Water Fingerprinting for community disease surveillance (Alternative Format Thesis)

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Water Fingerprinting for community disease surveillance

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Abstract

Water fingerprinting is an innovative surveillance tool, that can provide key data on both the environment and human health. It has significant relevance for research in the One Health domain, which is based on the concept that human health is closely entwined with animal and environmental health. In this thesis, wastewater epidemiology (WBE) was explored as a route for assessing many aspects of public health, including infectious disease spread and monitoring use of antimicrobials.

The next section of the thesis explored developing novel analytical methods for WBE, focusing on oxidative stress biomarkers as a biomarker for general public health. Liquid chromatography coupled to mass spectrometry was utilised to explore four endogenous biomarkers of public health in wastewater. Application of the developed method resulted in, for the first time, HNE-MA being successfully observed and quantified within wastewater over a study period of a week (displayed average daily loads per capita of 48.9 ± 4.1 mg//day/1000inh). This highlighted that HNE-MA could be used as a potential oxidative stress biomarker in future urban water fingerprinting studies.

The next two chapters explored the potential for WBE to be utilised for AMR surveillance. A longitudinal study of two urban catchment areas (one city and one small town) for a range of antimicrobials and their metabolites were investigated in this study. In total 17 parent antimicrobials and 8 metabolites were consistently quantifiable in the wastewater of both catchment areas across the 13-month period. ARGs levels in wastewater were also explored in the city of Bath. Data triangulation was undertaken to explore relationships between antimicrobial agents and corresponding ARGs. Results demonstrated that *ermB, sul1* and *intI1* observed no statistically significant loads in winter versus summer. In the second part of this study, correction factors were applied to antimicrobials to back-calculate consumption at the community level and compared with prescription data. This work has demonstrated the potential for WBE to be used to establish baselines for antimicrobial usage in communities, providing community-wide surveillance and evidence for informing public health interventions.

Finally, this thesis explored the environmental aspect of water fingerprinting, investigating river water sampling for global monitoring campaigns. This study explored use of a new integrated powerless, in-situ multi-mode extraction (iMME) sampler, with the aim of maintaining the integrity of a diverse range of >100 CECs via immobilization to polymeric and glass fibre materials, without access to a power supply or cold chain.

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Abbreviations

AA (Antimicrobial agent)

AB (Antibiotic)

BOD (Biological oxygen demand)

COD (Chemical oxygen demand)

DNA (Deoxyribonucleic acid)

DOC (Dissolved organic carbon)

dPCR (Digital polymerase chain reaction)

FA (Formic acid)

GLASS (Global antimicrobial resistance surveillance system (GLASS)

HDPE (High-density polyethylene)

IDL (instrumental detection limit)

IQL (instrumental quantification limit)

IS (labelled internal standard

LC-MS (Liquid chromatography coupled with mass spectrometry)

MDL (Method detection limit)

MeOH (Methanol)

MQL (Method quantification limit)

MS (Mass spectrometry)

NPS (Novel psychoactive substances)

PNDL

PNDP

PNEC (Predicted no effect concentration)

RNA (Ribonucleic acid)

SAPG (Scotland's Antimicrobial Prescription Group)

SARS-CoVID-2 (Severe acute respiratory syndrome coronavirus)

SD (Standard deviation)

SPE (Solid phase extraction)

TOC (Total organic carbon)

TSS (Total suspended solids)

UPLC-MS/MS (Ultra performance liquid chromatography coupled with tandem mass spectrometry)

WBE (Wastewater based epidemiology)

WHO (World Health Organisation)

WQI (Water quality indicator)

WW (Wastewater)

WWTP (Wastewater treatment plant)

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1 Overview and objectives of the thesis

1.1 Overview

The world is encountering multiple and urgent health challenges. Medical breakthroughs including the discovery and wide-spread dissemination of antimicrobials and development of novel vaccines have elevated society's ability to prevent and treat infectious diseases. Yet considerable threats and challenges still exist in modern medicine. The emergence and re-emergence of infectious diseases and accelerated rates of antimicrobial resistance are well-recognised public health threats. Alarming trends in the prevalence of non-communicable diseases have also been reported, with global rises in rates of cardiovascular diseases and diabetes. The novel coronavirus pandemic has had wide reaching and unprecedented impacts globally and has bought to the forefront questions on societies ability to respond rapidly to health threats and challenges. Furthermore, the changing landscape of the current world, with rapid and uncontrolled urbanisation, political strife and climate change further threaten public health and are recognised key drivers in the exacerbation of many diseases.

It is well-recognised that public health surveillance is critical for the prevention, intervention and control for both communicable and non-communicable diseases. Yet whilst current methods for public health surveillance exist, they are often lacking. They are often based on a small fraction of the population and not timely enough to respond to rapid changes. An important aspect of public health surveillance that is challenging for current systems is consideration of the environment. Public and environmental health are inherently linked, with individuals exposed to a diverse number of chemicals every day, with chronic long-term exposure effects not understood. The ability to monitor both the health of the environment and public health can allow exposure and effect to be explored as well as building an understanding of the anthropogenic input into our environment.

This thesis aims to investigate the potential of water fingerprinting as an effective tool for community disease surveillance, and the ability to provide complimentary evidence to current surveillance tools. Briefly, the analysis of chemical or biological markers in various water environments can provide a characteristic fingerprint that can inform on the environment in question. A popular branch of water fingerprinting is wastewater-based epidemiology (WBE), where influent (untreated) wastewater can be considered a pooled urine sample of a community that contributes. Several major advantages of this technique include population-wide data, the ability for timely results and the broad range of markers relating to public health to be investigated. The focus of water fingerprinting studies depends on the water in question, influent wastewater can reveal more about the specific health status of a community whereas surface waters for example, can reveal data about ecosystem health and potential community exposure. This thesis, with a focus on wastewater for infectious diseases and AMR, will set out to establish whether WBE is an effective tool for public health surveillance. To achieve this the following objectives have been established:

1.2 Objective 1

To give a critical perspective into how infectious disease surveillance is currently achieved and how novel WBE techniques could be utilised to provide complimentary infectious disease and antimicrobial resistance (AMR) data. To discuss recent advancements of WBE and to identify key gaps and challenges in current research. Finally, to propose recommendations into areas of work needing focus to apply WBE wide-scale for both infectious disease and AMR surveillance.

1.3 Objective 2

To establish a broader overview of WBE and how it could be used to support public health in the wake of the novel coronavirus (COVID-19) pandemic. This report will ascertain what has already been successfully applied in the field of WBE and how this has informed regional, national and international policy and public health making. To also identify active and developing areas of WBE, and how this could feed into current and future public health and policy issues.

1.4 Objective 3

To explore new analytical methodologies for novel biomarkers of health in wastewater, focusing on endogenous biomarkers (biomarkers formed in the body as a response to biological processes). Specifically, biomarkers linked to oxidative stress and inflammation, biomarkers more broadly associated with many diseases and lifestyle choices.

1.5 Objective 4

To explore the use of WBE for AMR surveillance and understand the relationships between AAs and ARGs. This will be achieved by a longitudinal study in two different catchment areas to investigate spatial differences and temporal trends of AAs in wastewater, and what impact, if any, has on corresponding ARGs. Novel analytical techniques will be utilised to explore AA and ARG presence in wastewater.

1.6 Objective 5

A case study to explore WBE as a tool for assessing AA consumption at the community level and whether variation in usages at the prescription level can be reflected in a community's wastewater. To investigate relationships between parent AAs and metabolites in wastewater to build baseline ratios and to determine any cases direct disposal of pharmaceuticals.

1.7 Objective 6

To investigate novel and cost-effective in-situ sampling methodologies for sampling rivers in under-resourced areas, and to explore limitations of analyte stability during sampling and transport. This is relevant for global sampling campaigns, where sampling in remote locations poses challenges with a lack of access to the cold chain for shipping and storage.

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2 Future perspectives of wastewater-based epidemiology: monitoring infectious disease spread and resistance to the community level

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2.1 Abstract

Infectious diseases are acknowledged as one of the most critical threats to global public health today. Climate change, unprecedented population growth with accelerated rates of antimicrobial resistance, have resulted in both the emergence of novel pathogenic organisms and the re-emergence of infections that were once controlled. The consequences have led to an increased vulnerability to infectious diseases globally. The ability to rapidly monitor the spread of diseases is key for prevention, intervention and control, however several limitations exist for current surveillance systems and the capacity to cope with the rapid population growth and environmental changes. Wastewater-Based Epidemiology (WBE) is a new epidemiology tool that has potential to act as a complementary approach for current infectious disease surveillance systems and an early warning system for disease outbreaks. WBE postulates that through the analysis of population pooled wastewater, infectious disease and resistance spread, the emergence of new disease outbreak to the community level can be monitored comprehensively and in real-time. This manuscript provides critical overview of current infectious disease surveillance status, as well as it introduces WBE and its recent advancements. It also provides recommendations for further development required for WBE application as an effective tool for infectious disease surveillance.

Key Words. Wastewater-based epidemiology, wastewater fingerprinting, infectious diseases, antimicrobial-resistance, public health

2.2 Introduction

Even with the advancement of infectious disease surveillance over the last century, communicable diseases still pose significant risks to public health. On the World Health Organisations (WHO) top 10 threats to global health in 2019, four are directly on infectious diseases: pandemic influenza, HIV, dengue and another for high-threat pathogens such as Ebola (World Health Organisation, 2019). Emerging infectious diseases caused by novel pathogenic organisms are of notable concern, it was highlighted by WHO that since the 1970s, over 1,500 new pathogens were discovered and nearly 40 new infectious diseases have been identified (World Health Organisation, 2018b). Many of these have

severely impacted communities, with several major outbreaks occurring within the last 20 years including severe acute respiratory syndrome (SARS) (2002-2003), Ebola (2014–2016), H1N1flu/swine flu (2009-2010), Zika virus (2015-2016) and COVID-19 (2019-2020) (World Health Organisation, 2020a). Two others on this list are regarding the prevention and treatment of infectious disease, one being hesitation to vaccinate and the other the rise in antimicrobial resistance (AMR), both have been linked to the re-emergence of communicable diseases.

There are a number of drivers affecting the emergence and re-emergence of infectious diseases (Woolhouse and Gowtage-Sequeria, 2005). These range from climate change, poverty and unprecedented population increases with uncontrolled urbanisation. Another driver is globalisation linked with tourism and trade, resulting in a strong network of air links. With regards to international flights it has been highlighted that the incubation period of any human disease is still longer than the lengthiest aviation time for any international flight (Frenk and Gómez-Dantés, 2002). Outbreaks are therefore not confined to one geographic location but are less than 24 hours away from being a threat somewhere else.

Another key factor for the re-emergence of infectious diseases has been linked with drug resistant pathogens. Whilst microbial evolution happens naturally, inappropriate usage of antimicrobials puts additional selective pressures and further facilitates rates of resistance (Allen *et al.*, 2010; Andersson and Hughes, 2014). Whilst antibiotics tends to be focused on in discussions of AMR, rising cases of both fungal and viral resistances still pose significant threats (Fisher *et al.*, 2018). For example, *Candida auris*, an emerging multidrug resistant yeast, is a cause of major hospital acquired infection with high associated mortality, having only first been identified in 2009 it has resistance to all clinically available antifungals (Lockhart *et al.*, 2017).

The rising rate of resistance has resulted in AMR being hailed as one of the biggest public health risks threatening medicine in the 21st century (O'Neill, 2014). Increasing concerns of AMR have led to the establishment of the Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 by WHO with the aims of sharing information on a global scale to strengthen data and aid decision making on national and international actions (World Health Organisation, 2015b). Whilst the recent report (2017-2018) has revealed detailed results with participation from over 60 countries, several limitations in the study were discussed (World Health Organisation, 2018a). It was recognized that there was a lack of sampling strategy leading to selection bias, also patient samples are typically taken from those that have sought out medical care and hence might not be representative for a population. It was further highlighted the need to move away from laboratory data to include epidemiological and population data.

2.3 Current Infection Disease Surveillance Techniques and their Limitations

Threats of (re)emerging infectious diseases along with rising rates of AMR reinforce that infectious disease surveillance is still an integral component of public health today. This has given rise to the ability to monitor spatial and temporal trends of diseases.

2.3.1 Disease monitoring

There are several techniques with a range of advantages and disadvantages currently used for infectious disease surveillance (Table 1). Disease monitoring (which is often disease specific), can vary significantly with country and will depend upon the resources and sophistication of the public health services and facilities available (Thacker *et al.*, 2006). The information collected can be provided to WHO, who have the authority to lead the global surveillance of infectious diseases. WHO have had an integral role in infectious disease surveillance, as well as leading international surveillance networks, e.g. influenza surveillance. They also provide international coordination of epidemic responses in diseases that pose significant public health risks. Examples of conventional routes to monitoring diseases are based upon existing resources, such as mortality and morbidity rates, prescription and hospital admission data. Whilst these are valuable source of information for surveillance purposes, they do suffer from bias, resource insensitivity and costs (Bauer, 2008).

Table 1. Routes to assessing public health and infectious disease surveillance techniques with advantages and disadvantages

Technique	Examples	Advantages	Disadvantages	References
Sentinel Surveillance	GP's reporting cases of influenza	Making use of an efficient system that is already in place Increase communication within communities Can help detecting larger health problems in a population	Rare and novel microbes occurrences are likely to be missed, e.g. new emerging virus Often focus on specific diseases	(Lee *et al.*, 2010)
Lab-based surveillance		Increased knowledge transfer between epidemiologists and microbiology laboratories Detailed information found on specific details of microbe e.g. virulence	Requires significant facilities, resources, trained staff and good communication links. Central reference laboratory is needed for standardisation and support If pathogens are rare, can lead to staff being complacent Selection bias on which samples are sent to the laboratory	(Choi, 2012)
Questionnaires or surveys	Recurrent or cross-sectional surveys	Can collect data for multiple diseases or exposures at one time Capability for local, national or international level Standardised methods utilised and high quality data often obtained Flexibility in questions asked Build up trends if survey is done repeatedly	Bias More information about public health Expensive – costs will vary on sample size, time period of survey Time delay to results If optional might not get a good response – might not be representative of whole populations Results can be difficult to interpret	(Thacker and Berkelman, 1988)
Search engine trends	Google Flu Trends (http://google.com/trends/)	Rapid obtainment of results Effective for large populations of web users Potential to track epidemics or diseases with high prevalence in a population	Difficult to determine if individuals searching are having symptoms or googling as concerned or to find out more Requires internet access, not as suitable for developing countries Differences in language backgrounds can lead to different words to describe symptoms being googled Diseases with low prevalence won't spike enough to notice	(Carneiro and Mylonakis, 2009)
Mortality and morbidity rates	Deaths recorded for diseases like Ebola or influenza	Inexpensive and well-established system of reporting Death certificates are legally required in most countries Can aid in monitoring the progression of an epidemic	If deaths from a particular cause are too low, mortality statistics potentially don't reflect accurate incidence of the disease Long delays in getting results Significant variation into how death certificates are filled Passive form of surveillance	(Choi, 2012)
Hospital admission data	ED-based surveillance for The Emerging Infectious Disease Surveillance Network	Can provide data on severity of injury, new emerging infectious disease and drug abuse Help identify if changes in healthcare are needed Potential early flagging of bioterrorism attack	Significant human and resource investment for setting up system and connecting with public health system Confidentiality challenges in sharing information to public health agencies Compliance of often busy emergency department staff to fill in data Need to standardise data collection	(Hirshon, 2000)

Many of the current systems in place are forms of passive surveillance that have disadvantages. For example, in countries with less developed health services, morbidity might be higher than assumed due to people failing to report to a healthcare service due to lack of access. This combined with the fact that sometimes in epidemics the laboratory facilities can become easily overwhelmed – the consequence of such is that these cases are not reported. The 2009 swine flu epidemic caused by a H1N1 influenza virus spread rapidly to more than 214 countries in the space of a few months. Whilst it was estimated that several million people were infected with over 18,400 confirmed laboratory deaths worldwide reported by August 2010 (World Health Organisation, 2010b), it is believed that this is a gross underestimation. Reported studies in the literature have estimated through modelling techniques that there could have been as many as 10-15 times this amount, with up to 203,000 respiratory deaths (Dawood *et al.*, 2012; Simonsen *et al.*, 2013). Dawood *et al.* projected around 80 % of these deaths occurring in Southeast Asia and Africa, the causes for underestimation have been attributed to poor reporting due to the overwhelming number of cases.

2.3.2 Infectious disease surveillance in growing urbanised nations.

The problems underlying infectious disease surveillance will only be exacerbated. Current predictions have estimated a global population growth of 26 % from 7.7 billion 2019 to 9.7 billion in 2050, with 68 % of the global population expected to be urban (United Nations Department of Economic and Social Affairs, 2019). With the current unprecedented rises in population size, there will undoubtedly be further challenges (but also opportunities) in rapid health surveillance and response.

Therefore there is a need for a surveillance technique that (i) provides comprehensive and objective data, (ii) gives results in real-time, (iii) is flexible, (iv) able to monitor multiple diseases, even those that are rare, (v) is scalable and cost effective (vi) could be applied in low resource settings. Furthermore, the surveillance system needs to have comprehensive data collection systems regarding emergence of new diseases and re-emergence of old diseases, the threat of imported diseases or pathogens, and the emergence of multidrug or pan-drug resistant organisms. It has also been highlighted in the literature that monitoring clinics and laboratories for informing on public health is not sufficient, and there should also be an aspect of environmental monitoring of potential hazards (Nsubuga *et al.*, 2006). Therefore, a surveillance technique that could also encompass environmental exposure would be invaluable in providing comprehensive exposure status and disease outcomes. A new surveillance technique utilising water fingerprinting is under the development to provide objective and comprehensive evaluation of both public and environmental health status in real-time.

2.4 Water Fingerprinting via Wastewater-Based Epidemiology – a new paradigm in public health assessment

Wastewater-Based Epidemiology (WBE) is a new approach utilised to give comprehensive health information on communities. The concept is primarily based upon the extraction, detection and then subsequent analysis and interpretation of chemical and/or biological compounds. These compounds, often referred to as biomarkers, could be harmful chemicals such as food toxicants and/or specific human excretion products (e.g. metabolites or endogenously formed chemicals as a result of exposure to and/or disease) that can be linked to the community as they are held within geographically defined water catchment areas (watersheds) to which whole populations contribute. Water sources that can be analysed are any that fall within the urban areas' catchment, and can include surface waters, domestic water sources and wastewaters. The results can then be used to give information on the community itself and its health, or environmental exposure. Wastewater is a popular and critical medium used in water fingerprinting. Often referred to as wastewater-based epidemiology (WBE), this technique can give an unbiased reflection on the community's health and lifestyle habits due to the rich source of biological and chemical information it contains (Kasprzyk-Hordern *et al.*, 2014).

2.4.1 Wastewater-based epidemiology (WBE) – the basics

WBE postulates that endogenous and exogenous urinary human biomarkers identified and quantified in wastewater can give a reflection of the population's health in (near)-real time (Figure 1). Wastewater (untreated) is usually collected from wastewater treatment plants (WWTP) as WWTPs serve communities located in well-defined geographical sewerage catchment areas. Usually, one WWTP serves a town or a city. Importantly, as a whole population contributes to wastewater collected by any WWTP, wastewater from this community can be considered as its pooled urine.

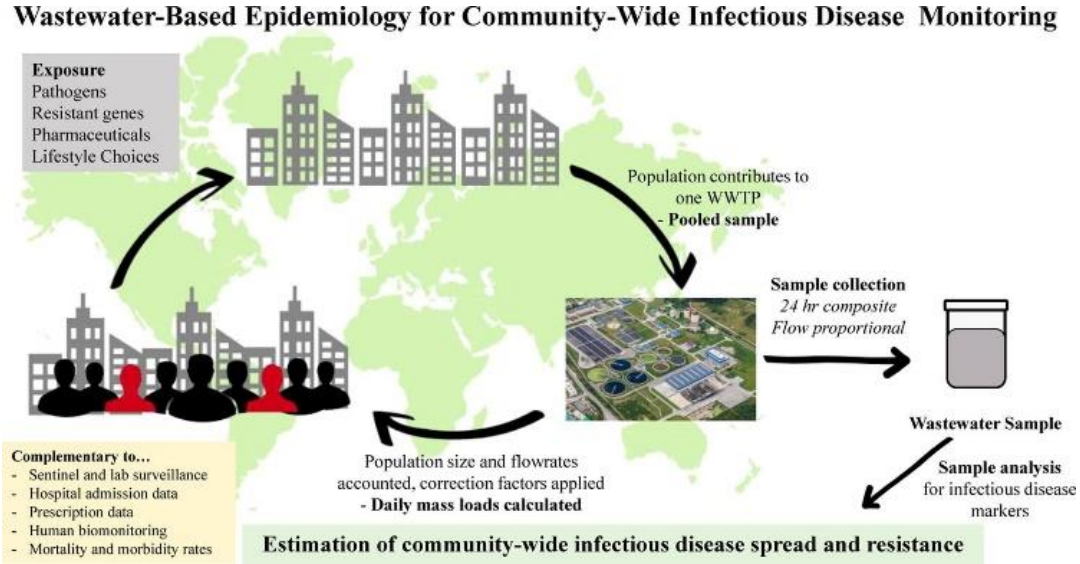

Figure 1. Graphical representation of the wastewater-based epidemiology (WBE) concept

A critical consideration in WBE are wastewater flow rates which are key to account for due to the wide variations in influent flows (e.g. wet weather causing dilution). The consequence is of such that when reporting upon the presence of a compound, it is typical to report as the daily loads in wastewater (mg/day). Furthermore, to normalise and allow comparisons for cities in different geographic locations, with varying population size, the daily loads per capita may be reported instead (mg/day/1000 inhabitants). This process of back calculation of community-wide drug consumption or exposure to chemical factors can provide un-biased reflection of key aspects of public health. For example, the monitoring of pharmaceutical or illicit drugs in wastewater can detect subtle changes in trends in usage and consumption in a community. Furthermore, not only could spatial and temporal trends be established but such data could be monitored in real time, allowing deviations from usual trends to be spotted early. This offers several advantages over biomonitoring techniques which focus on small target groups due to expenses and logistical challenges such as ethical considerations, as WBE is done on a population-wide scale, the anonymity of individuals is maintained. Water fingerprinting can also offer more timely analysis than other traditional based public health approaches. This would allow public services to respond more rapidly and potential health interventions to be employed.

2.4.2 WBE and international collaboration

The field of WBE is a rapidly growing one and has experienced enormous successes since the idea was first conceived by Daughton in 2001 who hypothesised that the analysis of drug residues in wastewater could be linked back to population usage (Daughton, 2001). This was then first achieved in 2005 by Zuccato who successfully extracted and quantified cocaine in both wastewater and surface water and to investigate cocaine usage in the community (Zuccato *et al.*, 2005).

A large number of international long-term monitoring initiative have since been established worldwide with the most active networks in Europe (Thomas *et al.*, 2012; European Monitoring Centre for Drugs and Drug Addiction, 2016), Australia (Lai *et al.*, 2016, 2018; Tscharke *et al.*, 2016; Choi *et al.*, 2019; O'Brien, Grant, *et al.*, 2019) and in the USA (Halden *et al.*, 2019). The successes of WBE that have been demonstrated on global scales have given rise to discussions on future outlooks for the technique (Thomas and Reid, 2011; Kasprzyk-Hordern *et al.*, 2014; Choi, Tscharke, *et al.*, 2018; Daughton, 2018). Initially, work was entirely focused upon illicit drug usage, including heroin, cocaine and methamphetamines (Castiglioni *et al.*, 2006; Boleda, Galceran and Ventura, 2007; Kasprzyk-Hordern, Dinsdale and Guwy, 2008; Zuccato *et al.*, 2008) but have since expanded to include a diverse range of other endogenous biomarkers, varying from ones linked to lifestyle choices such as alcohol consumption (Reid *et al.*, 2011; Rodríguez-Álvarez, Rodil, Cela, *et al.*, 2014; Boogaerts *et al.*, 2016), tobacco (Rodríguez-Álvarez, Rodil, Rico, *et al.*, 2014; Castiglioni *et al.*, 2015; Tscharke, White and Gerber, 2016; Lai *et al.*, 2017) and psychoactive substances (Mardal and Meyer, 2014; Reid, Derry and

Thomas, 2014; Kinyua *et al.*, 2015). Others have investigated general health through oxidative stress markers (Ryu, Reid and Thomas, 2015; Ryu *et al.*, 2016; Sims, Rice and Kasprzyk-Hordern, 2019).

Additionally, the analysis of metabolic urinary biomarkers of exposure in wastewater can reveal critical information upon community-wide exposure to external stressors accounted in everyday life. Examples of which can be exposure to chemical compounds such as endocrine disrupters, compounds that are known to effect hormone regulation, but that are typically not regulated (Testai *et al.*, 2013). Chemicals found in personal care products and consumer products, including UV filters in sunscreen, plasticizers, flame retardants and pesticides are suspected or known endocrine disruptors. Frameworks investigating community exposure to such compounds have been developed through analysis of exposure metabolites within wastewater, results of which have already provided comprehensive international population-wide exposure data for pesticides (Rousis *et al.*, 2017), flame retardants (Been *et al.*, 2017, 2018), carcinogens linked to tobacco (Lai *et al.*, 2017), UV filters (Lopardo *et al.*, 2018), mycotoxins (Gracia-Lor *et al.*, 2020) and BPA (Lopardo *et al.*, 2019).

2.5 Challenges of wastewater-based epidemiology

2.5.1 Complexity of wastewater matrix

Whilst conceptually WBE is very simple and clearly offers attractive advantages for the monitoring of public health, there are some challenges to be considered. For example, not only are the levels of biomarkers far more dilute in wastewater, especially in comparison to urine, but the wastewater matrix itself provides a complex environment to work in (Daughton, 2012). As previously mentioned, wastewater contains a diverse abundance of chemical and biological targets which can give incredibly detailed information about the population that contributes. However, a drawback to having such a large amount of information is in the successful extraction from the matrix itself and the subsequent analysis of specific targets can be difficult. Extraction methods such as solid phase extraction and immunoassay techniques along with sophisticated analytical tools such as advanced mass spectrometry have allowed for the analysis of a wide number of compounds (Petrie, Barden and Kasprzyk-Hordern, 2015). Recent developments in sensing approaches could enable measurements on site, which would allow the system to provide information on public health in real time (Yang, Kasprzyk-Hordern, *et al.*, 2015; Yang *et al.*, 2016, 2017).

2.5.2 Estimation of population size

Another challenge associated with WBE is the problem posed by dynamic populations (e.g. population fluctuations due to tourism and commuters) (Ort *et al.*, 2014). Typically the standard approach is for levels of certain endogenous biomarkers in humans, such as cortisol or cotinine, to be calculated as daily loads which have been normalised to the population. This enables inter-city comparison (Chen *et*

al., 2014). However, there are difficulties in estimating the population size of individual WWTP catchments. This can result in unaccounted for, unique population fluctuations that, whilst having negligible impact on the levels of biomarkers in large populations (>100,000 people), they might contribute to higher uncertainties in smaller populations.

There are several techniques that can be employed to reduce to the source of uncertainties associated with population size. Investigating certain hydrochemical parameters which have well-established methods of analysis, such as chemical oxygen demand (COD), biological oxygen demand (BOD) or ammonium (NH_4^+) can aid in estimating populations contributing to a WWTP catchment at a particular time period (van Nuijs *et al.*, 2011; Been *et al.*, 2014). These however can be influenced by the composition of wastewater. The other uncertainties associated with the technique briefly mentioned above with regards to sample collection and analytical variability amongst a couple of others, have all been extensively discussed in a number of reviews (Ort, Lawrence, Rieckermann, *et al.*, 2010; Castiglioni *et al.*, 2013; Ort *et al.*, 2014). However, SCORE and the EMDCCA have demonstrated that with recognition of the limitations of the technique, that the development and adoption of a reliable, standardised method will give reliability and credibility to the studies and allow spatial and temporal comparisons to be made.

Uncertainties within population size will also pose problems for infectious disease surveillance within wastewater, as the presence of tourists or commuters within a catchment area could make it challenging to monitor the actual emergence of an infection within that community For example it would be impossible to distinguish whether the presence of a virus in wastewater had stemmed from a visitor(s) passing though or from within the community itself. Arguably however, the presence of a virus in wastewater, whether from a resident in the catchment area or not, still provides critical information as members within the population may have been unknowingly exposed to the infected individual. This could indicate towards potential disease emergence within the community, allowing valuable time for appropriate preparation and response to be put into place.

3.5.3 Desirable Characteristics in Biomarkers

Endogenous and exogenous biomarkers in wastewater, when chosen carefully, can give key information with regards to health of a population. Along with some of the limitations of WBE touched upon above, there are also certain criteria that must be fulfilled for a biomarker to be considered in WBE techniques. For example, the biomarker in question must mostly be excreted via urine and concentrations of the biomarker must be in ng L^{-1} for downstream detection of the biomarker in wastewater (Chen *et al.*, 2014). Another vital characteristic is that the biomarker needs to be stable, not only in the sewage system but also during the process of sampling and storage (McCall *et al.*, 2016). Biomarkers also need to be unique to human metabolism and ideally the metabolism process involving the biomarker would be

well understood. This ensures that the biomarker in question has only originated from human sources, as opposed to exogenous ones (potential contamination of animals in the sewage system or from microbes present in wastewater) (Daughton, 2012). With regards to the sewage system, wastewater is home to an extensive range of complex microbial communities that are challenging to characterise and will vary geographically. As of such, there is a high risk of microbial degradation or transformation of chemical compounds. In fact, biological treatment in wastewater treatment plants, such as trickle bed filters, are home to these diverse microbial communities which play a key role for the breakdown of many organic compounds (Kraigher *et al.*, 2008).

2.6 Water fingerprinting for community-wide infectious disease diagnostics

WBE has already demonstrated successes in monitoring drug consumption, lifestyle choices and population-wide exposure. Several studies have discussed the future of WBE and the expansion to include biomarkers linked to other aspects of public health, including diet, stress, and biological based monitoring linked with illness (Gracia-Lor *et al.*, 2017; Choi, Tscharke, *et al.*, 2018; Daughton, 2018). Due to the wide array of endogenous chemical and biological urinary biomarkers linked with disease, there is clearly huge potential for WBE to be utilised to monitor infectious diseases and the spread of epidemics at the community level (Table 2).

Table 2. Proposed key biomarkers for use in WBE to monitor spread of infectious diseases to the community level.

Biomarker Groups	**Biomarker Examples**	**Treatment/indicator of**	**Reported Concentrations**	**Reference**
Biomarkers of intervention	**Antibiotics**			
e.g. Drugs and metabolites	Sulfamethoxazole *n-Acetyl sulfamethoxazole*	Urinary tract infections, bronchitis	<3 – 3100 ng/L (INF) 360 ± 110 ng/L (INF)	(Kasprzyk-Hordern, Dinsdale and Guwy, 2009; Hijosa-Valsero *et al.*, 2011; Guerra *et al.*, 2014)
	Azithromycin n-*Demethyl azithromycin*	Pneumonia. middle ear infections, strep throat and intestinal infectios	269- 22,730 ng/L (INF) <30-74 ng/L (INF)	(Senta *et al.*, 2019)
	Clarithromycin n- *Demethyl clarithromycin*	Pneumonia, skin infections, H. pylori infection, and Lyme disease.	111- 10,491 ng/L (INF) 13-1559 ng/L (INF)	(Senta *et al.*, 2019)
	Ciprofloxacin	Respiratory tract infections, skin infections, gastroenteritis	17-2500 ng/L (INF)	(Guerra *et al.*, 2014)
	Erythromycin	Respiratory tract infections	14 – 10,025 ng/L (INF)	(Kasprzyk-Hordern, Dinsdale and Guwy, 2009; Guerra *et al.*, 2014)
	Trimethoprim	Urinary tract infections	464–6796 ng/L (INF)	(Roberts and Thomas, 2006; Kasprzyk-Hordern, Dinsdale and Guwy, 2009)
	Antivirals			
	Oseltamivir phosphate *Oseltamivir carboxylate*	Flu virus (influenza)	5–529 ng/L (INF) 28–1213 ng/L (INF)	(Leknes, Sturtzel and Dye, 2012; Takanami *et al.*, 2012)
	Acyclovir *Carboxy-acyclovir*	Herpes simplex virus infections, chicken pox, shingles	1780 ng/L (INF) 490 - 3420 ng/L (INF)	(Prasse *et al.*, 2010; Funke, Prasse and Ternes, 2016)
	Emtricitabine *Carboxy-emtricitabine*	HIV	100 – 980 ng/L (INF) 24- 250 ng/L (INF)	(Funke, Prasse and Ternes, 2016)

	Lamivudine, *Carboxy lamivudine*	HIV/AIDs, hepatitis B	52 - 720 ng/L (INF) 25-84 ng/L (INF)	(Prasse *et al.*, 2010; Funke, Prasse and Ternes, 2016)
	Abacavir *Carboxy-abacavir*	HIV/AIDs	21- 140 ng/L (INF) 41 -560 ng/L (INF)	(Funke, Prasse and Ternes, 2016)
	Zanamivir	Flu virus (influenza)	16.3-27.8 ng/L (INF)	(Takanami *et al.*, 2012)
	Zidovudine	HIV/AIDs	310 - 380 ng/L (INF)	(Prasse *et al.*, 2010)
	Nevirapine	HIV/AIDs	4.8 - 21.8 ng/L (INF)	(Prasse *et al.*, 2010)
	Antifungals			
	Ketaconcazole	Skin infections	16 ng/L(INF)	(Huang *et al.*, 2010)
	Miconazole	Skin infections	5.2 – 1583 ng/L (INF)	(Kasprzyk-Hordern, Dinsdale and Guwy, 2009; Huang *et al.*, 2010; Guerra *et al.*, 2014)
	Clotrimazole	Skin and vaginal infections	23-33 ng/L (INF)	(Roberts and Thomas, 2006; Huang *et al.*, 2010)
	Painkillers			
	Acetaminophen	Painkiller	5529-500,000 ng/L(INF)	(Roberts and Thomas, 2006; Guerra *et al.*, 2014)
	Ibuprofen	Painkiller	968-45,000 ng/L(INF)	(Roberts and Thomas, 2006; Kasprzyk-Hordern, Dinsdale and Guwy, 2009; Guerra *et al.*, 2014)
Biochemical markers linked with physiological response	C-reactive protein (CRP)	Inflammation	0.54-2.76 μg/mL (Urine)	(Stuveling *et al.*, 2003)
e.g. Biomarkers of inflammation	Interlukin-6 (IL-6)	Inflammation in urinary tract infections	1.6-5.28 pg/mL(Urine)	(Roilides *et al.*, 1999; Renata *et al.*, 2013)

	Interlukin-8 (IL-8)	Inflammation in urinary tract infections	7-12 pg/mL (Urine)	(Taha, 2003)
	Lipoarabinomannan (LAM)	Potential indicator of tuberculosis in HIV infected patients	15 pg/mL to several hundred ng/mL(Urine)	(Boehme *et al.*, 2005; Savolainen *et al.*, 2013; Hamasur *et al.*, 2015)
	IP-10	Potential indicator of tuberculosis and pneumonia	5-110 pg/mL (Urine)	(Cannas *et al.*, 2010; Kim *et al.*, 2018)
Pathogenic organisms	**Bacterial DNA**			
e.g. Pathogenic genetic material/ DNA/RNA	*Klebsiella pneumoniae*	Pneumonia, UTI, bacteremia and endophthalmitis	6.31-6.56 log gene copies/100mL (INF)	(Shannon *et al.*, 2007)
	Pseudomonas aeruginosa	Pneumonia, UTI, gastrointestinal infections	4.31-4.38 log gene copies/100 mL (INF)	(Shannon *et al.*, 2007)
	Enterococcus faecalis	UTIs, bacteremia, septicemia	4.66-4.85 log gene copies/100 mL (INF)	(Shannon *et al.*, 2007)
	Viral DNA/RNA			
	Norovirus (GI)	Gastroenteritis	<10-3500 viral genomes/L (INF)	(Hellmér *et al.*, 2014)
	Norovirus (GII)	Gastroenteritis	12.4×10^3-320×10^3 viral genomes/L (INF)	(Hellmér *et al.*, 2014)
	Influenza A	Respiratory infection	2.6 × 105 genome copies/L (INF)	(Heijnen and Medema, 2011)
	Dengue	Severe flu-like illness	4-5 x 10-1 PFU/mL(Urine)	(Poloni *et al.*, 2010)
	Zika	Mild infection, microcephaly	0.7–220.106 copies/mL (Urine)	(Gourinat *et al.*, 2015)
	Hepatitis A	Liver infection	<10-1500 viral genomes/L (INF)	(Hellmér *et al.*, 2014)
	Severe acute respiratory syndrome (SARS CoV)	Respiratory infection	$<1x10^1$-$10^{6.5}$ (Faeces)	(Poon *et al.*, 2004)
	Fungal DNA			
	Candida species *Aspergillus (Aspergillus fumigatus, Aspergillus niger and Aspergillus flavus)*	Candidiasis Chronic pulmonary aspergillosis, pulmonary and nasal allergies, asthma, pneumonitis	Detected* (INF)	(Assress *et al.*, 2019)

	Parasites			
	Giardia lambli	Small intestine infections	2,653 - 13,408 cysts/litre (INF)	(Guy *et al.*, 2003)
	Cryptosporidium	Gastrointestinal illness	1 - 120 oocysts/litre (INF)	(Wallis *et al.*, 1996)
Biological response *e.g. Antibiotic resistant genes*	*mcr-1*	Colistin resistance	8.11 × 101 cell equivalents/100 ng DNA (INF)	(Hembach *et al.*, 2017)
	mecA	Methicillin resistance	$1x10^{1}$- ~$5x10^{4}$ genes/100 mL(INF)	(Börjesson *et al.*, 2009)
	ermB	Erythromycin resistance	$10^{5.2}$-10^{7} copies/mL(INF)	(Wang *et al.*, 2015)
	sul1	Sulphonamide resistance	$10^{5.46}$–$10^{7.54}$ copies/mL(INF)	(Munir, Wong and Xagoraraki, 2011; Wang *et al.*, 2015)
	bla_{OXA-1}	Beta-lactam resistance	$10^{5.4}$-$10^{7.3}$ copies/mL (INF)	(Wang *et al.*, 2015)
	tetW	Tetracycline resistance	$10^{4.2}$–$10^{7.4}$ copies/mL (INF)	(Munir, Wong and Xagoraraki, 2011; Wang *et al.*, 2015)

INF: Influent wastewater (U): Urine. PFU: Plaque forming units (measure of number of infectious particles).UTI: Urinary tract infection *Via sequencing

WBE could be utilised as a complementary surveillance technique which can give rapid, reliable information on a population that can inform what diseases are present in a community and could aid in monitoring disease outbreaks. It is of paramount importance to choose a wide-ranging panel of markers providing information on (i) pathogenic organisms (bacteria and viruses), (ii) biochemical markers linked with physiological response (endogenous markers e.g. biomarkers of inflammation including small molecules and proteins), (iii) markers of intervention (pharmaceuticals and their metabolites) biological response, (iv) markers of antimicrobial resistance.

2.6.1 Markers of pathogenic organisms

An example of a key **biological marker** are pathogenic DNA/RNA residues from bacteria, viruses and fungi. The detection in influent wastewater would suggest human sources and hence indicate what diseases are circulating within a population. Whilst risk factors for emerging infectious diseases have highlighted resistant bacteria as a concern, viruses pose a significant threat due to their high mutation rates and ability to adapt to new host, e.g. humans. This is particularly in the case of RNA viruses, where higher nucleotide substitution rates can result in this rapid adaption and spreading in new host populations (Woolhouse and Gowtage-Sequeria, 2005). The potential of wastewater to be used for viral surveillance has been discussed in literature (Wigginton, Ye and Ellenberg, 2015; Barras, 2018; O'Brien and Xagoraraki, 2019a). Wastewater surveillance has already demonstrated promising results with the potential for retrospective prediction of disease outbreaks of hepatitis A and norovirus (Hellmér *et al.*, 2014). Influenza in wastewater was also investigated during the H1N1 (swine) flu virus outbreak, whilst influenza A viruses were detected in sewage, the pandemic H1N1 virus however was not detected (Heijnen and Medema, 2011). Furthermore, environmental surveillance of polio in wastewater has been utilised since the 1980s, decades before when the term "wastewater-based epidemiology" was coined, with Finland (Hovi *et al.*, 2012) Israel (Roberts, 2013) and Senegal (Ndiaye, Diop and Diop, 2014) all successfully analysing sewage samples in order to assess polio circulating within populations. WHO have also released guidelines for employing environmental sampling to monitor polio in wastewater samples (World Health Organisation, 2003b).The complexity of a wastewater matrix is not only challenging for the extraction and quantification of chemical compounds, similar problems are apparent for biological biomarkers. Composition of wastewater contains a diverse range of PCR inhibitors including fats, proteins and humic and fulvic acids, which can cause problems later during downstream processing during PCR. The availability of different commercial extraction kits for DNA/RNA has demonstrated sometimes variable efficiencies and consistencies when extracting from PCR inhibitor rich samples, including wastewater and sediments (Mumy and Findlay, 2004; Walden, Carbonero and Zhang, 2017). This results in challenges when making meaningful comparisons across different studies and in establishing spatial and temporal trends. However, advancements of molecular biology techniques offer new routes for the analysis of genetic material, including digital PCR (dPCR) and next

generation sequencing techniques. In dPCR, the absolute quantification of target genes is calculated using Poisson distribution statistics via the partitioning of DNA/RNA samples into tens and thousands of reaction wells. Due to this partitioning effect, PCR inhibitory substances have demonstrated to have less of an effect in environmental samples, including wastewater, when analysed via dPCR (Rački *et al.*, 2014). Critical evaluation of dPCR and its suitability for certain sample types have been discussed by Salipante et al (Salipante and Jerome, 2020).

Next generation sequencing is another promising technology, providing a wealth of information on the complex microbial communities in samples, including identification of the diverse range of pathogens and resistance genes present. In particular, analysis of the viruses present via metagenomics has been highlighted as providing potentially key information on novel pathogens as well as re-emerging infectious diseases and AMR (Fernandez-Cassi *et al.*, 2018; Aarestrup and Woolhouse, 2020). Whilst standardisation of protocols of metagenomics remain a challenge, the continued advancements in the technology combined with decreasing costs sequencing have the potential to revolutionise both pathogen and resistance surveillance in wastewater.

2.6.2 Biochemical markers linked with physiological response

Protein based inflammation biomarkers represent a vital group of endogenous markers. Urine proteomics has attracted much interest in the last decade as has been evidenced to contain an abundant source of proteins. Urine for diagnosis purposes is desirable not only due to the non-invasive nature of testing but because of the previously untapped source of potential disease and health biomarkers (Zhao *et al.*, 2017). Some which could be sensitive to changes in the body and could be early indicators of disease. Whilst only a handful of proteins are currently utilised in clinics, it has been previously highlighted that this is not a limitation for WBE, as purposes here are not for diagnostic analysis (Daughton, 2018). Urinary inflammation biomarkers that are indicative of inflammation include C-reactive proteins (CRP) and interleukins including IL-6 and IL-8, have been highlighted as promising candidates for use in WBE (Rice and Kasprzyk-Hordern, 2019). Urinary CRP levels are routinely utilised in clinics and in human biomonitoring studies, e.g. to investigate renal function abnormalities within a population (Stuveling *et al.*, 2003). Other proteins that have previously been suggested for WBE include vitamin D binding proteins, which are prognostic biomarkers for kidney disease due to their significantly elevated levels occurring in the urine of infected individuals (Daughton, 2018).

Increased interest into urine proteomics for non-invasive clinical tests is a growing area and with it will bring greater understanding of the proteins present in urine. Whilst it is widely considered that proteomics in WBE would offer invaluable new insight into public health of communities, the analysis of proteins in wastewater however is still underexplored (Rice and Kasprzyk-Hordern, 2019). The extraction and analysis of these larger biomolecules from wastewater poses new analytical challenges

due to the complexity of the matrix, and questions regarding stability of proteins in the sewage systems are yet to be investigated.

2.6.3 Markers of pharmacological intervention

Biomarkers of intervention encompass pharmaceuticals used to treat infectious diseases or ones used to lessen the symptoms. As previously mentioned WBE has been successful at monitoring drug usage, and as many infectious diseases are seasonal, there are potentially interesting opportunities for trends to be established in wastewater. Regarding antibiotics, a handful of studies have demonstrated seasonal patterns for several antibiotics, including clarithromycin, erythromycin and ciprofloxacin with higher loads typically observed over winter (Coutu *et al.*, 2013; Golovko *et al.*, 2014). This is in line with the use of these antibiotics for respiratory infections where cases tend to peak in winter-early spring. In areas where prescription data is not widely available or antimicrobial medications can be bought over the counter with ease.

WBE could provide a route for monitoring antimicrobial usage within a community which otherwise might go unobserved. With rising rates of AMR, the importance of understanding consumption habits in a community is critical, one of the major advantages of WBE is the potential to distinguish differences between prescription and consumption of a pharmaceutical. Investigating ratios of parent compounds to respective metabolites or ratios between compound enantiomers in wastewater can inform on whether levels have originated from human excretion or from direct disposal of a pharmaceutical into the sewage system (Petrie *et al.*, 2016). Furthermore the availability of pharmacokinetics data and excretion rates can allow back calculation to the estimated amounts of a pharmaceutical that a population has ingested (Zuccato *et al.*, 2008). This ability to distinguish between prescribed, disposed and consumed is important as just because a pharmaceutical is prescribed does not necessarily mean it is used. Delayed prescribing is a strategy by which a general practitioner (GP) will make a prescription available but will ask the patient to delay from using in order to see if symptoms improve first. The initiative has been evidenced to successfully reduce antibiotic usage in a handful of countries, including New Zealand, Norway and England (Spurling *et al.*, 2013). The use of WBE could therefore give valuable insight into the amounts of antimicrobials a population has actually consumed.

It is well recognised that WWTPs are hotspots for resistance and the long term effects of exposing microbes to sub-inhibitory concentrations of antibiotics in wastewater streams is not well understood (Michael *et al.*, 2013; Andersson and Hughes, 2014). Furthermore, current antibiotic metabolites tend to be overlooked in wastewater analysis with parent compounds mainly focused on. The analysis of metabolites however could potentially provide information on antibiotic compliance in a community.

When compared to antibiotics, the prescription pattern of antivirals can differ as they are often less commonly prescribed on a day-to-day basis. For example, antivirals like Tamiflu® and Relenza®, are

stockpiled globally and are then deployed during pandemic periods which can result in high proportion of a community taking the drug in a short time window which is reflected in wastewater (Singer *et al.*, 2007). During the H1N1 influenza virus pandemic in 2009, Tamiflu® (oseltamivir phosphate) was heavily prescribed globally in response. It has been reported that oseltamivir carboxylate, a biologically active and persistent metabolite of oseltamivir phosphate, was observed in surface waters during peaks of the outbreak (Leknes, Sturtzel and Dye, 2012). This was due to increased loads of the metabolite in wastewater, which is widely known to not be readily removed by conventional WWTPs.

