul1* in winter compared to summer.
471
472 One site in this study; Coln2 showed a showed significantly increased prevalence of *sul2* in summer
473 despite having minimal concentrations of TMP and SMX, which contrasted with its low *sul1* prevalence.
474 *Sul2* is a sulfonamide resistant dihydropteroate synthase of gram-negative bacteria, generally found on
475 small plasmids, whereas *sul1* is generally found linked to other resistance genes on CL1s (Antunes et al,
476 2005; Jia et al, 2017). According to the National Office of Animal Health (NOAH), there are two different
477 sulfonamide classes in addition to SMX that can be prescribed in combination with TMP to treat
478 infections in farm animals: sulfadiazine (SDZ) and sulfadoxine (SX) (NOAH, 2020). TMP-SDZ is a short-
479 acting bacteriostatic antibiotic that is typically applied to treat infections in horses (Ensink et al, 2003).
480 TMP-SX is a bacteriostatic antibiotic typically used to treat respiratory tract infections in cattle (Dunkley,
481 1994). Previous studies have noted increased abundance of the *sul2* in agricultural samples containing
482 SDZ (Jechalke et al, 2012; Kopmann et al, 2012). As a result, is possible that the increased prevalence of
483 *sul2* shown at Coln2 in spite of the low SMX and TMP concentrations is driven by an agriculturally
484 relevant sulfonamide such as SDZ, which was not targeted by the chemical analysis of this study. This is
485 further emphasised by the fact that there was no relationship between *sul2* and *intI1* prevalence in this
486 study, as this shows that *sul2* genes are not emerging on CL1s alongside clinically relevant ARGs.
487 However, it must be acknowledged that the available UK antibiotic surveillance data for veterinary
488 applications only shows data for 'sulfonamides' as a general class, as opposed to distinguishing between
489 individual drugs such as SMX and SDZ (Bos et al, 2019). As a result, it is difficult to determine the actual
490 levels of SDZ that are being applied in agriculture. Also, despite the isolated high levels of *sul2* at Coln2,

this study showed that across all 20 sites on the Thames catchment, *sul1* was significantly more prevalent than *sul2.*

The fact that SMX and TMP can be co-prescribed potentially explains the correlative relationship between TMP residues and the prevalence of the *sul1* gene in our previous work (Holden et al, 2020), as SMX and TMP have been shown to be released in human waste and passed onto WWTPs (Nagulapally et al, 2009). This relationship is further demonstrated in this study by the fact that the river sites with the highest *sul1* gene coverage across the Thames catchment were also the sites which showed the highest TMP residue concentrations. However, antibiotic surveillance data suggests that TMP is predominantly prescribed as a single dose antibiotic for clinical purposes (Ashiru-Oredope et al, 2014). As a result, it is possible that the presence of TMP alone is increasing the prevalence of *sul1*, as *sul1* becomes incorporated into CL1s (Adelowo et al, 2018). Whilst it is possible that *sul1* genes from this study are carried by CL1s, data from this study shows that the prevalence of *intI1* did not vary significantly by season or by the concentration of TMP as *sul1* did. This shows that CL1s are not necessarily a 'proxy' for ARG load, which has been suggested in previous reports (Amos et al, 2018; Frank et al, 2007). CL1s which have been shown to confer resistance to a number of antibiotics, including SMX, TMP, amoxicillin (AMX), tetracycline (TCN) and erythromycin (ERY) (Langata et al, 2019; Ndi and Barton, 2011; Callejas et al, 2019). Our previous data showed no significant correlations between TCN and ERY concentrations with *sul1* prevalence, but we do not have chemical data available relating to AMX (Holden et al, 2020). It is also possible that concentrations of AMX are present at these river sites and they are also able to select for CL1s and therefore *sul1*. This effect has been noted in a previous study (Ironmonger et al, 2018).

The four sites with the highest TMP concentrations in this study were all located closest to WWTPs. Studies have noted the presence of TMP in raw waste-water at WWTPs, so the fact that TMP is

detected at river sites in this study suggests that the treatment process is not removing TMP before it is released in sewage effluent (Dan et al, 2013; Tran et al, 2018; Thiebault et al, 2019). At the four sites with the highest TMP concentrations, an increased prevalence of *sul1* was also shown. This is in spite of the fact that the prescribed dose of TMP/SMX for treating recurrent UTIs in adults is 200mg daily (NICE, 2020) and the concentration of TMP the highest concentration recorded in this study was 3000ng/L. As a result, it is possible that TMP concentrations that fall several-times below prescribed doses are able to drive positive selection for *sul1*. Sub-Minimum Inhibitory Concentration (MIC) doses of antibiotic residue driving selection for AMR genes has been indicated previously (Ter Kuile et al, 2016; Winstrand-Yuen et al, 2018; Opatowski et al, 2010). Bacterial exposure to antibiotics in any environment leads to resistance via three central mechanisms: (i) mutations at the DNA level (Händel et al, 2014) (ii) physiological adaptation (Händel et al, 2013) and (iii) HGT of genes that confer resistance (Davies and Davies, 2010). Sub-MIC levels have been shown to select for existing AMR, cause susceptible microbes to survive/adapt and also acquire sufficient resistance to survive subsequent clinical doses. Sub-MIC concentrations allow bacteria to induce protective mechanisms, such as the SOS response that enhances the mutation rate and therefore the chance that specific AMR mutations occur (Baharoglu and Mazel, 2014 & Long et al, 2016). As a result, it is possible that the TMP concentrations in this study are capable of selecting for ARGs such as *sul1*.

Conclusions

The average concentration of TMP shown across twenty sites on the Thames catchment was greater than the concentration of SMX. Nineteen of the twenty sites on the Thames catchment showed higher TMP concentrations than SMX concentrations, with the exception of Thame7. Of the twenty sites across the catchment, four of the sites showed particularly elevated concentrations of TMP. For three out of four sites with the highest TMP, TMP concentrations recorded were significantly higher in

winter compared to summer. However, limitations in the data described earlier mean that the variation in TMP levels might be a temporal effect, as opposed to a complete seasonal effect.

The population equivalence (PE) of four sites with the highest TMP concentrations was significantly higher than the PE for the four sites with the lowest TMP concentrations across twenty sites.

The land coverage of the 4 sites with the highest TMP concentrations was significantly more urban/suburban than those with the lowest TMP across twenty sites. By contrast, the land coverage of the 4 sites with the lowest TMP concentrations was significantly more arable than those with the highest.

The prevalence of the *sul1* gene across the twenty sites was significantly higher in winter compared to summer. By contrast, the prevalence of *sul2* at one particular site was significantly higher in summer compared to winter. However, no significant seasonal variation was shown any of the other genes relevant to TMP/SMX or class 1 integrons.

Future Work

Our previous work has shown evidence of a significant positive correlation between the concentration of TMP and the prevalence of *sul1* at sites across the Thames catchment (Holden et al, 2020). Data from this study pinpoints the sites with the highest concentrations of TMP and the highest prevalence of *sul1.* We have also shown that sites with the highest TMP concentrations and highest *sul1* prevalence are generally located closer to WWTPs than those with low TMP concentrations. Furthermore, sites with the highest TMP concentrations have a higher PE, are more urban/suburban and that their TMP concentration/*sul1* prevalence is higher in winter compared to summer. Despite this, there is still no definitive evidence of a causative impact of TMP concentrations on the prevalence of *sul1*. Future work will involve looking specifically at samples collected from a site of

interest highlighted in this study, in order to look at the impact that TMP concentrations at ng/L concentrations have upon *sul1* prevalence in a controlled experimental system.

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