The monitoring of drugs like antivirals and their metabolites not only informs upon drug compliance and the progression of an outbreak at the community level, but like with antibiotics, could provide critical information with regards to resistance. The presence of these drugs or their metabolised forms in low levels in the environment could cause irreversible effects to the viral genome resulting in resistant effects. For example, it has been highlighted the guts of wildfowl could be potential oseltamivir carboxylate-resistance hotspots due to exposure to the metabolite in surface waters (Singer *et al.*, 2007). The rapid spreading of the H1N1 virus and the ease of which viruses can become resistant to antivirals stress the importance of population-wide surveillance tools and again the importance of combining chemical analysis with biological. Furthermore, whilst a number of antiviral drugs have been detected in water bodies, there is still a knowledge gap of understanding the environmental and resistant impacts their presence has in wastewater streams, especially as they tend to pass through WWTP unchanged (Jain *et al.*, 2013).

2.6.4 Markers of antimicrobial resistance

Markers of antimicrobial resistance are another group of key biological biomarkers. The analysis of antimicrobial resistant genes in influent wastewater could provide a broader perspective of the resistant genes present within a population. This together with viral and bacterial monitoring arguably gives a more representative reflection of health of a community, as currently much of the understanding of both diseases and resistance circulating within in a community are based upon clinical samples. The results from clinics are often from a very small proportion of the population who are ill and hence not representative of the population as a whole, as many people can be carriers of a disease or a resistant gene and not experience symptoms (asymptomatic in case of diseases). As previously mentioned, it was highlighted by WHO's GLASS programme that a limitation is that current samples are focused on a clinical level and more epidemiological information on a population scale are needed for AMR surveillance purposes (World Health Organisation, 2018a).

WBE could aid in providing this population-wide information, to date a diverse range of ARGs have been studied and reported on in wastewater, typically through qPCR techniques (Zhang, Zhang and Fang, 2009; Mao *et al.*, 2015; Rodriguez-Mozaz *et al.*, 2015; Sun *et al.*, 2016). Only a handful of studies

to date have investigated relationships between the levels of antibiotics and abundance of ARGs in wastewater streams, Correlations observed between antibiotic and respective resistant gene levels have been antibiotic dependant with some correlations observed (Gao, Munir and Xagoraraki, 2012; Novo *et al.*, 2013; Rodriguez-Mozaz *et al.*, 2015; Xu *et al.*, 2015). However, it is generally recognised that the relationship between antibiotic concentrations and resistance in wastewater is complex with further studies needed. Furthermore, focus tends to be upon more common antibiotics resistances, such as sulphonamides, tetracyclines and quinolones, hence there is still a knowledge gap regarding other antimicrobial classes of AMR genes, including those associated with antifungal resistance. The effects of seasonality upon ARGs in wastewater is another underexplored area, though Caucci et al. reported strong seasonal abundances of ARGs within wastewater, with higher levels observed in Autumn and Winter which coincided with increased antibiotic prescribing in those months (Caucci *et al.*, 2016).

Further work is needed to consolidate the impacts of antimicrobial prescribing at the community level on the abundance of ARGs in wastewater, particularly if this is to be utilised for epidemiology purposes. Establishing this link is recognised as challenging as several factors will potentially influence the abundance of ARGs in sewers other than the selective pressures from antimicrobials being prescribed. For example the environmental conditions in sewers has been shown to potentially impact ARG abundance, including temperature, metal pollutants and changes in composition of microbial communities (Novo *et al.*, 2013; Sun *et al.*, 2016; Jiao *et al.*, 2018).

2.7 Ethical considerations

As with many other scientific innovations, WBE is not immune to misuse and misrepresentation. As WBE does not collect data on individuals, the ethical risks are low. However, it will be necessary to manage privacy issues and the potential for stigmatisation of certain societal groups. The ethical aspects of WBE have been discussed elsewhere ((http://score-cost.eu/ethical-guidelines-for-wbe/)). It is generally accepted that populations over >10,000 is enough to give anonymity and will pose no risk to smaller groups of people. This is also relevant in the case of publications to reduce any risk of media misinterpreting the publication's finding.

Expanding WBE to include infectious diseases will pose new challenges to the ethical considerations, particularly with regards to disease outbreaks. With regards to pathogen monitoring in wastewater, population size will be important. It has been highlighted by WHO for the case of monitoring polio in wastewater, that large populations may decrease sample sensitivity and therefore sampling from subgroups may be required (World Health Organisation, 2003b). As infectious diseases, such as polio, spread rapidly in urban areas, the sampling of subgroups might also provide faster interventions by public health authorities. However, sampling from smaller subgroups in cities could lead to stigmatism of vulnerable groups.

Furthermore, outbreaks and the subsequent handling of them will differ between developing and developed countries due to the availability of resources and the quality of health and regulatory infrastructures in place. However, any outbreak, regardless of geographic location are fragile situations. Thus, care must be taken in the reporting of diseases being investigated within a community and social understanding of the situations will be crucial. For example, fear-trigged behaviours have been attributed as one of the major contributing factors to the spread of Ebola in Western Africa (Shultz *et al.*, 2016). Stigmatism surrounding individuals infected with Ebola combined with a sense of distrust in health services and treatment centres resulted in efforts to hide cases. This exacerbated the Ebola spread, as there was a decreased chance of survival of those infected with home treatment and increased chances of infecting family members or carers which would in turn extended to the community.

Similar ethical issues have also been observed with outbreaks such as SARS, influenza and tuberculosis, which has resulted in WHO publishing the first comprehensive international ethics guidelines on public health surveillance in 2017 (World Health Organisation, 2017b). These can be appropriately adapted to different social, economic and epidemiological circumstances. As WBE continues to expand in the direction of disease monitoring, ethics should be considered and developed alongside. Ethic guidelines will need to be adaptable, and consider factors such geographic location, population and the biomarkers to be monitored to enable further development of this field.

2.8 Conclusions

It is widely acknowledged that effective surveillance systems are key for the rapid intervention and control of infectious disease outbreaks. There is also a requirement for population-wide surveillance information to compliment current clinical data. WBE has demonstrated significant promise in providing information on community-wide exposure and health status comprehensively and in near real-time. The importance of effective surveillance has been highlighted recently with the case of the novel coronavirus (COVID-19). On the 31 December 2019, a number of causes of pneumonia of an unknown cause were detected in Wuhan City in China. Just a week later on 7 January Chinese officials had reported a novel strain of the coronavirus (World Health Organisation, 2020b). Even with early intervention measures of quarantining cities in China and travel bans, on 3 March 2020 the number of confirmed cases were 90,892 across 73 countries with severe outbreaks occurring in South Korea, Iran and Italy (World Health Organisation, 2020c). Along with the current routes of global surveillance for the virus, WBE, if implemented, could track spread of the virus and, if linked with effective response system, could help with management. However, in order to successfully apply WBE in infectious disease surveillance, rapid advancements are required to tackle some of key challenges. These include:

- complexity of wastewater matrix and the need for new biomarker extraction techniques,

- difficulties in accurate estimation of population size to account for temporal population size fluctuations,
- non-existent biomarker discovery pipeline for both chemical and biological markers
- lack of analytical tools for cost-effective, sensitive, selective and multi-residue analysis of wide-ranging biomarker groups spanning from genes through to proteins and whole microorganisms.

2.9 References

Aarestrup, F. M. and Woolhouse, M. E. J. (2020) 'Using sewage for surveillance of antimicrobial resistance', *Science*, 367(6478), pp. 630–632. doi: 10.1126/science.aba3432.

Ahmed, F. *et al.* (2020) 'Wastewater-based estimation of the prevalence of gout in Australia', *Science of The Total Environment*, 715, p. 136925. doi: 10.1016/j.scitotenv.2020.136925.

Ahmed, W. *et al.* (2020) 'First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.138764.

Allen, H. K. *et al.* (2010) 'Call of the wild: antibiotic resistance genes in natural environments', *Nature Reviews Microbiology*. Nature Publishing Group, 8(4), pp. 251–259. doi: 10.1038/nrmicro2312.

Allen, M. T. and Levy, L. S. (2013) 'Parkinsons disease and pesticide exposure - A new assessment', *Critical Reviews in Toxicology*. doi: 10.3109/10408444.2013.798719.

Andersson, D. I. and Hughes, D. (2014) 'Microbiological effects of sublethal levels of antibiotics', *Nature Reviews Microbiology*, 12(7), pp. 465–478. doi: 10.1038/nrmicro3270.

Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) (2020) *Scottish One Health Antimicrobial Use and Antimicrobial Resistance in 2019 Annual Report*. Available at: https://hpspubsrepo.blob.core.windows.net/hps-website/nss/3133/documents/2_2020-11-17-sonaar-2019-report.pdf.

Aprea, M. C. (2012) 'Environmental and biological monitoring in the estimation of absorbed doses of pesticides', *Toxicology Letters*, 210(2), pp. 110–118. doi: 10.1016/j.toxlet.2011.08.008.

ARHAI Scotland (2020) *Scottish One Health Antimicrobial Use and Antimicrobial Resistance in 2019 Annual Report*. Available at: https://hpspubsrepo.blob.core.windows.net/hps-website/nss/3133/documents/2_2020-11-17-sonaar-2019-report.pdf.

Assress, H. A. *et al.* (2019) 'Diversity, Co-occurrence and Implications of Fungal Communities in Wastewater Treatment Plants', *Scientific Reports*, 9(1), p. 14056. doi: 10.1038/s41598-019-50624-z.

Bade, R. *et al.* (2020) 'Determination of 21 synthetic cathinones, phenethylamines, amphetamines and opioids in influent wastewater using liquid chromatography coupled to tandem mass spectrometry', *Talanta*, 208, p. 120479. doi: 10.1016/j.talanta.2019.120479.

Bade, R. *et al.* (2021) 'International snapshot of new psychoactive substance use: Case study of eight countries over the 2019/2020 new year period', *Water Research*. doi: 10.1016/j.watres.2021.116891.

Baker-Austin, C. *et al.* (2006) 'Co-selection of antibiotic and metal resistance', *Trends in Microbiology*, 14(4), pp. 176–182. doi: 10.1016/j.tim.2006.02.006.

Baker, D. R., Barron, L. and Kasprzyk-Hordern, B. (2014) 'Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates', *Science of the Total Environment*, 487(1), pp. 629–641. doi: 10.1016/j.scitotenv.2013.11.107.

Barr, D. B. (2008) 'Biomonitoring of exposure to pesticides', *Journal of Chemical Health and Safety*. doi: 10.1016/j.jchas.2008.07.001.

Barras, C. (2018) 'Going to waste', *Nature Medicine*, 24(10), pp. 1484–1487. doi: 10.1038/s41591-018-0218-0.

Bauer, M. *et al.* (2012) 'Genetic association between obstructive bronchitis and enzymes of oxidative stress', *Metabolism*, 61(12), pp. 1771–1779. doi: 10.1016/j.metabol.2012.05.013.

Bauer, S. (2008) 'Societal and ethical issues in human biomonitoring – a view from science studies', *Environmental Health*, 7(Suppl 1), p. S10. doi: 10.1186/1476-069X-7-S1-S10.

Baz-Lomba, J. A. *et al.* (2016) 'Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities', *BMC Public Health*, 16(1), p. 1035. doi: 10.1186/s12889-016-3686-5.

Been, F. *et al.* (2014) 'Population Normalization with Ammonium in Wastewater-Based Epidemiology: Application to Illicit Drug Monitoring', *Environmental Science & Technology*, 48(14), pp. 8162–8169. doi: 10.1021/es5008388.

Been, F. *et al.* (2017) 'Liquid Chromatography–Tandem Mass Spectrometry Analysis of Biomarkers of Exposure to Phosphorus Flame Retardants in Wastewater to Monitor Community-Wide Exposure', *Analytical Chemistry*, 89(18), pp. 10045–10053. doi: 10.1021/acs.analchem.7b02705.

Been, F. *et al.* (2018) 'Mining the Chemical Information on Urban Wastewater: Monitoring Human Exposure to Phosphorus Flame Retardants and Plasticizers', *Environmental Science & Technology*, 52(12), pp. 6996–7005. doi: 10.1021/acs.est.8b01279.

Berdeaux, O. *et al.* (2006) 'F-2-isoprostanes: Review of analytical methods', *Current Pharmaceutical Analysis*, 2(1), pp. 69–78. doi: 10.2174/157341206775474016.

Bhat, R. V. *et al.* (1997) 'A Foodborne Disease Outbreak Due to the Consumption of Moldy Sorghum

and Maize Containing Fumonisin Mycotoxins', *Journal of Toxicology: Clinical Toxicology*, 35(3), pp. 249–255. doi: 10.3109/15563659709001208.

Birben, E. *et al.* (2012) 'Oxidative Stress and Antioxidant Defense', *World Allergy Organization Journal*, 5(1), pp. 9–19. doi: 10.1097/WOX.0b013e3182439613.

Boehme, C. *et al.* (2005) 'Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 99(12), pp. 893–900. doi: 10.1016/j.trstmh.2005.04.014.

Boleda, M. R., Galceran, M. T. and Ventura, F. (2007) 'Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography–tandem mass spectrometry', *Journal of Chromatography A*, 1175(1), pp. 38–48. doi: 10.1016/j.chroma.2007.10.029.

Boogaerts, T. *et al.* (2016) 'Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach', *Drug and Alcohol Dependence*. Elsevier Ireland Ltd, 160, pp. 170–176. doi: 10.1016/j.drugalcdep.2016.01.002.

Boogaerts, T. *et al.* (2019) 'Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach', *Talanta*, 200, pp. 340–349. doi: 10.1016/j.talanta.2019.03.052.

Börjesson, S. *et al.* (2009) 'A seasonal study of the mecA gene and Staphylococcus aureus including methicillin-resistant S. aureus in a municipal wastewater treatment plant', *Water Research*, 43(4), pp. 925–932. doi: 10.1016/j.watres.2008.11.036.

Bowers, I. and Subedi, B. (2021) 'Isoprostanes in wastewater as biomarkers of oxidative stress during COVID-19 pandemic', *Chemosphere*, 271, p. 129489. doi: 10.1016/j.chemosphere.2020.129489.

Brewer, A. J. *et al.* (2012) 'Normalized diurnal and between-day trends in illicit and legal drug loads that account for changes in population', *Environmental Science and Technology*. doi: 10.1021/es202447r.

Bush, K. *et al.* (2011) 'Tackling antibiotic resistance', *Nature Reviews Microbiology*, 9(12), pp. 894–896. doi: 10.1038/nrmicro2693.

Cadarette, S. M. and Wong, L. (2015) 'An Introduction to Health Care Administrative Data', *The Canadian Journal of Hospital Pharmacy*. Canadian Society Of Hospital Pharmacists, 68(3), pp. 232–7. doi: 10.4212/cjhp.v68i3.1457.

Cannas, A. *et al.* (2010) 'IP-10 detection in urine is associated with lung diseases', *BMC Infectious Diseases*, 10(1), p. 333. doi: 10.1186/1471-2334-10-333.

Carneiro, H. A. and Mylonakis, E. (2009) ‘Google Trends: A Web-Based Tool for Real-Time Surveillance of Disease Outbreaks’, *Clinical Infectious Diseases*. Oxford University Press, 49(10), pp. 1557–1564. doi: 10.1086/630200.

Castiglioni, S. *et al.* (2006) ‘Identification and Measurement of Illicit Drugs and Their Metabolites in Urban Wastewater by Liquid Chromatography−Tandem Mass Spectrometry’, *Analytical Chemistry*, 78(24), pp. 8421–8429. doi: 10.1021/ac061095b.

Castiglioni, S. *et al.* (2013) ‘Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers’, *Environmental Science and Technology*, 47(3), pp. 1452–1460. doi: 10.1021/es302722f.

Castiglioni, S. *et al.* (2015) ‘A novel approach for monitoring tobacco use in local communities by wastewater analysis’, *Tobacco Control*, 24(1), pp. 38–42. doi: 10.1136/tobaccocontrol-2014-051553.

Castiglioni, S. *et al.* (2021) ‘New psychoactive substances in several European populations assessed by wastewater-based epidemiology’, *Water Research*. Elsevier Ltd, 195, p. 116983. doi: 10.1016/j.watres.2021.116983.

Castrignanò, E. *et al.* (2020) ‘Enantiomeric profiling of quinolones and quinolones resistance gene qnrS in European wastewaters’, *Water Research*, 175, p. 115653. doi: 10.1016/j.watres.2020.115653.

Caucci, S. *et al.* (2016) ‘Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow’, *FEMS Microbiology Ecology*. Edited by K. Smalla, 92(5), p. fiw060. doi: 10.1093/femsec/fiw060.

Le Chatelier, E. *et al.* (2013) ‘Richness of human gut microbiome correlates with metabolic markers’, *Nature*, 500(7464), pp. 541–546. doi: 10.1038/nature12506.

Chen, C. *et al.* (2014) ‘Towards finding a population biomarker for wastewater epidemiology studies’, *Science of The Total Environment*, 487(1), pp. 621–628. doi: 10.1016/j.scitotenv.2013.11.075.

Choi, B. C. K. (2012) ‘The Past, Present, and Future of Public Health Surveillance’, *Scientifica*, 2012(2), pp. 1–26. doi: 10.6064/2012/875253.

Choi, P. M., O’Brien, J. W., *et al.* (2018) ‘Population histamine burden assessed using wastewater-based epidemiology: The association of 1,4-methylimidazole acetic acid and fexofenadine’, *Environment International*. doi: 10.1016/j.envint.2018.08.009.

Choi, P. M., Tscharke, B. J., *et al.* (2018) ‘Wastewater-based epidemiology biomarkers: Past, present and future’, *TrAC Trends in Analytical Chemistry*, 105, pp. 453–469. doi: 10.1016/j.trac.2018.06.004.

Choi, P. M. *et al.* (2019) ‘Social, demographic, and economic correlates of food and chemical consumption measured by wastewater-based epidemiology’, *Proceedings of the National Academy of Sciences*, 116(43), pp. 21864–21873. doi: 10.1073/pnas.1910242116.

Choi, P. M. *et al.* (2020) 'Do food and stress biomarkers work for wastewater-based epidemiology? A critical evaluation', *Science of The Total Environment*, 736, p. 139654. doi: 10.1016/j.scitotenv.2020.139654.

Coutu, S. *et al.* (2013) 'Temporal dynamics of antibiotics in wastewater treatment plant influent', *Science of The Total Environment*, 458–460, pp. 20–26. doi: 10.1016/j.scitotenv.2013.04.017.

Cracowski, J. L. and Durand, T. (2006) 'Cardiovascular pharmacology and physiology of the isoprostanes', *Fundamental and Clinical Pharmacology*, 20(5), pp. 417–427. doi: 10.1111/j.1472-8206.2006.00435.x.

Daughton, C. G. (2001) 'Emerging pollutants, and communicating the science of environmental chemistry and mass spectrometry: Pharmaceuticals in the environment', *Journal of the American Society for Mass Spectrometry*, 12(10), pp. 1067–1076. doi: 10.1016/S1044-0305(01)00287-2.

Daughton, C. G. (2012) 'Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype', *Science of The Total Environment*, 424, pp. 16–38. doi: 10.1016/j.scitotenv.2012.02.038.

Daughton, C. G. (2018) 'Monitoring wastewater for assessing community health: Sewage Chemical-Information Mining (SCIM)', *Science of The Total Environment*, 619–620, pp. 748–764. doi: 10.1016/j.scitotenv.2017.11.102.

Daughton, C. G. (2020) 'Wastewater surveillance for population-wide Covid-19: The present and future', *Science of The Total Environment*, 736, p. 139631. doi: 10.1016/j.scitotenv.2020.139631.

Davies, S. S. *et al.* (2006) 'Quantification of dinor, dihydro metabolites of F2-isoprostanes in urine by liquid chromatography/tandem mass spectrometry.', *Analytical biochemistry*, 348(2), pp. 185–91. doi: 10.1016/j.ab.2005.10.012.

Dawood, F. S. *et al.* (2012) 'Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study', *The Lancet Infectious Diseases*, 12(9), pp. 687–695. doi: 10.1016/S1473-3099(12)70121-4.

Dekant, W. and Völkel, W. (2008) 'Human exposure to bisphenol A by biomonitoring: Methods, results and assessment of environmental exposures', *Toxicology and Applied Pharmacology*. doi: 10.1016/j.taap.2007.12.008.

Dhalla, N. (2000) 'Status of myocardial antioxidants in ischemia–reperfusion injury', *Cardiovascular Research*. Oxford University Press, 47(3), pp. 446–456. doi: 10.1016/S0008-6363(00)00078-X.

Diemert, S. and Yan, T. (2019) 'Clinically unreported salmonellosis outbreak detected via comparative genomic analysis of municipal wastewater Salmonella isolates', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.00139-19.

Dishaw, L. V. *et al.* (2011) 'Is the PentaBDE replacement, tris (1,3-dichloro-2-propyl) phosphate (TDCPP), a developmental neurotoxicant? Studies in PC12 cells', *Toxicology and Applied Pharmacology*, 256(3), pp. 281–289. doi: 10.1016/j.taap.2011.01.005.

Du, P. *et al.* (2018) 'Estimating population exposure to phthalate esters in major Chinese cities through wastewater-based epidemiology', *Science of The Total Environment*, 643, pp. 1602–1609. doi: 10.1016/j.scitotenv.2018.06.325.

Epplein, M. *et al.* (2009) 'Association of plasma micronutrient levels and urinary isoprostane with risk of lung cancer: the multiethnic cohort study.', *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 18(7), pp. 1962–70. doi: 10.1158/1055-9965.EPI-09-0003.

Escolà Casas, M. *et al.* (2021) 'Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution', *Environment International*, 150, p. 106404. doi: 10.1016/j.envint.2021.106404.

European Food Safety Authority (EFSA) (2015) *Scientific Opinion on Bisphenol A (2015) What is Bisphenol A and What Has EFSA Done? What Are the Main Results of EFSA's 2015 Risk Assessment of BPA? EFSA's Risk Assessment in More Detail What Potential Health Effects of BPA Has EFSA Identified?* doi: 10.2805/075460.

European Monitoring Centre for Drugs and Drug Addiction (2016) *European Drug Report 2016: Trends and Developments, European Monitoring of Drugs and Drugs Addiction*. doi: 10.2810/88175.

European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2016) *Assessing illicit drugs in wastewater: advances in wastewater-based drug epidemiology, Insight 22*. Available at: https://www.emcdda.europa.eu/system/files/publications/2273/TDXD16022ENC_4.pdf.

European Union (2006) *Commission Regulation 1881/2006, 2006.* Available at: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF.

Fattore, E. *et al.* (2016) 'Wastewater-based epidemiological evaluation of the effect of air pollution on short-acting beta-agonist consumption for acute asthma treatment', *Environmental Research*, 150, pp. 106–111. doi: 10.1016/j.envres.2016.05.051.

Fernandez-Cassi, X. *et al.* (2018) 'Metagenomics for the study of viruses in urban sewage as a tool for public health surveillance', *Science of The Total Environment*, 618, pp. 870–880. doi: 10.1016/j.scitotenv.2017.08.249.

Fisher, M. C. *et al.* (2018) 'Worldwide emergence of resistance to antifungal drugs challenges human health and food security', *Science*, 360(6390), pp. 739–742. doi: 10.1126/science.aap7999.

Foladori, P. *et al.* (2020) 'SARS-CoV-2 from faeces to wastewater treatment: What do we know? A

review.', *The Science of the total environment*, 743, p. 140444. doi: 10.1016/j.scitotenv.2020.140444.

Food Standards Scotland (2015) *Mycotoxins, Climate Change and Food Safety Workshop Report*. Available at: https://www.foodstandards.gov.scot/downloads/Chemical_contaminants_-_FSS_Mycotoxin_Workshop_report_final_-_December_2015.pdf.

Frenk, J. and Gómez-Dantés, O. (2002) 'Globalisation and the challenges to health systems.', *BMJ (Clinical research ed.)*. British Medical Journal Publishing Group, 325(7355), pp. 95–7. doi: 10.1136/bmj.325.7355.95.

Fung, F. and Clark, R. F. (2004) 'Health Effects of Mycotoxins: A Toxicological Overview', *Journal of Toxicology: Clinical Toxicology*, 42(2), pp. 217–234. doi: 10.1081/CLT-120030947.

Funke, J., Prasse, C. and Ternes, T. A. (2016) 'Identification of transformation products of antiviral drugs formed during biological wastewater treatment and their occurrence in the urban water cycle', *Water Research*, 98, pp. 75–83. doi: 10.1016/j.watres.2016.03.045.

Gable, L., Ram, N. and Ram, J. L. (2020) 'Legal and ethical implications of wastewater monitoring of SARS-CoV-2 for COVID-19 surveillance', *Journal of Law and the Biosciences*, 7(1), p. lsaa039. doi: 10.1093/jlb/lsaa039.

Galea, K. S. *et al.* (2015) 'Urinary biomarker concentrations of captan, chlormequat, chlorpyrifos and cypermethrin in UK adults and children living near agricultural land', *Journal of Exposure Science & Environmental Epidemiology*, 25(6), pp. 623–631. doi: 10.1038/jes.2015.54.

Gao, P., Munir, M. and Xagoraraki, I. (2012) 'Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant', *Science of The Total Environment*, 421–422, pp. 173–183. doi: 10.1016/j.scitotenv.2012.01.061.

Girones, R. *et al.* (2010) 'Molecular detection of pathogens in water – The pros and cons of molecular techniques', *Water Research*, 44(15), pp. 4325–4339. doi: 10.1016/j.watres.2010.06.030.

Golovko, O. *et al.* (2014) 'Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant', *Chemosphere*, 111, pp. 418–426. doi: 10.1016/j.chemosphere.2014.03.132.

González-Mariño, I. *et al.* (2016) 'Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries', *Environmental Science & Technology*, 50(18), pp. 10089–10096. doi: 10.1021/acs.est.6b02644.

González-Mariño, I. *et al.* (2017) 'Wastewater-Based Epidemiology as a New Tool for Estimating Population Exposure to Phthalate Plasticizers', *Environmental Science and Technology*. American Chemical Society, 51(7), pp. 3902–3910. doi: 10.1021/acs.est.6b05612.

González-Mariño, I. *et al.* (2020) 'Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring', *Addiction*. doi: 10.1111/add.14767.

González-Mariño, I. *et al.* (2021) 'Assessing population exposure to phthalate plasticizers in thirteen Spanish cities through the analysis of wastewater', *Journal of Hazardous Materials*. doi: 10.1016/j.jhazmat.2020.123272.

Gourinat, A.-C. *et al.* (2015) 'Detection of Zika Virus in Urine', *Emerging Infectious Diseases*, 21(1), pp. 84–86. doi: 10.3201/eid2101.140894.

GOV.UK (2017) *Health matters: obesity and the food environment*. Available at: https://www.gov.uk/government/publications/health-matters-obesity-and-the-food-environment/health-matters-obesity-and-the-food-environment--2 (Accessed: 16 March 2021).

Gracia-Lor, E. *et al.* (2017) 'Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives', *Environment International*, 99, pp. 131–150. doi: 10.1016/j.envint.2016.12.016.

Gracia-Lor, E. *et al.* (2020) 'Wastewater-based epidemiology for tracking human exposure to mycotoxins', *Journal of Hazardous Materials*, 382, p. 121108. doi: 10.1016/j.jhazmat.2019.121108.

Guerra, P. *et al.* (2014) 'Occurrence and fate of antibiotic, analgesic/anti-inflammatory, and antifungal compounds in five wastewater treatment processes', *Science of The Total Environment*, 473–474, pp. 235–243. doi: 10.1016/j.scitotenv.2013.12.008.

Guy, R. A. *et al.* (2003) 'Real-Time PCR for Quantification of Giardia and Cryptosporidium in Environmental Water Samples and Sewage', *Applied and Environmental Microbiology*, 69(9), pp. 5178–5185. doi: 10.1128/AEM.69.9.5178-5185.2003.

Halden, R. *et al.* (2019) 'Tracking harmful chemicals and pathogens using the Human Health Observatory at ASU', *Online Journal of Public Health Informatics*, 11(1). doi: 10.5210/ojphi.v11i1.9843.

Hall, W. *et al.* (2012) 'An analysis of ethical issues in using wastewater analysis to monitor illicit drug use', *Addiction*, 107(10), pp. 1767–1773. doi: 10.1111/j.1360-0443.2012.03887.x.

Halliwell, B. and Lee, C. Y. J. (2010) 'Using isoprostanes as biomarkers of oxidative stress: some rarely considered issues.', *Antioxidants & redox signaling*, 13(2), pp. 145–56. doi: 10.1089/ars.2009.2934.

Hamasur, B. *et al.* (2015) 'A Sensitive Urinary Lipoarabinomannan Test for Tuberculosis', *PLOS ONE*. Edited by J. Nigou, 10(4), p. e0123457. doi: 10.1371/journal.pone.0123457.

Harman, C., Reid, M. and Thomas, K. V. (2011) 'In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of

community drug usage', *Environmental Science and Technology*, 45(13), pp. 5676–5682. doi: 10.1021/es201124j.

Heijnen, L. and Medema, G. (2011) 'Surveillance of Influenza A and the pandemic influenza A (H1N1) 2009 in sewage and surface water in the Netherlands', *Journal of Water and Health*, 9(3), pp. 434–442. doi: 10.2166/wh.2011.019.

Hellmér, M. *et al.* (2014) 'Detection of Pathogenic Viruses in Sewage Provided Early Warnings of Hepatitis A Virus and Norovirus Outbreaks', *Applied and Environmental Microbiology*. Edited by D. W. Schaffner, 80(21), pp. 6771–6781. doi: 10.1128/AEM.01981-14.

Hembach, N. *et al.* (2017) 'Occurrence of the mcr-1 Colistin Resistance Gene and other Clinically Relevant Antibiotic Resistance Genes in Microbial Populations at Different Municipal Wastewater Treatment Plants in Germany', *Frontiers in Microbiology*. Frontiers, 8, p. 1282. doi: 10.3389/fmicb.2017.01282.

Hernández, F. *et al.* (2018) 'Mass spectrometric strategies for the investigation of biomarkers of illicit drug use in wastewater', *Mass Spectrometry Reviews*, 37(3), pp. 258–280. doi: 10.1002/mas.21525.

Hijosa-Valsero, M. *et al.* (2011) 'Removal of antibiotics from urban wastewater by constructed wetland optimization', *Chemosphere*, 83(5), pp. 713–719. doi: 10.1016/j.chemosphere.2011.02.004.

Hirshon, J. M. (2000) 'The Rationale for Developing Public Health Surveillance Systems Based on Emergency Department Data', *Academic Emergency Medicine*, 7(12), pp. 1428–1432. doi: 10.1111/j.1553-2712.2000.tb00503.x.

Hou, C. *et al.* (2020) 'Estimating the prevalence of hepatitis B by wastewater-based epidemiology in 19 cities in China', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.139696.

Hovi, T. *et al.* (2012) 'Role of environmental poliovirus surveillance in global polio eradication and beyond', *Epidemiology and Infection*, 140(1), pp. 1–13. doi: 10.1017/S095026881000316X.

Huang, Q. *et al.* (2010) 'Determination of commonly used azole antifungals in various waters and sewage sludge using ultra-high performance liquid chromatography–tandem mass spectrometry', *Journal of Chromatography A*, 1217(21), pp. 3481–3488. doi: 10.1016/j.chroma.2010.03.022.

Hughes, D. A. *et al.* (2001) 'The impact of non-compliance on the cost-effectiveness of pharmaceuticals: a review of the literature', *Health Economics*, 10(7), pp. 601–615. doi: 10.1002/hec.609.

Huijbers, P. M. C., Larsson, D. G. J. and Flach, C. F. (2020) 'Surveillance of antibiotic resistant Escherichia coli in human populations through urban wastewater in ten European countries', *Environmental Pollution*, 261, p. 114200. doi: 10.1016/j.envpol.2020.114200.

Hussein, H. S. and Brasel, J. M. (2001) 'Toxicity, metabolism, and impact of mycotoxins on humans

and animals', *Toxicology*. doi: 10.1016/S0300-483X(01)00471-1.

Jain, S. *et al.* (2013) 'Occurrence and Removal of Antiviral Drugs in Environment: A Review', *Water, Air, & Soil Pollution*, 224(2), p. 1410. doi: 10.1007/s11270-012-1410-3.

Jiao, Y.-N. *et al.* (2018) 'Biomarkers of antibiotic resistance genes during seasonal changes in wastewater treatment systems', *Environmental Pollution*, 234, pp. 79–87. doi: 10.1016/j.envpol.2017.11.048.

Joynt, G. M. and Wu, W. K. (2020) 'Understanding COVID-19: what does viral RNA load really mean?', *The Lancet Infectious Diseases*. doi: 10.1016/S1473-3099(20)30237-1.

Jury, K. L. *et al.* (2011) 'Are sewage treatment plants promoting antibiotic resistance?', *Critical Reviews in Environmental Science and Technology*. Taylor & Francis Group, 41(3), pp. 243–270. doi: 10.1080/10643380902772589.

Kardas, P. *et al.* (2005) 'A systematic review and meta-analysis of misuse of antibiotic therapies in the community', *International Journal of Antimicrobial Agents*, 26(2), pp. 106–113. doi: 10.1016/j.ijantimicag.2005.04.017.

Kasprzyk-Hordern, B. *et al.* (2014) 'Wastewater-based epidemiology for public health monitoring', *Water and Sewerage Journal*, (4), pp. 25–26. doi: 10.1136/tobaccocontrol-2014-051553.7.

Kasprzyk-Hordern, B. *et al.* (2021) 'Estimation of community-wide multi-chemical exposure via water-based chemical mining: Key research gaps drawn from a comprehensive multi-biomarker multi-city dataset', *Environment International*, 147, p. 106331. doi: 10.1016/j.envint.2020.106331.

Kasprzyk-Hordern, B., Dinsdale, R. M. and Guwy, A. J. (2008) 'Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography–electrospray tandem mass spectrometry', *Analytical and Bioanalytical Chemistry*, 391(4), pp. 1293–1308. doi: 10.1007/s00216-008-1854-x.

Kasprzyk-Hordern, B., Dinsdale, R. M. and Guwy, A. J. (2009) 'The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters', *Water Research*, 43(2), pp. 363–380. doi: 10.1016/j.watres.2008.10.047.

Kim, S. Y. *et al.* (2018) 'Urine IP-10 as a biomarker of therapeutic response in patients with active pulmonary tuberculosis', *BMC Infectious Diseases*, 18(1), p. 240. doi: 10.1186/s12879-018-3144-3.

Kinyua, J. *et al.* (2015) 'Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS', *Drug Testing and Analysis*, 7(9), pp. 812–818. doi: 10.1002/dta.1777.

Kitajima, M. *et al.* (2020) 'SARS-CoV-2 in wastewater: State of the knowledge and research needs', *Science of The Total Environment*, 739, p. 139076. doi: 10.1016/j.scitotenv.2020.139076.

Klawitter, Jelena *et al.* (2011) 'Quantification of 15-F2t-isoprostane in human plasma and urine: results from enzyme-linked immunoassay and liquid chromatography/tandem mass spectrometry cannot be compared.', *Rapid communications in mass spectrometry : RCM*, 25(4), pp. 463–8. doi: 10.1002/rcm.4871.

Kraigher, B. *et al.* (2008) 'Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors', *Water Research*. Pergamon, 42(17), pp. 4578–4588. doi: 10.1016/J.WATRES.2008.08.006.

Kraus, L. *et al.* (2003) 'Estimating prevalence of problem drug use at national level in countries of the European Union and Norway', *Addiction*. doi: 10.1046/j.1360-0443.2003.00326.x.

Kwak, Y.-K. *et al.* (2015) 'Surveillance of antimicrobial resistance among Escherichia coli in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society?', *International Journal of Antimicrobial Agents*, 45(1), pp. 25–32. doi: 10.1016/j.ijantimicag.2014.09.016.

Lai, F. Y. *et al.* (2011) 'Refining the estimation of illicit drug consumptions from wastewater analysis: Co-analysis of prescription pharmaceuticals and uncertainty assessment', *Water Research*, 45(15), pp. 4437–4448. doi: 10.1016/j.watres.2011.05.042.

Lai, F. Y. *et al.* (2016) 'Cocaine, MDMA and methamphetamine residues in wastewater: Consumption trends (2009–2015) in South East Queensland, Australia', *Science of The Total Environment*, 568, pp. 803–809. doi: 10.1016/j.scitotenv.2016.05.181.

Lai, F. Y. *et al.* (2017) 'Novel Wastewater-Based Epidemiology Approach Based on Liquid Chromatography–Tandem Mass Spectrometry for Assessing Population Exposure to Tobacco-Specific Toxicants and Carcinogens', *Analytical Chemistry*, 89(17), pp. 9268–9278. doi: 10.1021/acs.analchem.7b02052.

Lai, F. Y. *et al.* (2018) 'Measuring spatial and temporal trends of nicotine and alcohol consumption in Australia using wastewater-based epidemiology', *Addiction*, 113(6), pp. 1127–1136. doi: 10.1111/add.14157.

Lancaster, K. *et al.* (2019) 'A "promising tool"? A critical review of the social and ethico-political effects of wastewater analysis in the context of illicit drug epidemiology and drug policy', *Current Opinion in Environmental Science and Health*, 9, pp. 85–90. doi: 10.1016/j.coesh.2019.06.006.

Larsson, D. G. J. *et al.* (2018) 'Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance', *Environment International*. doi:

10.1016/j.envint.2018.04.041.

Lee, L. M. *et al.* (2010) *Principles & Practice of Public Health Surveillance*. Oxford University Press. doi: 10.1093/acprof:oso/9780195372922.001.0001.

Leknes, H., Sturtzel, I. E. and Dye, C. (2012) 'Environmental release of oseltamivir from a Norwegian sewage treatment plant during the 2009 influenza A (H1N1) pandemic', *Science of The Total Environment*, 414, pp. 632–638. doi: 10.1016/j.scitotenv.2011.11.004.

Lin, W. *et al.* (2019) 'Can water quality indicators and biomarkers be used to estimate real-time population?', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2018.12.390.

Liu, S. Y. *et al.* (2021) 'Tracing consumption patterns of stimulants, opioids, and ketamine in China by wastewater-based epidemiology', *Environmental Science and Pollution Research*. doi: 10.1007/s11356-020-12035-w.

Lockhart, S. R. *et al.* (2017) 'Simultaneous Emergence of Multidrug-Resistant Candida auris on 3 Continents Confirmed by Whole-Genome Sequencing and Epidemiological Analyses', *Clinical Infectious Diseases*, 64(2), pp. 134–140. doi: 10.1093/cid/ciw691.

Lodovici, M. and Bigagli, E. (2011) 'Oxidative stress and air pollution exposure', *Journal of Toxicology*, p. 9 pages. doi: 10.1155/2011/487074.

Lopardo, L. *et al.* (2018) 'Verifying community-wide exposure to endocrine disruptors in personal care products – In quest for metabolic biomarkers of exposure via in vitro studies and wastewater-based epidemiology', *Water Research*. Pergamon, 143, pp. 117–126. doi: 10.1016/j.watres.2018.06.028.

Lopardo, L. *et al.* (2019) 'Estimation of community-wide exposure to bisphenol A via water fingerprinting', *Environment International*, 125, pp. 1–8. doi: 10.1016/j.envint.2018.12.048.

Maguire, E. R. and Monsivais, P. (2015) 'Socio-economic dietary inequalities in UK adults: an updated picture of key food groups and nutrients from national surveillance data', *British Journal of Nutrition*, 113(1), pp. 181–189. doi: 10.1017/S0007114514002621.

Mao, D. *et al.* (2015) 'Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants', *Water Research*, 85, pp. 458–466. doi: 10.1016/j.watres.2015.09.010.

Mardal, M. and Meyer, M. R. (2014) 'Studies on the microbial biotransformation of the novel psychoactive substance methylenedioxypyrovalerone (MDPV) in wastewater by means of liquid chromatography-high resolution mass spectrometry/mass spectrometry', *Science of the Total Environment*. Elsevier, 493, pp. 588–595. doi: 10.1016/j.scitotenv.2014.06.016.

Mars, B. *et al.* (2017) 'Influences on antidepressant prescribing trends in the UK: 1995–2011', *Social Psychiatry and Psychiatric Epidemiology*. doi: 10.1007/s00127-016-1306-4.

Mastroianni, N., Lopez de Alda, M. and Barcelo, D. (2014) 'Analysis of ethyl sulfate in raw wastewater for estimation of alcohol consumption and its correlation with drugs of abuse in the city of Barcelona', *Journal of Chromatography A*, 1360, pp. 93–99. doi: 10.1016/j.chroma.2014.07.051.

McCall, A.-K. *et al.* (2016) 'Critical review on the stability of illicit drugs in sewers and wastewater samples', *Water Research*. Pergamon, 88, pp. 933–947. doi: 10.1016/J.WATRES.2015.10.040.

Meagher, E. A. *et al.* (1999) 'Alcohol-induced generation of lipid peroxidation products in humans', *Journal of Clinical Investigation*, 104(6), pp. 805–813. doi: 10.1172/JCI5584.

Medema, G. *et al.* (2020) 'Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands', *Environmental Science & Technology Letters*, 7(7), pp. 511–516. doi: 10.1021/acs.estlett.0c00357.

Michael, I. *et al.* (2013) 'Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review', *Water Research*, 47(3), pp. 957–995. doi: 10.1016/j.watres.2012.11.027.

Montuschi, P., Barnes, P. J. and Roberts, L. J. (2004) 'Isoprostanes: markers and mediators of oxidative stress.', *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 18(15), pp. 1791–1800. doi: 10.1096/fj.04-2330rev.

Mumy, K. L. and Findlay, R. H. (2004) 'Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR', *Journal of Microbiological Methods*, 57(2), pp. 259–268. doi: 10.1016/j.mimet.2004.01.013.

Munir, M., Wong, K. and Xagoraraki, I. (2011) 'Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan', *Water Research*, 45(2), pp. 681–693. doi: 10.1016/j.watres.2010.08.033.

Mustieles, V. *et al.* (2020) 'Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring', *Environment International*, 144, p. 105811. doi: 10.1016/j.envint.2020.105811.

Muszbek, N. *et al.* (2008) 'The economic consequences of noncompliance in cardiovascular disease and related conditions: a literature review', *International Journal of Clinical Practice*, 62(2), pp. 338–351. doi: 10.1111/j.1742-1241.2007.01683.x.

Nathwani, D. *et al.* (2011) 'Scottish Antimicrobial Prescribing Group (SAPG): Development and impact of the Scottish National Antimicrobial Stewardship Programme', *International Journal of Antimicrobial Agents*. doi: 10.1016/j.ijantimicag.2011.02.005.

National Records of Scotland (2020) *Drug-related deaths in Scotland in 2019*. Available at: https://www.nrscotland.gov.uk/files//statistics/drug-related-deaths/2019/drug-related-deaths-19-

pub.pdf.

Ndiaye, A. K., Diop, P. A. M. and Diop, O. M. (2014) 'Environmental surveillance of poliovirus and non-polio enterovirus in urban sewage in Dakar, Senegal (2007-2013)', *Pan African Medical Journal*. doi: 10.11604/pamj.2014.19.243.3538.

Needham, L. L., Calafat, A. M. and Barr, D. B. (2007) 'Uses and issues of biomonitoring', *International Journal of Hygiene and Environmental Health*, 210(3–4), pp. 229–238. doi: 10.1016/j.ijheh.2006.11.002.

Newton, R. J. *et al.* (2015) 'Sewage reflects the microbiomes of human populations', *mBio*, 6(2). doi: 10.1128/mBio.02574-14.

NHS Scotland Information Services Division (2019) *Prevalence of Problem Drug Use in Scotland: 2015/16 Estimates*. Available at: https://www.isdscotland.org/Health-Topics/Drugs-and-Alcohol-Misuse/Publications/2019-03-05/2019-03-05-Drug-Prevalence-2015-16-Report.pdf.

Nielsen, L. K. *et al.* (2014) 'The prevalence and impact of Fusarium head blight pathogens and mycotoxins on malting barley quality in UK', *International Journal of Food Microbiology*, 179, pp. 38–49. doi: 10.1016/j.ijfoodmicro.2014.03.023.

Nikolaidis, M. G., Kyparos, A. and Vrabas, I. S. (2011) 'F2-isoprostane formation, measurement and interpretation: The role of exercise', *Progress in Lipid Research*, pp. 89–103. doi: 10.1016/j.plipres.2010.10.002.

Nourooz-Zadeh, J. *et al.* (2006) 'Impact of diabetic polyneuropathy and cardiovascular autonomic neuropathy on the excretion of urinary 8-epi-PGF2αand its metabolites (2, 3-dinor and 2, 3-dinor-5, 6-dihydro)', *Free Radical Research*, 40(7), pp. 723–729. doi: 10.1080/10715760600615086.

Novo, A. *et al.* (2013) 'Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater', *Water Research*, 47(5), pp. 1875–1887. doi: 10.1016/j.watres.2013.01.010.

Nsubuga, P. *et al.* (2006) *Public Health Surveillance: A Tool for Targeting and Monitoring Interventions, Disease Control Priorities in Developing Countries*. The International Bank for Reconstruction and Development / The World Bank. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21250345 (Accessed: 21 August 2018).

van Nuijs, A. L. N. *et al.* (2011) 'Sewage epidemiology — A real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium', *Environment International*, 37(3), pp. 612–621. doi: 10.1016/j.envint.2010.12.006.

van Nuijs, A. L. N. *et al.* (2015) 'Do concentrations of pharmaceuticals in sewage reflect prescription figures?', *Environmental Science and Pollution Research*, 22(12), pp. 9110–9118. doi:

10.1007/s11356-014-4066-2.

O'Brien, E. and Xagoraraki, I. (2019a) 'A water-focused one-health approach for early detection and prevention of viral outbreaks', *One Health*, 7, p. 100094. doi: 10.1016/j.onehlt.2019.100094.

O'Brien, E. and Xagoraraki, I. (2019b) 'A water-focused one-health approach for early detection and prevention of viral outbreaks', *One Health*. doi: 10.1016/j.onehlt.2019.100094.

O'Brien, J. W. *et al.* (2014) 'A model to estimate the population contributing to the wastewater using samples collected on census day', *Environmental Science and Technology*, 48(1), pp. 517–525. doi: 10.1021/es403251g.

O'Brien, J. W. *et al.* (2015) 'Wastewater analysis of Census day samples to investigate per capita input of organophosphorus flame retardants and plasticizers into wastewater', *Chemosphere*. doi: 10.1016/j.chemosphere.2015.06.014.

O'Brien, J. W., Grant, S., *et al.* (2019) 'A National Wastewater Monitoring Program for a better understanding of public health: A case study using the Australian Census', *Environment International*, 122, pp. 400–411. doi: 10.1016/j.envint.2018.12.003.

O'Brien, J. W., Choi, P. M., *et al.* (2019) 'Evaluating the stability of three oxidative stress biomarkers under sewer conditions and potential impact for use in wastewater-based epidemiology', *Water Research*, 166, p. 115068. doi: 10.1016/j.watres.2019.115068.

O'Neill, J. (2014) *Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations*. Available at: https://amr-review.org/sites/default/files/AMR Review Paper - Tackling a crisis for the health and wealth of nations_1.pdf (Accessed: 26 September 2018).

Ort, C., Lawrence, M. G., Rieckermann, J., *et al.* (2010) 'Sampling for Pharmaceuticals and Personal Care Products (PPCPs) and Illicit Drugs in Wastewater Systems: Are Your Conclusions Valid? A Critical Review', *Environmental Science & Technology*, 44(16), pp. 6024–6035. doi: 10.1021/es100779n.

Ort, C., Lawrence, M. G., Reungoat, J., *et al.* (2010) 'Sampling for PPCPs in wastewater systems: Comparison of different sampling modes and optimization strategies', *Environmental Science and Technology*. doi: 10.1021/es100778d.

Ort, C. *et al.* (2014) 'Sewage-based Epidemiology Requires a Truly Transdisciplinary Approach', *GAIA - Ecological Perspectives for Science and Society*, 23(3), pp. 266–268. doi: 10.14512/gaia.23.3.12.

Ozkul, A. *et al.* (2007) 'Oxidative stress in acute ischemic stroke', *Journal of Clinical Neuroscience*, 14(11), pp. 1062–1066. doi: 10.1016/j.jocn.2006.11.008.

Pandopulos, A. J. *et al.* (2021) 'Application of catecholamine metabolites as endogenous population

biomarkers for wastewater-based epidemiology', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.142992.

Papageorgiou, M., Wells, L., Williams, C., White, K., *et al.* (2018) 'Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents', *Toxins*, 10(2), p. 50. doi: 10.3390/toxins10020050.

Papageorgiou, M., Wells, L., Williams, C., White, K. L. M., *et al.* (2018) 'Occurrence of deoxynivalenol in an elderly cohort in the UK: a biomonitoring approach', *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*. doi: 10.1080/19440049.2018.1508890.

Pärnänen, K. M. M. *et al.* (2019) 'Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence', *Science Advances*, 5(4), p. eaau9124. doi: 10.1126/sciadv.aau9124.

Patrono, C., Falco, A. and Davı, G. (2005) 'Isoprostane formation and inhibition in atherothrombosis', *Current opinion in pharmacology*, 5(2), pp. 198–203. doi: 10.1016/j.coph.2004.11.003.

Peacock, A. *et al.* (2019) 'New psychoactive substances: challenges for drug surveillance, control, and public health responses', *The Lancet*, 394(10209), pp. 1668–1684. doi: 10.1016/S0140-6736(19)32231-7.

Petrie, B. *et al.* (2015) 'Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry', *Journal of Chromatography A*, 1431, pp. 64–78. doi: 10.1016/j.chroma.2015.12.036.

Petrie, B. *et al.* (2016) 'New Framework To Diagnose the Direct Disposal of Prescribed Drugs in Wastewater - A Case Study of the Antidepressant Fluoxetine', *Environmental Science and Technology*, 50(7), pp. 3781–3789. doi: 10.1021/acs.est.6b00291.

Petrie, B., Barden, R. and Kasprzyk-Hordern, B. (2015) 'A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring', *Water Research*. Pergamon, 72, pp. 3–27. doi: 10.1016/j.watres.2014.08.053.

Phung, D. *et al.* (2017) 'Can wastewater-based epidemiology be used to evaluate the health impact of temperature? – An exploratory study in an Australian population', *Environmental Research*. doi: 10.1016/j.envres.2017.03.023.

Polo, D. *et al.* (2020) 'Making waves: Wastewater-based epidemiology for COVID-19 – approaches and challenges for surveillance and prediction', *Water Research*, 186, p. 116404. doi: 10.1016/j.watres.2020.116404.

Poloni, T. R. *et al.* (2010) 'Detection of dengue virus in saliva and urine by real time RT-PCR',

Virology Journal, 7(1), p. 22. doi: 10.1186/1743-422X-7-22.

Poon, L. L. M. *et al.* (2004) 'Detection of SARS Coronavirus in Patients with Severe Acute Respiratory Syndrome by Conventional and Real-Time Quantitative Reverse Transcription-PCR Assays', *Clinical Chemistry*, 50(1), pp. 67–72. doi: 10.1373/clinchem.2003.023663.

Prasse, C. *et al.* (2010) 'Antiviral Drugs in Wastewater and Surface Waters: A New Pharmaceutical Class of Environmental Relevance?', *Environmental Science & Technology*, 44(5), pp. 1728–1735. doi: 10.1021/es903216p.

Prichard, J. *et al.* (2014) 'Sewage epidemiology and illicit drug research: The development of ethical research guidelines', *Science of the Total Environment*, 472, pp. 550–555. doi: https://doi.org/10.1016/j.scitotenv.2013.11.039.

Prichard, J. *et al.* (2017) 'Wastewater analysis of substance use: Implications for law, policy and research', *Journal of Law and Medicine*, 24(4), pp. 837–849.

Proctor, K. *et al.* (2019) 'Multi-residue ultra-performance liquid chromatography coupled with tandem mass spectrometry method for comprehensive multi-class anthropogenic compounds of emerging concern analysis in a catchment-based exposure-driven study', *Analytical and Bioanalytical Chemistry*. doi: 10.1007/s00216-019-02091-8.

Public Health England (2019) *Obesity Profile*. Available at: https://fingertips.phe.org.uk/profile/national-child-measurement-programme (Accessed: 15 March 2021).

Public Health England (2020) *Air pollution: applying All Our Health*. Available at: https://www.gov.uk/government/publications/air-pollution-applying-all-our-health/air-pollution-applying-all-our-health#:~:text=In the UK%2C air pollution,and 36%2C000 deaths every year (Accessed: 21 March 2021).

Public Health England (2021) *COVID-19: mental health and wellbeing surveillance report - 2. Important Findings*. Available at: https://www.gov.uk/government/publications/covid-19-mental-health-and-wellbeing-surveillance-report/2-important-findings-so-far (Accessed: 16 March 2021).

Rački, N. *et al.* (2014) 'Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples', *Plant Methods*, 10(1), p. 42. doi: 10.1186/s13007-014-0042-6.

Raven, K. E. *et al.* (2019) 'Genomic surveillance of Escherichia coli in municipal wastewater treatment plants as an indicator of clinically relevant pathogens and their resistance genes', *Microbial Genomics*, 5(5). doi: 10.1099/mgen.0.000267.

Reid, M. J. *et al.* (2011) 'Analysis and Interpretation of Specific Ethanol Metabolites, Ethyl Sulfate,

and Ethyl Glucuronide in Sewage Effluent for the Quantitative Measurement of Regional Alcohol Consumption', *Alcoholism: Clinical and Experimental Research*, 35(9), p. no-no. doi: 10.1111/j.1530-0277.2011.01505.x.

Reid, M. J., Derry, L. and Thomas, K. V. (2014) 'Analysis of new classes of recreational drugs in sewage: Synthetic cannabinoids and amphetamine-like substances', *Drug Testing and Analysis*, 6(1–2), pp. 72–79. doi: 10.1002/dta.1461.

Renata, Y. *et al.* (2013) 'Urinary concentration of cytokines in children with acute pyelonephritis', *European Journal of Pediatrics*, 172(6), pp. 769–774. doi: 10.1007/s00431-012-1914-2.

Reuter, P. and Stevens, A. (2007) *Bringing Evidence and Analysis Together to Inform UK Drug Policy*.

Rice, J. *et al.* (2020) 'Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - A UK perspective', *Science of The Total Environment*, 735, p. 139433. doi: 10.1016/j.scitotenv.2020.139433.

Rice, J. and Kasprzyk-Hordern, B. (2019) 'A new paradigm in public health assessment: Water fingerprinting for protein markers of public health using mass spectrometry', *TrAC Trends in Analytical Chemistry*, 119, p. 115621. doi: 10.1016/j.trac.2019.115621.

Rico, M., Andrés-Costa, M. J. and Picó, Y. (2017) 'Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study', *Journal of Hazardous Materials*. doi: 10.1016/j.jhazmat.2016.05.079.

Risom, L., Møller, P. and Loft, S. (2005) 'Oxidative stress-induced DNA damage by particulate air pollution', *Mutat Res*, 592(1–2), pp. 119–137. doi: 10.1016/j.mrfmmm.2005.06.012.

Riva, F. *et al.* (2020) 'Testing urban wastewater to assess compliance with prescription data through wastewater-based epidemiology: First case study in Italy', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.139741.

Roberts, L. (2013) 'Israel's Silent Polio Epidemic Breaks All the Rules', *Science*, 342(6159), pp. 679–680. doi: 10.1126/science.342.6159.679.

Roberts, P. and Thomas, K. (2006) 'The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment', *Science of The Total Environment*, 356(1–3), pp. 143–153. doi: 10.1016/j.scitotenv.2005.04.031.

Rodríguez-Álvarez, T., Rodil, R., Rico, M., *et al.* (2014) 'Assessment of local tobacco consumption by liquid chromatography-tandem mass spectrometry sewage analysis of nicotine and its metabolites, cotinine and trans-3′-hydroxycotinine, after enzymatic deconjugation', *Analytical Chemistry*, 86(20), pp. 10274–10281. doi: 10.1021/ac503330c.

Rodríguez-Álvarez, T., Rodil, R., Cela, R., *et al.* (2014) 'Ion-pair reversed-phase liquid chromatography-quadrupole-time-of-flight and triple-quadrupole-mass spectrometry determination of ethyl sulfate in wastewater for alcohol consumption tracing', *Journal of Chromatography A*. Elsevier, 1328, pp. 35–42. doi: 10.1016/j.chroma.2013.12.076.

Rodriguez-Mozaz, S. *et al.* (2015) 'Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river', *Water Research*. Pergamon, 69, pp. 234–242. doi: 10.1016/j.watres.2014.11.021.

Roilides, E. *et al.* (1999) 'Increased Urine Interleukin-6 Concentrations Correlate with Pyelonephritic Changes on 99m Tc-Dimercaptosuccinic Acid Scans in Neonates with Urinary Tract Infections', *The Journal of Infectious Diseases*, 180(3), pp. 904–907. doi: 10.1086/314960.

Rousis, N. I. *et al.* (2017) 'Wastewater-based epidemiology to assess pan-European pesticide exposure', *Water Research*, 121, pp. 270–279. doi: 10.1016/j.watres.2017.05.044.

Rousis, N. I., Zuccato, E. and Castiglioni, S. (2016) 'Monitoring population exposure to pesticides based on liquid chromatography-tandem mass spectrometry measurement of their urinary metabolites in urban wastewater: A novel biomonitoring approach', *Science of The Total Environment*, 571, pp. 1349–1357. doi: 10.1016/j.scitotenv.2016.07.036.

Rousis, N. I., Zuccato, E. and Castiglioni, S. (2017) 'Wastewater-based epidemiology to assess human exposure to pyrethroid pesticides', *Environment International*, 99, pp. 213–220. doi: 10.1016/j.envint.2016.11.020.

Ryu, Y. *et al.* (2016) 'Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater associated with tobacco use', *Scientific Reports*, 6, p. 39055. doi: 10.1038/srep39055.

Ryu, Y., Reid, M. J. and Thomas, K. V. (2015) 'Liquid chromatography-high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater', *Journal of Chromatography A*, 1409, pp. 146–151. doi: 10.1016/j.chroma.2015.07.060.

Saillenfait, A.-M., Ndiaye, D. and Sabaté, J.-P. (2015) 'Pyrethroids: Exposure and health effects – An update', *International Journal of Hygiene and Environmental Health*, 218(3), pp. 281–292. doi: 10.1016/j.ijheh.2015.01.002.

Salipante, S. J. and Jerome, K. R. (2020) 'Digital PCR—An Emerging Technology with Broad Applications in Microbiology', *Clinical Chemistry*, 66(1), pp. 117–123. doi: 10.1373/clinchem.2019.304048.

Savolainen, L. *et al.* (2013) 'Modification of Clearview Tuberculosis (TB) Enzyme-Linked Immunosorbent Assay for TB Patients Not Infected with HIV', *Clinical and Vaccine Immunology*,

20(9), pp. 1479–1482. doi: 10.1128/CVI.00375-13.

Schwedhelm, E. *et al.* (2007) 'Mass Spectrometric Analysis of F2-Isoprostanes: Markers and Mediators in Human Disease', *Current Pharmaceutical Analysis*, 3(1), pp. 39–51. doi: 10.2174/157341207779802340.

ScotPHO Public Health Information for Scotland (2020) *Obesity*. Available at: https://www.scotpho.org.uk/clinical-risk-factors/obesity/key-points/ (Accessed: 15 March 2021).

Scottish Government (2019) *Pesticide Usage in Scotland*. Available at: https://www.gov.scot/news/pesticide-usage-in-scotland-5/.

Scottish Government (2020) *Cleaner Air for Scotland 2: Draft Air Quality Strategy Consultation*. Available at: https://www.gov.scot/publications/cleaner-air-scotland-2-draft-air-quality-strategy-consultation/pages/6/.

Senta, I. *et al.* (2019) 'Occurrence and Behavior of Macrolide Antibiotics in Municipal Wastewater Treatment: Possible Importance of Metabolites, Synthesis Byproducts, and Transformation Products', *Environmental Science & Technology*, 53(13), pp. 7463–7472. doi: 10.1021/acs.est.9b01420.

Shannon, K. E. *et al.* (2007) 'Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment', *Science of The Total Environment*, 382(1), pp. 121–129. doi: 10.1016/j.scitotenv.2007.02.039.

Shimanoe, C. *et al.* (2021) 'Perceived stress, depressive symptoms, and cortisol-to-cortisone ratio in spot urine in 6878 older adults', *Psychoneuroendocrinology*. doi: 10.1016/j.psyneuen.2020.105125.

Shultz, J. M. *et al.* (2016) 'The Role of Fear-Related Behaviors in the 2013–2016 West Africa Ebola Virus Disease Outbreak', *Current Psychiatry Reports*, 18(11), p. 104. doi: 10.1007/s11920-016-0741-y.

Simonsen, L. *et al.* (2013) 'Global Mortality Estimates for the 2009 Influenza Pandemic from the GLaMOR Project: A Modeling Study', *PLoS Medicine*. Edited by S. I. Hay, 10(11), p. e1001558. doi: 10.1371/journal.pmed.1001558.

Sims, N. and Kasprzyk-Hordern, B. (2020) 'Future perspectives of wastewater-based epidemiology: Monitoring infectious disease spread and resistance to the community level', *Environment International*, 139. doi: 10.1016/j.envint.2020.105689.

Sims, N., Rice, J. and Kasprzyk-Hordern, B. (2019) 'An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater', *Analytical and Bioanalytical Chemistry*, 411(11), pp. 2261–2271. doi: 10.1007/s00216-019-01667-8.

Singer, A. C. *et al.* (2007) 'Potential Risks Associated with the Proposed Widespread Use of

Tamiflu', *Environmental Health Perspectives*, 115(1), pp. 102–106. doi: 10.1289/ehp.9574.

Skelsey, P. and Newton, A. C. (2015) 'Future environmental and geographic risks of Fusarium head blight of wheat in Scotland', *European Journal of Plant Pathology*, 142(1), pp. 133–147. doi: 10.1007/s10658-015-0598-7.

Sleeuwenhoek, A. *et al.* (2007) *Biological monitoring of pesticide exposures. IOM Research Report TM/07/02.*

Spurling, G. K. P. *et al.* (2013) 'Delayed antibiotics for respiratory infections', *Cochrane Database of Systematic Reviews*. doi: 10.1002/14651858.CD004417.pub4.

Stephenson, G. and Richardson, A. (2014) *New Psychoactive Substances in England. A review of the evidence*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/368587/NPSevidenceReview.pdf.

Stuveling, E. M. *et al.* (2003) 'C-reactive protein is associated with renal function abnormalities in a non-diabetic population', *Kidney International*, 63(2), pp. 654–661. doi: 10.1046/j.1523-1755.2003.00762.x.

Sun, Y. *et al.* (2016) 'Multiple antibiotic resistance genes distribution in ten large-scale membrane bioreactors for municipal wastewater treatment', *Bioresource Technology*, 222, pp. 100–106. doi: 10.1016/j.biortech.2016.09.117.

Taha, A. S. (2003) 'Urinalysis for interleukin-8 in the non-invasive diagnosis of acute and chronic inflammatory diseases', *Postgraduate Medical Journal*, 79(929), pp. 159–163. doi: 10.1136/pmj.79.929.159.

Takanami, R. *et al.* (2012) 'Antiviral Drugs Zanamivir and Oseltamivir Found in Wastewater and Surface Water in Osaka, Japan', *Journal of Water and Environment Technology*, 10(1), pp. 57–68. doi: 10.2965/jwet.2012.57.

Tang, S. *et al.* (2020) 'Concentrations of phthalate metabolites in Australian urine samples and their contribution to the per capita loads in wastewater', *Environment International*. doi: 10.1016/j.envint.2020.105534.

Taylor, A. W., Bruno, R. S. and Traber, M. G. (2008) 'Women and Smokers Have Elevated Urinary F2-Isoprostane Metabolites: A Novel Extraction and LC–MS Methodology', *Lipids*, 43(10), pp. 925–936. doi: 10.1007/s11745-008-3222-1.

Tello, A., Austin, B. and Telfer, T. C. (2012) 'Selective Pressure of Antibiotic Pollution on Bacteria of Importance to Public Health', *Environmental Health Perspectives*, 120(8), pp. 1100–1106. doi: 10.1289/ehp.1104650.

Testai, E. *et al.* (2013) 'A plea for risk assessment of endocrine disrupting chemicals', *Toxicology*, 314(1), pp. 51–59. doi: 10.1016/j.tox.2013.07.018.

Thacker, S. B. *et al.* (2006) 'Measuring the public's health.', *Public Health Reports*, 121(1), pp. 14–22. doi: 10.1177/003335490612100107.

Thacker, S. B. and Berkelman, R. L. (1988) 'Public Health Surveillance in the United States', *Epidemiologic Reviews*, 10(1), pp. 164–190. doi: 10.1093/oxfordjournals.epirev.a036021.

Thai, P. K. *et al.* (2019) 'Evaluating the in-sewer stability of three potential population biomarkers for application in wastewater-based epidemiology', *Science of The Total Environment*, 671, pp. 248–253. doi: 10.1016/j.scitotenv.2019.03.231.

Thomaidis, N. S. *et al.* (2016) 'Reflection of Socioeconomic Changes in Wastewater: Licit and Illicit Drug Use Patterns', *Environmental Science & Technology*, 50(18), pp. 10065–10072. doi: 10.1021/acs.est.6b02417.

Thomas, K. V. *et al.* (2012) 'Comparing illicit drug use in 19 European cities through sewage analysis', *Science of The Total Environment*. Elsevier B.V., 432, pp. 432–439. doi: 10.1016/j.scitotenv.2012.06.069.

Thomas, K. V. and Reid, M. J. (2011) 'What else can the analysis of sewage for urinary biomarkers reveal about communities?', *Environmental Science and Technology*, 45(18), pp. 7611–7612. doi: 10.1021/es202522d.

Tong, A. Y. C., Peake, B. M. and Braund, R. (2011) 'Disposal practices for unused medications around the world', *Environment International*. doi: 10.1016/j.envint.2010.10.002.

Tscharke, B. J. *et al.* (2016) 'Temporal trends in drug use in Adelaide, South Australia by wastewater analysis', *Science of The Total Environment*, 565, pp. 384–391. doi: 10.1016/j.scitotenv.2016.04.183.

Tscharke, B. J., White, J. M. and Gerber, J. P. (2016) 'Estimates of tobacco use by wastewater analysis of anabasine and anatabine', *Drug Testing and Analysis*, 8(7), pp. 702–707. doi: 10.1002/dta.1842.

Tsikas, D. *et al.* (2003) 'Divergence in urinary 8-iso-PGF2α(iPF2α-III, 15-F2t-IsoP) levels from gas chromatography-tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-iso-PGF2α', *Journal of Chromatography B*, 794(2), pp. 237–255. doi: 10.1016/S1570-0232(03)00457-4.

Tuanny Franco, L. *et al.* (2019) 'Biomonitoring of mycotoxin exposure using urinary biomarker approaches: a review', *Toxin Reviews*. doi: 10.1080/15569543.2019.1619086.

UK Government (2019a) *Health Matters: tobacco and alcohol CQUIN*. Available at: https://www.gov.uk/government/publications/health-matters-preventing-ill-health-from-alcohol-and-

tobacco/health-matters-preventing-ill-health-from-alcohol-and-tobacco-use (Accessed: 16 March 2021).

UK Government (2019b) *Tackling antimicrobial resistance 2019–2024. The UK's five year national action plan*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/784894/UK_AMR_5_year_national_action_plan.pdf.

United Nations Department of Economic and Social Affairs (2019) *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423).* Available at: https://population.un.org/wpp/Publications/Files/WPP2019_Highlights.pdf.

van der Veen, I. and de Boer, J. (2012) 'Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis', *Chemosphere*, 88(10), pp. 1119–1153. doi: 10.1016/j.chemosphere.2012.03.067.

Vitale, D., Morales Suárez-Varela, M. and Picó, Y. (2021) 'Wastewater-based epidemiology, a tool to bridge biomarkers of exposure, contaminants, and human health', *Current Opinion in Environmental Science & Health*, 20, p. 100229. doi: 10.1016/j.coesh.2021.100229.

Walden, C., Carbonero, F. and Zhang, W. (2017) 'Assessing impacts of DNA extraction methods on next generation sequencing of water and wastewater samples', *Journal of Microbiological Methods*, 141, pp. 10–16. doi: 10.1016/j.mimet.2017.07.007.

Wallis, P. M. *et al.* (1996) 'Prevalence of Giardia cysts and Cryptosporidium oocysts and characterization of Giardia spp. isolated from drinking water in Canada', *Applied and Environmental Microbiology*, 62, pp. 2789–2797.

Wang, H. *et al.* (2020) 'Human exposure of bisphenol A and its analogues: understandings from human urinary excretion data and wastewater-based epidemiology', *Environmental Science and Pollution Research*, 27(3), pp. 3247–3256. doi: 10.1007/s11356-019-07111-9.

Wang, J. *et al.* (2015) 'Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants', *Science of The Total Environment*, 526, pp. 366–373. doi: 10.1016/j.scitotenv.2015.05.046.

Wells, L. *et al.* (2017) 'Deoxynivalenol biomarkers in the urine of UK vegetarians', *Toxins*. doi: 10.3390/toxins9070196.

Wigginton, K. R., Ye, Y. and Ellenberg, R. M. (2015) 'Emerging investigators series: the source and fate of pandemic viruses in the urban water cycle', *Environmental Science: Water Research & Technology*. The Royal Society of Chemistry, 1(6), pp. 735–746. doi: 10.1039/C5EW00125K.

Woolhouse, M. E. J. and Gowtage-Sequeria, S. (2005) 'Host Range and Emerging and Reemerging

Pathogens', *Emerging Infectious Diseases*, 11(12), pp. 1842–1847. doi: 10.3201/eid1112.050997.

World Health Organisation (2001) *WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation*. Available at: https://apps.who.int/iris/handle/10665/42363.

World Health Organisation (2003a) *Adherence to long-term therapies: evidence for action.* Available at: https://www.who.int/chp/knowledge/publications/adherence_full_report.pdf.

World Health Organisation (2003b) *Guidelines for environmental surveillance of poliovirus circulation (No. WHO/V&B/03.03)*. Available at: http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf.

World Health Organisation (2010a) 'Toxicological and Health Aspects of Bisphenol A Joint FAO/WHO Expert Meeting', in. Available at: http://apps.who.int/.../44624.

World Health Organisation (2010b) *WHO Pandemic (H1N1) 2009–update 112*. Available at: https://www.who.int/csr/don/2010_08_06/en/ (Accessed: 7 August 2019).

World Health Organisation (2015a) *Global action plan on antimicrobial resistance, World Health Organisation*. doi: ISBN 978 92 4 150976 3.

World Health Organisation (2015b) *Global Antimicrobial Resistance Surveillance System: Manual for Early Implementation.* Available at: https://www.who.int/antimicrobial-resistance/publications/surveillance-system-manual/en/.

World Health Organisation (2017a) *Antimicrobial Resistance*. Available at: https://www.who.int/news-room/q-a-detail/antimicrobial-resistance#:~:text=Antimicrobial resistance occurs when microorganisms,referred to as "superbugs". (Accessed: 10 March 2021).

World Health Organisation (2017b) *World Health Organization. WHO guidelines on ethical issues in public health surveillance*. Available at: https://www.who.int/ethics/publications/public-health-surveillance/en/ (Accessed: 13 August 2019).

World Health Organisation (2018a) *Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017-2018*. Available at: https://apps.who.int/iris/bitstream/handle/10665/279656/9789241515061-eng.pdf?ua=1.

World Health Organisation (2018b) *Managing epidemics Key facts about major deadly diseases*. Available at: https://www.who.int/emergencies/diseases/managing-epidemics/en/.

World Health Organisation (2019) *Ten Threats to Global Health in 2019*. Available at: https://www.who.int/news-room/feature-stories/ten-threats-to-global-health-in-2019 (Accessed: 31 January 2020).

World Health Organisation (2020a) *Disease outbreaks by year*. Available at: https://www.who.int/csr/don/archive/year/en/.

World Health Organisation (2020b) *Novel Coronavirus (2019-nCoV) Situation Report 1*. Available at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200121-sitrep-1-2019-ncov.pdf.

World Health Organisation (2020c) *Novel Coronavirus (COVID-19) Situation*. Available at: https://experience.arcgis.com/experience/685d0ace521648f8a5beeeee1b9125cd.

Wu, C. *et al.* (2016) 'Concurrent quantification of multiple biomarkers indicative of oxidative stress status using liquid chromatography-tandem mass spectrometry', *Analytical Biochemistry*. Elsevier Inc, 512, pp. 26–35. doi: 10.1016/j.ab.2016.07.030.

Wu, L. L. *et al.* (2004) 'Urinary 8-OHdG: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics', *Clinica Chimica Acta*, pp. 1–9. doi: 10.1016/j.cccn.2003.09.010.

Xiao, Y. *et al.* (2019) 'Assessing the trend of diabetes mellitus by analyzing metformin as a biomarker in wastewater', *Science of The Total Environment*, 688, pp. 281–287. doi: 10.1016/j.scitotenv.2019.06.117.

Xu, J. *et al.* (2015) 'Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river', *Chemosphere*, 119, pp. 1379–1385. doi: 10.1016/j.chemosphere.2014.02.040.

Yan, T. *et al.* (2018) 'Municipal Wastewater as a Microbial Surveillance Platform for Enteric Diseases: A Case Study for Salmonella and Salmonellosis', *Environmental Science and Technology*. doi: 10.1021/acs.est.8b00163.

Yang, Z., D'Auriac, M. A., *et al.* (2015) 'A Novel DNA Biosensor Using a Ferrocenyl Intercalator Applied to the Potential Detection of Human Population Biomarkers in Wastewater', *Environmental Science & Technology*, 49(9), pp. 5609–5617. doi: 10.1021/acs.est.5b00637.

Yang, Z., Kasprzyk-Hordern, B., *et al.* (2015) 'Community sewage sensors for monitoring public health', *Environmental Science and Technology*, 49(10), pp. 5845–5846. doi: 10.1021/acs.est.5b01434.

Yang, Z. *et al.* (2016) 'Community Sewage Sensors towards Evaluation of Drug Use Trends: Detection of Cocaine in Wastewater with DNA-Directed Immobilization Aptamer Sensors.', *Scientific reports*. Nature Publishing Group, 6(October 2015), p. 21024. doi: 10.1038/srep21024.

Yang, Z. *et al.* (2017) 'Monitoring Genetic Population Biomarkers for Wastewater-Based Epidemiology', *Analytical Chemistry*, 89(18), pp. 9941–9945. doi: 10.1021/acs.analchem.7b02257.

Zhang, X.-X., Zhang, T. and Fang, H. H. P. (2009) 'Antibiotic resistance genes in water environment', *Applied Microbiology and Biotechnology*. Springer-Verlag, 82(3), pp. 397–414. doi:

10.1007/s00253-008-1829-z.

Zhao, M. *et al.* (2017) 'A comprehensive analysis and annotation of human normal urinary proteome', *Scientific Reports*, 7(1), p. 3024. doi: 10.1038/s41598-017-03226-6.

Zheng, P. *et al.* (2013) 'Novel urinary biomarkers for diagnosing bipolar disorder', *Metabolomics*. doi: 10.1007/s11306-013-0508-y.

Zheng, P. *et al.* (2016) 'Identification of sex-specific urinary biomarkers for major depressive disorder by combined application of NMR- and GC-MS-based metabonomics', *Translational psychiatry*. doi: 10.1038/tp.2016.188.

Zuccato, E. *et al.* (2005) 'Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse', *Environmental Health*, 4(1), p. 14. doi: 10.1186/1476-069X-4-14.

Zuccato, E. *et al.* (2008) 'Estimating Community Drug Abuse by Wastewater Analysis', *Environmental Health Perspectives*, 116(8), pp. 1027–1032. doi: 10.1289/ehp.11022.

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3 Review of wastewater monitoring applications for public health and novel aspects of environmental quality

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3.2 Executive Summary

The rapid spread of severe acute respiratory syndrome coronavirus (SARS-CoVID-2) has devasted and overwhelmed public health systems across the world. The analysis of SARS-CoVID-2 in wastewater, via a technique known as wastewater-based epidemiology (WBE), has shown significant promise for monitoring disease spread. As a result, rapid and considerable infrastructure has been established to sample and analyse wastewater in the UK and internationally to track outbreaks. Whilst WBE is a relatively new field, the technique has expanded and developed over the past couple of decades to inform on varying aspects of public health. The aim of this project was to review the literature on what other areas WBE could be used to inform on public health.

3.2.1 Aims and objectives

1) Determine the existing and potential applications for WBE
2) Outline where wastewater-monitoring is already being used, and for what purpose
3) Highlight applications that are well-established, and which are potentially promising
4) Identify the research gaps
5) Determine the ethical considerations of WBE, what has been done already and what needs to be done

3.2.2 Key findings

The review of the national and international literature on WBE found:

- WBE is a relatively new field and many of the studies to date have been proof of concept. Only very few applications of WBE exist that are ready to be implemented now 1) estimation of community wide illicit drug usage 2) estimation of lifestyle chemical usage, e.g. alcohol, nicotine and caffeine

- Infectious disease tracking has historically been successful for monitoring potential polio cases within communities. WBE networks for SARS-CoVID-2 surveillance have been established regionally, nationally and internationally. Further research is needed to establish what virus loads indicate

- Disease prevalence in a community for both non-communicable and communicable diseases have been estimated by pharmaceutical usage. Key to this is the analysis of both parent compounds and metabolites to determine consumption or direct disposal into a sewaer system

- Recent studies have shown promise for WBE to monitor prevalence of allergies or asthma burden in a population linked with environmental factors

- WBE has clear potential to estimate of community-wide exposure to hazardous chemicals, including pesticides and plasticizers

- The analysis of endogenous biomarkers (e.g. markers of inflammation or stress) in WBE would give valuable information on many aspects of public health and community lifestyles. However, very few endogenous biomarkers have been studied in WBE. Further work is needed in this area to identify suitable and representative biomarkers

- Research gaps in WBE include a lack of understanding regarding stability, representative metabolites in wastewater and a lack of understanding of metabolism to undergo back-calculation of exposure

- As WBE continues to advance, the ethical considerations must be established and kept up-to-date with developments

3.2.3 Recommendations

WBE is a relatively new field. There are only a very few technology ready applications. These include estimation of community wide illicit drugs, lifestyle chemical usage (alcohol, nicotine and caffeine), infectious disease tracking and estimation of disease prevalence based on pharmaceutical usage (for diabetes, cardiovascular disease or mental health conditions). Required infrastructure includes specialised staff to undertake sampling and sample preparation as well as an investment in instrumentation. There is a clear potential to apply WBE to estimate community-wide exposure to hazardous chemicals (including pesticides and industrial chemicals) or the prevalence of non-communicable disease (including asthma). However, further essential research is required to fully appreciate WBE's potential to transform community-wide heath assessment. These include: (1) Fundamental research on a new biomarker base to inform public health status. (2) Novel approaches towards population equivalent estimation. (3) Novel approaches towards sampling. (4) Novel approaches towards analysis and sensing. (5) Modelling and statistical analysis are required to fully appreciate spatiotemporal variability in large scale datasets.

3.3 Introduction

Wastewater-based epidemiology (WBE) has received increasing attention over the past year across the world. In the UK, local, regional and national wastewater monitoring programmes were established in 2020 to detect severe acute respiratory syndrome coronavirus (SARS-CoVID-2) patterns in human sewage to monitor outbreaks. This concerted effort between UK, Scottish and Welsh government, water companies, universities and research institutes has seen considerable investment into establishing the infrastructure, methodology and resources needed to sample, analyse, and interpret data from WBE. Whilst coronavirus has so far been the primary focus of these programmes, it is widely acknowledged that wastewater contains a diverse amount of chemical and biological information that can be used for wider public health purposes. The aim of this project was to review the literature on where else WBE could be utilised to inform public health.

3.3.1 What is wastewater-based epidemiology

WBE is a field that combines multiple disciplines, bringing together scientists and engineers. It is a technique where wastewater is analysed to give information on the communities within a wastewater catchment. Epidemiology is the study of the distribution and patterns of disease and health in defined populations, and WBE is using wastewater for this purpose. WBE is achieved through the analysis of indicators of health and disease, known as biomarkers, that have been excreted from individuals in a community into the sewer system. Biomarkers are broadly defined as a characteristic that can be objectively measured and quantified as an indicator of biological response (World Health Organisation, 2001). These can be: (i) exogenous biomarkers (also known as external agents, stressors) such as pharmaceuticals consumed to treat diseases, air contaminants, food toxicants, or genetic material (e.g. DNA or RNA) from bacteria or viruses causing an infection and (ii) endogenous biomarkers (formed in humans) such as markers of inflammation or stress. They can be found in elevated or reduced levels in the body, for example in blood, tissues, faeces or urine.

3.3.2 The concept of wastewater-based epidemiology

WBE is conceptionally very simple. Influent wastewater (untreated, raw sewage) can be considered a pooled urine and faeces sample of the community that contributes (figure 1). The total amounts of analysed biomarkers in wastewater can be linked back to the community via back-calculations to calculate the daily amounts or daily doses per 1000 people. Pharmaceuticals are popular biomarkers in WBE, as many pass through the body unchanged or excreted as metabolites. It is for this reason that the inclusion of metabolites of drugs in WBE can add a further dimension. The ratios between parent drugs and their corresponding metabolites in wastewater can be used to see if drugs have been consumed rather than directly disposed of (Petrie *et al.*, 2016). This is particularly the case when the metabolism of a drug is well-understood.

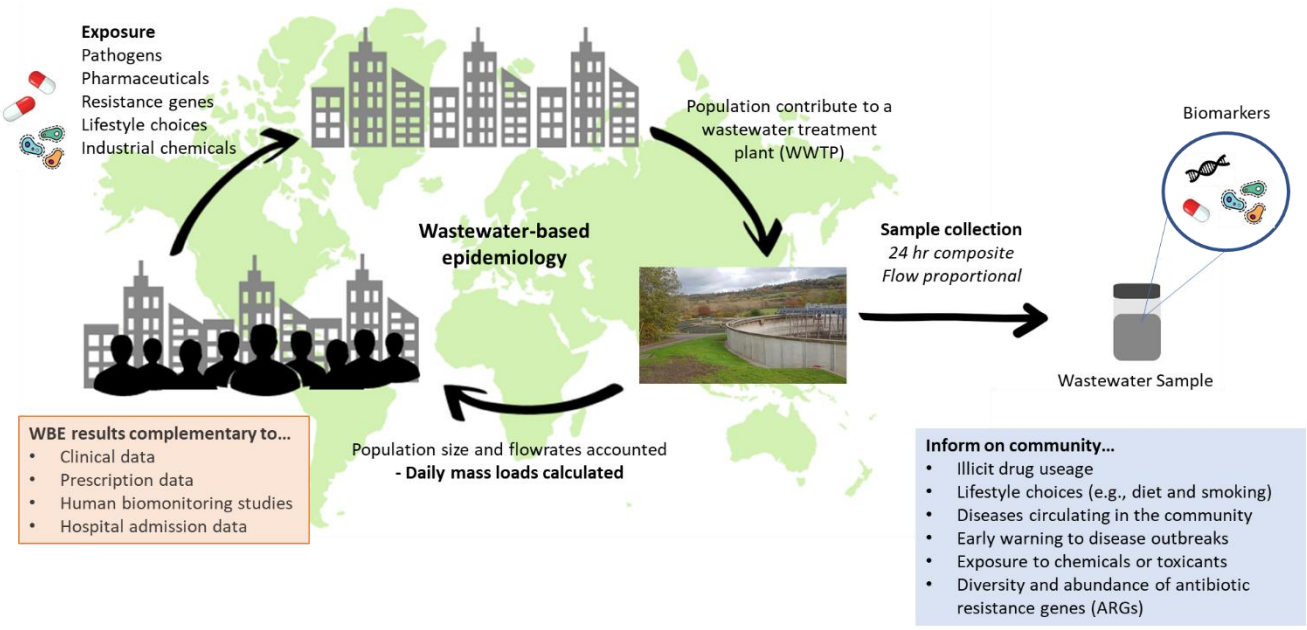

Figure 1. Graphical representation of the wastewater-based epidemiology (WBE) concept

There are several requirements for the quantification of biomarkers in WBE to be achieved. Firstly, that samples collected are 24-hour composite samples (Ort, Lawrence, Reungoat, *et al.*, 2010). This is where individual samples are taken at hourly time intervals over a 24-hour period using autosamplers. These hourly samples are combined to represent community's wastewater over a calendar day. In contrast, grab samples are the process of taking a single sample from a time point and this can give valuable information about a specific snapshot in a moment of time. In the case of viruses, grab samples can be a cost-effective route of estimating disease prevalence in a community at a particular moment, potentially acting as an early warning system.

Secondly, is an understanding of the population size of the community. Population estimates are considered one of the largest uncertainties in WBE (Castiglioni *et al.*, 2013). Wastewater treatment plants (WWTPs) treat a well-defined catchment area and have an understanding of general population size. However, fluctuations in catchment area including commuting and travel, result in challenges identifying number of individuals contributing to a WWTP. Accurate population estimates are vital for back calculations in WBE, for example intake of particular pharmaceuticals or exposure to certain toxins per 1000 people. Population sizes are also essential for normalising biomarker loads in wastewater to allow comparisons between different-sized communities. The uncertainties surrounding population estimates have led to an active and developing field of WBE (van Nuijs *et al.*, 2011; Been *et al.*, 2014; Thai *et al.*, 2019; Pandopulos *et al.*, 2021).

Many biomarkers have been proposed as potentials to estimate population size. Hydrochemical parameters, including biological and chemical oxygen demand and ammonia have been traditionally used (van Nuijs *et al.*, 2011; Been *et al.*, 2014). Potential candidates have been expanded to include

certain pharmaceuticals (O'Brien *et al.*, 2014; Rico, Andrés-Costa and Picó, 2017), artificial sweeteners (O'Brien *et al.*, 2014; Rico, Andrés-Costa and Picó, 2017), creatinine (Brewer *et al.*, 2012) and even DNA (Yang, D'Auriac, *et al.*, 2015). However, limitations still exist for many of the proposed population biomarkers. These have included stability issues, plus amounts in wastewater can be influenced by industrial discharge and cultural behaviours in communities (Lin *et al.*, 2019). This can lead to potentially misrepresentative estimates on population size.

Finally the flow rates of influent wastewater are essential, as concentrations of a specific biomarker in wastewater can be instead calculated as normalised daily mass loads. Concentrations of biomarkers could appear more dilute due to higher volumes of water in the sewage systems (e.g. increased rainfall or increased community water usage). Higher flow rates could therefore skew biomarkers concentrations to read lower than they are. Accounting for both population size and flowrates allows the comparison of different sized communities to be achieved.

The ability to analyse diverse and numerous biomarkers from a single influent sample allows for many different aspects of public health to be studied. Furthermore, sampling wastewater periodically can allow long-term trends to be established for a community. The analysis of which could therefore reveal critical information for public health that is complimentary to current routes, for example assessing the effectiveness of public health interventions.

Part of WBEs growing popularity is due to its ability to overcome challenges that conventional routes to assessing public health have. Examples of conventional routes include existing sources such as mortality and morbidity rates, prescription and hospital and admission data. Another is human biomonitoring studies (HBMs), where small representative samples from a population give samples (e.g., blood or urine) (Barr, 2008). Whilst all are valuable sources of public health information, there are several limitations. They can be biased, resource intensive and may involve lengthy ethical procedures (Bauer, 2008). Furthermore, these existing strategies are fragmented with different health issues considered distinct areas. There is often no easy mechanism for sharing of data between or being able to respond rapidly to upcoming health threats. WBE on the other hand can allow for real-time monitoring, with the ability to address the changing landscape of public health. Plus, as wastewater is from all the sewered community an overall reflection of health can be achieved.

3.3.3 Methodology for wastewater-based epidemiology

In WBE, biomarkers need to be extracted from wastewater before analysis. This poses several extraction and analytical challenges. Biomarkers are often in low concentration in wastewater plus the complex composition of wastewater, containing a diverse source of chemical and biological biomarkers, can pose problems for extraction and analysis (Daughton, 2012; Chen *et al.*, 2014). Sample preparation usually includes a filtering and preconcentration step to ensure biomarker concentrations are in high enough levels to be quantified. This is usually achieved for chemical target biomarkers via methods

including solid phase extraction and immunoassay approaches. The analytical technique of choice for WBE has been liquid chromatography-tandem mass spectrometry (Hernández *et al.*, 2018). This technique has demonstrated the ability to create diverse multi-compound methods for analysis in water environments (Petrie *et al.*, 2015; Proctor *et al.*, 2019). For the extraction of biological based biomarkers, such as DNA and RNA, numerous commercial kits exist on the market allowing for the extraction of genetic material from samples. Analysis for these has been achieved through next generation DNA sequencing and quantitative PCR (q-PCR). These have allowed for non-targeted and targeted screening of a wide range of genetic targets, including viruses, bacteria and resistance genes (Hellmér *et al.*, 2014; Newton *et al.*, 2015; Diemert and Yan, 2019; Huijbers, Larsson and Flach, 2020).

3.3.4 Biomarker selection

For biomarkers to be used in WBE there are several key criteria that need to be met (Daughton, 2012; Chen *et al.*, 2014) 1) biomarker levels need to be high enough to quantify in wastewater 2) biomarkers need to be characteristic to the exposure or disease/health status in question, for example they are released via human excretion and not formed in the sewer system 3) Excretion from humans is well understood, this is essential for back calculations 4) Stable in wastewater and in transport and storage.

3.3.5 Background of wastewater-based epidemiology

WBE has become a well-established field over the past two decades. The concept that the analysis of drug residues in wastewater could be linked back to a community was first theorised by Daughton in 2001 (Daughton, 2001). This was achieved by Zuccato in 2005 (Zuccato *et al.*, 2005), who focused on cocaine in both wastewater and linked it back to the community. The first international network, SCORE (https://score-cost.eu/) was established in 2010. The approach was adopted by EMCDDA (https://www.emcdda.europa.eu/topics/pods/waste-water-analysis_en) to enable measurement of illicit drugs in Europe. This has led to a global standardised system with regular sampling campaigns taking place on annual basis (https://score-cost.eu/monitoring/interlab/) (https://score-cost.eu/monitoring/). Since then, many global networks of long-term wastewater monitoring have been established. These include: Australia Network, Underworld, The Spanish Network of Wastewater-Based Epidemiology.

Recently, WBE has seen a surge of attention for its ability to monitor genetic material from SARS-CoVID-2 as a means for tracking infections in a community (Daughton, 2020; Foladori *et al.*, 2020; Kitajima *et al.*, 2020; Polo *et al.*, 2020). Popularity has grown largely due to the ability to give a whole community perspective from capturing input from asymptomatic carriers, and the potential to predict outbreaks. Several national and international collaborations have been established to further knowledge in using coronavirus in wastewater to monitor community spread.

Whilst popularity to WBE has increased, other benefits for widespread monitoring for wastewater have not always received as much attention as they could outside the academic community. Whilst the potential uses and benefits of WBE for public health have been widely acknowledged in the scientific

literature (Thomas and Reid, 2011; Kasprzyk-Hordern *et al.*, 2014; Choi, Tscharke, *et al.*, 2018; Daughton, 2018; Vitale, Morales Suárez-Varela and Picó, 2021), there has sometimes been a gap between translating this research to implementing programmes nationally. The wide-spread implementation of the infrastructure to monitor SARS-CoVID-2 provides an opportunity to expand out on the analysis on wastewater samples collected to other recognised biomarkers. Not only could this provide key public health information by complementing current surveillance techniques, but also provide novel routes to assess public health interventions.

3.4 Current Research

The review presents the broad and diverse field of WBE and what has been achieved to date, Table 1 presents a small selection of biomarkers examples that have been used in WBE in each area of use. This is by no means an extensive list and only includes a very small example of biomarkers for each section.

Table 1: Example biomarkers and usage in WBE

Biomarkers of…	**Potential Biomarkers**	**Biomarker Examples**	**Treatment/indicator of**	**Reference of wastewater study**
Infectious Disease	Antimicrobials and metabolites	Clarithromycin	Pneumonia, skin infections treatment	(Proctor *et al.*, 2019; Senta *et al.*, 2019; Escolà Casas *et al.*, 2021)
		n-Desmethyl clarithromycin	Metabolite of clarithromycin	(Senta *et al.*, 2019)
		Sulfamethoxole	Urinary tract infections, bronchitis	(Kasprzyk-Hordern, Dinsdale and Guwy, 2009; Guerra *et al.*, 2014; Proctor *et al.*, 2019; Escolà Casas *et al.*, 2021)
		n-Acetyl sulfamethoxazole	Metabolite of sulfamethoxazole	(Escolà Casas *et al.*, 2021)
		Oseltamivir phosphate	Influenza treatment	(Leknes, Sturtzel and Dye, 2012; Takanami *et al.*, 2012)
		Oseltamivir carboxylate	Metabolite of oseltamivir phosphate	(Leknes, Sturtzel and Dye, 2012; Takanami *et al.*, 2012)
		Acyclovir	Herpes simplex virus infections and shingles	(Prasse *et al.*, 2010; Funke, Prasse and Ternes, 2016)
		Carboxy-acyclovi	Metabolite of acyclovir	(Funke, Prasse and Ternes, 2016)
		Ketoconazole	Skin infection fungal treatment	(Huang *et al.*, 2010; Proctor *et al.*, 2019)
		Miconazole	Skin infection fungal treatment	(Kasprzyk-Hordern, Dinsdale and Guwy, 2009; Guerra *et al.*, 2014)
	Pathogenic DNA/RNA	Poliomyelitis (polio)	Infection that can affect the central nervous system (viral)	(Ndiaye, Diop and Diop, 2014)
		Severe acute respiratory syndrome (SARS-CoVID-2)	Respiratory infection (viral)	(Medema *et al.*, 2020; W. Ahmed *et al.*, 2020)
		Norovirus (GI/GII)	Gastroenteritis (viral)	(Hellmér *et al.*, 2014)
		Influenza A	Respiratory infection (viral)	(Heijnen and Medema, 2011)
		Klebsiella pneumoniae	Pneumonia, UTI, bacteraemia and endophthalmitis (bacteria)	(Shannon *et al.*, 2007)
		Enterococcus faecalis	UTIs, bacteraemia, septicaemia (bacteria)	(Shannon *et al.*, 2007)

		Salmonella enterica	Fever, vomiting and abdominal pain (bacteria)	(Yan *et al.*, 2018; Diemert and Yan, 2019)
		Candida spp	Candidiasis (fungal)	(Assress *et al.*, 2019)
		Cryptosporidium	Gastrointestinal illness (parasite)	(Wallis *et al.*, 1996)
		Giardia lambli	Small intestine infections (parasite)	(Guy *et al.*, 2003)
Antimicrobial resistance (AMR)	Antibiotic resistant genes	*ermB*	Erythromycin resistance	(Wang *et al.*, 2015)
		sul1	Sulphonamide resistance	(Wang *et al.*, 2015)
		mcr-1	Colistin resistance	(Hembach *et al.*, 2017)
		qnrS	Quinolone resistance	(Castrignanò *et al.*, 2020)
Mental Health and well-being	Drugs and metabolites	Fluoxetine	SSRI antidepressant	(Petrie *et al.*, 2015; van Nuijs *et al.*, 2015; Boogaerts *et al.*, 2019; Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021)
		Norfluoxetine	Metabolite of fluoxetine	(Petrie *et al.*, 2016; Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021)
		Venlafaxine	SSRI antidepressant	(Lai *et al.*, 2011; van Nuijs *et al.*, 2015; Boogaerts *et al.*, 2019; Rice *et al.*, 2020; Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021)
		Desmethylvenlafaxine	Metabolite of venlafaxine	(Boogaerts *et al.*, 2019; Kasprzyk-Hordern *et al.*, 2021)
		Citalopram	SSRI antidepressant	(van Nuijs *et al.*, 2015; Boogaerts *et al.*, 2019; Riva *et al.*, 2020; Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021)
		Norcitalopram	Metabolite of citalopram	(Boogaerts *et al.*, 2019)
		n-Desmethylcitalopram	Metabolite of citalopram	(Riva *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
		Mirtazapine	Antidepressant	(Boogaerts *et al.*, 2019)

Obesity and Cardiovascular disease	Drugs and metabolites	Atenolol	High blood pressure	(Lai *et al.*, 2011; Petrie *et al.*, 2015; van Nuijs *et al.*, 2015; Proctor *et al.*, 2019; Escolà Casas *et al.*, 2021)
		Metformin	Diabetes drug	(van Nuijs *et al.*, 2015; Proctor *et al.*, 2019; Xiao *et al.*, 2019; Kasprzyk-Hordern *et al.*, 2021)
		Oxypurinol	Metabolite of allopurinol (gout treatment)	(F. Ahmed *et al.*, 2020)
	DNA	*Bacteroides spp.* *Faecalibacterium spp*	Faecal bacteria	(Newton *et al.*, 2015)
Asthma/allergies	Drugs and metabolites	Salbutamol	Preventative inhalers for asthma	(Fattore *et al.*, 2016)
		Cetirizine	Antihistamine, used to relieve mild allergy symptoms	(Harman, Reid and Thomas, 2011; Proctor *et al.*, 2019)
		Fexofenadine	Antihistamine, used to relieve mild allergy symptoms	(Choi, O'Brien, *et al.*, 2018; Proctor *et al.*, 2019)
	Endogenous biomarker	1,4-methylimidazole acetic acid	Indicator of histamine turnover	(Choi, O'Brien, *et al.*, 2018)
Lifestyle Factors	Illicit drugs	Cocaine	Stimulant	(Zuccato *et al.*, 2005, 2008; van Nuijs *et al.*, 2011; González-Mariño *et al.*, 2020; Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
		Benzoylecgonine	Metabolite of cocaine	(Zuccato *et al.*, 2008; van Nuijs *et al.*, 2011; González-Mariño *et al.*, 2020; Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
		Cocaethylene	Metabolite formed when cocaine and ethanol are consumed together	(Mastroianni, Lopez de Alda and Barcelo, 2014)
		Amphetamine	Stimulant	(Zuccato *et al.*, 2008; González-Mariño *et al.*, 2020; Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
	New Psychoactive substances (NPS)	Methcathinone Mephedrone	Stimulant, similar activity to amphetamine (synthetic cathinones)	(González-Mariño *et al.*, 2016; Castiglioni *et al.*, 2021) (Rice *et al.*, 2020)

		Methoxetamine	Stimulant, similar activity to ketamine	(Rice *et al.*, 2020)
		Para-methoxyamphetamine (PMA)	Psychoactive drug with similar effects to MDMA (phenethylamine)	(Castiglioni *et al.*, 2021)
	Smoking	Nicotine	Predominantly found in tobacco	(Proctor *et al.*, 2019; Rice *et al.*, 2020)
		Cotinine	Metabolite of nicotine	(Rodríguez-Álvarez, Rodil, Rico, *et al.*, 2014; Castiglioni *et al.*, 2015; Proctor *et al.*, 2019; Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
		Hydroxy-cotinine	Metabolite of nicotine	(Rodríguez-Álvarez, Rodil, Rico, *et al.*, 2014; Castiglioni *et al.*, 2015; Kasprzyk-Hordern *et al.*, 2021)
		Anatabine (ANATA)	Tobacco related toxicant/carcinogens	(Tscharke, White and Gerber, 2016)
		Anabasine (ANABA)	Tobacco related toxicant/carcinogens	(Tscharke, White and Gerber, 2016)
	Alcohol	Ethyl sulphate	Metabolite of alcohol consumption	(Mastroianni, Lopez de Alda and Barcelo, 2014; Baz-Lomba *et al.*, 2016; Boogaerts *et al.*, 2016)
	Diet	Caffeine	Stimulant, found in coffee	(Proctor *et al.*, 2019; Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
		1,7-dimethylxanthine	Metabolite of caffeine	(Proctor *et al.*, 2019; Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)
		Enterodiol and enterolactones	Fibre indicator	(Choi *et al.*, 2019, 2020)
		Proline betaine	Citrus consumption indicator	(Choi *et al.*, 2019, 2020)
		N-methyl-2-pyridone-5-carboxamide (2PY) and N-methyl-4-pyridone3-carboxamide (4PY)	Vitamin B metabolite, formed via consumption of nicotinamide (a major B3 vitamer)	(Choi *et al.*, 2019, 2020)
		4-pyridoxic acid	Dietary vitamin B6 intake biomarker	(Choi *et al.*, 2019, 2020)
		Acesulfame, saccharin, and sucralose	Artificial sweeteners	(Choi *et al.*, 2019)
Exposure	Plasticizers/ phthalates	Bisphenol A (BPA)	Plasticizer	(Lopardo *et al.*, 2018, 2019; Wang *et al.*, 2020)
		BPA sulphate	Metabolite of BPA	(Lopardo *et al.*, 2019; Wang *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021)

		Monoethyl phthalate (MEP)	Metabolite of diethyl phthalate (DEP) exposure	(González-Mariño *et al.*, 2017, 2021; Du *et al.*, 2018; Tang *et al.*, 2020)
		Monomethyl phthalate (MMP)	Metabolite of dimethyl phthalate (DMP) exposure	(González-Mariño *et al.*, 2017, 2021; Du *et al.*, 2018; Tang *et al.*, 2020)
		Mono-i-butyl phthalate (MiBP)	Metabolite of di-iso-butyl phthalate (DiBP) exposure	(González-Mariño *et al.*, 2017, 2021; Du *et al.*, 2018; Tang *et al.*, 2020)
	Pesticides	Atrazine desisopropyl (DIA)	Metabolite of triazine pesticide exposure	(Rousis, Zuccato and Castiglioni, 2016, 2017)
		Dimethyl thiophosphate (DMTP)	Metabolite of organophosphate pesticide exposure	(Rousis, Zuccato and Castiglioni, 2016, 2017)
		3-phenoxybenzoic acid (3-PBA)	Metabolite of pyrethroid pesticide exposure	(Rousis, Zuccato and Castiglioni, 2016, 2017; Rousis *et al.*, 2017; Kasprzyk-Hordern *et al.*, 2021)
	Flame retardants	Tris (2-butoxyethyl) phosphate (TBOEP)	Parent flame retardant	(O'Brien *et al.*, 2015)
		Bis(2-butoxyethyl) phosphate (BBOEP)	Metabolite of TBOEP exposure	(Been *et al.*, 2017)
		Bis(2-butoxyetyl) 3'-hydroxy-2-butoxyethyl phosphate (HO-TBOEP)	Metabolite of TBOEP exposure	(Been *et al.*, 2017)
	Mycotoxins	Deoxynivalenol (DON) Fumonisins B1, B2 and B3	Toxicants commonly in grains (e.g., corn, wheat, oats)	(Gracia-Lor *et al.*, 2020)
Endogenous Biomarkers	Oxidative stress	8-iso-PGF2α	Indicator of oxidative stress	(Ryu, Reid and Thomas, 2015; Ryu *et al.*, 2016; Bowers and Subedi, 2021)
Population size	Hydrochemical markers	Chemical oxygen demand (COD) and Biological oxygen demand (BOD)	Population equivalent	(van Nuijs *et al.*, 2011)
		Ammonia (NH_4^+)	Population equivalent	(Been *et al.*, 2014)
	Endogenous biomarkers	Homovanillic acid (HVA) and vanillylmandelic acid (VMA)	Metabolites of dopamine, adrenaline and noradrenaline	(Pandopulos *et al.*, 2021)

Abbreviations: UTI: urinary tract infection. SSRI: Selective serotonin reuptake inhibitor. *ssp*: species

3.5 Lifestyle choices

3.5.1 Illicit drugs

Scotland's rising illicit drug usage has been highlighted as a public health crisis. There was an estimated 55,800-58,900 people in Scotland with a drug usage problem in 2018 (NHS Scotland Information Services Division, 2019). Furthermore, an estimated 1,264 people died from drug related causes in 2019, a rise from 1,187 in 2018 (National Records of Scotland, 2020). This value was highlighted as higher than any other European country and over three times that of the UK as a whole. Estimating illicit drug prevalence in a community is widely acknowledged as being challenging, particularly as drug usage is associated with hidden and stigmatised behaviours. Traditional assessment of illicit drug usage in a community includes surveys, police seizures and hospital admissions (Kraus *et al.*, 2003). Whilst these methods provide key public health information, they have several limitations. They can be time consuming and struggle to deliver up-to-date information in the changeable nature of illicit drug usage. Relying on these methods alone may result in the full picture of a community's drug usage problem being missed. These concerns have been highlighted by the UK Drug Policy Commission (UKDPC), it is recognised that drug usage is likely underreported and the full scope of a community's drug usage problem is underestimated (Reuter and Stevens, 2007).

The analysis of illicit drug residues in wastewater has demonstrated to be a dynamic and robust drug monitoring tool, with the ability to provide timely information on drug use patterns. The successes of WBE in this field have resulted in the EMCDDA establishing this technique as a novel and established drug use indicator (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2016). A broad number of illicit drugs and metabolites have been investigated in wastewater, including cocaine, heroin, methamphetamines and the respective metabolites across the world (Castiglioni *et al.*, 2006; Boleda, Galceran and Ventura, 2007; Kasprzyk-Hordern, Dinsdale and Guwy, 2008; Zuccato *et al.*, 2008). Studies have not only demonstrated drug use trends that have been consistent with other drug monitoring approaches but the ability to coordinate international studies through standardised approaches.

3.5.2 New psychoactive substances

WBE has continued to grow in this field, informing on the rising trend in usage of novel psychoactive substances (NPS). NPS, previously named 'legal highs' are drugs designed to mimic the effects of illegal substances such as cocaine or ecstasy (Stephenson and Richardson, 2014). It is estimated that NPS first appeared in the UK drug market in 2008/2009. The challenges associated with NPS, compared to more "traditional" illicit drugs, are the large number of new compounds and the rapidness that these can enter and leave the market (Peacock *et al.*, 2019).

Several studies have analysed NPS in wastewater to link back with community (González-Mariño *et al.*, 2016; Bade *et al.*, 2020, 2021). One of the most extensive studies to date, published in 2021, reported

wastewater results from 22 cities across 14 European countries over two years (Castiglioni *et al.*, 2021). This study investigated 30 NPS, including synthetic cathinones and phenethylamines alongside the more "traditional" illicit drugs (e.g. cocaine, MDMA and methamphetamine). Results demonstrated that spatial and temporal trends of different NPS could be established and that WBE could inform on the rapid changes in drug usage in the community. It has been highlighted there are challenges back-calculating NPS consumption in a community due to the limited information on human metabolism for NPS (Castiglioni *et al.*, 2021). This is contrast to many illicit drugs where metabolism is well known and reported. Further studies are therefore needed to identify the most suitable biomarkers for NPS consumption. However, initial work has clearly demonstrated WBE's ability to reflect trends of NPS usage over time and in different catchment areas. Thereby overcoming some of the challenges that traditional techniques experience in monitoring the dynamic and complex behaviours of drug usage.

3.5.3 Monitoring illicit drug and new psychoactive trends

The ability to monitor long-term trends in WBE for lifestyle factors can allow the effectiveness of policy interventions to be assessed. One UK-based study investigated in wastewater the trends of illicit and licit drug consumption between 2014-2018 in one city (Rice *et al.*, 2020). Mephedrone was classified as a class B drug in 2010. Whilst mephedrone was quantified in wastewater the first two years of this study, after 2015 it was no longer detected. The lack of mephedrone detected could demonstrate a delayed shift upon illicit drug choice after implementing the new drug classification. Another policy intervention was the regulation of NPS in 2016. Results in wastewater here observed an increased amount of more "traditional" drugs of abuse including cocaine and ketamine. WBE can reflect the complexities of regulation with regards to illicit drugs, as restrictions of one class can lead to increased use of another, as potentially observed in this study. WBE provides valuable near-real time monitoring for illicit drug consumption that can aid in complementing and filling gaps for current routes of assessing usage. This can provide evidence to allow policy makers to make informed decisions.

It should be noted that WBE can aid in identifying correlations in both licit and illicit drugs, indicating poly-drug usage. One recent study in China identified strong correlations indicting polydrug usage patterns between several pairs of illicit drugs, including heroin and cocaine, methcathione and ketamine (Liu *et al.*, 2021). Another study observed correlations between antidepressants and opioids (methadone, codeine and tramadol), potentially reflecting polydrug use of patients prescribed opioids also prescribed antidepressants (Choi *et al.*, 2019). Certain metabolites could also indicate co-consumption. Benzoylecgonine is the main metabolite for cocaine, but cocaethylene can be formed too when cocaine and ethanol (alcohol) are consumed together. Wastewater loads of cocaethylene has shown strong positive correlations to other metabolites of alcohol and cocaine in wastewater (Mastroianni, Lopez de Alda and Barcelo, 2014). This relevance is highlighted as one of the causes of increased drug deaths in Scotland is attributed to the consumption of two or more drugs at the same time. The Scottish Government has reported that of the 1,264 deaths linked to illicit drugs in 2019, 94

% of these deaths were of people who took more than one substance (National Records of Scotland, 2020).

3.5.4 Alcohol consumption and smoking

WBE has been used to investigate other lifestyle choices, including for alcohol consumption and prevalence of smoking. It is recognised that smoking and alcohol consumption are amongst the most significant risk factors for disease burden in the UK (UK Government, 2019a). Increased disease risk includes cancers, heart and liver diseases. Both alcohol consumption and smoking rates are typically assessed by general population surveys and sale statistics. In the case of alcohol, stockpiling, international buying and consumption of illegal alcohol cannot be assessed by these routes.

WBE analysis for these two areas relies on the analysis of metabolites in wastewater. For alcohol consumption, the metabolite of ethanol, ethyl sulphate, has mostly been applied in WBE (Mastroianni, Lopez de Alda and Barcelo, 2014; Baz-Lomba *et al.*, 2016; Boogaerts *et al.*, 2016). Back calculations of ethyl sulphate in wastewater can give values of L per 1000 people per day. Monitoring these levels over longer periods of time can demonstrate strong weekly trends, with clear differences between weekdays and weekends and variations in alcohol consumption can be observed in different cities.

Smoking on the other hand uses nicotine and its metabolites, cotinine and hydroxycotinine in WBE (Rodríguez-Álvarez, Rodil, Rico, *et al.*, 2014; Castiglioni *et al.*, 2015). Back-calculations here can calculate number of cigarettes per 1000 people per day. Recent research has also identified two further biomarkers linked to tobacco, anatabine and anabasine (Tscharke, White and Gerber, 2016). These are urinary biomarkers that are excreted as a by-product of smoking and are specific to tobacco (unlike nicotine which can be found in nicotine patches and gum). WBE monitoring for both tobacco and alcohol consumption has successfully demonstrated its applicability in monitoring spatio-temporal trends in both local, national and international scales. The accurate monitoring of alcohol consumption and smoking in a community is essential if related health policies are to be evaluated.

3.5.5 Diet

Diet is another area of WBE that has been proposed in the last decade (Thomas and Reid, 2011; Daughton, 2012, 2018; Choi, Tscharke, *et al.*, 2018). Diet is key for maintaining good health and well-being, a poor diet been associated with many health conditions and diseases. It is also linked to with sociodemographic patterns, with lower socioeconomic groups having poorer diets, for example lower fruit and vegetable intake (Maguire and Monsivais, 2015). Several biomarkers associated with diet in wastewater, including metabolites of fibre and fruits consumption and vitamins has been investigated in Australia (Choi *et al.*, 2019). However a follow up study highlighted that whilst many urinary biomarkers associated with diet have been identified, many have been found to not be stable enough to be used in WBE and many experience significant degradation in the sewer system (Choi *et al.*, 2020). Instead, several biomarkers were identified to be useful for qualitative/semi-quantitative work and back-

calculations were in good agreement with literature values. These included enterodiol and enterolactone (indicators of fibre intake) and proline beta (indicator of citrus consumption) which could be used as a proxy for a healthy diet.

3.6 Monitoring behavioural changes and external stresses

WBE reveals unique insight and perspective of a community's lifestyle choices. Recent studies of WBE have been utilised to monitor how communities cope with certain environmental and social stresses. With one study investigating trends and correlations of both licit and illicit drugs in a defined period of economic stress and social strain (Thomaidis *et al.*, 2016). Another study identified correlations between increased environmental temperature with increased usage of artificial sweeteners (e.g. acesulfame) in wastewater (Phung *et al.*, 2017).

Furthermore, WBE not only gives insight upon behavioural patterns associated with drug consumption, but also with attitudes surrounding disposal of medications. Unused medications should be returned to pharmacies to be incinerated and disposed of. It is known however that people can incorrectly dispose of unused medications, ending up in landfill or put down sink or toilet to end up in wastewater treatment system (Tong, Peake and Braund, 2011). The ability to distinguish between consumption and direct disposal of the commonly prescribed antidepressant, fluoxetine has been investigated in wastewater (Petrie *et al.*, 2016). This is done by understanding the chemical structures in the prescribed formulation of the parent drug and the ratios between parent and metabolite (norfluoxetine) excreted by an individual. It is expected the ratios between fluoxetine and norfluoxetine in wastewater will be at a constant level when the parent drug has been consumed. An irregularly high level of fluoxetine in wastewater one day where the norfluoxetine levels remains low, indicates a significant amount of the parent drug has been dumped rather than consumed. Back-calculation done in this study estimated the equivalent fluoxetine loads for around 900 tablets directly dumped into sewage system.

3.7 Communicable Diseases

Whilst monitoring for SARS-CoVID-2 in wastewater has attracted increasing attention during the past year. Using wastewater for disease surveillance is not a new concept and has been well discussed in the literature (Wigginton, Ye and Ellenberg, 2015; Barras, 2018; O'Brien and Xagoraraki, 2019b). Environmental surveillance of polio in wastewater for example has been established (World Health Organisation, 2003b; Hovi *et al.*, 2012; Roberts, 2013). Others have retrospectively predicted outbreaks of hepatitis A and norovirus in wastewater (Hellmér *et al.*, 2014) and influenza has also been detected in wastewater (Heijnen and Medema, 2011). Though notably less common, WBE has also been applied to monitor bacterial infections (Yan *et al.*, 2018). For example, prevalence of enteric *Salmonella* in a population, causing sickness and diarrhoea, was monitored in Hawaii (Diemert and Yan, 2019). Researchers could observe elevated levels of a particular *Salmonella* strain in wastewater simultaneously with a clinically reported outbreak. Results also showed same strain re-emerged as a

dominant species in wastewater a year later, potentially indicating a new outbreak of *Salmonella* in the community that was not detected by clinics.

Many of these mentioned studies have used genetic material from pathogens, e.g. DNA or RNA, to monitor spread in a community. However the benefit of including the analysis of other relevant biomarkers with genetic material of pathogen in question has been highlighted as more effective route to assessing disease spread at the community level (Daughton, 2020). Whilst the genetic material of a pathogen can be a very specific biomarker and have the potential to act as an early warning system, there are limitations as recognised by the literature (Daughton, 2020; Foladori *et al.*, 2020; Kitajima *et al.*, 2020; Polo *et al.*, 2020). For example, with regards to coronavirus RNA, the amounts individuals excrete can be variable (Joynt and Wu, 2020). This can cause challenges with back-calculating RNA levels quantified in wastewater to the number of individuals potentially infected at the community level (Kitajima *et al.*, 2020). This can be further complicated with the questionability on whether the genetic material present in wastewater has come from an active virus or from someone who has recently recovered from the virus (Daughton, 2020). This could result in overestimation of prevalence of the disease at the community level. Whilst these discussions have focused on coronavirus, they are applicable for other diseases monitored via WBE too. Other potential limitations that have been previously discussed prior to coronavirus are stability of the genetic material in wastewater, variability in sampling approaches and the low efficiency for virus concentration methods (Girones *et al.*, 2010).

It is considered that analysing other biomarkers associated with diseases, including pharmaceuticals and endogenous biomarkers (e.g. inflammation) could help overcome some of these discussed limitations (Daughton, 2020). For example, pharmaceuticals used to treat specific diseases and corresponding metabolites can be a good reflection for disease presence in a community. Prevalence of hepatitis B has been done using the antiviral drug lamivudine (Hou *et al.*, 2020). The limitations are recognised that prescribed drugs may not always be disease specific, in the case of lamivudine it is also prescribed for human immunodeficiency viruses (HIV) treatment, and it is impossible to distinguish whether treatment is from hepatitis B or HIV. This study also did not look at metabolites of lamivudine. Therefore, some of the levels of lamivudine observed could have resulted from direct disposal of lamivudine into the sewage system via toilet or sink rather than consumption. Endogenous biomarkers of disease and health are discussed later in this review.

3.8 Antimicrobial Resistance

Antimicrobial resistance (AMR) has been hailed as one of the greatest threats to public health risks threatening medicine in the 21st century (O'Neill, 2014). Antimicrobial resistance is defined by WHO as "microorganisms such as bacteria, viruses, fungi and parasites change in ways that render the medications used to cure the infections they cause ineffective"(World Health Organisation, 2017a). This process occurs naturally but is further accelerated by inappropriate use of medicines. The

consequences of AMR are significant, and could result in easily treated infections being fatal (Bush *et al.*, 2011). It has been estimated by 2050 there could be as many as 10 million deaths per year attributed to AMR (O'Neill, 2014). Poor surveillance has been highlighted as one of the critical problems regarding AMR. The Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 by WHO was established with the aims of sharing information on the global scale to strengthen data and aid decision making on national and international actions (World Health Organisation, 2015a). Several limitations of current AMR surveillance were highlighted, including selection bias in samples and inconsistent global coverage. The result was a call to include AMR data from whole populations and not just from clinical studies alone.

Scotland's Antimicrobial Prescription Group (SAPG) (https://www.sapg.scot/) was established in 2008 with a primary aim to coordinate a national framework for antimicrobial stewardship. SAPG have developed surveillance systems and ensured standardised information on antimicrobial use and resistance that is accessible to NHS boards. In 2019, the Scottish One Health Antimicrobial Use and Antimicrobial Resistance report was published by Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) Scotland (Antimicrobial Resistance and Healthcare Associated Infection (ARHAI), 2020). This report provides information on antibiotic use and resistance to antibiotics in Scotland during 2019. It supports the five-year United Kingdom National Action Plan and a 20-year vision for containing and controlling AMR (UK Government, 2019b). Currently, much surveillance is based on clinical data and prescription data. WBE provides an opportunity as a complimentary technique to achieve a whole population approach to tackling AMR.

3.8.1 Patient compliance to pharmaceuticals

Prescription data in the UK is easily accessible, however just because a medication has been prescribed does not necessarily mean it was consumed. Patient compliance to medical treatments is known to vary. In the case of antibiotics, individuals often feel recovered before the end of a prescribed dose. The result this can be individuals do not complete the course of antibiotics, potentially leading to both stockpiling of leftover doses and self-prescribing at a later date. It has been previously highlighted that up to a third of patients do not comply to antibiotic treatment instructions and a quarter use doses leftover from previous treatment (Kardas *et al.*, 2005). Leftover antibiotics are a key driver for AMR and non-compliance not only promotes resistance but has serious implications of costs of healthcare (Hughes *et al.*, 2001). Estimating patient compliance is challenging, as it relies on self-reported questionnaires and counting leftover doses in clinics but results from these can be biased.

Whist numerous WBE studies exist for investigating licit and illicit drug consumption, only a handful of studies to date have compared spatial and temporal trends of pharmaceuticals and matched these to predicted concentrations (Lai *et al.*, 2011; Baz-Lomba *et al.*, 2016; Rice *et al.*, 2020; Riva *et al.*, 2020; Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021). This requires a knowledge of prescription

data, formulations, excretion rates and can be utilised to investigate overall adherence of medications in a population. Whilst these studies have looked at a broad range of pharmaceuticals, a couple of these studies have included antibiotics (Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021). It is key that both parent compounds and metabolites are considered in wastewater analysis for two reasons 1) in the case for antimicrobials specifically both have implications for promoting resistance in bacteria in WWTPs 2) in the case of pharmaceuticals more generally, the ratio between parent and metabolite is key for assessing if a pharmaceutical has been consumed (e.g. compliance can be investigated) or if it has directly been disposed of. An understanding of antibiotics in wastewater is important, as unlike other contaminants where cut off points can be identified, sub-lethal concentrations of antibiotics in wastewater can promote resistance (Jury *et al.*, 2011).

3.8.2 Antimicrobial resistance surveillance

It has been proposed that the combined wastewater surveillance of antibiotics and metabolites with pathogens and antibiotic resistance genes (ARGs) could be a proxy for regional AMR and how changes can occur overtime (Kwak *et al.*, 2015; Larsson *et al.*, 2018). The presence of ARGs in wastewater will be complex, some genes may be excreted from the human community contributing and others will be from the diverse microbial communities found in wastewater. These microbial communities will be influenced from years of exposure to sub-lethal concentrations of multiple antibiotics and metabolites in wastewater. Furthermore, the varying consumption patterns in human populations will result in unique microbial communities in wastewater between different geographic locations. WBE also allows analysis of cofactors in wastewater. These include other micro-contaminants such as heavy metals and biocides (e.g., disinfectants), that can have a role in promoting or facilitating antimicrobial resistance in microorganisms (Baker-Austin *et al.*, 2006; Tello, Austin and Telfer, 2012).

There are numerous studies in the literature investigating AMR surveillance in wastewater (Gao, Munir and Xagoraraki, 2012; Novo *et al.*, 2013; Rodriguez-Mozaz *et al.*, 2015; Raven *et al.*, 2019; Castrignanò *et al.*, 2020). Recent studies have also demonstrated strong relationships existing between wastewater and clinical resistance prevalence, indicating wastewaters ability to potentially predict resistance in clinics (Pärnänen *et al.*, 2019; Huijbers, Larsson and Flach, 2020).

Due to the complexities of antibiotic pollution and impacts on resistance, robust surveillance methods are needed to assess resistance on a clinical level. Overall whilst total antibiotic usage has decreased by 7.6 % since 2015 in Scotland (ARHAI Scotland, 2020), it is recognised that there is more to be done if the UK's overall goal to reduce antibiotic usage in humans by 15 % by 2024 is to be achieved (UK Government, 2019b). The ability of WBE to monitor long term trends will be key for AMR surveillance, as observing population-wide trends over several years will be required. Whilst the direct impact of interventions may be observed via reduced antimicrobial and metabolite levels in wastewater, to see the impacts interventions have on resistance in microbial communities in wastewater and environment may

occur over many years. Having effective WBE in place to monitor antimicrobials, genes and cofactors will not only allow effective population-wide surveillance to be achieved but also allow interventions on the community scale to be monitored. Such data would be complimentary to current surveillance in Scotland where a national surveillance programme for monitoring AMR in clinically important pathogens was established in 2009 (based on the European Antimicrobial Resistance Surveillance System) (Nathwani *et al.*, 2011).

3.9 Non-communicable diseases and well-being

3.9.1 Mental health

Mental health has been identified as one of the main causes of disease burden worldwide. The COVID-19 pandemic has exacerbated mental health issues in the UK. Evidence has indicated a worsening of mental health in the first national lockdown on the 23rd March 2020, with UK government identifying psychological distress, anxiety and depressive symptoms peaking in April 2020 (Public Health England, 2021). Isolation was identified as a significant contributor to rising rates of mental health services and access to medications. Antidepressant usage in a population is typically based on prescription data and general population surveys (Thacker and Berkelman, 1988; Cadarette and Wong, 2015). Whilst UK is fortunate to have easily accessible prescription data, the limitations with current routes include reporting bias for population surveys and as previously touched upon with antibiotics, whilst a drug has been prescribed does not necessarily mean it has been consumed. These limitations can potentially be attributed to fears of stigmatism surrounding issues with mental health.

Many studies have included antidepressants in multi-compound analytical methods in wastewater (Petrie *et al.*, 2015; Thomaidis *et al.*, 2016; Boogaerts *et al.*, 2019; Choi *et al.*, 2019; Proctor *et al.*, 2019). One recent study focused entirely on antidepressants for WBE, developing an analytical method for 27 commonly prescribed antidepressants with several metabolites (Boogaerts *et al.*, 2019). Back calculations for popular antidepressants including citalopram, venlafaxine and mirtazapine from wastewater sampled from four WWTPs in Belgium demonstrated the mass loads agreed with prescription data. Two UK-based studies investigated a broad selection of pharmaceuticals including antidepressants in WBE and linked back to prescription data (Rice *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021).

One of these UK-based studies monitored wastewater trends of both licit and illicit drugs in one city in the South-West of England between 2014-2018 (Rice *et al.*, 2020). The ability to monitor long-term consumption trends by WBE were demonstrated here. For example, significantly increased levels of the antidepressant venlafaxine in wastewater reflected increases in prescriptions at the catchment level. Interestingly, wastewater loads of venlafaxine were in higher amounts then the concentrations predicted from prescription data. Rates of antidepressant prescription were rising in the decades prior to the study period and were expected to continue increasing (Mars *et al.*, 2017). The UK has been experiencing a

period of political economic austerity since 2010 including across the period of 2014–2018, and under similar circumstances in other European countries this has led to an increase in prescriptions of antidepressants and other drugs to treat mental health (Thomaidis *et al.*, 2016). In line with expectations, venlafaxine prescription rates did increase significantly each year, although prescription rates of other antidepressants (amitriptyline and fluoxetine) did not change significantly from 2014 to 2018. Increasing demand for antidepressants coupled with the online availability of venlafaxine and its relatively lower cost, compared to NHS prescriptions, could explain the mismatch between prescription and wastewater data. Ultimately, the reason behind the discrepancy in wastewater and prescription data is unclear, but what is important is that the trends in both of them are the same, which helps to provide important context to the results.

A significant advantage of WBE over conventional routes is the ability to gain health information from the whole population. A limitation of monitoring pharmaceuticals alone in wastewater for estimating disease prevalence is that a portion of the population could be missed. For example, those who are experiencing mental health problems but have not sought out medical help or have chosen non-pharmaceutical therapies. Residues of antidepressants and metabolites in wastewater would imply an origin of an individual who has sought out help from a medical health professional and had antidepressants prescribed. However due to the stigmatism that still surrounds mental health problems today, it is highly likely a portion of the population who experience mental health problems will be missed.

The analysis of endogenous biomarkers that are elevated or decreased in urine when an individual is experiencing mental stress would be valuable for WBE (Daughton, 2012). It should be noted there are no routine diagnostics on urine analysis for mental health disorders. At a clinical level, several studies have investigated urinary metabolites that could be linked to mental health (Zheng *et al.*, 2013, 2016; Shimanoe *et al.*, 2021). However there has currently been no studies of endogenous biomarkers of mental health in wastewater, further research is needed to identify urinary biomarkers indicative of mental health that are suitable for WBE.

3.9.2 Obesity and cardiovascular diseases

The UK is experiencing growing problems of obesity with an estimated 63 % of adults over a healthy weight and a half of these living with obesity (Public Health England, 2019). Scotland has been identified as having some of the highest obesity levels among the Organisation of Economic Co-operation and Development (OECD) countries (ScotPHO Public Health Information for Scotland, 2020). Obesity increases risks of developing certain diseases, including type 2 diabetes, high blood pressure and certain cancers (GOV.UK, 2017). Higher levels of obesity have been correlated to lower socio-economic status and links between lower self-esteem and mental wellbeing have been identified. Failure to address growing rates of obesity results in greater pressure on the NHS. It was estimated that

the NHS spent £6.1 billion on overweight and obesity-related health in 2014-2015, with these UK-wide NHS costs projected to reach £9.7 billion by 2050 (GOV.UK, 2017).

Wastewater monitoring provides a multifaceted approach to assessing population-wide cardiovascular diseases and associated conditions. For example, analysis of antidiabetic drugs in wastewater have been good indicators of disease prevalence in the community. Metformin is a first-line medication for type 2 diabetes and helps lower blood sugar levels. One study used metformin as a biomarker in wastewater to assess trends of type 2 diabetes over a period of four years, with results showing increasing trends that matched prevalence results estimated from traditional surveys (Xiao *et al.*, 2019). Linking into earlier discussions of how wastewater can provide information on a communities' diet, a large-scale study in Australia assessed the prevalence of gout in a population using WBE (F. Ahmed *et al.*, 2020). Gout is a type of arthritis that can cause severe joint pain and has many risk factors including obesity and diet. Researchers analysed oxypurinol, a main urinary metabolite of the first-line gout treatment allopurinol, in the wastewater collected from 75 WWTPs. Many multi-residue analysis methods have been developed that include pharmaceuticals associated with cardiovascular diseases in wastewater exist in the literature (Petrie *et al.*, 2015; Choi *et al.*, 2019; Proctor *et al.*, 2019; Escolà Casas *et al.*, 2021).

It is not just pharmaceuticals and metabolites in wastewater that can give key population-wide information on cardiovascular diseases. The analysis of fragments of DNA from bacteria that reside in an individual's gut can also be used. It has been previously highlighted in the literature that several bacterial species that reside in the human gut microbiome (the community of microorganisms living together), are enough to differentiate between an obese individual and a lean one (Le Chatelier *et al.*, 2013). Following on from this it was theorised that the human faecal microbiome could therefore potentially act as proxy to the human gut microbiome. One study analysed the bacterial communities in wastewater via gene sequencing from 71 cities in the US and found good predictors of estimated levels of obesity within the community (Newton *et al.*, 2015). The analysis of biomarkers associated with obesity including pharmaceuticals and gut bacteria via WBE can act as an additional epidemiology tool to provide real time monitoring of community health. Disease prevalence and trends can be monitored long-term allowing the effectiveness of public health interventions to be assessed, providing up-to-date evidence for policy makers to make informed decisions.

As with antibiotics, patient compliance to medication is also another area of concern. On average, a course of antibiotics will typically last 5 days, in contrast many treatments associated with non-communicable diseases, including diabetes, tend to be long-term (Kardas *et al.*, 2005; Muszbek *et al.*, 2008). It was previously highlighted that nearly 9 % of all cardiovascular diseases in Europe are attributed to poor adherence with medical treatments (Muszbek *et al.*, 2008). It has further been estimated that in developed countries, compliance to long-term treatments does not succeed 50 %

(World Health Organisation, 2003a). As previously mentioned, only a handful of WBE studies to date have matched long-term trends and spatial differences with predicted concentrations calculated from prescription and excretion data (Lai *et al.*, 2011; Baz-Lomba *et al.*, 2016; Rice *et al.*, 2020; Riva *et al.*, 2020; Escolà Casas *et al.*, 2021; Kasprzyk-Hordern *et al.*, 2021). Whilst a relatively new area to WBE, these studies have shown potential with matching wastewater data to predicted values. Results could be invaluable for measuring community compliance for both long-term conditions, including diabetes and mental health problems and short-term treatments, such as antimicrobials.

3.9.3 Asthma and allergies

WBE has also been applied to monitor both asthma and allergies and have correlated levels with environmental factors. One example of this is estimating a populations burden to hay fever (allergic rhinitis) through wastewater. This can be achieved by monitoring medications used to treat hay fever. For example, cetirizine and fexofenadine are antihistamines and are common ingredients in over-the-counter hay fever medications. One study in Oslo demonstrated positive correlations between seasonal pollen and cetirizine levels in wastewater, with much higher loads observed in summer when compared to winter (Harman, Reid and Thomas, 2011). Another study used WBE to investigate population hay fever burden with fexofenadine and 1,4-methylimidazole acetic acid (MIAA) (Choi, O'Brien, *et al.*, 2018). MIAA is an endogenous urinary biomarker released by the body in response to histamine. Results demonstrated strong correlations between the two, indicating histamine burden is linked with fexofenadine intake.

A community's asthma burden associated with air pollution is another area of WBE that has shown promise in recent years. In the UK, air pollution has been identified as one of the largest risks to public health, with the annual mortality of human-made air pollution in the UK is roughly equivalent to between 28,000 and 36,000 deaths every year (Public Health England, 2020). It has been estimated that between 2017 and 2025, the total cost to the NHS and social care system of air pollutants will be £1.6 billion (Public Health England, 2020). Air pollution has been linked to many health conditions, including exacerbation of asthma, increases in respiratory and cardiovascular diseases, lung cancer and recent research affecting the brain causing dementia. Growing concerns of air pollution have led to clean air initiatives across the UK, including Scotland's Cleaner air for Scotland strategy for the next five years to improve air quality (Scottish Government, 2020).

Whilst particulate matter is one way to directly monitor effects of interventions for air pollution, there is not always a clear link to assessing the public health impacts in real time. There are often delays in getting information of hospital admissions or prescriptions associated with air pollution and this may reflect only a small percentage of the population effected. WBE have demonstrated asthma burden in a population using the medication salbutamol as an indicator for asthma. Salbutamol is the active pharmaceutical in inhalers, acting as a bronchodilator which helps relax the muscles of the airways in

the lungs. Salbutamol inhalers are known as reliver inhalers, as they give quick relief from breathing problems when required. As asthma is exacerbated by air pollution, salbutamol in wastewater is therefore a good indicator of when someone might have experienced symptoms and used one to relieve them. Inhaler usage is challenging to assess from prescription data alone as whilst inhalers have been prescribed, there is no information to when they have been used. This is added to the fact that NHS advice is to replace inhalers every six months, even if not empty. Salbutamol levels in wastewater can therefore reflect in near-real time a community to relieve symptoms via salbutamol inhalers. The relationship between salbutamol in wastewater and air pollution was investigated via a study in Milan (Fattore *et al.*, 2016). Increased levels of airborne particulate matter with increased levels of salbutamol in wastewater were observed, indicating exacerbated asthma symptoms on days with higher levels of air pollution. WBE could therefore provide novel insight into estimation of allergy and asthma burden in a population much quicker than current public health monitoring tools. Further work has been identified as broadening the medications to analyse in wastewater to cover more hay fever and asthma medications.

3.10 Exposure of chemicals

An area of WBE that is currently under development is for monitoring community exposure to various chemicals. Currently human biomonitoring (HBM) studies are the main tool for assessing exposure for many classes of compounds, including pesticides and bisphenol A (BPA) (Barr, 2008; Dekant and Völkel, 2008). Limitations to HBM include stringent and lengthy ethical procedures, samples can be invasive (e.g., sampling blood) and excretion profiles of biomarkers can vary throughout the day (Bauer, 2008). The results from HBM will also only provide a snapshot of population exposure at a particular moment of time. In contrast, WBE can help overcome some of these limitations by being reflective of whole populations over a period of time. Whilst WBE would not replace HBMs, it can provide an efficient and cost-effective approach to complement them.

3.10.1 Pesticide exposure

Exposure to pesticides has been associated with neurological conditions including Parkinson's disease, cancer and sperm DNA damage (Allen and Levy, 2013; Saillenfait, Ndiaye and Sabaté, 2015). Whilst it has been reported that overall pesticide usage has declined in Scotland since 2018, 98 % of arable crops were still treated with a pesticide in 2018 (Scottish Government, 2019). There is currently a lack of pesticide exposure information for the general population in the UK, as many pesticide exposure studies have been focused on exposure farm workers might experience (Sleeuwenhoek *et al.*, 2007). Whilst farmers may experience exposure from direct application of pesticide, the general population is exposed to pesticides through diet and through living close to agricultural areas where spraying occurs (Aprea, 2012). One study to date has investigated urinary biomarkers of pesticide exposure of residents in the UK, including East Lothian in Scotland (a major arable area) (Galea *et al.*, 2015). Results demonstrated that were was no evidence of increased urinary biomarker excretion in residents following

spray events. However, levels observed in urine were in agreement with other studies done internationally, indicating diet is a likely source of pesticide exposure. It was recognised however that pesticides have short half-lives in the body which presents a challenge for HBM data, as urine samples would need to be collected within a 24-hour period from a spray event ideally.

WBE could again be used to overcome this limitation. The first studies using WBE to investigate population exposure to pesticides was in 2016 and 2017 and have demonstrated regional differences and comparable results with HBMs (Rousis, Zuccato and Castiglioni, 2016, 2017). The most extensive study to date investigated population exposure to three classes of pesticides (triazines, organophosphates and pyrethroids) across eight cities across Europe (Rousis *et al.*, 2017). A selection of 15 urinary metabolites of pesticide exposure were evaluated in wastewater. Back-calculated intake of pesticides were compared with national statistics on insecticide sales for each country. The results indicated higher levels of a countries insecticide sales can lead to higher population exposure to pesticides. The back-calculated pesticide intake values were compared to results from HBM studies previously done, demonstrating comparable results and indicating WBE could be a cost-effective solution to population monitoring of pesticide exposure.

Another UK-based study assessed pesticide exposure via WBE for pyrethroid pesticides on five cities in the South-West (Kasprzyk-Hordern *et al.*, 2021). This study demonstrated geographic differences between cities indicating different levels of pesticide exposure depending upon location. Whilst there is a push for reducing reliance of pesticide usage on crops, unprecedented population growth and climate change continues to put stress on food production. Due to the variance in exposure experienced in cities observed in WBE studies so far, the importance of assessing pesticide exposure across multiple locations is key to highlighting vulnerable populations. In the UK, studies utilising WBE to investigate pesticide exposure in communities has only been achieved in the South-west of England. Expanding out to multiple-cities could provide complementary data and build upon HBM work previously done in the UK. Long-term monitoring trends could expand on knowledge on population exposure through both diet and potential spraying effects.

3.10.2 Industrial chemicals exposure (bisphenol A, phthalates and flame retardants)

Exposure to industrial chemicals that individuals come across in everyday life is another area showing promise for WBE. Bisphenol A (BPA) is a common plasticizer which has been evidenced to have endocrine disrupting properties. Endocrine disrupting chemicals interfere with hormone regulation which can affect health and reproduction in both humans and animals (World Health Organisation, 2010a) Diet has been highlighted as a major source of exposure, e.g. due to leaching from plastic packaging into food (Mustieles *et al.*, 2020). Flame retardants are another class of chemicals used increasingly in consumer products yet have been associated with several human health problems including suspected carcinogens and concerns of neurodevelopment issues (Dishaw *et al.*, 2011; van

der Veen and de Boer, 2012). There is a lack of evidence on exposure to such chemicals and long-term effects of exposure are not fully understood. Growing evidence of the negative effects of BPA have led to replacements by other bisphenols, such as bisphenol S (Mustieles *et al.*, 2020). However, these have also demonstrated to have endocrine disrupting properties. The growing areas of concern have catalysed Scotland's environmental charity, Fidra, to have designated projects for tackling both bisphenols and flame retardants in everyday products (https://www.fidra.org.uk/projects/). It is recognised there is an urgent need for cost-effective monitoring tools to timely assess human exposure for a range of chemicals. Not only can this inform upon current risks on exposure to chemicals in everyday use, but also assess exposure to potential chemical replacements.

There have been several studies of WBE for assessing community exposure of BPA (Lopardo *et al.*, 2018, 2019; Wang *et al.*, 2020; Kasprzyk-Hordern *et al.*, 2021), phthalates (González-Mariño *et al.*, 2017, 2021; Du *et al.*, 2018; Tang *et al.*, 2020) and for flame retardants (O'Brien *et al.*, 2015; Been *et al.*, 2017). One study investigated community exposure of certain flame retardants and plasticizers across five cities in Europe (Been *et al.*, 2018). In the UK, several studies have been done investigative community exposure for BPA via WBE, similar to pesticides these studies have only been done in the South-west of the UK (Lopardo *et al.*, 2018, 2019; Kasprzyk-Hordern *et al.*, 2021). One study investigated BPA sulphate as a characteristic urinary metabolite of BPA exposure in wastewater of five major WWTPs. Results from this study demonstrated varying levels of BPA exposure between sites, with two of the five observing higher BPA sulphate loads corresponding to higher intakes of BPA. These were estimated to be well above the tolerable daily intake threshold set by the European Food Safety Authority (European Food Safety Authority (EFSA), 2015). As there are limited studies currently on chemical exposure to varying classes of compounds in the UK more evidence is needed for effective policy interventions. WBE could be applied as a cost-effective and timely tool to help identify vulnerable populations to chemical exposure.

3.10.3 Mycotoxin exposure

Exposure to mycotoxins in a community's diet is another area of promise for WBE. Mycotoxins are toxic compounds naturally produced via funguses that grow on food like cereals. Exposure to mycotoxins have shown harmful effects on both human and animal health, with links to cancers, birth defects and gastrointestinal disorders (Bhat *et al.*, 1997; Hussein and Brasel, 2001; Fung and Clark, 2004). Due to the associated risks, maximum acceptable limits have been established for some mycotoxins in food (European Union, 2006). However, it is widely acknowledged gaps on the impacts of climate change and the prevalence of mycotoxins. Altered temperatures, increased rainfall could allow fungal species to be more prevalent or allow strains to evolve (Skelsey and Newton, 2015).

The main cereals grown in Scotland are barely (malting purposes) and oats (food and animal feed). It has been previously highlighted by that the main mycotoxin producing fungi of concern from a Scotland

and a wider UK perspective are the *Fusarium spp* (Food Standards Scotland, 2015). Infection of *Fusarium spp* causes *Fusarium* head blight, producing the mycotoxins deoxynivalenol (DON) and zearalenone (ZON). Due to the robust nature and stability of mycotoxins they have been reported to pass into, fermented products including beer but not distilled products like whiskey (Food Standards Scotland, 2015). This has caused concern for malting brewers regarding fungal contamination as this can impact quality and flavour of final product (Nielsen *et al.*, 2014). Due to growing concerns of mycotoxin exposure, a number of urinary HBM studies have been investigated across the world (Tuanny Franco *et al.*, 2019). In the UK, a handful of HBM studies have investigated mycotoxins in urine, these studies have focused on DON and urinary metabolites (Wells *et al.*, 2017; Papageorgiou, Wells, Williams, K. L. M. White, *et al.*, 2018; Papageorgiou, Wells, Williams, K. White, *et al.*, 2018). Results from these studies reported certain groups in the UK, including young children and adolescents may be exceeding current limits of DON. Limitations with studies have been recognised as uncertainties with estimating mycotoxin dietary intake and the small number of communities been investigated. There is therefore a need for larger-scale and longer-term studies to address population exposure to a range of mycotoxins.

WBE has recently been applied to assess community exposure mycotoxins in four cities in Spain and Italy (Gracia-Lor *et al.*, 2020). A selection of eleven urinary mycotoxins, including DON and fumonisins B1, B2 and B3 were investigated. It was reported that DON intake estimates that were back-calculated by WBE, were close to reported values in HBM studies. Whilst this new area of work for WBE, has study has demonstrated initial promise of using wastewater to assess as a community intake of mycotoxins, complimentary to current HBM approaches.

3.11 Endogenous biomarkers linked with disease or health status

As mentioned previously, endogenous biomarkers are produced by an individual's metabolism in response to either a disease or health status. For example, these could be biomarkers of inflammation or stress that are produced in the body in response to a disease. An earlier example of an endogenous biomarker applied in WBE was MIAA, a urinary biomarker released by the body in response to histamine. This was analysed alongside the hay fever medications in wastewater to estimate hay fever burden in a population. (Choi, O'Brien, *et al.*, 2018) The benefits of broadening WBE to include endogenous biomarkers has been well-discussed in the literature (Choi, Tscharke, *et al.*, 2018; Daughton, 2020; Rice *et al.*, 2020; Sims and Kasprzyk-Hordern, 2020). Daughton presented the Sewage Chemical-Information mining (SCIM) approach for this purpose (Daughton, 2012, 2018). Here the analysis of endogenous biomarkers in wastewater could reveal novel insight into general health or disease status in the community. Biomarkers analysed could be indicative of certain states, such as stress, inflammation or disease and the types of biomarkers could vary, including small molecules, proteins, sugars and lipids.

Oxidative stress has previously been highlighted as promising biomarkers for use in WBE, with isoprostanes highlighted as ideal candidates (Daughton, 2012). Oxidative stress is when there is an imbalance of free radicals and antioxidants in the body, the result of which can lead to cell and tissue damage. Whilst it's involved in natural processes such as aging, it is also linked to many diseases and lifestyle choices (e.g. smoking). Oxidative stress is a relatively new area for WBE, with a few studies discussing its potential use in WBE (Daughton, 2012; Ryu, Reid and Thomas, 2015). One study has investigated the isoprostane oxidative stress biomarker, 8-iso-PGF2α, for its suitability as a marker of health (Ryu *et al.*, 2016). Wastewater from 11 cities in Europe was analysed alongside metabolites of tobacco smoking (hydroxycotinine) and alcohol consumption (ethyl sulphate) to investigate potential correlations. Results reported strong correlations of 8-iso-PGF2α with tobacco consumption across studied cities. A follow up study investigated in-sewer stability of several isoprostanes and confirmed suitable stability for WBE (O'Brien, Choi, *et al.*, 2019). A more recent study in the US investigated several isoprostane isomers in wastewater to monitor community stress during the COVID-19 pandemic (Bowers and Subedi, 2021).

With regards to monitoring infectious disease spread at the community level via WBE, it has been highlighted by Daughton that WBE should not be limited to monitoring the infectious genetic material alone. Instead WBE should be expanded out to targeting endogenous biomarkers that are significantly elevated in a diseased state (Daughton, 2020). The benefits of expanding out WBE to indirect, more generic markers of infection were highlighted as reduced costs for analysis and potentially such biomarkers could be better indicators of infection, possibly resulting in a better early warning system. Furthermore, in the case of COVID-19 much uncertainty lies in variability of viral excretion (Joynt and Wu, 2020). Analysing indirect biomarkers of inflammation alongside genetic material could therefore help account for this. This information would be valuable as many diseases, both infectious and non-communicable, involve inflammatory damage and oxidative stress. There is still much work to be done in this field for expanding out WBE to include multiple endogenous markers to link back to public health. There is currently a lack of endogenous biomarkers reflecting chronic disease state (e.g. diabetes) and for well-being. However, with the growing field of metabolomics, it is expected more urinary endogenous biomarkers will continue to be identified.

3.12 Future Outlook

3.12.1 Environmental considerations

WBE uses analytical tools, infrastructure and knowledge base that were developed with environmental monitoring in mind. These include: liquid chromatography and mass spectrometry instrumentation that is widely used for quantitative analysis of regulated and emerging contaminants in water or composite samplers that are widely used at inlets and outlets of WWTPs to determine efficiency of wastewater treatment processes. There comes an opportunity for the development of an integrated local, regional

or a national monitoring system focussed on whole river catchments to deliver critical information on both environmental and public health. Historically environmental health was evaluated independently of public health issues. This is counterproductive as environmental health is directly interlinked with public health, ie. environmental deterioration including pollution and loss of biodiversity has direct impact on public health.

As an example, a recent project undertaken in South-West of England designed an integrated sampling regime and developed analytical methods focussed on >100 chemicals in the River Avon Catchment in South West England (Kasprzyk-Hordern *et al.*, 2021). A total of five WWTPs serving five towns and cities, covering an area of approximately 2,000 km^2 and the population of ~1.5 million (this constitutes >75% of the overall population in the catchment). Samples collected from wastewater influent and effluent allowed for verification of efficiency of treatment processes and identified problematic pollutants (also those of emerging nature or recommended for regulation, i.e. included on EU watchlists) that might require further attention. Samples from receiving river water allowed for the evaluation of environmental risks (paper in preparation). Wastewater influent was used to inform public health status: pharmaceuticals from wide-ranging groups were used as proxies to inform prevalence of NCD (i.e. cardiovascular disease, diabetes, asthma) (paper in preparation) as well as antibiotic usage and prevalence of resistance genes. Spatiotemporal trends in chemical intake were observed as a result of occupational exposure (higher bisphenol A (BPA) intake during weekday), and lifestyle choices (higher cocaine and pyrethroid pesticides intake during weekend). WBE is not intended to estimate individual exposure to chemicals. It can however provide estimates at a community level, and as a result, it has the potential to be developed into an early warning system, a powerful tool for large scale screening studies identifying communities at risk and in need of high-resolution individual testing at a localised scale.

3.12.2 Ethical Considerations

WBE is currently subject to rapid developments. Is has been successfully applied in national and international SARS-CoV-2 surveillance. WBE is now acknowledged as one of epidemiology tools, hence, ethical considerations should also apply, especially when applying near-source tracking. Ethical guidelines exist only for WBE's first application, estimation of illicit drug usage via WBE, currently utilised by EMCDDA to estimate drug use trends in the EU (https://score-cost.eu/wp-content/uploads/sites/118/2016/11/WBE-ethical-guidelines-FINAL-March-2016-.pdf).

There are key issues that need careful consideration especially in the context of near source tracking that is widely applied in SARS-CoV2 surveillance. For example, sampling from small communities could lead to stigmatisation of vulnerable groups. If data is made available to the public, this could lead to results being misused and misconstrued in the media. The ethical protocols first established for WBE regarding illicit drugs, established that sampling from population sizes >10,000 was large enough to

ensure stigmatisation of smaller groups is avoided. This same protection might therefore not be extended for population sizes under this number. Sampling wastewater from small communities is possible but ethical procedures need to be put in place and it is essential that data is anonymised. To mitigate the risks of stigmatisation the following key points need to be established before sampling takes place i) clear aims and objectives of the sampling, ii) what biomarkers will be investigated and what possible implications these have, iii) population size of the community iv) who will have access to this data and how will this data be used.

The concerns regarding the ethics and legalities surrounding WBE are valid, several discussions exist upon ethics on illicit drugs in WBE (Hall *et al.*, 2012; Prichard *et al.*, 2014, 2017; Lancaster *et al.*, 2019) and more recently on SARS-CoV-2 (Gable, Ram and Ram, 2020). It is important that WBE should be treated and utilised as any other epidemiological tool and subjected to ethical review and scrutiny. In the context of the rapidly developing surveillance of SARS-CoV2 in wastewater, national and international ethical guidelines are urgently needed. Furthermore, these will need to be reviewed and updated, if wastewater samples collected are analysed for a broader set biomarkers. WBE as a tool to rapidly inform on public health can offer significant benefits and attraction, as observed by the recent surge of popularity in monitoring COVID-19 outbreaks. It is vital however that with these rapid developments occurring in WBE, that ethical considerations are not left behind.

3.13 Policy Relevance

WBE is relevant to a number of policy areas within Scottish Government, primarily health-related, but also with links to other fields. This section provides a brief (nonexhaustive) overview of where WBE and existing policies may interact.

Lifestyle choices including use of illicit drugs, alcohol and tobacco as well as diet and mental health all feature significantly in Scotland's Public Health Priorities strategy (Scottish Government, 2018a) implemented by the Population Health Directorate.

Data on illicit drug use currently focuses on information relating to drug offences and court proceedings, the use of drugs in prisons and prevalence studies and surveys (ScotPHO, 2021). By its nature, illicit drug use is likely to be underreported and difficult to monitor effectively. The ability to detect drug metabolites through WBE could provide enhanced surveillance at a population or even community level and thus provide additional data reinforcing the existing drug use policy framework.

A key policy document with respect to alcohol consumption is the "The Alcohol Framework 2018" (Scottish Government, 2018b). The potential for WBE to strengthen framework lies in the need for "establishment of a research and evidence network" (Alcohol Focus Scotland, 2017). Policy documents highlight the need to seek to improve sources of data, noting limitations of that currently gathered and

the need for it to be rationalised against other data sources. WBE provides an opportunity to augment this existing monitoring of alcohol markers.

The Scottish Government is committed to reducing tobacco smoking and associated disease. The current relevant policy document in this respect is the five- year "Raising Scotland's Tobacco-free Generation: our tobacco control action plan 2018" (Scottish Government, 2018c), with an ambition for a tobacco-free Scotland by 2034 (Priority 4) (Scottish Government, 2018a).

The use of WBE to identify populations with elevated usage of illicit drugs, alcohol or tobacco may help to target priority geographical areas for intervention, feeding into Public Health Priority 4 – "A Scotland where we reduce the use of and harm from alcohol, tobacco andother drugs". The success of this priority depends upon understanding what drives consumption – thus WBEbased markers for these lifestyle choices could facilitate linkage with potential socioeconomic determinants of a given wastewater-producing population. Further, it feeds into Priority 2 "A Scotland where we flourish in our early years" and Priority 1 "A Scotland where we live in vibrant, health and safe places and communities" with on-going risk to children's health and wellbeing through substance abuse, indoor air quality and parental modelling of smoking behaviour.

WBE-based indicators for diet could help to underpin the Scottish Government's obesity strategy, published in 2018: "A Healthier Future: Scotland's Diet and Healthy Weight Delivery Plan" (ScotPHO, 2020). This specifically mentions a commitment to evidence-based policy (Scottish Government, 2018d) which could be further supported by WBE-monitoring of dietary markers for obesity or metabolites associated with specific food types, for example, enhancing the existing list of obesity indicators (Scottish Government, 2018e). It also highlights the need for both population-wide measures and targeted support to families most at risk. Similarly, the detection of metformin as a biomarker for Type 2 diabetes may feed into the strategy plan "A Healthier Future: type 2 Diabetes prevention, early detection and intervention framework" (Scottish Government, 2018f). WBE could potentially contribute to understanding both the national picture for such health issues and identifying at risk populations. Since dietary markers can highlight other lifestyle choices which may be influenced by wider issues (for example the relationship identified between increased prevalence of artificial sweeteners and environmental temperature (Phung et al., 2017) use of WBE may even link into environmental policy areas such as climate change targets (Scottish Government, 2020b). Other dietary markers may be relevant to the Heart Disease Improvement Plan – particularly as consideration is given to at risk (e.g. socially deprived) populations (Scottish Government, 2014a), the identification and surveillance of which may be supported by WBE.

Monitoring the prevalence of metabolites associated with antidepressants, anti-anxiety drugs and other prescription medications for mental health conditions, or indeed biomarkers directly associated with mental stress, is applicable to the Scottish Government's Mental Health Strategy (Scottish Government,

2017) in particular a key policy objective identified under the "What Research Matters for Mental Health Policy in Scotland" paper which is to "Achieve better outcomes which can be measured" (Mitchell and Kearney, 2015).

Scotland's Public Health Priority 1 includes making "improvements to the quality of the air we breathe" (Scottish Government, 2018a). Because air pollution is harmful to health, markers for asthma drug metabolites in wastewater not only support health policies but also have the potential to support strategies such as "Cleaner air for Scotland - 2" (Scottish Government, 2020a) accurately predicting the health benefits of further reducing air pollution is complex, therefore additional sources of data in this arena may be helpful.

Communicable diseases are a major policy area in which WBE could be pertinent to data collection. Health Protection Scotland (HPS) undertakes surveillance of a number of key bacterial and viral pathogens which cause communicable disease and are also shed in faeces. Examples include Norovirus, Campylobacter, E. coli O157, Toxoplasma and Hepatitis (Heath Protection Scotland, 2015). Outbreak identification and control is also undertaken by HPS and HPS integrates with animal and environmental health organisations to forms part of the "One-Health" approach to protecting Scotland from infection hazards. WBE offers the potential to apply either DNA-based or isolation/whole genome sequence-based approaches to assist outbreak investigation or as a means of population-level surveillance of specific organisms and/ or strains, thus feeding directly into the Scottish Health Protection Network's "A Public Health Microbiology Strategy for Scotland" (Health Protection Scotland, 2018) and The Human Animal Infections and Risk Surveillance (HAIRS) (a multi-agency cross-government horizon scanning and risk assessment group of which Scottish Government and Health Protection Scotland are a part (Public Health England, 2015).

Scotland's Antimicrobial Prescription Group (SAPG, 2021) was established in 2008 with a primary aim to coordinate a national framework for antimicrobial stewardship. SAPG have developed surveillance systems and ensured standardised information on antimicrobial use and resistance that is accessible to NHS boards. In 2019, the Scottish One Health Antimicrobial Use and Antimicrobial Resistance report was published by Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) Scotland (Antimicrobial Resistance and Healthcare Associated Infection (ARHAI), 2020). This report provided information on antibiotic use and resistance to antibiotics in Scotland during 2019. Alongside initiatives such as the One Health Breakthrough Partnership (a collaboration between NHS Highland, Scottish Water, SEPA, MedSmart, James Hutton Institute, University of the Highlands and Islands, Glasgow Caledonian University) which reports to Scottish Government (Scottish Parliament, 2020) - these provide join-up between public health, veterinary and environmental aspects of AMR. They support the five-year United Kingdom National Action Plan and a 20-year vision for containing and controlling AMR (UK Government, 2019b). Currently, much surveillance is based on clinical data and prescription

data. WBE provides an opportunity as a complimentary technique to achieve a whole population approach to tackling AMR in a multifaceted way because it is possible to detect antimicrobial resistance genes, antibiotic residues and metabolites and co-selecting compounds such as heavy metals or personal care products. Further, if employed to better understand patient compliance with pharmaceutical prescriptions including antibiotics, through detection of parent compound-metabolite ratios in wastewater, WBE can further underpin the AMR strategies mentioned above.

WBE has potential to complement current practices in identifying and monitoring exposure to chemicals, for example accompanying the SASA Pesticide Survey data, relevant to the code of practice for using plant protection products in Scotland (Scottish Executive and Health and Safety Commission, 2007). In addition, understanding the prevalence of endocrine disrupting chemicals such as bisphenol A (common in food packaging), phthalates (household and personal care products) flame retardants (furnishings) could support related statutory instruments and policy groups. Examples include the Materials and Articles in Contact with Food (Scotland) Amendment Regulations (Scottish Statutory Instruments, 2019) and the Scottish Chemical Policy Network (UK Chemicals Stakeholder Forum, 2020), supporting understanding of dietary mycotoxin exposure may augment surveillance of food products undertaken by Food Standards Scotland (Munro and Gratz, 2018) and is pertinent to the Contaminants in Food (Scotland) Regulation (Scottish Statutory Instruments, 2013). This review focusses primarily on the association of wastewater-based determinants as markers for population-based health status. However, many wastewater associated markers and chemicals directly or indirectly impact receiving waters and as such are relevant to a range of water-related environmental policies and plans including the Scotland River Basins Standards Directive (Scottish Government, 2014b), River Basin Management Plans (SEPA, 2021) and a raft of regulations under the water environment legislation (Scottish Government, 2018g).

3.14 Recommendations and conclusions

WBE is a relatively new field. There are only a very few technology ready applications. These include:

1. Estimation of community wide illicit drug usage (currently applied by EMCDDA in the EU and internationally (e.g. in Australia and in the US). Required infrastructure includes specialised staff to undertake sampling and sample preparation as well as an investment in instrumentation (liquid chromatography coupled with mass spectrometry).
2. Estimation of lifestyle chemical usage: alcohol, nicotine and caffeine. Required infrastructure: as above
3. Infectious disease tracking (e.g. polio, SARS-CoV-2). Required infrastructure includes specialised staff to undertake sampling and sample preparation as well as analysis with PCR and sequencing

4 Estimation of disease prevalence based on pharmaceutical usage. Required infrastructure includes specialised staff to undertake sampling and sample preparation as well as an investment in instrumentation (liquid chromatography coupled with mass spectrometry)
5 There are research gaps though. These include lack of understanding of stability of pharmaceuticals and their metabolites in wastewater, lack of understanding of metabolism to undertake back-calculation of exposure

There is a clear potential to apply WBE in:

1. Estimation of community-wide exposure to hazardous chemicals. Some initial work indicates that wastewater can provide information on community wide exposure to pesticides and industrial chemicals, which are linked with either occupational exposure or lifestyle choices.
2. Prevalence of non-communicable disease (NCD). Current WBE approaches allow for estimation of pharmaceutical usage to treat, e.g. diabetes, cardiovascular disease or mental health conditions.

However, further research is required to fully appreciate WBE's potential to transform community-wide heath assessment. These include:

1. Research on a new biomarker base to inform public health status (to expand WBE applications)
2. Novel approaches towards population equivalent estimation. These are required to undertake spatiotemporal quantitative analysis of community wide exposure or public health status (to provide more accurate WBE measurements)
3. Investment in new infrastructure might be required in terms of sampling and wastewater flow measurements to enable meaningful quantitative analysis of chemical markers (to provide more accurate WBE measurements).
4. Novel approaches towards sampling to allow for truly representative sample to be obtained. Current approaches utilise 24h composite samplers. These samplers are mainly deployed at wastewater treatment plants. New sampling approaches might be required when sampling near source (to provide more accurate WBE measurements).
5. Novel approaches towards analysis and sensing as wastewater analysis of biomarkers required highly selective and sensitive techniques (to provide more accurate WBE measurements and to expand WBE applications).
6. Modelling and statistical analysis required to fully appreciate spatiotemporal variability in large scale datasets (to provide more accurate WBE measurements).

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3.38 References

Aarestrup, F. M. and Woolhouse, M. E. J. (2020) 'Using sewage for surveillance of antimicrobial resistance', *Science*, 367(6478), pp. 630–632. doi: 10.1126/science.aba3432.

Ahmed, F. *et al.* (2020) 'Wastewater-based estimation of the prevalence of gout in Australia', *Science of The Total Environment*, 715, p. 136925. doi: 10.1016/j.scitotenv.2020.136925.

Ahmed, W. *et al.* (2020) 'First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.138764.

Allen, H. K. *et al.* (2010) 'Call of the wild: antibiotic resistance genes in natural environments', *Nature Reviews Microbiology*. Nature Publishing Group, 8(4), pp. 251–259. doi: 10.1038/nrmicro2312.

Allen, M. T. and Levy, L. S. (2013) 'Parkinsons disease and pesticide exposure - A new assessment', *Critical Reviews in Toxicology*. doi: 10.3109/10408444.2013.798719.

Andersson, D. I. and Hughes, D. (2014) 'Microbiological effects of sublethal levels of antibiotics', *Nature Reviews Microbiology*, 12(7), pp. 465–478. doi: 10.1038/nrmicro3270.

Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) (2020) *Scottish One Health Antimicrobial Use and Antimicrobial Resistance in 2019 Annual Report*. Available at: https://hpspubsrepo.blob.core.windows.net/hps-website/nss/3133/documents/2_2020-11-17-sonaar-2019-report.pdf.

Aprea, M. C. (2012) 'Environmental and biological monitoring in the estimation of absorbed doses of pesticides', *Toxicology Letters*, 210(2), pp. 110–118. doi: 10.1016/j.toxlet.2011.08.008.

ARHAI Scotland (2020) *Scottish One Health Antimicrobial Use and Antimicrobial Resistance in 2019 Annual Report*. Available at: https://hpspubsrepo.blob.core.windows.net/hps-website/nss/3133/documents/2_2020-11-17-sonaar-2019-report.pdf.

Assress, H. A. *et al.* (2019) 'Diversity, Co-occurrence and Implications of Fungal Communities in Wastewater Treatment Plants', *Scientific Reports*, 9(1), p. 14056. doi: 10.1038/s41598-019-50624-z.

Bade, R. *et al.* (2020) 'Determination of 21 synthetic cathinones, phenethylamines, amphetamines and opioids in influent wastewater using liquid chromatography coupled to tandem mass spectrometry', *Talanta*, 208, p. 120479. doi: 10.1016/j.talanta.2019.120479.

Bade, R. *et al.* (2021) 'International snapshot of new psychoactive substance use: Case study of eight countries over the 2019/2020 new year period', *Water Research*. doi: 10.1016/j.watres.2021.116891.

Baker-Austin, C. *et al.* (2006) 'Co-selection of antibiotic and metal resistance', *Trends in Microbiology*, 14(4), pp. 176–182. doi: 10.1016/j.tim.2006.02.006.

Baker, D. R., Barron, L. and Kasprzyk-Hordern, B. (2014) 'Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates', *Science of the Total Environment*, 487(1), pp. 629–641. doi: 10.1016/j.scitotenv.2013.11.107.

Barr, D. B. (2008) 'Biomonitoring of exposure to pesticides', *Journal of Chemical Health and Safety*. doi: 10.1016/j.jchas.2008.07.001.

Barras, C. (2018) 'Going to waste', *Nature Medicine*, 24(10), pp. 1484–1487. doi: 10.1038/s41591-018-0218-0.

Bauer, M. *et al.* (2012) 'Genetic association between obstructive bronchitis and enzymes of oxidative stress', *Metabolism*, 61(12), pp. 1771–1779. doi: 10.1016/j.metabol.2012.05.013.

Bauer, S. (2008) 'Societal and ethical issues in human biomonitoring – a view from science studies', *Environmental Health*, 7(Suppl 1), p. S10. doi: 10.1186/1476-069X-7-S1-S10.

Baz-Lomba, J. A. *et al.* (2016) 'Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities', *BMC Public Health*, 16(1), p. 1035. doi: 10.1186/s12889-016-3686-5.

Been, F. *et al.* (2014) 'Population Normalization with Ammonium in Wastewater-Based Epidemiology: Application to Illicit Drug Monitoring', *Environmental Science & Technology*, 48(14), pp. 8162–8169. doi: 10.1021/es5008388.

Been, F. *et al.* (2017) 'Liquid Chromatography–Tandem Mass Spectrometry Analysis of Biomarkers of Exposure to Phosphorus Flame Retardants in Wastewater to Monitor Community-Wide Exposure', *Analytical Chemistry*, 89(18), pp. 10045–10053. doi: 10.1021/acs.analchem.7b02705.

Been, F. *et al.* (2018) 'Mining the Chemical Information on Urban Wastewater: Monitoring Human Exposure to Phosphorus Flame Retardants and Plasticizers', *Environmental Science & Technology*, 52(12), pp. 6996–7005. doi: 10.1021/acs.est.8b01279.

Berdeaux, O. *et al.* (2006) 'F-2-isoprostanes: Review of analytical methods', *Current Pharmaceutical Analysis*, 2(1), pp. 69–78. doi: 10.2174/157341206775474016.

Bhat, R. V. *et al.* (1997) 'A Foodborne Disease Outbreak Due to the Consumption of Moldy Sorghum and Maize Containing Fumonisin Mycotoxins', *Journal of Toxicology: Clinical Toxicology*, 35(3), pp. 249–255. doi: 10.3109/15563659709001208.

Birben, E. *et al.* (2012) ‘Oxidative Stress and Antioxidant Defense’, *World Allergy Organization Journal*, 5(1), pp. 9–19. doi: 10.1097/WOX.0b013e3182439613.

Boehme, C. *et al.* (2005) ‘Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis’, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 99(12), pp. 893–900. doi: 10.1016/j.trstmh.2005.04.014.

Boleda, M. R., Galceran, M. T. and Ventura, F. (2007) ‘Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography–tandem mass spectrometry’, *Journal of Chromatography A*, 1175(1), pp. 38–48. doi: 10.1016/j.chroma.2007.10.029.

Boogaerts, T. *et al.* (2016) ‘Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach’, *Drug and Alcohol Dependence*. Elsevier Ireland Ltd, 160, pp. 170–176. doi: 10.1016/j.drugalcdep.2016.01.002.

Boogaerts, T. *et al.* (2019) ‘Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach’, *Talanta*, 200, pp. 340–349. doi: 10.1016/j.talanta.2019.03.052.

Börjesson, S. *et al.* (2009) ‘A seasonal study of the mecA gene and Staphylococcus aureus including methicillin-resistant S. aureus in a municipal wastewater treatment plant’, *Water Research*, 43(4), pp. 925–932. doi: 10.1016/j.watres.2008.11.036.

Bowers, I. and Subedi, B. (2021) ‘Isoprostanes in wastewater as biomarkers of oxidative stress during COVID-19 pandemic’, *Chemosphere*, 271, p. 129489. doi: 10.1016/j.chemosphere.2020.129489.

Brewer, A. J. *et al.* (2012) ‘Normalized diurnal and between-day trends in illicit and legal drug loads that account for changes in population’, *Environmental Science and Technology*. doi: 10.1021/es202447r.

Bush, K. *et al.* (2011) ‘Tackling antibiotic resistance’, *Nature Reviews Microbiology*, 9(12), pp. 894–896. doi: 10.1038/nrmicro2693.

Cadarette, S. M. and Wong, L. (2015) ‘An Introduction to Health Care Administrative Data’, *The Canadian Journal of Hospital Pharmacy*. Canadian Society Of Hospital Pharmacists, 68(3), pp. 232–7. doi: 10.4212/cjhp.v68i3.1457.

Cannas, A. *et al.* (2010) ‘IP-10 detection in urine is associated with lung diseases’, *BMC Infectious Diseases*, 10(1), p. 333. doi: 10.1186/1471-2334-10-333.

Carneiro, H. A. and Mylonakis, E. (2009) ‘Google Trends: A Web-Based Tool for Real-Time Surveillance of Disease Outbreaks’, *Clinical Infectious Diseases*. Oxford University Press, 49(10), pp. 1557–1564. doi: 10.1086/630200.

Castiglioni, S. *et al.* (2006) ‘Identification and Measurement of Illicit Drugs and Their Metabolites in Urban Wastewater by Liquid Chromatography−Tandem Mass Spectrometry’, *Analytical Chemistry*, 78(24), pp. 8421–8429. doi: 10.1021/ac061095b.

Castiglioni, S. *et al.* (2013) ‘Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers’, *Environmental Science and Technology*, 47(3), pp. 1452–1460. doi: 10.1021/es302722f.

Castiglioni, S. *et al.* (2015) ‘A novel approach for monitoring tobacco use in local communities by wastewater analysis’, *Tobacco Control*, 24(1), pp. 38–42. doi: 10.1136/tobaccocontrol-2014-051553.

Castiglioni, S. *et al.* (2021) ‘New psychoactive substances in several European populations assessed by wastewater-based epidemiology’, *Water Research*. Elsevier Ltd, 195, p. 116983. doi: 10.1016/j.watres.2021.116983.

Castrignanò, E. *et al.* (2020) ‘Enantiomeric profiling of quinolones and quinolones resistance gene qnrS in European wastewaters’, *Water Research*, 175, p. 115653. doi: 10.1016/j.watres.2020.115653.

Caucci, S. *et al.* (2016) ‘Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow’, *FEMS Microbiology Ecology*. Edited by K. Smalla, 92(5), p. fiw060. doi: 10.1093/femsec/fiw060.

Le Chatelier, E. *et al.* (2013) ‘Richness of human gut microbiome correlates with metabolic markers’, *Nature*, 500(7464), pp. 541–546. doi: 10.1038/nature12506.

Chen, C. *et al.* (2014) ‘Towards finding a population biomarker for wastewater epidemiology studies’, *Science of The Total Environment*, 487(1), pp. 621–628. doi: 10.1016/j.scitotenv.2013.11.075.

Choi, B. C. K. (2012) ‘The Past, Present, and Future of Public Health Surveillance’, *Scientifica*, 2012(2), pp. 1–26. doi: 10.6064/2012/875253.

Choi, P. M., O’Brien, J. W., *et al.* (2018) ‘Population histamine burden assessed using wastewater-based epidemiology: The association of 1,4-methylimidazole acetic acid and fexofenadine’, *Environment International*. doi: 10.1016/j.envint.2018.08.009.

Choi, P. M., Tscharke, B. J., *et al.* (2018) ‘Wastewater-based epidemiology biomarkers: Past, present and future’, *TrAC Trends in Analytical Chemistry*, 105, pp. 453–469. doi: 10.1016/j.trac.2018.06.004.

Choi, P. M. *et al.* (2019) 'Social, demographic, and economic correlates of food and chemical consumption measured by wastewater-based epidemiology', *Proceedings of the National Academy of Sciences*, 116(43), pp. 21864–21873. doi: 10.1073/pnas.1910242116.

Choi, P. M. *et al.* (2020) 'Do food and stress biomarkers work for wastewater-based epidemiology? A critical evaluation', *Science of The Total Environment*, 736, p. 139654. doi: 10.1016/j.scitotenv.2020.139654.

Coutu, S. *et al.* (2013) 'Temporal dynamics of antibiotics in wastewater treatment plant influent', *Science of The Total Environment*, 458–460, pp. 20–26. doi: 10.1016/j.scitotenv.2013.04.017.

Cracowski, J. L. and Durand, T. (2006) 'Cardiovascular pharmacology and physiology of the isoprostanes', *Fundamental and Clinical Pharmacology*, 20(5), pp. 417–427. doi: 10.1111/j.1472-8206.2006.00435.x.

Daughton, C. G. (2001) 'Emerging pollutants, and communicating the science of environmental chemistry and mass spectrometry: Pharmaceuticals in the environment', *Journal of the American Society for Mass Spectrometry*, 12(10), pp. 1067–1076. doi: 10.1016/S1044-0305(01)00287-2.

Daughton, C. G. (2012) 'Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype', *Science of The Total Environment*, 424, pp. 16–38. doi: 10.1016/j.scitotenv.2012.02.038.

Daughton, C. G. (2018) 'Monitoring wastewater for assessing community health: Sewage Chemical-Information Mining (SCIM)', *Science of The Total Environment*, 619–620, pp. 748–764. doi: 10.1016/j.scitotenv.2017.11.102.

Daughton, C. G. (2020) 'Wastewater surveillance for population-wide Covid-19: The present and future', *Science of The Total Environment*, 736, p. 139631. doi: 10.1016/j.scitotenv.2020.139631.

Davies, S. S. *et al.* (2006) 'Quantification of dinor, dihydro metabolites of F2-isoprostanes in urine by liquid chromatography/tandem mass spectrometry.', *Analytical biochemistry*, 348(2), pp. 185–91. doi: 10.1016/j.ab.2005.10.012.

Dawood, F. S. *et al.* (2012) 'Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study', *The Lancet Infectious Diseases*, 12(9), pp. 687–695. doi: 10.1016/S1473-3099(12)70121-4.

Dekant, W. and Völkel, W. (2008) 'Human exposure to bisphenol A by biomonitoring: Methods, results and assessment of environmental exposures', *Toxicology and Applied Pharmacology*. doi: 10.1016/j.taap.2007.12.008.

Dhalla, N. (2000) ‘Status of myocardial antioxidants in ischemia–reperfusion injury’, *Cardiovascular Research*. Oxford University Press, 47(3), pp. 446–456. doi: 10.1016/S0008-6363(00)00078-X.

Diemert, S. and Yan, T. (2019) ‘Clinically unreported salmonellosis outbreak detected via comparative genomic analysis of municipal wastewater Salmonella isolates’, *Applied and Environmental Microbiology*. doi: 10.1128/AEM.00139-19.

Dishaw, L. V. *et al.* (2011) ‘Is the PentaBDE replacement, tris (1,3-dichloro-2-propyl) phosphate (TDCPP), a developmental neurotoxicant? Studies in PC12 cells’, *Toxicology and Applied Pharmacology*, 256(3), pp. 281–289. doi: 10.1016/j.taap.2011.01.005.

Du, P. *et al.* (2018) ‘Estimating population exposure to phthalate esters in major Chinese cities through wastewater-based epidemiology’, *Science of The Total Environment*, 643, pp. 1602–1609. doi: 10.1016/j.scitotenv.2018.06.325.

Epplein, M. *et al.* (2009) ‘Association of plasma micronutrient levels and urinary isoprostane with risk of lung cancer: the multiethnic cohort study.’, *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 18(7), pp. 1962–70. doi: 10.1158/1055-9965.EPI-09-0003.

Escolà Casas, M. *et al.* (2021) ‘Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution’, *Environment International*, 150, p. 106404. doi: 10.1016/j.envint.2021.106404.

European Food Safety Authority (EFSA) (2015) *Scientific Opinion on Bisphenol A (2015) What is Bisphenol A and What Has EFSA Done? What Are the Main Results of EFSA’s 2015 Risk Assessment of BPA? EFSA’s Risk Assessment in More Detail What Potential Health Effects of BPA Has EFSA Identified?* doi: 10.2805/075460.

European Monitoring Centre for Drugs and Drug Addiction (2016) *European Drug Report 2016: Trends and Developments, European Monitoring of Drugs and Drugs Addiction*. doi: 10.2810/88175.

European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2016) *Assessing illicit drugs in wastewater: advances in wastewater-based drug epidemiology, Insight 22*. Available at: https://www.emcdda.europa.eu/system/files/publications/2273/TDXD16022ENC_4.pdf.

European Union (2006) *Commission Regulation 1881/2006, 2006.* Available at: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF.

Fattore, E. *et al.* (2016) ‘Wastewater-based epidemiological evaluation of the effect of air pollution on short-acting beta-agonist consumption for acute asthma treatment’, *Environmental Research*, 150, pp.

106–111. doi: 10.1016/j.envres.2016.05.051.

Fernandez-Cassi, X. *et al.* (2018) 'Metagenomics for the study of viruses in urban sewage as a tool for public health surveillance', *Science of The Total Environment*, 618, pp. 870–880. doi: 10.1016/j.scitotenv.2017.08.249.

Fisher, M. C. *et al.* (2018) 'Worldwide emergence of resistance to antifungal drugs challenges human health and food security', *Science*, 360(6390), pp. 739–742. doi: 10.1126/science.aap7999.

Foladori, P. *et al.* (2020) 'SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review.', *The Science of the total environment*, 743, p. 140444. doi: 10.1016/j.scitotenv.2020.140444.

Food Standards Scotland (2015) *Mycotoxins, Climate Change and Food Safety Workshop Report*. Available at: https://www.foodstandards.gov.scot/downloads/Chemical_contaminants_-_FSS_Mycotoxin_Workshop_report_final_-_December_2015.pdf.

Frenk, J. and Gómez-Dantés, O. (2002) 'Globalisation and the challenges to health systems.', *BMJ (Clinical research ed.)*. British Medical Journal Publishing Group, 325(7355), pp. 95–7. doi: 10.1136/bmj.325.7355.95.

Fung, F. and Clark, R. F. (2004) 'Health Effects of Mycotoxins: A Toxicological Overview', *Journal of Toxicology: Clinical Toxicology*, 42(2), pp. 217–234. doi: 10.1081/CLT-120030947.

Funke, J., Prasse, C. and Ternes, T. A. (2016) 'Identification of transformation products of antiviral drugs formed during biological wastewater treatment and their occurrence in the urban water cycle', *Water Research*, 98, pp. 75–83. doi: 10.1016/j.watres.2016.03.045.

Gable, L., Ram, N. and Ram, J. L. (2020) 'Legal and ethical implications of wastewater monitoring of SARS-CoV-2 for COVID-19 surveillance', *Journal of Law and the Biosciences*, 7(1), p. lsaa039. doi: 10.1093/jlb/lsaa039.

Galea, K. S. *et al.* (2015) 'Urinary biomarker concentrations of captan, chlormequat, chlorpyrifos and cypermethrin in UK adults and children living near agricultural land', *Journal of Exposure Science & Environmental Epidemiology*, 25(6), pp. 623–631. doi: 10.1038/jes.2015.54.

Gao, P., Munir, M. and Xagoraraki, I. (2012) 'Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant', *Science of The Total Environment*, 421–422, pp. 173–183. doi: 10.1016/j.scitotenv.2012.01.061.

Girones, R. *et al.* (2010) 'Molecular detection of pathogens in water – The pros and cons of molecular techniques', *Water Research*, 44(15), pp. 4325–4339. doi: 10.1016/j.watres.2010.06.030.

Golovko, O. *et al.* (2014) 'Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant', *Chemosphere*, 111, pp. 418–426. doi: 10.1016/j.chemosphere.2014.03.132.

González-Mariño, I. *et al.* (2016) 'Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries', *Environmental Science & Technology*, 50(18), pp. 10089–10096. doi: 10.1021/acs.est.6b02644.

González-Mariño, I. *et al.* (2017) 'Wastewater-Based Epidemiology as a New Tool for Estimating Population Exposure to Phthalate Plasticizers', *Environmental Science and Technology*. American Chemical Society, 51(7), pp. 3902–3910. doi: 10.1021/acs.est.6b05612.

González-Mariño, I. *et al.* (2020) 'Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring', *Addiction*. doi: 10.1111/add.14767.

González-Mariño, I. *et al.* (2021) 'Assessing population exposure to phthalate plasticizers in thirteen Spanish cities through the analysis of wastewater', *Journal of Hazardous Materials*. doi: 10.1016/j.jhazmat.2020.123272.

Gourinat, A.-C. *et al.* (2015) 'Detection of Zika Virus in Urine', *Emerging Infectious Diseases*, 21(1), pp. 84–86. doi: 10.3201/eid2101.140894.

GOV.UK (2017) *Health matters: obesity and the food environment*. Available at: https://www.gov.uk/government/publications/health-matters-obesity-and-the-food-environment/health-matters-obesity-and-the-food-environment--2 (Accessed: 16 March 2021).

Gracia-Lor, E. *et al.* (2017) 'Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives', *Environment International*, 99, pp. 131–150. doi: 10.1016/j.envint.2016.12.016.

Gracia-Lor, E. *et al.* (2020) 'Wastewater-based epidemiology for tracking human exposure to mycotoxins', *Journal of Hazardous Materials*, 382, p. 121108. doi: 10.1016/j.jhazmat.2019.121108.

Guerra, P. *et al.* (2014) 'Occurrence and fate of antibiotic, analgesic/anti-inflammatory, and antifungal compounds in five wastewater treatment processes', *Science of The Total Environment*, 473–474, pp. 235–243. doi: 10.1016/j.scitotenv.2013.12.008.

Guy, R. A. *et al.* (2003) 'Real-Time PCR for Quantification of Giardia and Cryptosporidium in Environmental Water Samples and Sewage', *Applied and Environmental Microbiology*, 69(9), pp. 5178–5185. doi: 10.1128/AEM.69.9.5178-5185.2003.

Halden, R. *et al.* (2019) 'Tracking harmful chemicals and pathogens using the Human Health

Observatory at ASU', *Online Journal of Public Health Informatics*, 11(1). doi: 10.5210/ojphi.v11i1.9843.

Hall, W. *et al.* (2012) 'An analysis of ethical issues in using wastewater analysis to monitor illicit drug use', *Addiction*, 107(10), pp. 1767–1773. doi: 10.1111/j.1360-0443.2012.03887.x.

Halliwell, B. and Lee, C. Y. J. (2010) 'Using isoprostanes as biomarkers of oxidative stress: some rarely considered issues.', *Antioxidants & redox signaling*, 13(2), pp. 145–56. doi: 10.1089/ars.2009.2934.

Hamasur, B. *et al.* (2015) 'A Sensitive Urinary Lipoarabinomannan Test for Tuberculosis', *PLOS ONE*. Edited by J. Nigou, 10(4), p. e0123457. doi: 10.1371/journal.pone.0123457.

Harman, C., Reid, M. and Thomas, K. V. (2011) 'In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage', *Environmental Science and Technology*, 45(13), pp. 5676–5682. doi: 10.1021/es201124j.

Heijnen, L. and Medema, G. (2011) 'Surveillance of Influenza A and the pandemic influenza A (H1N1) 2009 in sewage and surface water in the Netherlands', *Journal of Water and Health*, 9(3), pp. 434–442. doi: 10.2166/wh.2011.019.

Hellmér, M. *et al.* (2014) 'Detection of Pathogenic Viruses in Sewage Provided Early Warnings of Hepatitis A Virus and Norovirus Outbreaks', *Applied and Environmental Microbiology*. Edited by D. W. Schaffner, 80(21), pp. 6771–6781. doi: 10.1128/AEM.01981-14.

Hembach, N. *et al.* (2017) 'Occurrence of the mcr-1 Colistin Resistance Gene and other Clinically Relevant Antibiotic Resistance Genes in Microbial Populations at Different Municipal Wastewater Treatment Plants in Germany', *Frontiers in Microbiology*. Frontiers, 8, p. 1282. doi: 10.3389/fmicb.2017.01282.

Hernández, F. *et al.* (2018) 'Mass spectrometric strategies for the investigation of biomarkers of illicit drug use in wastewater', *Mass Spectrometry Reviews*, 37(3), pp. 258–280. doi: 10.1002/mas.21525.

Hijosa-Valsero, M. *et al.* (2011) 'Removal of antibiotics from urban wastewater by constructed wetland optimization', *Chemosphere*, 83(5), pp. 713–719. doi: 10.1016/j.chemosphere.2011.02.004.

Hirshon, J. M. (2000) 'The Rationale for Developing Public Health Surveillance Systems Based on Emergency Department Data', *Academic Emergency Medicine*, 7(12), pp. 1428–1432. doi: 10.1111/j.1553-2712.2000.tb00503.x.

Hou, C. *et al.* (2020) 'Estimating the prevalence of hepatitis B by wastewater-based epidemiology in

19 cities in China', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.139696.

Hovi, T. *et al.* (2012) 'Role of environmental poliovirus surveillance in global polio eradication and beyond', *Epidemiology and Infection*, 140(1), pp. 1–13. doi: 10.1017/S095026881000316X.

Huang, Q. *et al.* (2010) 'Determination of commonly used azole antifungals in various waters and sewage sludge using ultra-high performance liquid chromatography–tandem mass spectrometry', *Journal of Chromatography A*, 1217(21), pp. 3481–3488. doi: 10.1016/j.chroma.2010.03.022.

Hughes, D. A. *et al.* (2001) 'The impact of non-compliance on the cost-effectiveness of pharmaceuticals: a review of the literature', *Health Economics*, 10(7), pp. 601–615. doi: 10.1002/hec.609.

Huijbers, P. M. C., Larsson, D. G. J. and Flach, C. F. (2020) 'Surveillance of antibiotic resistant Escherichia coli in human populations through urban wastewater in ten European countries', *Environmental Pollution*, 261, p. 114200. doi: 10.1016/j.envpol.2020.114200.

Hussein, H. S. and Brasel, J. M. (2001) 'Toxicity, metabolism, and impact of mycotoxins on humans and animals', *Toxicology*. doi: 10.1016/S0300-483X(01)00471-1.

Jain, S. *et al.* (2013) 'Occurrence and Removal of Antiviral Drugs in Environment: A Review', *Water, Air, & Soil Pollution*, 224(2), p. 1410. doi: 10.1007/s11270-012-1410-3.

Jiao, Y.-N. *et al.* (2018) 'Biomarkers of antibiotic resistance genes during seasonal changes in wastewater treatment systems', *Environmental Pollution*, 234, pp. 79–87. doi: 10.1016/j.envpol.2017.11.048.

Joynt, G. M. and Wu, W. K. (2020) 'Understanding COVID-19: what does viral RNA load really mean?', *The Lancet Infectious Diseases*. doi: 10.1016/S1473-3099(20)30237-1.

Jury, K. L. *et al.* (2011) 'Are sewage treatment plants promoting antibiotic resistance?', *Critical Reviews in Environmental Science and Technology*. Taylor & Francis Group, 41(3), pp. 243–270. doi: 10.1080/10643380902772589.

Kardas, P. *et al.* (2005) 'A systematic review and meta-analysis of misuse of antibiotic therapies in the community', *International Journal of Antimicrobial Agents*, 26(2), pp. 106–113. doi: 10.1016/j.ijantimicag.2005.04.017.

Kasprzyk-Hordern, B. *et al.* (2014) 'Wastewater-based epidemiology for public health monitoring', *Water and Sewerage Journal*, (4), pp. 25–26. doi: 10.1136/tobaccocontrol-2014-051553.7.

Kasprzyk-Hordern, B. *et al.* (2021) 'Estimation of community-wide multi-chemical exposure via

water-based chemical mining: Key research gaps drawn from a comprehensive multi-biomarker multi-city dataset', *Environment International*, 147, p. 106331. doi: 10.1016/j.envint.2020.106331.

Kasprzyk-Hordern, B., Dinsdale, R. M. and Guwy, A. J. (2008) 'Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography–electrospray tandem mass spectrometry', *Analytical and Bioanalytical Chemistry*, 391(4), pp. 1293–1308. doi: 10.1007/s00216-008-1854-x.

Kasprzyk-Hordern, B., Dinsdale, R. M. and Guwy, A. J. (2009) 'The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters', *Water Research*, 43(2), pp. 363–380. doi: 10.1016/j.watres.2008.10.047.

Kim, S. Y. *et al.* (2018) 'Urine IP-10 as a biomarker of therapeutic response in patients with active pulmonary tuberculosis', *BMC Infectious Diseases*, 18(1), p. 240. doi: 10.1186/s12879-018-3144-3.

Kinyua, J. *et al.* (2015) 'Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS', *Drug Testing and Analysis*, 7(9), pp. 812–818. doi: 10.1002/dta.1777.

Kitajima, M. *et al.* (2020) 'SARS-CoV-2 in wastewater: State of the knowledge and research needs', *Science of The Total Environment*, 739, p. 139076. doi: 10.1016/j.scitotenv.2020.139076.

Klawitter, Jelena *et al.* (2011) 'Quantification of 15-F2t-isoprostane in human plasma and urine: results from enzyme-linked immunoassay and liquid chromatography/tandem mass spectrometry cannot be compared.', *Rapid communications in mass spectrometry : RCM*, 25(4), pp. 463–8. doi: 10.1002/rcm.4871.

Kraigher, B. *et al.* (2008) 'Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors', *Water Research*. Pergamon, 42(17), pp. 4578–4588. doi: 10.1016/J.WATRES.2008.08.006.

Kraus, L. *et al.* (2003) 'Estimating prevalence of problem drug use at national level in countries of the European Union and Norway', *Addiction*. doi: 10.1046/j.1360-0443.2003.00326.x.

Kwak, Y.-K. *et al.* (2015) 'Surveillance of antimicrobial resistance among Escherichia coli in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society?', *International Journal of Antimicrobial Agents*, 45(1), pp. 25–32. doi: 10.1016/j.ijantimicag.2014.09.016.

Lai, F. Y. *et al.* (2011) 'Refining the estimation of illicit drug consumptions from wastewater analysis: Co-analysis of prescription pharmaceuticals and uncertainty assessment', *Water Research*, 45(15), pp. 4437–4448. doi: 10.1016/j.watres.2011.05.042.

Lai, F. Y. *et al.* (2016) 'Cocaine, MDMA and methamphetamine residues in wastewater: Consumption trends (2009–2015) in South East Queensland, Australia', *Science of The Total Environment*, 568, pp. 803–809. doi: 10.1016/j.scitotenv.2016.05.181.

Lai, F. Y. *et al.* (2017) 'Novel Wastewater-Based Epidemiology Approach Based on Liquid Chromatography–Tandem Mass Spectrometry for Assessing Population Exposure to Tobacco-Specific Toxicants and Carcinogens', *Analytical Chemistry*, 89(17), pp. 9268–9278. doi: 10.1021/acs.analchem.7b02052.

Lai, F. Y. *et al.* (2018) 'Measuring spatial and temporal trends of nicotine and alcohol consumption in Australia using wastewater-based epidemiology', *Addiction*, 113(6), pp. 1127–1136. doi: 10.1111/add.14157.

Lancaster, K. *et al.* (2019) 'A "promising tool"? A critical review of the social and ethico-political effects of wastewater analysis in the context of illicit drug epidemiology and drug policy', *Current Opinion in Environmental Science and Health*, 9, pp. 85–90. doi: 10.1016/j.coesh.2019.06.006.

Larsson, D. G. J. *et al.* (2018) 'Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance', *Environment International*. doi: 10.1016/j.envint.2018.04.041.

Lee, L. M. *et al.* (2010) *Principles & Practice of Public Health Surveillance*. Oxford University Press. doi: 10.1093/acprof:oso/9780195372922.001.0001.

Leknes, H., Sturtzel, I. E. and Dye, C. (2012) 'Environmental release of oseltamivir from a Norwegian sewage treatment plant during the 2009 influenza A (H1N1) pandemic', *Science of The Total Environment*, 414, pp. 632–638. doi: 10.1016/j.scitotenv.2011.11.004.

Lin, W. *et al.* (2019) 'Can water quality indicators and biomarkers be used to estimate real-time population?', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2018.12.390.

Liu, S. Y. *et al.* (2021) 'Tracing consumption patterns of stimulants, opioids, and ketamine in China by wastewater-based epidemiology', *Environmental Science and Pollution Research*. doi: 10.1007/s11356-020-12035-w.

Lockhart, S. R. *et al.* (2017) 'Simultaneous Emergence of Multidrug-Resistant Candida auris on 3 Continents Confirmed by Whole-Genome Sequencing and Epidemiological Analyses', *Clinical*

Infectious Diseases, 64(2), pp. 134–140. doi: 10.1093/cid/ciw691.

Lodovici, M. and Bigagli, E. (2011) 'Oxidative stress and air pollution exposure', *Journal of Toxicology*, p. 9 pages. doi: 10.1155/2011/487074.

Lopardo, L. *et al.* (2018) 'Verifying community-wide exposure to endocrine disruptors in personal care products – In quest for metabolic biomarkers of exposure via in vitro studies and wastewater-based epidemiology', *Water Research*. Pergamon, 143, pp. 117–126. doi: 10.1016/j.watres.2018.06.028.

Lopardo, L. *et al.* (2019) 'Estimation of community-wide exposure to bisphenol A via water fingerprinting', *Environment International*, 125, pp. 1–8. doi: 10.1016/j.envint.2018.12.048.

Maguire, E. R. and Monsivais, P. (2015) 'Socio-economic dietary inequalities in UK adults: an updated picture of key food groups and nutrients from national surveillance data', *British Journal of Nutrition*, 113(1), pp. 181–189. doi: 10.1017/S0007114514002621.

Mao, D. *et al.* (2015) 'Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants', *Water Research*, 85, pp. 458–466. doi: 10.1016/j.watres.2015.09.010.

Mardal, M. and Meyer, M. R. (2014) 'Studies on the microbial biotransformation of the novel psychoactive substance methylenedioxypyrovalerone (MDPV) in wastewater by means of liquid chromatography-high resolution mass spectrometry/mass spectrometry', *Science of the Total Environment*. Elsevier, 493, pp. 588–595. doi: 10.1016/j.scitotenv.2014.06.016.

Mars, B. *et al.* (2017) 'Influences on antidepressant prescribing trends in the UK: 1995–2011', *Social Psychiatry and Psychiatric Epidemiology*. doi: 10.1007/s00127-016-1306-4.

Mastroianni, N., Lopez de Alda, M. and Barcelo, D. (2014) 'Analysis of ethyl sulfate in raw wastewater for estimation of alcohol consumption and its correlation with drugs of abuse in the city of Barcelona', *Journal of Chromatography A*, 1360, pp. 93–99. doi: 10.1016/j.chroma.2014.07.051.

McCall, A.-K. *et al.* (2016) 'Critical review on the stability of illicit drugs in sewers and wastewater samples', *Water Research*. Pergamon, 88, pp. 933–947. doi: 10.1016/J.WATRES.2015.10.040.

Meagher, E. A. *et al.* (1999) 'Alcohol-induced generation of lipid peroxidation products in humans', *Journal of Clinical Investigation*, 104(6), pp. 805–813. doi: 10.1172/JCI5584.

Medema, G. *et al.* (2020) 'Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands', *Environmental Science & Technology Letters*, 7(7), pp. 511–516. doi: 10.1021/acs.estlett.0c00357.

Michael, I. *et al.* (2013) 'Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review', *Water Research*, 47(3), pp. 957–995. doi: 10.1016/j.watres.2012.11.027.

Montuschi, P., Barnes, P. J. and Roberts, L. J. (2004) 'Isoprostanes: markers and mediators of oxidative stress.', *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 18(15), pp. 1791–1800. doi: 10.1096/fj.04-2330rev.

Mumy, K. L. and Findlay, R. H. (2004) 'Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR', *Journal of Microbiological Methods*, 57(2), pp. 259–268. doi: 10.1016/j.mimet.2004.01.013.

Munir, M., Wong, K. and Xagoraraki, I. (2011) 'Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan', *Water Research*, 45(2), pp. 681–693. doi: 10.1016/j.watres.2010.08.033.

Mustieles, V. *et al.* (2020) 'Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring', *Environment International*, 144, p. 105811. doi: 10.1016/j.envint.2020.105811.

Muszbek, N. *et al.* (2008) 'The economic consequences of noncompliance in cardiovascular disease and related conditions: a literature review', *International Journal of Clinical Practice*, 62(2), pp. 338–351. doi: 10.1111/j.1742-1241.2007.01683.x.

Nathwani, D. *et al.* (2011) 'Scottish Antimicrobial Prescribing Group (SAPG): Development and impact of the Scottish National Antimicrobial Stewardship Programme', *International Journal of Antimicrobial Agents*. doi: 10.1016/j.ijantimicag.2011.02.005.

National Records of Scotland (2020) *Drug-related deaths in Scotland in 2019*. Available at: https://www.nrscotland.gov.uk/files//statistics/drug-related-deaths/2019/drug-related-deaths-19-pub.pdf.

Ndiaye, A. K., Diop, P. A. M. and Diop, O. M. (2014) 'Environmental surveillance of poliovirus and non-polio enterovirus in urban sewage in Dakar, Senegal (2007-2013)', *Pan African Medical Journal*. doi: 10.11604/pamj.2014.19.243.3538.

Needham, L. L., Calafat, A. M. and Barr, D. B. (2007) 'Uses and issues of biomonitoring', *International Journal of Hygiene and Environmental Health*, 210(3–4), pp. 229–238. doi: 10.1016/j.ijheh.2006.11.002.

Newton, R. J. *et al.* (2015) 'Sewage reflects the microbiomes of human populations', *mBio*, 6(2). doi:

10.1128/mBio.02574-14.

NHS Scotland Information Services Division (2019) *Prevalence of Problem Drug Use in Scotland: 2015/16 Estimates*. Available at: https://www.isdscotland.org/Health-Topics/Drugs-and-Alcohol-Misuse/Publications/2019-03-05/2019-03-05-Drug-Prevalence-2015-16-Report.pdf.

Nielsen, L. K. *et al.* (2014) 'The prevalence and impact of Fusarium head blight pathogens and mycotoxins on malting barley quality in UK', *International Journal of Food Microbiology*, 179, pp. 38–49. doi: 10.1016/j.ijfoodmicro.2014.03.023.

Nikolaidis, M. G., Kyparos, A. and Vrabas, I. S. (2011) 'F2-isoprostane formation, measurement and interpretation: The role of exercise', *Progress in Lipid Research*, pp. 89–103. doi: 10.1016/j.plipres.2010.10.002.

Nourooz-Zadeh, J. *et al.* (2006) 'Impact of diabetic polyneuropathy and cardiovascular autonomic neuropathy on the excretion of urinary 8-epi-PGF2αand its metabolites (2, 3-dinor and 2, 3-dinor-5, 6-dihydro)', *Free Radical Research*, 40(7), pp. 723–729. doi: 10.1080/10715760600615086.

Novo, A. *et al.* (2013) 'Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater', *Water Research*, 47(5), pp. 1875–1887. doi: 10.1016/j.watres.2013.01.010.

Nsubuga, P. *et al.* (2006) *Public Health Surveillance: A Tool for Targeting and Monitoring Interventions, Disease Control Priorities in Developing Countries*. The International Bank for Reconstruction and Development / The World Bank. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21250345 (Accessed: 21 August 2018).

van Nuijs, A. L. N. *et al.* (2011) 'Sewage epidemiology — A real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium', *Environment International*, 37(3), pp. 612–621. doi: 10.1016/j.envint.2010.12.006.

van Nuijs, A. L. N. *et al.* (2015) 'Do concentrations of pharmaceuticals in sewage reflect prescription figures?', *Environmental Science and Pollution Research*, 22(12), pp. 9110–9118. doi: 10.1007/s11356-014-4066-2.

O'Brien, E. and Xagoraraki, I. (2019a) 'A water-focused one-health approach for early detection and prevention of viral outbreaks', *One Health*, 7, p. 100094. doi: 10.1016/j.onehlt.2019.100094.

O'Brien, E. and Xagoraraki, I. (2019b) 'A water-focused one-health approach for early detection and prevention of viral outbreaks', *One Health*. doi: 10.1016/j.onehlt.2019.100094.

O'Brien, J. W. *et al.* (2014) 'A model to estimate the population contributing to the wastewater using

samples collected on census day', *Environmental Science and Technology*, 48(1), pp. 517–525. doi: 10.1021/es403251g.

O'Brien, J. W. *et al.* (2015) 'Wastewater analysis of Census day samples to investigate per capita input of organophosphorus flame retardants and plasticizers into wastewater', *Chemosphere*. doi: 10.1016/j.chemosphere.2015.06.014.

O'Brien, J. W., Grant, S., *et al.* (2019) 'A National Wastewater Monitoring Program for a better understanding of public health: A case study using the Australian Census', *Environment International*, 122, pp. 400–411. doi: 10.1016/j.envint.2018.12.003.

O'Brien, J. W., Choi, P. M., *et al.* (2019) 'Evaluating the stability of three oxidative stress biomarkers under sewer conditions and potential impact for use in wastewater-based epidemiology', *Water Research*, 166, p. 115068. doi: 10.1016/j.watres.2019.115068.

O'Neill, J. (2014) *Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations*. Available at: https://amr-review.org/sites/default/files/AMR Review Paper - Tackling a crisis for the health and wealth of nations_1.pdf (Accessed: 26 September 2018).

Ort, C., Lawrence, M. G., Rieckermann, J., *et al.* (2010) 'Sampling for Pharmaceuticals and Personal Care Products (PPCPs) and Illicit Drugs in Wastewater Systems: Are Your Conclusions Valid? A Critical Review', *Environmental Science & Technology*, 44(16), pp. 6024–6035. doi: 10.1021/es100779n.

Ort, C., Lawrence, M. G., Reungoat, J., *et al.* (2010) 'Sampling for PPCPs in wastewater systems: Comparison of different sampling modes and optimization strategies', *Environmental Science and Technology*. doi: 10.1021/es100778d.

Ort, C. *et al.* (2014) 'Sewage-based Epidemiology Requires a Truly Transdisciplinary Approach', *GAIA - Ecological Perspectives for Science and Society*, 23(3), pp. 266–268. doi: 10.14512/gaia.23.3.12.

Ozkul, A. *et al.* (2007) 'Oxidative stress in acute ischemic stroke', *Journal of Clinical Neuroscience*, 14(11), pp. 1062–1066. doi: 10.1016/j.jocn.2006.11.008.

Pandopulos, A. J. *et al.* (2021) 'Application of catecholamine metabolites as endogenous population biomarkers for wastewater-based epidemiology', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.142992.

Papageorgiou, M., Wells, L., Williams, C., White, K., *et al.* (2018) 'Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents', *Toxins*, 10(2), p. 50. doi:

10.3390/toxins10020050.

Papageorgiou, M., Wells, L., Williams, C., White, K. L. M., *et al.* (2018) 'Occurrence of deoxynivalenol in an elderly cohort in the UK: a biomonitoring approach', *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*. doi: 10.1080/19440049.2018.1508890.

Pärnänen, K. M. M. *et al.* (2019) 'Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence', *Science Advances*, 5(4), p. eaau9124. doi: 10.1126/sciadv.aau9124.

Patrono, C., Falco, A. and Davı, G. (2005) 'Isoprostane formation and inhibition in atherothrombosis', *Current opinion in pharmacology*, 5(2), pp. 198–203. doi: 10.1016/j.coph.2004.11.003.

Peacock, A. *et al.* (2019) 'New psychoactive substances: challenges for drug surveillance, control, and public health responses', *The Lancet*, 394(10209), pp. 1668–1684. doi: 10.1016/S0140-6736(19)32231-7.

Petrie, B. *et al.* (2015) 'Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry', *Journal of Chromatography A*, 1431, pp. 64–78. doi: 10.1016/j.chroma.2015.12.036.

Petrie, B. *et al.* (2016) 'New Framework To Diagnose the Direct Disposal of Prescribed Drugs in Wastewater - A Case Study of the Antidepressant Fluoxetine', *Environmental Science and Technology*, 50(7), pp. 3781–3789. doi: 10.1021/acs.est.6b00291.

Petrie, B., Barden, R. and Kasprzyk-Hordern, B. (2015) 'A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring', *Water Research*. Pergamon, 72, pp. 3–27. doi: 10.1016/j.watres.2014.08.053.

Phung, D. *et al.* (2017) 'Can wastewater-based epidemiology be used to evaluate the health impact of temperature? – An exploratory study in an Australian population', *Environmental Research*. doi: 10.1016/j.envres.2017.03.023.

Polo, D. *et al.* (2020) 'Making waves: Wastewater-based epidemiology for COVID-19 – approaches and challenges for surveillance and prediction', *Water Research*, 186, p. 116404. doi: 10.1016/j.watres.2020.116404.

Poloni, T. R. *et al.* (2010) 'Detection of dengue virus in saliva and urine by real time RT-PCR', *Virology Journal*, 7(1), p. 22. doi: 10.1186/1743-422X-7-22.

Poon, L. L. M. *et al.* (2004) 'Detection of SARS Coronavirus in Patients with Severe Acute

Respiratory Syndrome by Conventional and Real-Time Quantitative Reverse Transcription-PCR Assays', *Clinical Chemistry*, 50(1), pp. 67–72. doi: 10.1373/clinchem.2003.023663.

Prasse, C. *et al.* (2010) 'Antiviral Drugs in Wastewater and Surface Waters: A New Pharmaceutical Class of Environmental Relevance?', *Environmental Science & Technology*, 44(5), pp. 1728–1735. doi: 10.1021/es903216p.

Prichard, J. *et al.* (2014) 'Sewage epidemiology and illicit drug research: The development of ethical research guidelines', *Science of the Total Environment*, 472, pp. 550–555. doi: https://doi.org/10.1016/j.scitotenv.2013.11.039.

Prichard, J. *et al.* (2017) 'Wastewater analysis of substance use: Implications for law, policy and research', *Journal of Law and Medicine*, 24(4), pp. 837–849.

Proctor, K. *et al.* (2019) 'Multi-residue ultra-performance liquid chromatography coupled with tandem mass spectrometry method for comprehensive multi-class anthropogenic compounds of emerging concern analysis in a catchment-based exposure-driven study', *Analytical and Bioanalytical Chemistry*. doi: 10.1007/s00216-019-02091-8.

Public Health England (2019) *Obesity Profile*. Available at: https://fingertips.phe.org.uk/profile/national-child-measurement-programme (Accessed: 15 March 2021).

Public Health England (2020) *Air pollution: applying All Our Health*. Available at: https://www.gov.uk/government/publications/air-pollution-applying-all-our-health/air-pollution-applying-all-our-health#:~:text=In the UK%2C air pollution,and 36%2C000 deaths every year (Accessed: 21 March 2021).

Public Health England (2021) *COVID-19: mental health and wellbeing surveillance report - 2. Important Findings.* Available at: https://www.gov.uk/government/publications/covid-19-mental-health-and-wellbeing-surveillance-report/2-important-findings-so-far (Accessed: 16 March 2021).

Rački, N. *et al.* (2014) 'Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples', *Plant Methods*, 10(1), p. 42. doi: 10.1186/s13007-014-0042-6.

Raven, K. E. *et al.* (2019) 'Genomic surveillance of Escherichia coli in municipal wastewater treatment plants as an indicator of clinically relevant pathogens and their resistance genes', *Microbial Genomics*, 5(5). doi: 10.1099/mgen.0.000267.

Reid, M. J. *et al.* (2011) 'Analysis and Interpretation of Specific Ethanol Metabolites, Ethyl Sulfate,

and Ethyl Glucuronide in Sewage Effluent for the Quantitative Measurement of Regional Alcohol Consumption', *Alcoholism: Clinical and Experimental Research*, 35(9), p. no-no. doi: 10.1111/j.1530-0277.2011.01505.x.

Reid, M. J., Derry, L. and Thomas, K. V. (2014) 'Analysis of new classes of recreational drugs in sewage: Synthetic cannabinoids and amphetamine-like substances', *Drug Testing and Analysis*, 6(1–2), pp. 72–79. doi: 10.1002/dta.1461.

Renata, Y. *et al.* (2013) 'Urinary concentration of cytokines in children with acute pyelonephritis', *European Journal of Pediatrics*, 172(6), pp. 769–774. doi: 10.1007/s00431-012-1914-2.

Reuter, P. and Stevens, A. (2007) *Bringing Evidence and Analysis Together to Inform UK Drug Policy*.

Rice, J. *et al.* (2020) 'Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - A UK perspective', *Science of The Total Environment*, 735, p. 139433. doi: 10.1016/j.scitotenv.2020.139433.

Rice, J. and Kasprzyk-Hordern, B. (2019) 'A new paradigm in public health assessment: Water fingerprinting for protein markers of public health using mass spectrometry', *TrAC Trends in Analytical Chemistry*, 119, p. 115621. doi: 10.1016/j.trac.2019.115621.

Rico, M., Andrés-Costa, M. J. and Picó, Y. (2017) 'Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study', *Journal of Hazardous Materials*. doi: 10.1016/j.jhazmat.2016.05.079.

Risom, L., Møller, P. and Loft, S. (2005) 'Oxidative stress-induced DNA damage by particulate air pollution', *Mutat Res*, 592(1–2), pp. 119–137. doi: 10.1016/j.mrfmmm.2005.06.012.

Riva, F. *et al.* (2020) 'Testing urban wastewater to assess compliance with prescription data through wastewater-based epidemiology: First case study in Italy', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2020.139741.

Roberts, L. (2013) 'Israel's Silent Polio Epidemic Breaks All the Rules', *Science*, 342(6159), pp. 679–680. doi: 10.1126/science.342.6159.679.

Roberts, P. and Thomas, K. (2006) 'The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment', *Science of The Total Environment*, 356(1–3), pp. 143–153. doi: 10.1016/j.scitotenv.2005.04.031.

Rodríguez-Álvarez, T., Rodil, R., Rico, M., *et al.* (2014) 'Assessment of local tobacco consumption by liquid chromatography-tandem mass spectrometry sewage analysis of nicotine and its metabolites,

cotinine and trans-3′-hydroxycotinine, after enzymatic deconjugation', *Analytical Chemistry*, 86(20), pp. 10274–10281. doi: 10.1021/ac503330c.

Rodríguez-Álvarez, T., Rodil, R., Cela, R., *et al.* (2014) 'Ion-pair reversed-phase liquid chromatography-quadrupole-time-of-flight and triple-quadrupole-mass spectrometry determination of ethyl sulfate in wastewater for alcohol consumption tracing', *Journal of Chromatography A*. Elsevier, 1328, pp. 35–42. doi: 10.1016/j.chroma.2013.12.076.

Rodriguez-Mozaz, S. *et al.* (2015) 'Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river', *Water Research*. Pergamon, 69, pp. 234–242. doi: 10.1016/j.watres.2014.11.021.

Roilides, E. *et al.* (1999) 'Increased Urine Interleukin-6 Concentrations Correlate with Pyelonephritic Changes on 99m Tc-Dimercaptosuccinic Acid Scans in Neonates with Urinary Tract Infections', *The Journal of Infectious Diseases*, 180(3), pp. 904–907. doi: 10.1086/314960.

Rousis, N. I. *et al.* (2017) 'Wastewater-based epidemiology to assess pan-European pesticide exposure', *Water Research*, 121, pp. 270–279. doi: 10.1016/j.watres.2017.05.044.

Rousis, N. I., Zuccato, E. and Castiglioni, S. (2016) 'Monitoring population exposure to pesticides based on liquid chromatography-tandem mass spectrometry measurement of their urinary metabolites in urban wastewater: A novel biomonitoring approach', *Science of The Total Environment*, 571, pp. 1349–1357. doi: 10.1016/j.scitotenv.2016.07.036.

Rousis, N. I., Zuccato, E. and Castiglioni, S. (2017) 'Wastewater-based epidemiology to assess human exposure to pyrethroid pesticides', *Environment International*, 99, pp. 213–220. doi: 10.1016/j.envint.2016.11.020.

Ryu, Y. *et al.* (2016) 'Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater associated with tobacco use', *Scientific Reports*, 6, p. 39055. doi: 10.1038/srep39055.

Ryu, Y., Reid, M. J. and Thomas, K. V. (2015) 'Liquid chromatography-high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater', *Journal of Chromatography A*, 1409, pp. 146–151. doi: 10.1016/j.chroma.2015.07.060.

Saillenfait, A.-M., Ndiaye, D. and Sabaté, J.-P. (2015) 'Pyrethroids: Exposure and health effects – An update', *International Journal of Hygiene and Environmental Health*, 218(3), pp. 281–292. doi: 10.1016/j.ijheh.2015.01.002.

Salipante, S. J. and Jerome, K. R. (2020) 'Digital PCR—An Emerging Technology with Broad

Applications in Microbiology', *Clinical Chemistry*, 66(1), pp. 117–123. doi: 10.1373/clinchem.2019.304048.

Savolainen, L. *et al.* (2013) 'Modification of Clearview Tuberculosis (TB) Enzyme-Linked Immunosorbent Assay for TB Patients Not Infected with HIV', *Clinical and Vaccine Immunology*, 20(9), pp. 1479–1482. doi: 10.1128/CVI.00375-13.

Schwedhelm, E. *et al.* (2007) 'Mass Spectrometric Analysis of F2-Isoprostanes: Markers and Mediators in Human Disease', *Current Pharmaceutical Analysis*, 3(1), pp. 39–51. doi: 10.2174/157341207779802340.

ScotPHO Public Health Information for Scotland (2020) *Obesity*. Available at: https://www.scotpho.org.uk/clinical-risk-factors/obesity/key-points/ (Accessed: 15 March 2021).

Scottish Government (2019) *Pesticide Usage in Scotland*. Available at: https://www.gov.scot/news/pesticide-usage-in-scotland-5/.

Scottish Government (2020) *Cleaner Air for Scotland 2: Draft Air Quality Strategy Consultation*. Available at: https://www.gov.scot/publications/cleaner-air-scotland-2-draft-air-quality-strategy-consultation/pages/6/.

Senta, I. *et al.* (2019) 'Occurrence and Behavior of Macrolide Antibiotics in Municipal Wastewater Treatment: Possible Importance of Metabolites, Synthesis Byproducts, and Transformation Products', *Environmental Science & Technology*, 53(13), pp. 7463–7472. doi: 10.1021/acs.est.9b01420.

Shannon, K. E. *et al.* (2007) 'Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment', *Science of The Total Environment*, 382(1), pp. 121–129. doi: 10.1016/j.scitotenv.2007.02.039.

Shimanoe, C. *et al.* (2021) 'Perceived stress, depressive symptoms, and cortisol-to-cortisone ratio in spot urine in 6878 older adults', *Psychoneuroendocrinology*. doi: 10.1016/j.psyneuen.2020.105125.

Shultz, J. M. *et al.* (2016) 'The Role of Fear-Related Behaviors in the 2013–2016 West Africa Ebola Virus Disease Outbreak', *Current Psychiatry Reports*, 18(11), p. 104. doi: 10.1007/s11920-016-0741-y.

Simonsen, L. *et al.* (2013) 'Global Mortality Estimates for the 2009 Influenza Pandemic from the GLaMOR Project: A Modeling Study', *PLoS Medicine*. Edited by S. I. Hay, 10(11), p. e1001558. doi: 10.1371/journal.pmed.1001558.

Sims, N. and Kasprzyk-Hordern, B. (2020) 'Future perspectives of wastewater-based epidemiology: Monitoring infectious disease spread and resistance to the community level', *Environment*

International, 139. doi: 10.1016/j.envint.2020.105689.

Sims, N., Rice, J. and Kasprzyk-Hordern, B. (2019) 'An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater', *Analytical and Bioanalytical Chemistry*, 411(11), pp. 2261–2271. doi: 10.1007/s00216-019-01667-8.

Singer, A. C. *et al.* (2007) 'Potential Risks Associated with the Proposed Widespread Use of Tamiflu', *Environmental Health Perspectives*, 115(1), pp. 102–106. doi: 10.1289/ehp.9574.

Skelsey, P. and Newton, A. C. (2015) 'Future environmental and geographic risks of Fusarium head blight of wheat in Scotland', *European Journal of Plant Pathology*, 142(1), pp. 133–147. doi: 10.1007/s10658-015-0598-7.

Sleeuwenhoek, A. *et al.* (2007) *Biological monitoring of pesticide exposures. IOM Research Report TM/07/02.*

Spurling, G. K. P. *et al.* (2013) 'Delayed antibiotics for respiratory infections', *Cochrane Database of Systematic Reviews*. doi: 10.1002/14651858.CD004417.pub4.

Stephenson, G. and Richardson, A. (2014) *New Psychoactive Substances in England. A review of the evidence*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/368587/NPSevidenceReview.pdf.

Stuveling, E. M. *et al.* (2003) 'C-reactive protein is associated with renal function abnormalities in a non-diabetic population', *Kidney International*, 63(2), pp. 654–661. doi: 10.1046/j.1523-1755.2003.00762.x.

Sun, Y. *et al.* (2016) 'Multiple antibiotic resistance genes distribution in ten large-scale membrane bioreactors for municipal wastewater treatment', *Bioresource Technology*, 222, pp. 100–106. doi: 10.1016/j.biortech.2016.09.117.

Taha, A. S. (2003) 'Urinalysis for interleukin-8 in the non-invasive diagnosis of acute and chronic inflammatory diseases', *Postgraduate Medical Journal*, 79(929), pp. 159–163. doi: 10.1136/pmj.79.929.159.

Takanami, R. *et al.* (2012) 'Antiviral Drugs Zanamivir and Oseltamivir Found in Wastewater and Surface Water in Osaka, Japan', *Journal of Water and Environment Technology*, 10(1), pp. 57–68. doi: 10.2965/jwet.2012.57.

Tang, S. *et al.* (2020) 'Concentrations of phthalate metabolites in Australian urine samples and their

contribution to the per capita loads in wastewater', *Environment International*. doi: 10.1016/j.envint.2020.105534.

Taylor, A. W., Bruno, R. S. and Traber, M. G. (2008) 'Women and Smokers Have Elevated Urinary F2-Isoprostane Metabolites: A Novel Extraction and LC–MS Methodology', *Lipids*, 43(10), pp. 925–936. doi: 10.1007/s11745-008-3222-1.

Tello, A., Austin, B. and Telfer, T. C. (2012) 'Selective Pressure of Antibiotic Pollution on Bacteria of Importance to Public Health', *Environmental Health Perspectives*, 120(8), pp. 1100–1106. doi: 10.1289/ehp.1104650.

Testai, E. *et al.* (2013) 'A plea for risk assessment of endocrine disrupting chemicals', *Toxicology*, 314(1), pp. 51–59. doi: 10.1016/j.tox.2013.07.018.

Thacker, S. B. *et al.* (2006) 'Measuring the public's health.', *Public Health Reports*, 121(1), pp. 14–22. doi: 10.1177/003335490612100107.

Thacker, S. B. and Berkelman, R. L. (1988) 'Public Health Surveillance in the United States', *Epidemiologic Reviews*, 10(1), pp. 164–190. doi: 10.1093/oxfordjournals.epirev.a036021.

Thai, P. K. *et al.* (2019) 'Evaluating the in-sewer stability of three potential population biomarkers for application in wastewater-based epidemiology', *Science of The Total Environment*, 671, pp. 248–253. doi: 10.1016/j.scitotenv.2019.03.231.

Thomaidis, N. S. *et al.* (2016) 'Reflection of Socioeconomic Changes in Wastewater: Licit and Illicit Drug Use Patterns', *Environmental Science & Technology*, 50(18), pp. 10065–10072. doi: 10.1021/acs.est.6b02417.

Thomas, K. V. *et al.* (2012) 'Comparing illicit drug use in 19 European cities through sewage analysis', *Science of The Total Environment*. Elsevier B.V., 432, pp. 432–439. doi: 10.1016/j.scitotenv.2012.06.069.

Thomas, K. V. and Reid, M. J. (2011) 'What else can the analysis of sewage for urinary biomarkers reveal about communities?', *Environmental Science and Technology*, 45(18), pp. 7611–7612. doi: 10.1021/es202522d.

Tong, A. Y. C., Peake, B. M. and Braund, R. (2011) 'Disposal practices for unused medications around the world', *Environment International*. doi: 10.1016/j.envint.2010.10.002.

Tscharke, B. J. *et al.* (2016) 'Temporal trends in drug use in Adelaide, South Australia by wastewater analysis', *Science of The Total Environment*, 565, pp. 384–391. doi: 10.1016/j.scitotenv.2016.04.183.

Tscharke, B. J., White, J. M. and Gerber, J. P. (2016) 'Estimates of tobacco use by wastewater analysis of anabasine and anatabine', *Drug Testing and Analysis*, 8(7), pp. 702–707. doi: 10.1002/dta.1842.

Tsikas, D. *et al.* (2003) 'Divergence in urinary 8-iso-PGF2α(iPF2α-III, 15-F2t-IsoP) levels from gas chromatography-tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-iso-PGF2α', *Journal of Chromatography B*, 794(2), pp. 237–255. doi: 10.1016/S1570-0232(03)00457-4.

Tuanny Franco, L. *et al.* (2019) 'Biomonitoring of mycotoxin exposure using urinary biomarker approaches: a review', *Toxin Reviews*. doi: 10.1080/15569543.2019.1619086.

UK Government (2019a) *Health Matters: tobacco and alcohol CQUIN*. Available at: https://www.gov.uk/government/publications/health-matters-preventing-ill-health-from-alcohol-and-tobacco/health-matters-preventing-ill-health-from-alcohol-and-tobacco-use (Accessed: 16 March 2021).

UK Government (2019b) *Tackling antimicrobial resistance 2019–2024. The UK's five year national action plan*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/784894/UK_AMR_5_year_national_action_plan.pdf.

United Nations Department of Economic and Social Affairs (2019) *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423).* Available at: https://population.un.org/wpp/Publications/Files/WPP2019_Highlights.pdf.

van der Veen, I. and de Boer, J. (2012) 'Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis', *Chemosphere*, 88(10), pp. 1119–1153. doi: 10.1016/j.chemosphere.2012.03.067.

Vitale, D., Morales Suárez-Varela, M. and Picó, Y. (2021) 'Wastewater-based epidemiology, a tool to bridge biomarkers of exposure, contaminants, and human health', *Current Opinion in Environmental Science & Health*, 20, p. 100229. doi: 10.1016/j.coesh.2021.100229.

Walden, C., Carbonero, F. and Zhang, W. (2017) 'Assessing impacts of DNA extraction methods on next generation sequencing of water and wastewater samples', *Journal of Microbiological Methods*, 141, pp. 10–16. doi: 10.1016/j.mimet.2017.07.007.

Wallis, P. M. *et al.* (1996) 'Prevalence of Giardia cysts and Cryptosporidium oocysts and characterization of Giardia spp. isolated from drinking water in Canada', *Applied and Environmental Microbiology*, 62, pp. 2789–2797.

Wang, H. *et al.* (2020) 'Human exposure of bisphenol A and its analogues: understandings from human urinary excretion data and wastewater-based epidemiology', *Environmental Science and Pollution Research*, 27(3), pp. 3247–3256. doi: 10.1007/s11356-019-07111-9.

Wang, J. *et al.* (2015) 'Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants', *Science of The Total Environment*, 526, pp. 366–373. doi: 10.1016/j.scitotenv.2015.05.046.

Wells, L. *et al.* (2017) 'Deoxynivalenol biomarkers in the urine of UK vegetarians', *Toxins*. doi: 10.3390/toxins9070196.

Wigginton, K. R., Ye, Y. and Ellenberg, R. M. (2015) 'Emerging investigators series: the source and fate of pandemic viruses in the urban water cycle', *Environmental Science: Water Research & Technology*. The Royal Society of Chemistry, 1(6), pp. 735–746. doi: 10.1039/C5EW00125K.

Woolhouse, M. E. J. and Gowtage-Sequeria, S. (2005) 'Host Range and Emerging and Reemerging Pathogens', *Emerging Infectious Diseases*, 11(12), pp. 1842–1847. doi: 10.3201/eid1112.050997.

World Health Organisation (2001) *WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation*. Available at: https://apps.who.int/iris/handle/10665/42363.

World Health Organisation (2003a) *Adherence to long-term therapies: evidence for action.* Available at: https://www.who.int/chp/knowledge/publications/adherence_full_report.pdf.

World Health Organisation (2003b) *Guidelines for environmental surveillance of poliovirus circulation (No. WHO/V&B/03.03)*. Available at: http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf.

World Health Organisation (2010a) 'Toxicological and Health Aspects of Bisphenol A Joint FAO/WHO Expert Meeting', in. Available at: http://apps.who.int/.../44624.

World Health Organisation (2010b) *WHO Pandemic (H1N1) 2009–update 112*. Available at: https://www.who.int/csr/don/2010_08_06/en/ (Accessed: 7 August 2019).

World Health Organisation (2015a) *Global action plan on antimicrobial resistance, World Health Organisation*. doi: ISBN 978 92 4 150976 3.

World Health Organisation (2015b) *Global Antimicrobial Resistance Surveillance System: Manual for Early Implementation.* Available at: https://www.who.int/antimicrobial-resistance/publications/surveillance-system-manual/en/.

World Health Organisation (2017a) *Antimicrobial Resistance*. Available at:

https://www.who.int/news-room/q-a-detail/antimicrobial-resistance#:~:text=Antimicrobial resistance occurs when microorganisms,referred to as “superbugs”. (Accessed: 10 March 2021).

World Health Organisation (2017b) *World Health Organization. WHO guidelines on ethical issues in public health surveillance*. Available at: https://www.who.int/ethics/publications/public-health-surveillance/en/ (Accessed: 13 August 2019).

World Health Organisation (2018a) *Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017-2018*. Available at: https://apps.who.int/iris/bitstream/handle/10665/279656/9789241515061-eng.pdf?ua=1.

World Health Organisation (2018b) *Managing epidemics Key facts about major deadly diseases*. Available at: https://www.who.int/emergencies/diseases/managing-epidemics/en/.

World Health Organisation (2019) *Ten Threats to Global Health in 2019*. Available at: https://www.who.int/news-room/feature-stories/ten-threats-to-global-health-in-2019 (Accessed: 31 January 2020).

World Health Organisation (2020a) *Disease outbreaks by year*. Available at: https://www.who.int/csr/don/archive/year/en/.

World Health Organisation (2020b) *Novel Coronavirus (2019-nCoV) Situation Report 1*. Available at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200121-sitrep-1-2019-ncov.pdf.

World Health Organisation (2020c) *Novel Coronavirus (COVID-19) Situation*. Available at: https://experience.arcgis.com/experience/685d0ace521648f8a5beeeee1b9125cd.

Wu, C. *et al.* (2016) ‘Concurrent quantification of multiple biomarkers indicative of oxidative stress status using liquid chromatography-tandem mass spectrometry’, *Analytical Biochemistry*. Elsevier Inc, 512, pp. 26–35. doi: 10.1016/j.ab.2016.07.030.

Wu, L. L. *et al.* (2004) ‘Urinary 8-OHdG: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics’, *Clinica Chimica Acta*, pp. 1–9. doi: 10.1016/j.cccn.2003.09.010.

Xiao, Y. *et al.* (2019) ‘Assessing the trend of diabetes mellitus by analyzing metformin as a biomarker in wastewater’, *Science of The Total Environment*, 688, pp. 281–287. doi: 10.1016/j.scitotenv.2019.06.117.

Xu, J. *et al.* (2015) ‘Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river’, *Chemosphere*, 119, pp. 1379–1385. doi:

10.1016/j.chemosphere.2014.02.040.

Yan, T. *et al.* (2018) 'Municipal Wastewater as a Microbial Surveillance Platform for Enteric Diseases: A Case Study for Salmonella and Salmonellosis', *Environmental Science and Technology*. doi: 10.1021/acs.est.8b00163.

Yang, Z., D'Auriac, M. A., *et al.* (2015) 'A Novel DNA Biosensor Using a Ferrocenyl Intercalator Applied to the Potential Detection of Human Population Biomarkers in Wastewater', *Environmental Science & Technology*, 49(9), pp. 5609–5617. doi: 10.1021/acs.est.5b00637.

Yang, Z., Kasprzyk-Hordern, B., *et al.* (2015) 'Community sewage sensors for monitoring public health', *Environmental Science and Technology*, 49(10), pp. 5845–5846. doi: 10.1021/acs.est.5b01434.

Yang, Z. *et al.* (2016) 'Community Sewage Sensors towards Evaluation of Drug Use Trends: Detection of Cocaine in Wastewater with DNA-Directed Immobilization Aptamer Sensors.', *Scientific reports*. Nature Publishing Group, 6(October 2015), p. 21024. doi: 10.1038/srep21024.

Yang, Z. *et al.* (2017) 'Monitoring Genetic Population Biomarkers for Wastewater-Based Epidemiology', *Analytical Chemistry*, 89(18), pp. 9941–9945. doi: 10.1021/acs.analchem.7b02257.

Zhang, X.-X., Zhang, T. and Fang, H. H. P. (2009) 'Antibiotic resistance genes in water environment', *Applied Microbiology and Biotechnology*. Springer-Verlag, 82(3), pp. 397–414. doi: 10.1007/s00253-008-1829-z.

Zhao, M. *et al.* (2017) 'A comprehensive analysis and annotation of human normal urinary proteome', *Scientific Reports*, 7(1), p. 3024. doi: 10.1038/s41598-017-03226-6.

Zheng, P. *et al.* (2013) 'Novel urinary biomarkers for diagnosing bipolar disorder', *Metabolomics*. doi: 10.1007/s11306-013-0508-y.

Zheng, P. *et al.* (2016) 'Identification of sex-specific urinary biomarkers for major depressive disorder by combined application of NMR- and GC-MS-based metabonomics', *Translational psychiatry*. doi: 10.1038/tp.2016.188.

Zuccato, E. *et al.* (2005) 'Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse', *Environmental Health*, 4(1), p. 14. doi: 10.1186/1476-069X-4-14.

Zuccato, E. *et al.* (2008) 'Estimating Community Drug Abuse by Wastewater Analysis', *Environmental Health Perspectives*, 116(8), pp. 1027–1032. doi: 10.1289/ehp.11022.

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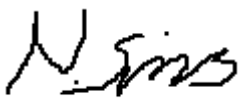
Signed							**Date**	15/03/2022		

4 An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater

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4.1 Abstract

Reported herein is the development of an analytical method for the detection of four oxidative stress biomarkers in wastewater using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) and solid phase extraction (SPE). The following four biomarkers of oxidative stress and lipid peroxidation have been investigated: hydroxynonenal - mercapturic acid (HNE-MA), 8-*iso*-prostglandin F2beta (8-*iso*-$PGF_{2\beta}$), 8-nitroguanine (8-NO_2Gua) and 8-hydroxy-2-deoxyguanosine (8-OHdG). The method showed very good performance: accuracy (>87 %), precision (>90 %), method quantification limits (1.3 -3.0 ng L^{-1}) and biomarker stability in wastewater in the case of HNE-MA, 8-OHdG and 8-*iso*-$PGF_{2\beta}$. In contrast, 8-NO_2Gua was found to be less stable in wastewater, which affected its method performance: accuracy (>63 %), precision (>91 %) and method quantification limits (85.3 ng L^{-1}). Application of the developed method resulted in, for the first time, HNE-MA being successfully observed and quantified within wastewater over a study period of a week (displayed average daily loads per capita of 48.9 ± 4.1 mg/1000/people/day). 8-*iso*-$PGF_{2\beta}$ was detected with good intensity but could not be quantified due to co-elution with other isomers. 8-OHdG was detected, albeit at <MQL. This study demonstrates the potential for expanding on the possible endogenous biomarkers of health used in urban water fingerprinting to aid in measuring health in near-real time on a community-wide scale.

Keywords: oxidative stress; biomarkers; wastewater; urban water fingerprinting

4.2 Introduction

Wastewater-based epidemiology is a rapidly developing and innovative technique that analyses human metabolic excretion products in the wastewater of a defined population (Daughton, 2001). The wastewater of a community is an incredibly valuable, yet traditionally under-estimated, source of knowledge. The analysis of targeted aspects of biological and chemical information wastewater contains can offer a unique reflection of health upon the population that contributes. The concept of

WBE has already experienced enormous successes from communities to international scales to evaluate and compare trends in illicit drug usage (Zuccato *et al.*, 2005; Castiglioni *et al.*, 2006; Thomas *et al.*, 2012), pharmaceuticals (Baker, Barron and Kasprzyk-Hordern, 2014), alcohol (Reid *et al.*, 2011; Mastroianni, Lopez de Alda and Barcelo, 2014; Boogaerts *et al.*, 2016) and tobacco consumption (Rodríguez-Álvarez, Rodil, Rico, *et al.*, 2014; Castiglioni *et al.*, 2015; Tscharke, White and Gerber, 2016). Recently the potential for WBE to evaluate and monitor community-wide public health has been highlighted by analysing endogenous urinary biomarkers of human health and disease (Daughton, 2012; Kasprzyk-Hordern *et al.*, 2014; Gracia-Lor *et al.*, 2017).

Currently monitoring public health is done via conventional epidemiological studies. These are based upon existing resources including morbidity data, prescription rates and questionnaires (Thacker *et al.*, 2006; Daughton, 2012). However the results from such sources of information can be subject to bias and are not always representative of a whole community, hence can give misleading results. One of the crucial disadvantages of current approaches is there is no capacity for real-time monitoring of health on a community-scale. This results in difficulties in establishing trends in a population's health and causes serious issues in implementing appropriate and effective healthcare interventions.

An increasingly popular branch of epidemiology studies based upon the assessment of human exposure to external factors such as environmental pollution is human biomonitoring. This technique involves the detection and analysis of biomarkers of interest in biological samples of individuals. Such samples can include saliva, blood, tissue or excretion products. (Needham, Calafat and Barr, 2007). However this process is expensive and time-consuming and results in only a small portion of a population being investigated which might not be representative of a population as a whole (Bauer, 2008). Furthermore such techniques require samples from thousands of patients in a defined geographic location and the selection of a control group for comparison can be challenging.

A possible solution to these drawbacks is to use WBE as complementary tool to conventional public health assessments (Kasprzyk-Hordern *et al.*, 2014). The ability to analyse and monitor endogenous biomarkers of disease within the wastewater of a community in near-real time could offer an unbiased, reflection of the health of the population that contributes. It has been proposed that the evaluation of oxidative stress biomarkers could give key information upon the health status of a community (Daughton, 2012). Oxidative stress is defined as the imbalance between reactive oxygen species and the ability of the body to counteract with antioxidants (Birben *et al.*, 2012). It is a key characteristic of many acute and chronic diseases including stroke (Ozkul *et al.*, 2007), heart disease (Dhalla, 2000), cancers and respiratory infections (Bauer *et al.*, 2012) as well as being an indicator for certain lifestyle factors such as excessive smoking and alcohol consumption (Meagher *et al.*, 1999; Epplein *et al.*, 2009). Indications of oxidative stress are often reflected through elevated levels of specific biomarkers within parts of body, including blood plasma, and urine. Higher levels of oxidative stress biomarkers in individuals within populations have not only been linked with various diseases and lifestyle factors but

have also been correlated with environmental exposure, for example air pollution (Risom, Møller and Loft, 2005; Lodovici and Bigagli, 2011). As a result, not only could measurement of cumulative stress give information about the general health of a community but could also give valuable data on the exposure to external factors such as anthropogenic pollution, an area of study where still very little is known.

In particular, a handful of key oxidative stress biomarkers have been well-studied within urine, with various analytical methods developed for 8-*iso*-prostaglandin F2alpha (8-*iso*-$PGF_{2\alpha}$), 8-nitroguaninne (8-NO_2Gua) and 8-hydroxy-2-deoxyguanosine (8-OHdG) and hydroxynonenal - mercapturic acid (HNE-MA) (Berdeaux *et al.*, 2006; Klawitter *et al.*, 2011; Wu *et al.*, 2016). However, to date only one biomarker of oxidative stress has been observed and quantified by WBE techniques in wastewater (Ryu, Reid and Thomas, 2015). Wastewater analysis poses many challenges as the matrix itself has significantly higher complexity and interchangeability in comparison to urine. Furthermore with regards to the biomarkers themselves the concentrations in wastewater are far lower (sub-ppt levels) than those observed in urine (e.g. ng/mg of creatinine for 8-OHdG(Wu *et al.*, 2004)). Urinary 8-*iso*-$PGF_{2\alpha}$ is formed within the body from the oxidation of arachidonic acid and is widely-recognised reliable biomarker of oxidative stress with elevated levels typically observed within urine [12, 31–33]. In a unique study by Ryu *et al.* 8-*iso*-$PGF_{2\alpha}$ was successfully extracted from wastewater samples using highly specific immunoassay approaches (Ryu, Reid and Thomas, 2015). A further study demonstrated 8-*iso*-$PGF_{2\alpha}$ correlated with the major metabolite of smoking in wastewater across 11 cities in Europe (Ryu *et al.*, 2016).

This paper aimed to develop an analytical method using ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS) to analyse, for the first time, four biomarkers of oxidative stress 8-*iso*-prostglandin F2beta (8-*iso*-$PGF_{2\beta}$), HNE-MA, 8-NO_2Gua and 8-OHdG from wastewater through application of solid phase extraction (SPE) techniques. 8-OHdG and 8-NO_2Gua are reliable markers of oxidative DNA and nitrative DNA damage respectively. Reactive oxygen species (ROS) produced as a result of oxidative stress can not only damage DNA but also cause destruction of the cell membranes in a process known as lipid peroxidation. The urinary biomarker HNE-MA is a key indicator of cell membrane damage and 8-*iso*-$PGF_{2\beta}$ in an isomer of the reliable oxidative stress marker 8-*iso*-$PGF_{2\alpha}$.

4.3 Materials and methods

4.3.1 Materials

A total of four biomarkers were selected for method development due to their acknowledged indication of oxidative stress within urine (Wu *et al.*, 2016). The standard 8-OHdG was bought from Sigma Aldrich (UK), its respective internal standard $^{15}N_5$-8-OHdG along with 8-NO_2Gua were purchased from Santa Cruz Biotechnologies (UK). The standards 8-*iso*-$PGF_{2\beta}$, HNE-MA and the internal standard HNE-MA-d3 were bought from Cayman Chemicals (US). Stock solutions of selected biomarkers were

made up by dissolving solid samples in MeOH and all stock solutions were kept in the dark at – 80 °C. Working solutions were diluted from the stock solutions to make up the desired concentrations in 80:20 H_2O:MeOH. Solvents such as MeOH and toluene were HPLC grade and purchased from Sigma Aldrich. To remove the risk of basic functional groups reacting with silanols on glass surfaces, all glassware was deactivated using 5% dimethylchlorosilane (DMDCS) in toluene. The silanisation of glass occurred by rinsing with DMDCS before washing twice with toluene and three times with MeOH.

4.3.2 Solid-phase extraction

The solid-phase extraction (SPE) procedure followed the protocol previously published by Petrie *et al.* (Petrie *et al.*, 2015). For all extractions, Oasis HLB (Waters, hydrophilic-lipophilic-balanced) cartridges (60 mg, 3 mL) were conditioned with 2 mL of MeOH followed by 2 mL of deionised water (pH 7.5) for equilibration. Influent wastewater was aliquoted into 100 mL samples before spiking with internal standard solutions (final concentrations of 100 ng and 500 ng for HNE-MA-d3 and $^{15}N_5$-8-OHdG respectively). Spiked influent wastewater samples were then filtered through GF/F filters before loading onto the pre-conditioned HLB cartridges at a flow rate of < 1 mL min^{-1}. Once loaded, cartridges were left to dry under vacuum for 30 minutes. Elution of analytes occurred using 4 mL of MeOH at a steady flow rate of 1 mL min^{-1}. Once eluted, samples were evaporated till dry under N_2, 40 °C using TurboVap evaporator (Caliper, UK) this was then followed by reconstitution with 500 µL of 80:20 H_2O: MeOH. Samples were transferred to polypropylene vials and 20 µL of sample were injected into the Waters Acquity UPLC system. A graphical representation of the extraction procedures and analytical methodology has been detailed (Figure 1).

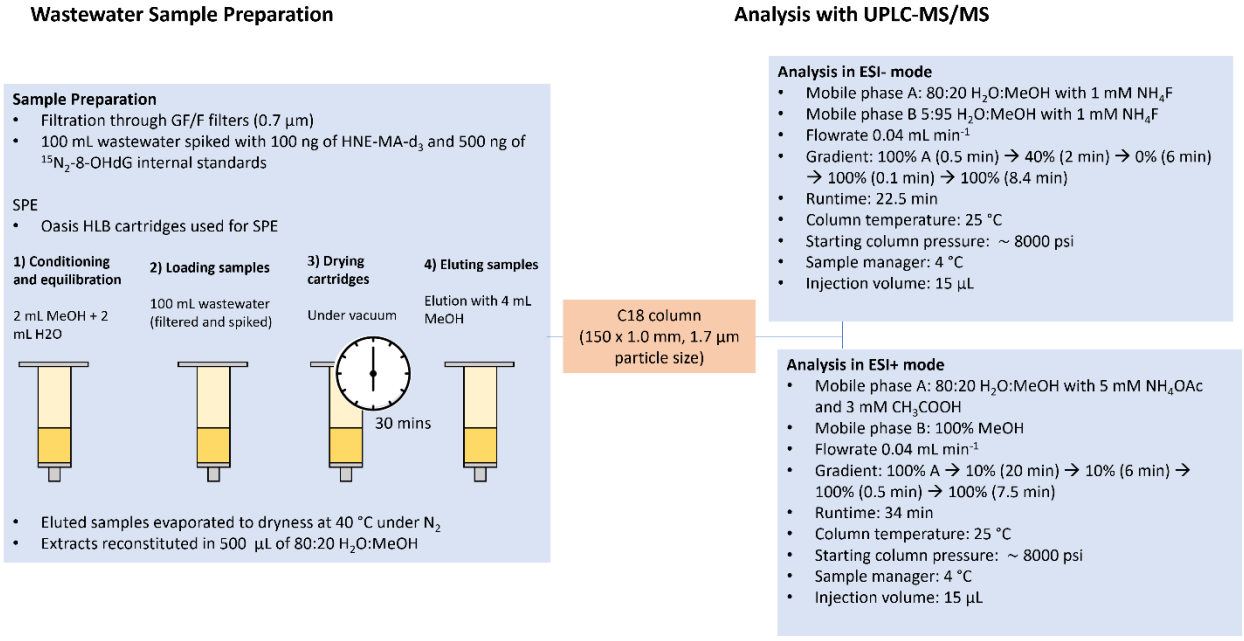

Fig. 1. Summary of the wastewater sample preparation and extraction followed by analytical method details.

4.4 Liquid chromatography coupled with tandem mass spectrometry

Liquid chromatography was performed using a Waters Acquity UPLC system which was coupled to the Xevo TQD Triple Quadrupole Mass Spectrometer. (Waters, UK) Due to ionisation preference of the chosen biomarkers, two methods have been developed for this study. 8-OHdG ionised in ESI positive mode whereas HNE-MA, 8-*iso*-$PGF_{2\beta}$ and 8-NO_2Gua ionised preferentially in ESI negative mode (Table 1). Both methods used a reversed-phase BEH C18 column (150 × 1.0 mm, 1.7 µm particle size) (Waters, UK) with a 0.2 µm, 2.1 mm in-line column filter (Water, UK) maintained at 25 °C. Mobile phase used in ESI negative was as follows: A; 80:20 H_2O:MeOH with 1 mM NH4F (mobile phase A) and 5:95 H_2O:MeOH with 1 mM NH_4F (mobile phase B) with the following gradient: 100 %A (0.5 min) – 40 % (2 min) – 0 %A (5.5 min). Mobile phase used in ESI positive was as follows: A; 80:20 H_2O:MeOH with 5 mM NH_4OAc and 0.3 % CH_3COOH (mobile phase A) and MeOH (mobile phase B) with the following gradient: 100 %A reduced to 10 % over 20 min. The mobile phase flow rate was kept constant at 0.04 mL min^{-1} and a 20 µL injection volume was used in both methods.

Table 1 Target biomarkers with MS parameter details and fragment details plus internal standards used

Compound/Internal Standard	**Biomarker of…**	**MRM Mass transition (m/z)**	**Cone Voltage (v)**	**Collision Energy (v)**	**ESI**
8-OHdG	Oxidative DNA damage	284.0 → 168.1 284.0 → 140.2	45	18 30	Positive
$^{15}N_5$-8-OHdG		289.1 → 173.2		18	
HNE-MA	Lipid peroxidation	318.1 → 171.1 318.1 → 143.1	32	22 24	Negative
HNE-MA (d3)		321.5 → 174.2		22	
8-NO_2Gua	Nitrative DNA damage	194.9 → 178.1 194.9 → 153.1	40	15 15	Negative
8-*Iso*-$PGF_{2\beta}$	Lipid peroxidation	353.4 → 193.2 353.4 → 247.3	53	22 22	Negative

MassLynx 4.1 (Waters, UK) was used to control the LCMS system. TargetLynx (Waters, Manchester, UK) was used for data processing. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. $[M-H]^-$ and $[M+H]^+$ were selected as molecular ions in ESI- and ESI+ respectively. MRM transitions and ESI parameters were obtained after direct infusion of each standard at a concentration of 100 µg L^{-1} in the mass spectrometer. Optimised ESI parameters were as follows: capillary voltage 3.0 kV in ESI positive and 3.2 kV in ESI negative. The source temperature was 150 ^oC and the desolvation temperature was 400 ^oC. Nitrogen was used as nebulising and desolvation gas. The cone gas flow was 100 L h^{-1} and the desolvation gas flow was 550 L h^{-1}. Argon was used as the collision gas. Optimised MS/MS parameters can be found in Table 1. Two MRM transitions, one for

quantification and one for confirmation) were chosen for each compound. Only one MRM transition was selected for labelled internal standards.

The chosen methods were successful in the identification of 8-OHdG and achieved good separation of the negatively ionised biomarkers with elution at different retention times all within the first 10 minutes of the run. Separation and identification of the quantifying mass fragment for each biomarker were successfully observed (Figure 2). With regards to internal standards to allow quantification of target biomarkers in samples, a deuterated form of HNE-MA (HNE-MA-d3) was used for all three of the biomarkers ionised in negative mode. For 8-OHdG, a nitrogen labelled 8-OHdG ($^{15}N_5$-8-OHdG) was used as the internal standard in positive mode.

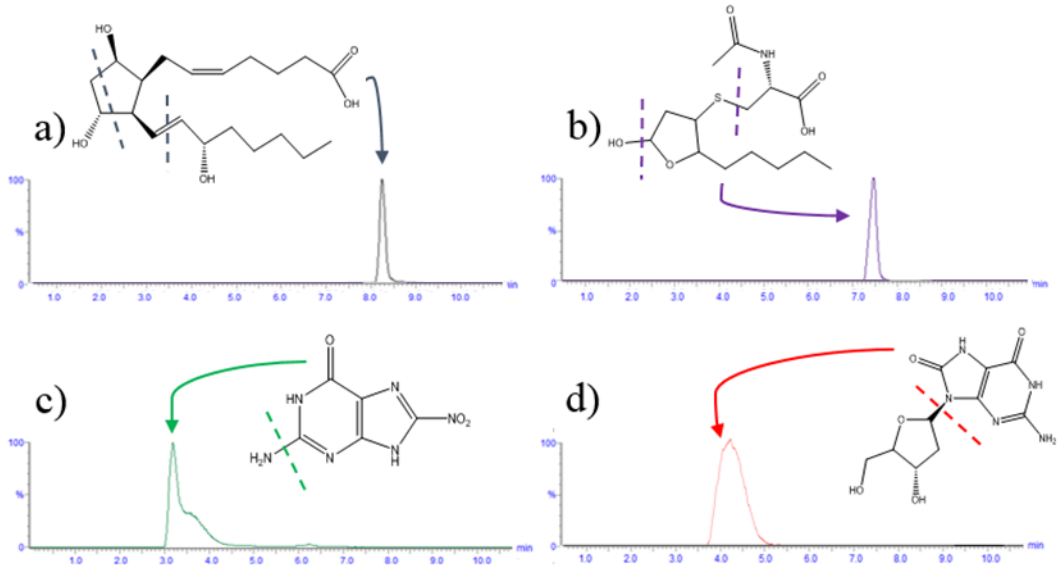

Fig. 2. LC chromatograms and proposed structures of the quantifying mass fragment ions in mobile phase (80:20 H_2O:MeOH). **a)** 8-*iso*-$PGF_{2\beta}$, m/z: 353.4 → 193.2 **b)** HNE-MA, m/z: 318.1 → 171.1 **c)** 8-NO_2Gua, m/z: 194.9 → 177.8 **d)** 8-OHdG, m/z: 284.0 → 168.1. Target analytes spiked at 500 µg L^{-1}.

4.5 Wastewater Sample Collection

Influent wastewater samples were collected via 24 h composite samples across a seven day period from a wastewater treatment plant in the South-West of England serving a population equivalent ~ 886,650. Once collected, samples were stored and transported upon ice to the laboratory for extraction and analysis. To account for both flows and population equivalent, daily loads per capita in mg/1000/people/day were calculated (Equation 1).

$$Daily\ Load\ per\ Capita = Concentration\ x\ flow\ (m^3) x\ 1000\ x\ \left(\frac{1000}{population\ equivalent}\right) \quad \text{Equation 1}$$

4.6 Method Validation

To establish regions of linearity, a triplicate injection of a 17-point calibration curve with concentrations ranging from 0.01-1000 µg L^{-1} was done for each compound. To determine inter- and intra- day

accuracy and precision triplicate injections of three different concentrations were prepared within a 24 h period across three separate days. New solutions were made up for each separate day, the three different concentrations were 10 µg L^{-1} (50 µg L^{-1} for 8-OHdG), 100 µg L^{-1} and 500 µg L^{-1}. Precision was calculated using relative standard deviation (RSD) for replicate injections (n=6). Method inter- and intra-day accuracy and precision were also established via the spiking of target biomarkers at initial concentrations of 0.5 µg L^{-1} and 1.25 µg L^{-1} into 100 mL of influent wastewater before the SPE step. Extracted samples were then injected in duplicate across a 24-hour period and averages across the two concentrations were used to establish intra-day method accuracy and precision (n=3 injected in duplicate). To determine method inter-day accuracy and precision, fresh influent wastewater samples were prepared by spiking again at 0.5 µg L^{-1} and 1.25 µg L^{-1} and extracting before injecting as described above, across a further two 24 hour periods.

Instrument detection limits (IDL) and instrument quantification limits (IQL) were established by the lowest concentrations which gave signal to noise ratios ≥ 3 and ≥ 10 respectively. The method detection limit (MDL) was calculated using the following:

$$MDL = \frac{IDL\ x\ 100}{Rec\ x\ CF} \qquad \text{Equation 2}$$

where IDL is the instrumental limit of detection, Rec is the relative SPE recovery of the analyte in wastewater and CF in the SPE concentration factor.

Method recoveries for each compound were determined by spiking of known amounts of analytes before extraction into 100 mL allotted influent wastewater samples at two different concentrations of individual analytes (0.5 µg L^{-1} and 1.25 µg L^{-1}) with internal standards spiked into each sample at 100 ng and 500 ng for HNE-MA-d3 and $^{15}N_5$-8-OHdG respectively. Method recoveries have been calculated as corrected recoveries (i.e. taking the internal standard concentration into consideration). This is calculated by the ratio of the concentration of target analytes in wastewater solutions when spiked before SPE (minus the concentration of analyte in the blank wastewater sample), divided by the standard mobile phase concentration (Equation 3).

$$Method\ Recoveries_{corrected} = \left(\frac{A_{spiked\ before\ SPE} - A_{blank}}{A_{mobile\ phase}}\right) x\ 100\ \% \qquad \text{Equation 3}$$

To determine matrix suppression, the ratio of the concentration of target analytes in wastewater samples spiked after SPE (minus the concentration of the analyte in the blank wastewater sample) is divided with the standard mobile phase sample concentration (Equation 4).

$$Matrix\ Supression = \left(1 - \frac{A_{spiked\ after\ SPE} - A_{blank}}{A_{mobile\ phase}}\right) x\ 100\ \% \qquad \text{Equation 4}$$

4.7 Biomarker stability in wastewater

To assess the stability of the target analytes within wastewater a 24 h wastewater stability study was performed. A total of four 2 L reactors of influent wastewater was used, two of which were kept at 17

°C with the other two kept at 4 °C to determine if any degradation occurred at two different temperatures. Each reactor was spiked with each target analyte to determine a final concentration of 250 µg L^{-1}. After initial analyte spiking, 2 x 50 mL samples were taken from each reactor and spiked with respective internal standards before filtering and SPE extraction to give concentration at time 0. After which a further five sampling points were taken across the 24 h (0, 2, 4, 6, 12, 24 h) with 2 x 50 mL samples taken from each reactor at the time point. For calculating average concentration of target analytes at each sampling point, the average of both the two samples was taken at each time point along with the average across duplicate reactors. Errors were calculated using the standard deviation of concentrations across duplicate reactors and duplicate samples (n=4).

4.8 Results and discussion

4.8.1 Method Validation

4.8.1.1 LC-MS Validation Parameters

Regarding the calibration curves, the mean coefficients of determination (R^2) gave excellent linearity with values ≥0.997 for all four biomarkers over the concentration range investigated (0–500 µg L^{-1} or 0–1000 µg L^{-1}) (Table 2). However, not all biomarkers displayed acceptable linearity (R^2 ≥0.997) across the entire concentration range studied. 8-NO_2Gua required two calibration curves, 0.1-100 µg L^{-1} and 100-1000 µg L^{-1} at R^2 at 0.998 and 0.999 respectively. Intra- and inter-day accuracy exhibited acceptable ranges of 94-107 % for all biomarkers. Regarding intra- and inter-day precision, all four biomarkers displayed very small deviations giving >97 % for all biomarkers investigated.

Instrument detection limits (IDL) were as low as 0.01 µg L^{-1} for both HNE-MA and 8-NO_2Gua and 0.05 µg L^{-1} for 8-*iso*-$PGF_{2\beta}$. Instrument quantification limits (IQLs) were generally low at <0.5 µg $L^{-1.}$ 8-OHdG displayed slightly poorer sensitivities at 1 µg L^{-1} for detection and 5 µg L^{-1} for quantification.

Table 2 Instrument performance data detailing linearity including instrument detection limits (IDLs) and instrument quantification limits (IQLs) and intra- and inter- day accuracy and precision for all biomarkers studied

Compound	Internal Standard	Linearity		IDL [µg L^{-1}]	IQL [µg L^{-1}]	Intra-day instrument performance		Inter-day instrument performance	
		Range [µg L^{-1}]	R^2			Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]
8-OHdG	$^{15}N_5$-8-OHdG	5-500	0.997	1	5	95.6	97.7	97.1	97.5
HNE-MA	HNE-MA-d3	0.5-1000	0.999	0.01	0.5	103	98.4	106	98.1
8-NO_2Gua	HNE-MA-d3	0.1-100 100-1000	0.998 0.997	0.01	0.1	107	97.7	94.1	97.3
8-*Iso*-$PGF_{2\beta}$	HNE-MA-d3	0.5-1000	0.999	0.05	0.5	99.2	97.8	101	98.8

4.8.1.2 SPE-LC-MS Validation Parameters

Regarding method sensitivity within wastewater matrices, method detection limits (MDLs) of <0.2 ng L^{-1} were achieved for HNE-MA, 8-*iso*-$PGF_{2\beta}$ and 8-NO_2Gua, . HNE-MA in particular gave excellent method sensitivity with an MDL at 0.0590 ng L^{-1}. Method quantification limits (MQLs) for the same three biomarkers were also <3 ng L^{-1}. 8-OHdG on the other hand gave slightly poorer method sensitivity (17.1 ng L^{-1} and 85.3 ng L^{-1} for MDL and MQL respectively),

Results from method recoveries are all reported as corrected recoveries (i.e. the internal standards have been considered). HNE-MA gave excellent recoveries with minimal matrix suppression across the two concentrations studied (85 % and 17 % averages respectively over 0.5 and 1.25 µg L^{-1}). Signal enhancement was observed for 8-*iso*-$PGF_{2\beta}$ (-67 % and -55 % at 0.5 and 1.25 µg L^{-1} respectively) and high method recoveries were exhibited (142 % and 147 % at 0.5 and 1.25 µg L^{-1} respectively). This is attributed to the challenges in identifying 8-*iso*-$PGF_{2\beta}$ amongst the peaks it occurs in within the unspiked wastewater sample. With regards to 8-OHdG and 8-NO_2Gua, both had lower but reproducible Method recoveries (average recoveries of 32 % and 65 % respectively across 0.5 and 1.25 µg L^{-1}). Furthermore both compounds were moderately susceptible to a wastewater matrix, with 8-OHdG average of 47 % signal suppression and 8-NO_2Gua at 46 % across 0.5 and 1.25 µg L^{-1}. Regarding method accuracy, HNE-MA had excellent method accuracy results across the three days studied with 101 % for inter-day accuracy (Table 3). The higher but consistent method accuracies observed for 8-*iso*-$PGF_{2\beta}$ at 140 % and 134 % for intra- and inter-day are attributed to again being unable to identify the biomarker peak in the un-spiked wastewater samples. Therefore concentrations of this biomarker already present in real wastewater samples were not accounted for in calculations. 8-NO_2Gua exhibited acceptable method accuracies at 88.9 % for inter-day whilst 8-OHdG displayed low method accuracies at 64.1 % for inter-day. The lower method accuracies observed for 8-OHdG is a reflection of the challenges of analysing this biomarker in real wastewater samples, further evidenced by the results of matrix effects and method recoveries (Table 3). Regarding method precision, both inter- and intra-day precision gave acceptable ranges of 90-96 % for all four biomarkers studied.

Table 3 Method performance data detailing method detection limits (MDLs) and method quantification limits (MQLs), Method recoveries and matrix effects, intra- and inter-day accuracy and precision for all biomarkers studied (n = 3 injected in duplicate)

Compound	Method linearity [ng L^{-1}]	MDL [ng L^{-1}]	MQL [ng L^{-1}]	Method recoveries [%]		Matrix effects [%]		Intra-day method performance		Inter-day method performance	
				0.5 [µg L^{-1}]	1.25 [µg L^{-1}]	0.5 [µg L^{-1}]	1.25 [µg L^{-1}]	Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]
HNE-MA	3.0-5903	0.06	3.0	83.4	86.0	18.0	15.9	91.2	95.1	101	95.6
8-*Iso*-$PGF_{2\beta}$	1.7-3455	0.17	1.7	142	147	-67.2	-55.2	140	95.2	134	94.4
8-NO_2Gua	1.3-13123	0.13	1.3	67.9	61.3	48.6	44.2	86.5	90.4	88.9	90.2
8-OHdG	85.3-8532	17.1	85.3	29.0	35.1	53.0	40.5	63.2	91.8	64.1	92.5

4.8.2 8-*Iso*-$PGF_{2\beta}$ and its isomers

Interestingly when studying 8-*iso*-$PGF_{2\beta}$ in wastewater, instead of a clearly resolved peak that is observed within the mobile phase, there is a broad, poorly resolved series of peaks eluting between 6-10 min in wastewater. However when spiked with the target analyte at initial concentrations of 0.5 µg L^{-1} and 1.25 µg L^{-1}, 8- *iso*-$PGF_{2\beta}$ can be identified amongst the series of peaks (Figure 3). A possible explanation for this observation could be due to the presence of a wide range of F2-isoprostane compounds in wastewater. 8-*iso*-$PGF_{2\beta}$ belongs to a large family of prostaglandin-like isomers known as the isoprostanes. The isoprostanes are metabolic products of the peroxidation of the arachidonic acid via a free radical catalysed mechanism (Cracowski and Durand, 2006). Fatty acids like arachidonic acid occur with relative abundance in human cells and are crucial components as they facilitate the proper formation and function of cell membranes. From the peroxidation of arachidonic acid, four classes of F2-isoprostanes may be formed (Berdeaux *et al.*, 2006). The F2-isoprostane regioisomer compromises of eight diastereoisomers that arise to 64 different F2-isoprostanes. So if 8-*iso*-$PGF_{2\beta}$ is present in wastewater then it is highly likely the other isomers excreted in urine will also be present. SPE is widely recognised as a non-specific extraction technique, and with the combination of Oasis HLB cartridges will ultimately result in the extraction of a wide number of compounds including those with similar chemistries. Furthermore such similar isomers are likely to have matching mass fragment peaks hence the potential elution of different fragment ions around the target analytes potentially resulting in the interference observed.

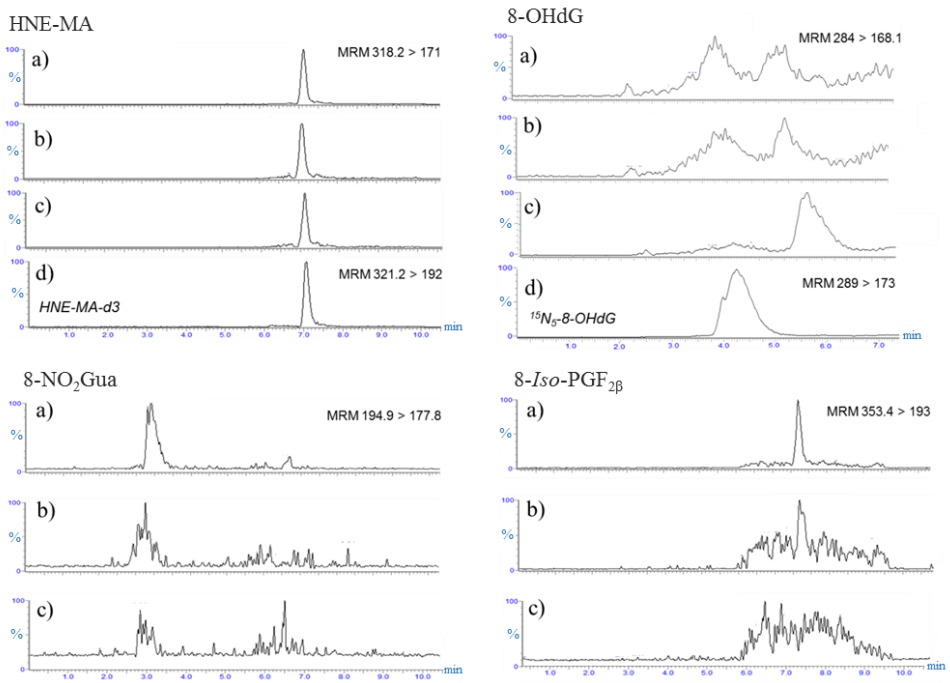

Fig. 3. LC chromatograms of the quantifying mass fragment ions of each target biomarker in influent wastewater samples a) initial spiked analyte concentration of 0.5 µg L^{-1} b) initial spiked analyte concentration of 0.5 µg L^{-1} c) unspiked wastewater d) internal standards: HNE-MA-d3 (spiked at 100 ng L^{-1}) or $^{15}N_5$-8-OHdG (spiked at 500 ng L^{-1}).

It should be noted that it is recognised in the literature of the lack of clarity in whether a number of analytical methods for F2-IsoPs in biological matrices are specific for a single isomer or whether it is capturing numerous isobaric species (Tsikas *et al.*, 2003; Schwedhelm *et al.*, 2007; Halliwell and Lee, 2010). For example, Davies *et al.* demonstrated various dinor, dihydro F2-IsoP metabolite species being captured within a single chromatographic peak in urine samples via tandem LC-MS techniques (Davies *et al.*, 2006). Due to the significant number of various stereo- and regio-isomers of the F2-IsoP family, the analytical challenges of separation and reliability of peaks given are well recognised within biological matrices such as urine. It is unsurprising therefore that such difficulties are similarly observed within more complex matrices such as wastewater. However such challenges have been overcome in WBE, as previously mentioned Ryu *et al.* used highly selective immunoassay techniques to capture 8-*iso*-$PGF_{2\alpha}$ from wastewater to give a single isomer species (Ryu, Reid and Thomas, 2015).

However it is important to study the isoPs as a group in WBE, particularly as it is not currently known which isomer indicates oxidative stress the best or even which isomer is most abundant in urine. This idea was partially explored in an extensive review by Daughton reviewing the potential of isoPs for use in WBE, in particular it was highlighted that F2t-IsoPs including 8-*iso*-$PGF_{2\alpha}$ was one of the first ones to became widely available to purchase, hence much of the early studies are based upon this (Daughton, 2012). Indeed it has been widely agreed that the study of isoPs as a marker of oxidative stress in clinical studies should be studied as a group and metabolites should also be included (Nourooz-Zadeh *et al.*,

2006; Taylor, Bruno and Traber, 2008; Nikolaidis, Kyparos and Vrabas, 2011). This would not only reduce complications of variability of excretion amounts thereby improving reliability, but by capturing and studying the F2-isoprostanes could help in creating a standardised analytical method for use both in clinical fields and WBE. Further work is currently undertaken by the authors to identify and quantify all relevant F2-isoprostanes.

4.8.3 Stability of biomarkers in wastewater

Whilst the behaviours of target biomarkers in clinical matrices, for example in urine, are well reported (Wu *et al.*, 2016), the stability and presence of such compounds have not been previously reported in wastewater (with the exception of 8-*iso*-$PGF_{2\alpha}$). Results from the 24-hour wastewater biomarker stability study displayed positive results for the majority of the biomarkers studied (Figure 4). HNE-MA, 8-OHdG and 8-*iso*-$PGF_{2\beta}$ all demonstrated high stabilities with little degradation at both 4 °C and 17 °C in wastewater over the 24 hour period (>90 % of the concentration spiked at t 0 h remaining at time period 24 hours). Interestingly for 8-NO_2Gua, significant degradation was observed over the 24 hour period with both reactors at 4 °C and 17 °C with 68 % and 10 % of the concentration spiked at t 0 h remaining at 24 hours respectively. This indicates that 8-NO_2Gua, however useful as a biomarker, might show low stability in wastewater. Conversely initial screening of the biomarkers in wastewater has shown 8-NO_2Gua to be successfully detected and quantifiable at a concentration of 0.0832 ± 0.519 µg L^{-1}, n=3 (Figure 3).

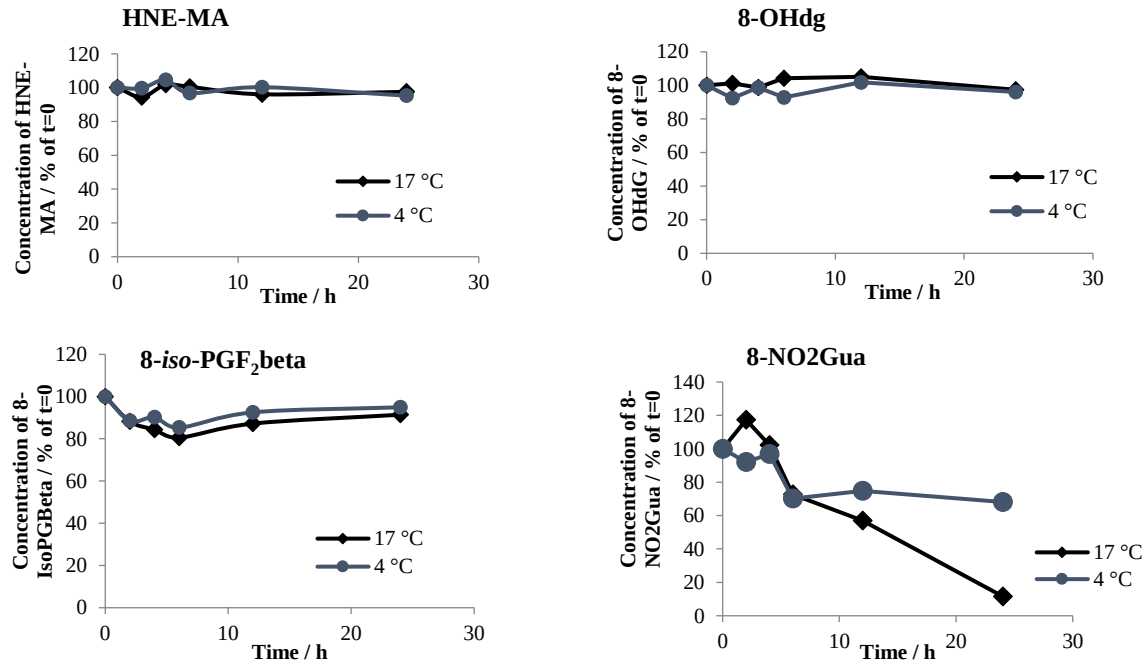

Fig. 4. Stability of each target biomarker in influent wastewater incubated at 17 °C and 4 °C (n=4) over 24 hours. Initial analyte spiking of 1.25 µg L^{-1} within each 2 L reactor (final concentration in 500 µL at t=0, 250 µg L^{-1}).

4.8.4 Wastewater analysis

When spiked into wastewater, all four biomarkers were detected and quantified at their characteristic retention times of 7.45, 3.5, 8.22 and 4.11 min for HNE-MA, 8-NO_2Gua, 8-*iso*-$PGF_{2\beta}$ and 8-OHdG respectively (Figure 3). To further test the validated method, a sampling campaign compromising of 24-hour composite influent wastewater samples were studied over seven days. As markers of oxidative stress and lipid peroxidation, it was assumed that daily loads of target analytes would not experience significant weekly variations and should give relatively stable concentrations across the sampling period. Results demonstrated that HNE-MA gave excellent resolved peaks on all days of the campaign and could be quantified every day. Using influent flowrates and the population of the WWTP, daily loads per capita of HNE-MA were calculated (Figure 5). Observed levels of HNE-MA averaged at 48.9 ± 4.07 mg/1000/people/day across seven days sampled. 8-*iso*-$PGF_{2\beta}$ was detected with good intensity but was found amongst the broad series of peaks as previously mentioned. 8-OHdG was detected, albeit at <LOQ. 8-NO_2Gua on the other hand was not detected on any of the seven days investigated, as previously mentioned this might be attributed to its low stability within wastewater samples. A potential factor of why this biomarker was observed in previous screening of wastewater samples could be dilution (e.g. wetter weather causing variable flows). This shall be addressed in future work to verify the results, in particular due to the stability of biomarker investigation into whether grab sample over composite samples would be more appropriate.

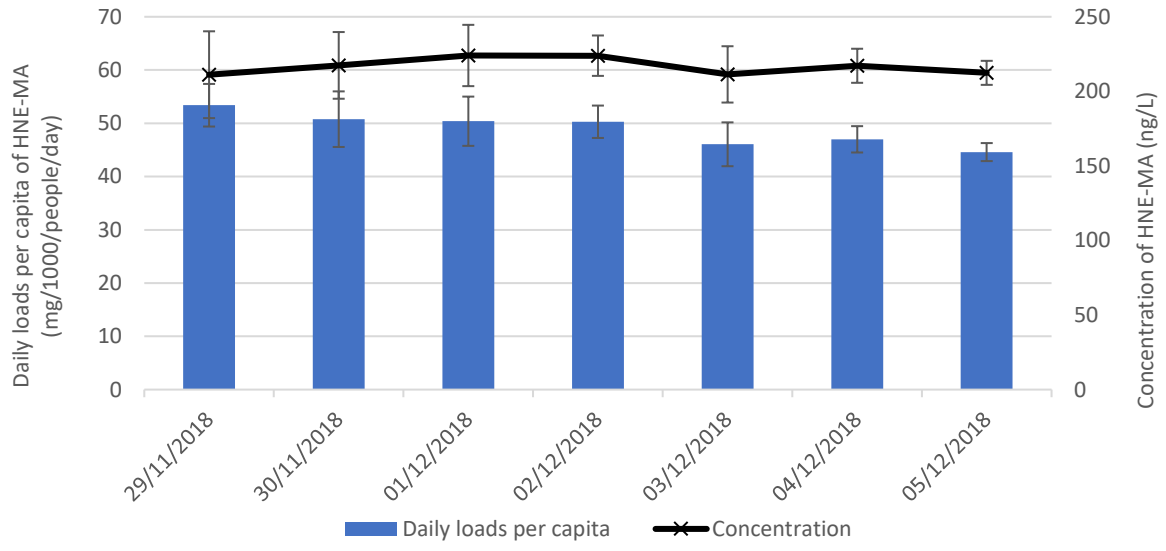

Fig. 5. Daily loads per capita and concentration of HNE-MA in influent wastewater over period of 29/11/2017-05/11/2017. 24 h composite samples used with errors calculated by standard deviation (n=4).

4.9 Conclusion

To conclude, we have reported, for the first time, the development of an analytical method using SPE and UHPLC-MS/MS techniques for the detection and quantification of four biomarkers of oxidative stress in wastewater. The method showed very good performance: accuracy (>87 %), precision (>90 %), method quantification limits (1.3 -3.0 ng L^{-1}) and biomarker stability in wastewater for HNE-MA, 8-OHdG and 8-*iso*-$PGF_{2\beta}$. In contrast, 8-NO_2Gua was found to be less stable in wastewater (68 % and 10 % of the concentration spiked at t 0 h remaining at 24 hours respectively at 4 °C and 17 °C), which affected its method performance: accuracy (>63 %), precision (>91 %), method quantification limits (85.3 ng L^{-1}). All four biomarkers were detected within wastewater samples but full quantification of only HNE-MA was carried out. HNE-MA was quantified in wastewater at levels averaging at 48.9 ± 4.1 mg/1000/people/day. 8-*iso*-$PGF_{2\beta}$ was detected within the broad series of peaks as previously mentioned, further work is required in order to investigate separation. 8-OHdG was detected, albeit at <MQL due to relatively low MQL for this biomarker. To the authors' knowledge, HNE-MA has never been observed and quantified successfully in wastewater before. Further work is required to fully evaluate suitability of 8-NO_2Gua as a biomarker due to its low stability. More extensive sample preparation utilising sorbents of higher selectivity and higher concentration factor should be also considered to allow for full quantification of 8-OHdG and 8-*iso*-$PGF_{2\beta}$

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Compliance with ethical standards

The study was performed in accordance with the ethical standards.

Conflict of interest

The authors declare that they have no conflict of interest.

4.10 References

Baker, D.R., Barron, L., Kasprzyk-Hordern, B., 2014. Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates. Sci. Total Environ. 487, 629–641. https://doi.org/10.1016/j.scitotenv.2013.11.107

Bauer, M., Gräbsch, C., Schlink, U., Klopp, N., Illig, T., Krämer, U., von Berg, A., Schaaf, B., Borte, M., Heinrich, J., Herbarth, O., Lehmann, I., Röder, S., 2012. Genetic association between

obstructive bronchitis and enzymes of oxidative stress. Metabolism 61, 1771–1779. https://doi.org/10.1016/j.metabol.2012.05.013

Bauer, S., 2008. Societal and ethical issues in human biomonitoring – a view from science studies. Environ. Heal. 7, S10. https://doi.org/10.1186/1476-069X-7-S1-S10

Berdeaux, O., Scruel, O., Cracowski, J.L., Durand, T., 2006. F-2-isoprostanes: Review of analytical methods. Curr. Pharm. Anal. 2, 69–78. https://doi.org/10.2174/157341206775474016

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative Stress and Antioxidant Defense. World Allergy Organ. J. 5, 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Boogaerts, T., Covaci, A., Kinyua, J., Neels, H., van Nuijs, A.L.N., 2016. Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach. Drug Alcohol Depend. 160, 170–176. https://doi.org/10.1016/j.drugalcdep.2016.01.002

Castiglioni, S., Senta, I., Borsotti, A., Davoli, E., Zuccato, E., 2015. A novel approach for monitoring tobacco use in local communities by wastewater analysis. Tob. Control 24, 38–42. https://doi.org/10.1136/tobaccocontrol-2014-051553

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2006. Identification and Measurement of Illicit Drugs and Their Metabolites in Urban Wastewater by Liquid Chromatography−Tandem Mass Spectrometry. Anal. Chem. 78, 8421–8429. https://doi.org/10.1021/ac061095b

Cracowski, J.L., Durand, T., 2006. Cardiovascular pharmacology and physiology of the isoprostanes. Fundam. Clin. Pharmacol. 20, 417–427. https://doi.org/10.1111/j.1472-8206.2006.00435.x

Daughton, C.G., 2012. Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype. Sci. Total Environ. 424, 16–38. https://doi.org/10.1016/j.scitotenv.2012.02.038

Daughton, C.G., 2001. Emerging pollutants, and communicating the science of environmental chemistry and mass spectrometry: Pharmaceuticals in the environment. J. Am. Soc. Mass

Spectrom. 12, 1067–1076. https://doi.org/10.1016/S1044-0305(01)00287-2

Davies, S.S., Zackert, W., Luo, Y., Cunningham, C.C., Frisard, M., Roberts, L.J., 2006. Quantification of dinor, dihydro metabolites of F2-isoprostanes in urine by liquid chromatography/tandem mass spectrometry. Anal. Biochem. 348, 185–91. https://doi.org/10.1016/j.ab.2005.10.012

Dhalla, N., 2000. Status of myocardial antioxidants in ischemia–reperfusion injury. Cardiovasc. Res. 47, 446–456. https://doi.org/10.1016/S0008-6363(00)00078-X

Epplein, M., Franke, A.A., Cooney, R. V, Morris, J.S., Wilkens, L.R., Goodman, M.T., Murphy, S.P., Henderson, B.E., Kolonel, L.N., Le Marchand, L., 2009. Association of plasma micronutrient levels and urinary isoprostane with risk of lung cancer: the multiethnic cohort study. Cancer Epidemiol. Biomarkers Prev. 18, 1962–70. https://doi.org/10.1158/1055-9965.EPI-09-0003

Gracia-Lor, E., Castiglioni, S., Bade, R., Been, F., Castrignanò, E., Covaci, A., González-Mariño, I., Hapeshi, E., Kasprzyk-Hordern, B., Kinyua, J., Lai, F.Y., Letzel, T., Lopardo, L., Meyer, M.R., O'Brien, J., Ramin, P., Rousis, N.I., Rydevik, A., Ryu, Y., Santos, M.M., Senta, I., Thomaidis, N.S., Veloutsou, S., Yang, Z., Zuccato, E., Bijlsma, L., 2017. Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives. Environ. Int. 99, 131–150. https://doi.org/10.1016/j.envint.2016.12.016

Halliwell, B., Lee, C.Y.J., 2010. Using isoprostanes as biomarkers of oxidative stress: some rarely considered issues. Antioxid. Redox Signal. 13, 145–56. https://doi.org/10.1089/ars.2009.2934

Kasprzyk-Hordern, B., Bijlsma, L., Castiglioni, S., Covaci, A., de Voogt, P., Emke, E., Hernández, F., Ort, C., Reid, M., Nuijs, A.L.N. Van, Thomas, K. V., 2014. Wastewater-based epidemiology for public health monitoring. Water Sewerage J. 25–26. https://doi.org/10.1136/tobaccocontrol-2014-051553.7.

Klawitter, Jelena, Haschke, M., Shokati, T., Klawitter, Jost, Christians, U., 2011. Quantification of 15-F2t-isoprostane in human plasma and urine: results from enzyme-linked immunoassay and liquid chromatography/tandem mass spectrometry cannot be compared. Rapid Commun. Mass

Spectrom. 25, 463–8. https://doi.org/10.1002/rcm.4871

Lodovici, M., Bigagli, E., 2011. Oxidative stress and air pollution exposure. J. Toxicol. https://doi.org/10.1155/2011/487074

Mastroianni, N., Lopez de Alda, M., Barcelo, D., 2014. Analysis of ethyl sulfate in raw wastewater for estimation of alcohol consumption and its correlation with drugs of abuse in the city of Barcelona. J. Chromatogr. A 1360, 93–99. https://doi.org/10.1016/j.chroma.2014.07.051

Meagher, E.A., Barry, O.P., Burke, A., Lucey, M.R., Lawson, J.A., Rokach, J., FitzGerald, G.A., 1999. Alcohol-induced generation of lipid peroxidation products in humans. J. Clin. Invest. 104, 805–813. https://doi.org/10.1172/JCI5584

Montuschi, P., Barnes, P.J., Roberts, L.J., 2004. Isoprostanes: markers and mediators of oxidative stress. FASEB J. 18, 1791–1800. https://doi.org/10.1096/fj.04-2330rev

Needham, L.L., Calafat, A.M., Barr, D.B., 2007. Uses and issues of biomonitoring. Int. J. Hyg. Environ. Health 210, 229–238. https://doi.org/10.1016/j.ijheh.2006.11.002

Nikolaidis, M.G., Kyparos, A., Vrabas, I.S., 2011. F2-isoprostane formation, measurement and interpretation: The role of exercise. Prog. Lipid Res. https://doi.org/10.1016/j.plipres.2010.10.002

Nourooz-Zadeh, J., Sohr, C.G., Durand, T., Ziegler, D., 2006. Impact of diabetic polyneuropathy and cardiovascular autonomic neuropathy on the excretion of urinary 8-epi-PGF2αand its metabolites (2, 3-dinor and 2, 3-dinor-5, 6-dihydro). Free Radic. Res. 40, 723–729. https://doi.org/10.1080/10715760600615086

Ozkul, A., Akyol, A., Yenisey, C., Arpaci, E., Kiylioglu, N., Tataroglu, C., 2007. Oxidative stress in acute ischemic stroke. J. Clin. Neurosci. 14, 1062–1066. https://doi.org/10.1016/j.jocn.2006.11.008

Patrono, C., Falco, A., Davı, G., 2005. Isoprostane formation and inhibition in atherothrombosis. Curr. Opin. Pharmacol. 5, 198–203. https://doi.org/10.1016/j.coph.2004.11.003

Petrie, B., Youdan, J., Barden, R., Kasprzyk-Hordern, B., 2015. Multi-residue analysis of 90

emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1431, 64–78. https://doi.org/10.1016/j.chroma.2015.12.036

Reid, M.J., Langford, K.H., Mørland, J., Thomas, K. V., 2011. Analysis and Interpretation of Specific Ethanol Metabolites, Ethyl Sulfate, and Ethyl Glucuronide in Sewage Effluent for the Quantitative Measurement of Regional Alcohol Consumption. Alcohol. Clin. Exp. Res. 35, no-no. https://doi.org/10.1111/j.1530-0277.2011.01505.x

Risom, L., Møller, P., Loft, S., 2005. Oxidative stress-induced DNA damage by particulate air pollution. Mutat Res 592, 119–137. https://doi.org/10.1016/j.mrfmmm.2005.06.012

Rodríguez-Álvarez, T., Rodil, R., Rico, M., Cela, R., Quintana, J.B., 2014. Assessment of local tobacco consumption by liquid chromatography-tandem mass spectrometry sewage analysis of nicotine and its metabolites, cotinine and trans-3′-hydroxycotinine, after enzymatic deconjugation. Anal. Chem. 86, 10274–10281. https://doi.org/10.1021/ac503330c

Ryu, Y., Gracia-Lor, E., Baz-Lomba, J.A., Bramness, J.G., Castiglioni, S., Castrignanò, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.-K., Ort, C., Plósz, B.G., Ramin, P., Rousis, N.I., Reid, M.J., Thomas, K. V., 2016. Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater associated with tobacco use. Sci. Rep. 6, 39055. https://doi.org/10.1038/srep39055

Ryu, Y., Reid, M.J., Thomas, K. V., 2015. Liquid chromatography-high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater. J. Chromatogr. A 1409, 146–151. https://doi.org/10.1016/j.chroma.2015.07.060

Schwedhelm, E., Benndorf, R., Boger, R., Tsikas, D., 2007. Mass Spectrometric Analysis of F2-Isoprostanes: Markers and Mediators in Human Disease. Curr. Pharm. Anal. 3, 39–51. https://doi.org/10.2174/157341207779802340

Taylor, A.W., Bruno, R.S., Traber, M.G., 2008. Women and Smokers Have Elevated Urinary F2-

Isoprostane Metabolites: A Novel Extraction and LC–MS Methodology. Lipids 43, 925–936. https://doi.org/10.1007/s11745-008-3222-1

Thacker, S.B., Stroup, D.F., Carande-Kulis, V., Marks, J.S., Roy, K., Gerberding, J.L., 2006. Measuring the public's health. Public Health Rep. 121, 14–22. https://doi.org/10.1177/003335490612100107

Thomas, K. V., Bijlsma, L., Castiglioni, S., Covaci, A., Emke, E., Grabic, R., Hernández, F., Karolak, S., Kasprzyk-Hordern, B., Lindberg, R.H., Lopez de Alda, M., Meierjohann, A., Ort, C., Pico, Y., Quintana, J.B., Reid, M., Rieckermann, J., Terzic, S., van Nuijs, A.L.N., de Voogt, P., 2012. Comparing illicit drug use in 19 European cities through sewage analysis. Sci. Total Environ. 432, 432–439. https://doi.org/10.1016/j.scitotenv.2012.06.069

Tscharke, B.J., White, J.M., Gerber, J.P., 2016. Estimates of tobacco use by wastewater analysis of anabasine and anatabine. Drug Test. Anal. 8, 702–707. https://doi.org/10.1002/dta.1842

Tsikas, D., Schwedhelm, E., Suchy, M.T., Niemann, J., Gutzki, F.M., Erpenbeck, V.J., Hohlfeld, J.M., Surdacki, A., Frölich, J.C., 2003. Divergence in urinary 8-iso-PGF2α(iPF2α-III, 15-F2t-IsoP) levels from gas chromatography-tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-iso-PGF2α. J. Chromatogr. B 794, 237–255. https://doi.org/10.1016/S1570-0232(03)00457-4

Wu, C., Chen, S., Peng, K., Cheng, T., Wu, K., 2016. Concurrent quantification of multiple biomarkers indicative of oxidative stress status using liquid chromatography-tandem mass spectrometry. Anal. Biochem. 512, 26–35. https://doi.org/10.1016/j.ab.2016.07.030

Wu, L.L., Chiou, C.C., Chang, P.Y., Wu, J.T., 2004. Urinary 8-OHdG: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin. Chim. Acta. https://doi.org/10.1016/j.cccn.2003.09.010

Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S., Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. Environ. Heal. 4, 14. https://doi.org/10.1186/1476-069X-4-14

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Community and hospital derived antimicrobials and resistance genes in a two-city one-year longitudinal study

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Abstract

This longitudinal study provides insight into antimicrobial agents (AA) usage within two communities in the Southwest of the UK, one city (Bath) and one town (Keynsham) over 13 months randomised monitoring programme of community and hospital wastewater. AAs, including metabolites, from a range of different classes were quantified over the study period. Average loads of AAs were higher in Bath than for Keynsham which reflected the larger population. Several AAs experienced seasonal fluctuations, such as the macrolides erythromycin and clarithromycin that were found in higher loads in the winter, whilst other AA levels, including sulfamethoxazole and sulfapyridine, stayed consistent over the study period. A small number of antimicrobial resistant genes (ARGs) were also studied within the city area, in order to determine how closely the abundance of these genes correlates with the levels of relevant AAs. Interestingly, and as opposed to antibiotics, ARGs were found to be less variable, which indicates that fluctuations in antibiotic usage might either not directly affect ARG levels or this process spans beyond the 13-month monitoring period. However, it is important to note that weekly positive correlations between individual associated AAs and ARGs were observed where seasonal variability in AA use was reported: *ermB* and macrolides clarithromycin and N-desmethyl clarithromycin, N-acetyl sulfapyridine and *sul1* and ofloxacin and *qnrS*. Gene loads normalised to *16S rRNA* (gene load per microbe) were positively correlated to the gene loads normalised to the human population (gene load per capita), which indicates, yet again, that the abundance of microorganisms is proportional to the size of human population and that the community size is a major driver of ARG levels in wastewater. Comparison of hospital and community wastewater showed higher number of AAs and their metabolites, their frequency of occurrence and concentrations in hospital wastewater. This is likely due to, with shorter sewage residence time, lower volume of flow and concentration of individuals at the source requiring AAs when compared to community wastewater. Examples include: linezolid (used only in severe bacterial infections) and amoxicillin (widely used, also in community but with very low wastewater stability) that were found only in hospital wastewater. ciprofloxacin, sulfamethoxazole, trimethoprim, and metronidazole, macrolides were found at much higher concentrations in hospital wastewater while tetracycline and oxytetracycline, as well as antiretrovirals, had an opposite trend. In contrast, comparable concentrations of resistant genes were observed in both community and hospital wastewater. This supports the hypothesis that AMR levels are more of an endemic nature, developing over time in individual communities. Both hospital and community wastewater had AAs that exceeded PNEC values (e.g. clarithromycin, ciprofloxacin. In general, though, hospital effluents had a greater number of quantifiable AAs exceeding PNECs (e.g. sulfamethoxazole, erythromycin, trimethoprim). Hospitals are therefore an important consideration in AMR surveillance as could be high risk areas for AMR.

Keywords: Wastewater-based epidemiology, antimicrobials, antibiotics, resistance genes, ARGs, hospital effluent

1. Introduction

The evolution and spread of antimicrobial resistance (AMR) limits therapeutic options for a broad range of infectious diseases, and poses a global threat to public health (World Health Organisation, 2020). Whilst genes conferring resistance have evolved naturally prior to the administration of antimicrobial agents (AAs), the inappropriate use of these drugs dramatically accelerated the spread of resistance genes across different species, settings and geographical locations. It has been estimated in 2019 that there were 4.95 million deaths associated with bacterial AMR infections, including 1.27 million deaths directly attributed to resistant bacteria (Murray et al., 2022). These rising global rates of AMR have stressed the importance of effective surveillance systems for understanding the burden of resistance and identifying new or re-emerging threats. Effective surveillance can also feedback into evidence-based policy making and evaluate the effectiveness of public health interventions. As highlighted by WHO's GLASS report (World Health Organisation, 2018), there are clear disparities in AMR surveillance between different countries, due to limited resources and infrastructure. There is also a recognised lack of population-wide surveillance data regarding AMR, highlighting a need to develop both cost-effective and standardised population-wide surveillance AMR systems.

A promising tool for monitoring community-wide AMR surveillance is wastewater-based epidemiology (WBE). Wastewater treatment plants (WWTPs) serve a well-defined community, capturing all the excretion products of this population. These excretion products contain biomarkers of pathogens, pharmaceuticals and other indicators of population health. Estimates of exposure to pathogens, or consumption of pharmaceuticals, can be made by quantifying these biomarkers in influent (untreated) wastewater, whilst accounting for flow rates and population size. The resulting data complement evidence generated through traditional public health approaches.

WBE as a public health tool has clear advantages; it not only provides anonymous population-wide data but is also relatively inexpensive and gives rapid results. In the case of AMR, analysis of AA residues in wastewater can inform on consumption within the community. This can be valuable where prescription data is not easily obtainable, or where AAs are available over-the-counter or online. Complimentary to the analysis of AAs, the analysis of resistance genes or co-factors (constituents in wastewater that can co-select for resistance) can also provide key information on the presence of resistant bacterial communities in wastewater. The ability of WBE to give rapid results provides the potential for rapid responses. In the case of AMR, WBE also holds to the promise of evaluating the effectiveness of antimicrobial policy. However, WBE is not without its challenges. Estimation of population sizes and biomarker suitability pose problems, and remain an active area of research (Been et al., 2014; Chen et al., 2014; Choi et al., 2020; Daughton, 2018).

An overview of WBE studies focusing on the presence of AAs is given in Table 1. WBE has been used to investigate AA usage in flu season (Zhang et al., 2019) and even to demonstrate changing consumption patterns in the wake of the COVID-19 pandemic (Galani et al., 2021). Furthermore, combining analysis of AAs with ARGs can reveal how AA usage impacts on the presence of ARGs. For example, areas with higher quinolone consumption, driven by population size, were found to have a higher prevalence of *qnrS* gene, which encodes resistance to this antibiotic (Castrignanò et al., 2020). Similar results were observed for other AAs and their respective resistance genes (macrolides and *ermB*, sulfamethoxazole and *sul1*, chloramphenicol and *catA*) (Elder et al., 2021).

The development of advanced DNA sequencing techniques offers a promising approach for monitoring the abundance of ARGs in the environment (Guo et al., 2017; Hendriksen et al., 2019; Lanza et al., 2018; Petrovich et al., 2020; Riquelme et al., 2021). High throughput quantitative polymerase chain reaction (qPCR) has also been utilised to reflect expected clinical resistance trends across Europe

(Pärnänen et al., 2019). Complexities of AMR require all proxies (e.g. antimicrobials, genes, co-factors) to be studied simultaneously to give a full picture of AMR prevalence at a community level.

Table 1. Overview of studies focused primarily on AAs in WBE

AAs investigated	**Metabolites investigated**	**Genes investigated**	**Number of sites and location**	**Sampling duration**	**Reference**
21 AAs	-	-	8 WWTPs, Beijing, China	1 day	(Zhang et al., 2019)
6 AAs	-	-	4 megacities, China		(Yuan et al., 2016)
11 AAs (mainly quinolones)	Desethylene-ciprofloxacin,	*qnrS*	7 European cities	7 days	(Castrignanò et al., 2020)
16 AAs (4 classes)	-	*qnrS, ermB, sul1* and *catA*	5 cities, South-West, UK	7 days	(Elder et al., 2021)
25 AAs	-	-	Athens, Greece	7 days, pre-pandemic 15 days in 2020	(Galani et al., 2021)
45 AAs	Anhydro erythromycin Acetylsulfamethoxazole	Metagenomic untargeted shotgun sequencing	12 WWTPs, 6 countries	14 influent samples each site	(Riquelme et al., 2021; Singh et al., 2019)

Due to the multifaceted nature of AMR, the One Health approach has proved a valuable framework for tackling this complex issue. One Health aims at holistic understanding and management of public and environmental health, and has been successfully adopted in AMR research with considerable global human and animal health, food security, and safety impacts. One Health studies aim to incorporate a dynamic set of biological, chemical, and socioeconomic indicators that are difficult to unravel. Whilst AA and ARG analysis have been undertaken internationally (Hendriksen et al., 2019; Pärnänen et al., 2019; Petrovich et al., 2020) many studies focus on shorter sampling periods with inconclusive results regarding AA-ARG correlations. Longer term wastewater monitoring of AAs will capture both fluctuating and consistent consumption patterns between seasons, and can therefore complement e prescription data. Evidence can also be generated on the relationship between the abundance of AAs and associated ARGs in these communities.

The aim of this paper is to:

1. Provide better understanding of AA-ARG associations in the context of a longitudinal 1-year monitoring of two contrasting communities; Bath: 120,113 inhabitants (inh) and Keynsham: 21,247 inh, for a suite of AAs and their metabolites (AA/met), and corresponding ARGs.
2. Undertake data triangulation to understand relationships between AA/met levels and corresponding ARGs; as well as water quality indicators (WQIs-see SI) in the context of seasonal AA use.
3. Provie better understanding of AA and ARG contributions in hospital vs community wastewater and their associations in the two types of wastewater.
4. To understand the role of wastewater in the dissemination of AMR, and to explore measured vs predicted no effect concentrations (PNECs) of AAs in both community and hospital wastewater.

2. Materials and Methods

2.1 Materials and target compounds

AAs were selected for this study as they cover a broad and diverse range of classes with both parent compounds and metabolites. Table 1 shows the AAs and metabolites investigated during this project along with AA abbreviations used in this paper. More detailed information regarding AAs and metabolites, along with internal standards used, may be found in the supplementary information (Table S1 and S2). This method has previously been developed by Holton and Kasprzyk-Hordern (2021), and full method development details may be found there. Analytical standards and deuterated (stable isotope–labelled) standards were obtained from Sigma-Aldrich (Gillingham, UK), TRC (Toronto, Canada), LGC (Middlesex, UK), or MCE (Cambridge, UK). The methanol used was HPLC-grade (Sigma-Aldrich), the water was of 18.2 MΩ quality (Elga, Marlow, UK); and the purity of formic acid, used as the mobile-phase additive, was >95% (Sigma-Aldrich). Glassware was deactivated using 5% dimethylchlorosilane in toluene (Sigma-Aldrich) to avoid losses via adsorption. Oasis HLB (60 mg, 3 mL) SPE cartridges, polypropylene LC vials, and Whatman GF/F 0.7-µm filters were purchased from Waters (Manchester, UK).

Table 1: AA targets investigated in this study, ordered by class groupings, table adapted from Holton and Kasprzyk-Hordern, 2021, https://rdcu.be/cxqhT

Grouping	Chemical	Abbrev
Sulphonamide &	Sulfadiazine	SDZ
Trimethoprim	Sulfapyridine	SPY
	Sulfamethoxazole	SMX
	Sulfasalazine	SLZ
	Trimethoprim	TMP
	N-acetyl sulfadiazine	aSDZ
	N-acetyl sulfapyridine	aSPY
	N-acetyl sulfamethoxazole	aSMX
	4-hydroxy-trimethoprim	hTMP
Macrolide	Azithromycin	AZM
& Lincosamide	Erythromycin	ERY
	Clarithromycin	CLR
	Clindamycin	CLI
	N-desmethyl azithromycin	dmAZM
	N-desmethyl erythromycin A	dmERY
	N-desmethyl clarithromycin	dmCLR
	N-desmethyl clindamycin	dmCLI
β-lactams		
Penicillin	Amoxicillin	AMX
	Ampicillin	AMP
	Flucloxacillin	FLX
	Penicillin G	PenG
	Penicillin V	PenV
	Amoxicilloic acid	AMXa
	Ampicilloic acid	AMPa
	Penicilloic G acid	PenGa
Cephalosporin	Cefalexin	LEX
	Cefixime	CFM
	Ceftiofur	CTF
	Ceftriaxone	CRO
Monobactam	Aztreonam	ATM
Carbapenem	Imipenem	IPM
	Meropenem	MEM
Quinolone	Besifloxacin	BSF
	Ciprofloxacin	CIP
	Danofloxacin	DFX
	Enrofloxacin	ENR
	Flumequine	FLU
	Gatifloxacin	GAT
	Lomefloxacin	LOM
	Moxifloxacin	MXF
	Nadifloxacin	NAD
	Nalidixic acid	NAL
	Norfloxacin	NOR
	Ofloxacin (Levofloxacin) *	OFX

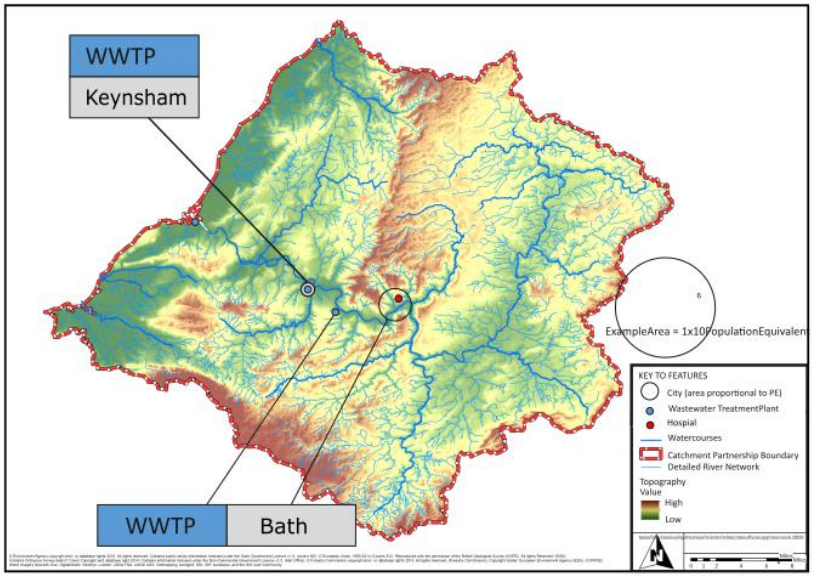
	Prulifloxacin	PFLX
	Sarafloxacin	SRF
	Desethylene ciprofloxacin	deCIP
	Hydroxy-norfloxacin	hNOR
	Ofloxacin N-oxide	OFXo
	Desmethyl-ofloxacin	dmOFX
	Ulifloxacin	UFX
TB (1st line)	Isoniazid	INH
	Pyrazinamide	PZA
	Ethambutol	EMB
	Rifampicin	RMP
	Rifabutin	RFB
	Isonicotinic acid	INa
	Acetyl-isoniazid	aINH
	5-Hydroxy-pyrazinoic acid	hPZA
	25-desacetyl rifampicin	daRMP
	25-O-desacetyl rifabutin	daRFB
TB (MDR)	Capreomycin IA	CAPIa
	Capreomycin IB	CAPIb
	Gentamycin C1	GEN1
	Gentamycin C1a	GEN1a
	Gentamycin C2 C2a C2b	GEN2
	Kanamycin A	KAN
	Streptomycin A	STR
	D-cycloserine	DCS
TB (other)	Delamanid	DMD
	Bedaquiline	BDQ
	Linezolid	LZD
	Thalidomide	THAL
OTHER		
Amphenicol	Chloramphenicol	CHL
	Florfenicol	FLO
	2-Amino-1-(4-nitrophenyl)-1,3-propanediol	ANP
Cycline	Doxycycline	DOX
	Oxytetracycline	OTC
	Tetracycline	TET
Nitrofuran	Nitrofurantoin	NIT
	1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione	NPAHD
Azole	Metronidazole	MTZ
	Ketoconazole	KTC
	Hydroxy-metronidazole	hMTZ
	Deacetyl-ketoconazole	daKTC
Antiretroviral	Emtricitabine	FTC
	Lamivudine	3TC

Multi-drug resistant (MDR), tuberculosis (TB), LC-MS method is not chiral (*)

2.2 Sampling

2.2.1 Wastewater Treatment Plants

This study investigated two wastewater treatment (WWTP) sites in the South West of the UK over a sampling period between 2018-2019 (figure 1). The WWTPs serve the city of Bath, with a population equivalent of 120,113 and the town of Keynsham with a population equivalent of 21,247. Both sites are surrounded by agricultural areas and have limited contribution from industrial input (<1%). WWTP Bath does have input from a major hospital in the catchment area. 24-hour composite samplers, set up for flow proportional sampling every 15 minutes, were used to sample screened, but untreated, influent wastewater. Samples were transported on ice to the laboratory for processing.



Site	Sewer residence time[a] (h)	Population served (population equivalents)	Industrial contributions to population equivalents	Mean flow ($m^3 d^{-1}$)
Bath	<0.5-9	120,113	1.2 %	33178
Keynsham	<0.5-2	21,247	0.1 %	3316

[a]under typical summer flows

Figure 1. Catchment map and site information of studied WWTPs

2.2.2 Hospital effluent sampling

Effluent wastewater samples were collected from a hospital within the catchment area of Bath. Samples were collected over five consecutive days (5^{th}-9^{th} August 2019) from a hospital outlet that accounts for approximately 60% of the total wastewater originating from this hospital. This is a medium-sized hospital (>700 beds and a catchment of 500,000 people). Between the hospital and the WWTP serving Bath is 10 km of sewer pipeline. Hospital effluent samples were collected by 24 h composite samplers set to time-proportional, with 50 mL collected every 15 minutes. Collected samples were transported on ice to the laboratory to be processed (< 1 h).

2.2.3 Water quality indicators and flow measured, in WWTPs

A range of water quality/sanitary indicators (WQIs: BOD, COD, Suspended solids, Chloride, Ortophos, T Phosphorous, suspended organic carbon, Ammonia as N, metals: Al, Fe, Mn) were analysed at Wessex Water at certain sampling points over the studied period in both Bath and Keynsham. Full experimental details may be found in the supplementary. Influent wastewater flows and rainfall over the studied period in both catchment areas were also reported (figure S1). Discussion on WQI, AA and ARG associations is available in SI.

2.2.4 Population equivalent estimation

The population equivalent of those served by the WWTP (PE-WW) was estimated by Wessex Water (Table 2). By multiplying the number of properties in the catchment area by occupancy rate (set at district level), the resident population estimate was determined. The resident population also considers the number of multi-occupancy residencies that fall within the catchment area, including care homes, residential schools, university halls, and military bases. As a UNESCO world heritage site, Bath has a thriving tourism input. Tourists were considered as non-resident population, and due to challenges estimating input of day-trippers to the catchment area, they were not included.

By utilising WQIs, other inputs to the wastewater stream could be calculated. This included commercial waste; determined by considering supply flow to commercial properties and estimates of 60 g BOD per capita per day. Tankard waste imports were determined via the amount of COD present in the known volume of waste and, assuming 120 g COD per capita per day.

Table 2. Populations equivalents used in the study (2018/9)

Wessex Water Population Data	**Calculation of PE-WW**			
City/Town served	**Bath**		**Keynsham**	
Year	**2018**	**2019**	**2018**	**2019**
Domestic-Billed Properties	43,611	45,274	8,028	9,144
Average Household Size	2.23	2.25	2.23	2.25
Resident Population Estimate	97,253	101,866	17,902	20,572
Adjustment for Care Homes	1,411	1,411	315	315
Adjustment for Universities	5,800	5,800	0	0
Adjustment for Schools/Colleges	800	800	0	0
Non-Resident Population	7,250	7,250	123	123
Commercial PE	2,026	2,006	222	222
Trade Effluent PE	1,139	980	0	13
Tankered Waste PE	0	0	0	0
Total PE served by WWTP	**115,679**	**120,113**	**18,562**	**21,247**

2.3 Chemical analysis – AA and metabolite quantification

2.3.1. Sample preparation

Collected influent wastewater samples were transported on ice to the lab (<1 hr). On arrival, samples were portioned into 50 mL and spiked with 50 ng of each internal standard and shaken. Samples were then filtered through GF/F filters (Whatman, UK). Solid phase extraction (SPE) was used to extract target AAs from wastewater, Oasis HLB cartridges were preconditioned using 2 mL of MeOH followed by 2 mL of MilLi-Q H_2O at a flowrate of 1 mL min^{-1}. Wastewater filtrates were then loaded onto the preconditioned cartridges at a rate of 5 mL min^{-1}. Cartridges were dried for at least 30 minutes under vacuum. For the elution step, 4 mL of MeOH at a rate of 1 mL min^{-1} with the eluate collected in silanised glass vials. Eluates were then dried at 40 °C under N_2 via a TurboVap evaporator. Dried residues were reconstituted with 500 µL of 80:20 H_2O: MeOH and transferred into polypropylene vials and kept at -18 °C until analysis. Further details on the method can be found in Holton and Kasprzyk-Hordern, 2021.

2.3.2 Analyte Quantification

Full analytical method validation and instrument conditions may be found in Holton and Kasprzyk-Hordern (Holton and Kasprzyk-Hordern, 2021). For the analysis of target AAs, ultra-performance liquid chromatography (UPLC) coupled with a XEVO triple quadrupole mass spectrometer (TQD-MS). The analytical method is in total 19 minutes long and the column used was a reverse phase BEH C18 column (50 x 2.1 mm, 1.7 µm). For separation of target AAs, mobile phase A consisted of 95:5 H_2O:MeOH with 0.1 % formic acid with mobile phase B as 100 % MeOH. Flowrate was set at 0.2 mL min^{-1} and injection volume was 20 µL. Mobile phase starting conditions were 0 % B (held for 1 minute), then a gradual gradient to 40 % B (8.5 minutes), gradient up to 100 % B (3.5 minutes), hold of 100 % B (3 minutes) and finally dropping back to 0 % B (0.5 minutes).

Regarding mass spectrometry conditions, briefly the method was achieved in ESI positive mode with the source desolvation temperature at 400 °C. Nitrogen was used as the nebulising and desolvation gas and argon was used as the collision gas. For gas flows, the desolvation gas was at 1000 L h^{-1} and the cone gas was at 100 L h^{-1}.

2.4. Biological analysis – ARG quantification

Four ARGs were selected to monitor in Bath, according to previous work done in the catchment area for both genes and associated AAs and due to their clinical importance (Elder et al., 2021). The selected genes were *ermB* (macrolide resistance), *sul1* (sulphonamide resistance), *qnrS* (fluoroquinolone resistance), and *intI1* (potential marker of anthropogenic pollution). An additional two genes were investigated in hospital effluent, *tetW* (tetracycline resistance) and *blaTEM* (resistance to β-lactams), to explore the relationships between AAs and ARGs. To quantify these genes, digital PCR (dPCR) was utilised. An additional couple of wastewater samples were analysed by DNA sequencing, an untargeted approach to provide comprehensive metagenomic information and to characterise the bacterial communities and genes present in the selected wastewater samples

2.4.1 DNA extraction

To investigate temporal trends of ARGs, four wastewater samples per month from Bath were chosen to investigate selected ARGs using digital PCR (dPCR). Of these samples, two were selected to further investigate using DNA sequencing (one wastewater sample from November 2018 and one sample from March 2019). Influent wastewater samples (100 mL) were filtered through Nalgene™ Sterile Analytical Filter Units (Thermo Scientific, UK) containing 0.2 μm cellulose filter papers. DNA was then extracted from the membrane directly from the filter paper using FastDNA SPIN Kit for Soil (MP Bio, UK). The amount of extracted DNA was determined using a Qubit 4 Fluorometer (Thermo Scientific, UK). DNA was kept at -20 °C before further analysis.

2.4.2 DNA sequencing and metagenomic analysis

Extracted DNA from the samples was sent to MicrobesNG (Birmingham, UK) where Illumina HiSeq sequencing (rapid run, 2×250 bp paired end reads) was conducted with in-house quality control (adapter trimming with Trimmomatic v0.30, with a sliding window quality score cut-off value of Q15). Taxonomical profiling was performed with Kraken2 2.0.8 and Bracken 2.5 (Lu et al., 2017) using the Genome taxonomy database for improved performance (GTDB) (Méric et al., 2019). ARG relevant to the antibiotics chosen to select for antibiotic resistant bacteria in this study were identified using the Comprehensive Antibiotic Resistance Database (CARD) and Resistance Gene Identifier (RGI; 5.1.1). An inhouse R script was used to process the metagenomic data. Low abundance taxa were filtered using a pre-processing step by removing Operational Taxonomic Units (OTUs) that had non-zero values in ≤ 10% of samples.

2.4.3 Digital PCR

Digital PCR (dPCR) analysis was performed using a the QuantStudio® 3D Digital PCR System (Thermo Scientific, UK). The dPCR reaction was made up according to manufacturer instructions of QuantStudio® 3D Digital PCR Master Mix, appropriate TaqMan™ primers with MGB probes, sterile water, and DNA sample. Due to similarities in gene abundance in wastewater samples, *intI1* and *sul1* were duplexed allowing quantification of both genes on one chip with *intI1* having this in FAM™ dye and *sul1* with VIC® dye. The mixture was then portioned onto dPCR chip wells and sealed.

The thermo cycling conditions chosen for PCR involved: a temperature ramp to 95 °C, 10 min hold; a reduction to 60 °C for 2 min; before increasing to 98 °C for 30 sec. This cycle between 60 °C and 98 °C was repeated 40 times to allow for efficient gene amplification. The system was then lowered to 60 °C and held for 2 min, before cooling to room temperature. After cooling, each chip was processed using the QuantStudio 3D Digital PCR system chip reader. To analyse chips, the AnalysisSuite™ software was used for quantification of the target gene. Each DNA sample was run in duplicate for each gene investigated.

2.4.4 Quality control dPCR

Negative controls for dPCR were achieved using sterile water (DNA blanks) to confirm non-amplification. For positive process controls, 10 µL of TaqMan™ Universal DNA Spike in Control (Thermo Fisher) was spiked into the lysis step of the DNA extraction kit. Six extracts of the same wastewater sample were spiked to assess recovery efficiency of the extraction kit, giving an average recovery of 41 ± 12 % (figure S2).

2.5 Statistical calculations

P values were calculated via paired sample T tests, to investigate any seasonal effects of AAs and ARGs. By combining sample data, the seasons considered were winter (November ’18, December ‘18 and January ’19), spring (February ’19, March ’19 and April ’19) and summer (May ’19, June ’19 and July ’19). Statistical significance in all tests was defined as $p \leq 0.05$. Pearson correlation coefficients were used to investigate potential relationships between ARG and AA abundance. Positive correlations were considered >0.5 and negative correlations were considered <-0.05. Statistical significance testing was performed to highlight correlation results that were significant.

3. Results and discussion

3.1 AAs and their metabolites in a longitudinal study of two contrasting urban areas

3.1.1 Absolute AA concentrations

A diverse range of AAs and metabolites were observed and quantified across the 13-month sampling period. In total, 17 parent AAs and 8 metabolites were quantified consistently in wastewater in both sites during the sampling period. AAs and metabolites from the macrolide and sulphonamide classes were well represented, with AAs from the aminoglycoside not detected once during the sampling period. Average wastewater concentrations for the consistently quantified AAs and metabolites were reported at 0.50 ± 0.58 ng/L for Bath and 0.52 ± 0.75 ng/L for Keynsham over all studied months. Whilst the concentration of AAs and respective metabolites in wastewater will be impacted by consumption patterns at the community level, variable wastewater flowrates will also have an impact, highlighting the importance of normalising concentrations with flowrates when monitoring both spatial and temporal trends.

3.1.2 AA loads

3.1.2.1 Spatial variability

To account for variable flows, daily loads of AAs were calculated. This allowed for further comparisons between different months e.g., AA seasonality (figure 2). Full breakdowns of AA loads in both Bath and Keynsham may be found in the SI for individual points and average monthly loads. In general, AA average loads were higher in Bath than for Keynsham which coincides with a larger population. The estimated population equivalent for Bath is ~5.7 x bigger than compared to Keynsham, which indicates that AA loads are population size driven.

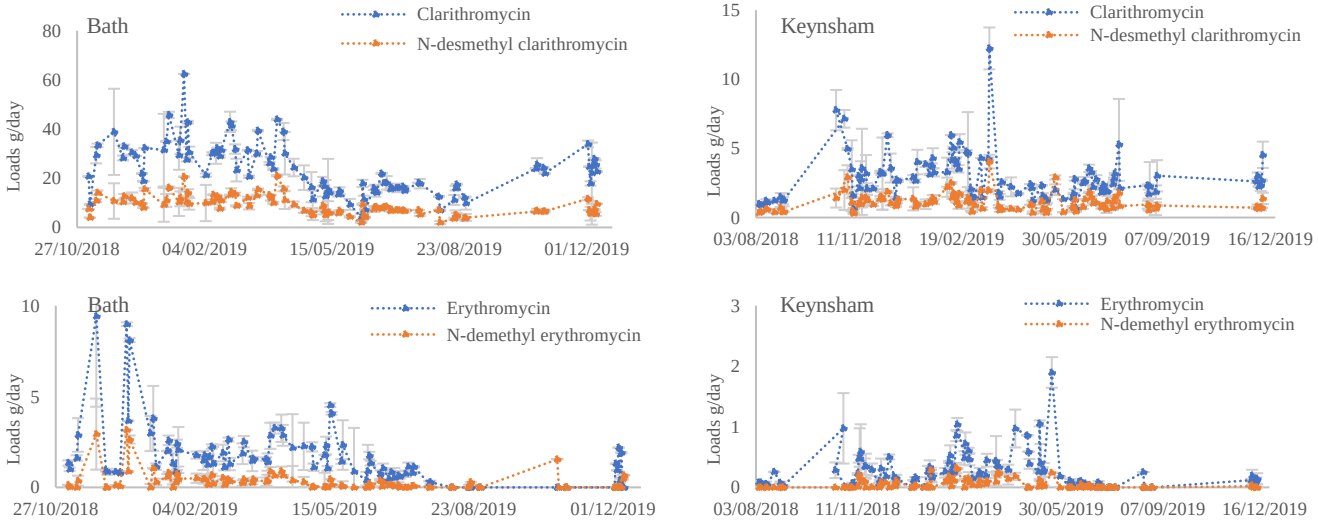
Regarding high abundance in wastewater, SPY was observed in high levels in both Bath and Keynsham respectively (43.1 ± 16.4 g/day and 5.6 ± 3.1 g/day). This corresponded with high levels of aSPY, with 32.0 ± 13.0 and 1.4 ± 1.13 g/day in the two sites respectively. As previously mentioned, SPY is no longer prescribed for human use in the UK, but is continued to be used as a veterinary medication. Both SPY and aSPY however are major metabolites of SLZ, which is prescribed for humans as an anti-inflammatory. In wastewater, SLZ had lower average loads than its respective metabolites, at 7.41 ± 5.10 and 0.94 ± 0.63 g/day in Bath and Keynsham, previous work has demonstrated SLZ has lower recovery and stability comparatively to its two metabolites (Holton et al., 2022). It is likely that SPY

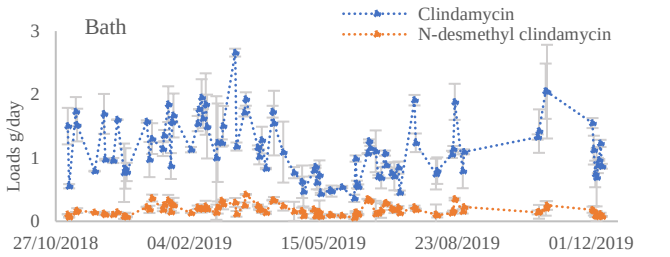
and aSPY residues will be present in influent from a combination of consumption of SLZ on the community level and other sources, including agricultural run-off of SPY into the sewage system.

3.1.2.2 Temporal variability

Total loads of AAs and metabolites in wastewater were generally higher in winter when compared to summer (figure 2). Statistical significance was gauged using T-tests to compare the three seasons for which most data was available; winter 2018/19, spring 2019 and summer 2019 (table S3). SPY had one of the lowest temporal variabilities across the three seasons in both Bath and Keynsham (average loads, winter: 47.1 ± 17.6 and 6.6 ± 2.9 g/day, spring: 39.6 ± 15.8 and 5.0 ± 3.8 g/day, summer: 43.5 ± 16.9 and 6.7 ± 2.9 g/day for Bath and Keynsham respectively, all $p>0.05$). In Bath, SMX also had low temporal variability across the three seasons, with 14.7 ± 5.0 g/day, 14.3 ± 5.7 g/day and 15.5 ± 4.8 g/day in winter, spring, and summer respectively (on average a 5 % change between seasons, $p > 0.05$). In contrast, slightly higher loads of SMX were observed in Keynsham in summer than in winter (0.68 ± 0.54 g/day vs 1.40 ± 0.71 g/day, $p\leq0.05$), which may reflect differences in prescribing practices across the catchment.

AAs that displayed statistically significant higher loads in winter than in summer included MTZ, SLZ, LEX, TET, OTC, CIP, TMP, and the metabolites hMTZ and AMXa (table S3). The AAs with some of the highest variabilities across the three seasons in Bath and Keynsham were the macrolides CLR and ERY. Statistically significant differences were observed when loads peaked in winter 2018/19 at an average load of 32.8 ± 8.7 and 2.4 ± 2.0 g/day for Bath and Keynsham respectively, compared to summer loads of 14.5 ± 4.2 and 0.8 ± 0.4 g/day ($p\leq0.05$). Similar observations could be observed for CLR and ERY's major metabolites dmCLR and dmERY. As mentioned previously, CLR and ERY are known to have higher prescribing in winter months, when respiratory illness peaks in the UK. Respiratory infections tend to follow predictable seasonal patterns in temperate climates (Price et al., 2019). In colder months, individuals spend more time in enclosed spaces indoors which can lead to increases in the spread of infectious diseases. As a result, the total prescribed AA mass is often higher in winter- particularly for macrolide, penicillins and cephalosporin classes (Curtis et al., 2019). Furthermore, lower loads of ERY in wastewater, compared to CLR, may be due to the preferences of prescribing CLR over ERY due to better tissue penetration, fewer side effects and greater patient compliance (Amsden, 1996).




Bath
Clindamycin
N-desmethyl clindamycin
Loads g/day
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2
1
0
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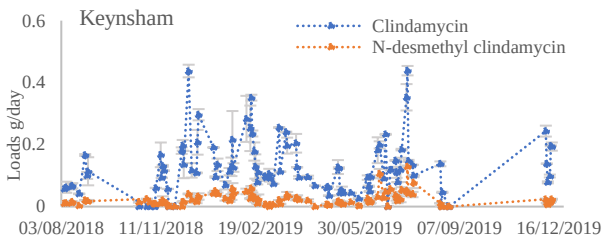
04/02/2019
15/05/2019
23/08/2019
01/12/2019


Keynsham
Clindamycin
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Loads g/day
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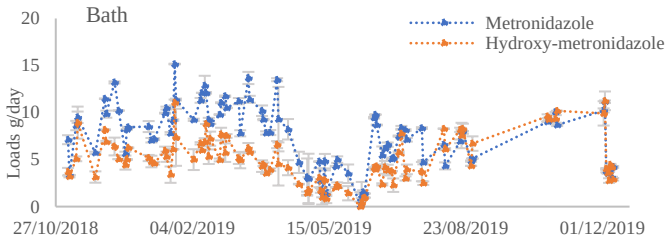
11/11/2018
19/02/2019
30/05/2019
07/09/2019
16/12/2019


Bath
Metronidazole
Hydroxy-metronidazole
Loads g/day
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10
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04/02/2019
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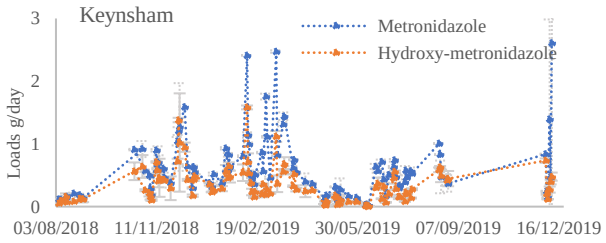
01/12/2019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Loads g/day
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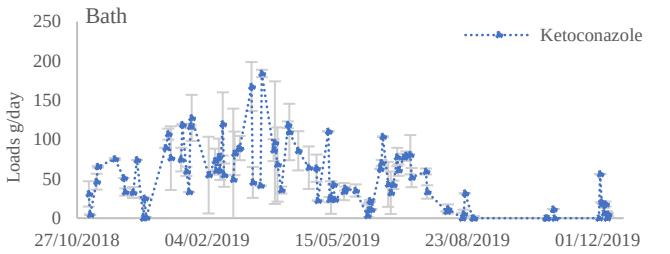
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15/05/2019
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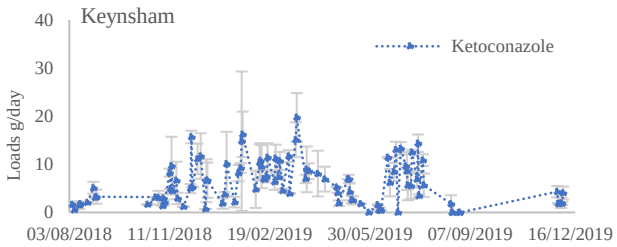
01/12/2019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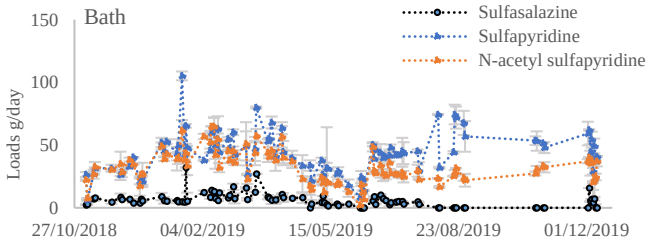
30/05/2019
07/09/2019
16/12/2019


Bath
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Sulfapyridine
N-acetyl sulfapyridine
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100
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27/10/2018
04/02/2019
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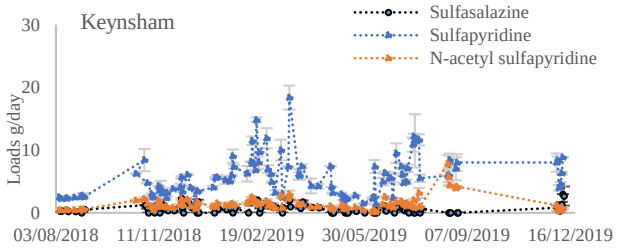
23/08/2019
01/12/2019


Keynsham
Sulfasalazine
Sulfapyridine
N-acetyl sulfapyridine
Loads g/day
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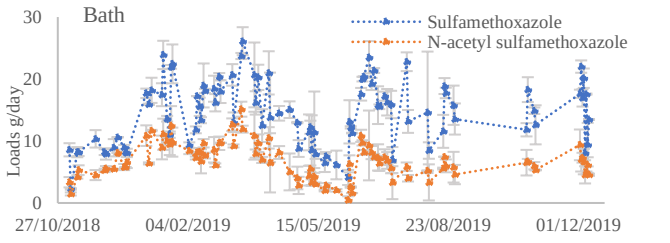
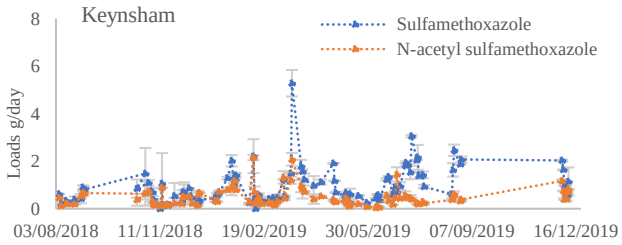
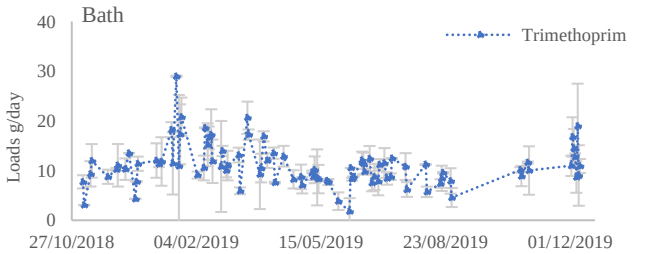
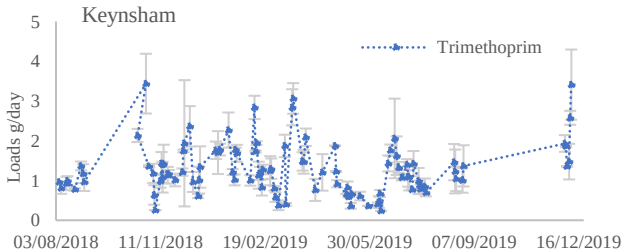
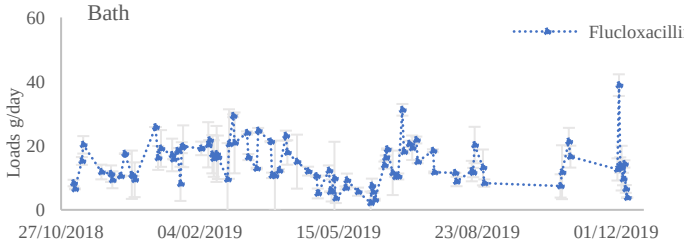
03/08/2018
11/11/2018
19/02/2019
30/05/2019
07/09/2019
16/12/2019

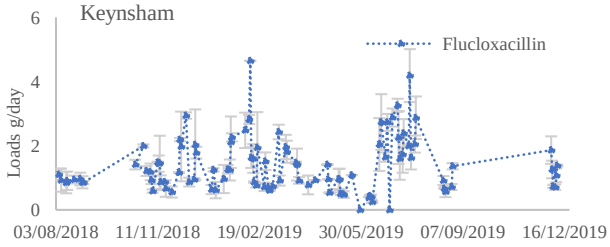

Bath
Sulfamethoxazole
N-acetyl sulfamethoxazole
Loads g/day
30
20
10
0
27/10/2018
04/02/2019
15/05/2019
23/08/2019
01/12/2019


Keynsham
Sulfamethoxazole
N-acetyl sulfamethoxazole
Loads g/day
8
6
4
2
0
03/08/2018
11/11/2018
19/02/2019
30/05/2019
07/09/2019
16/12/2019


Bath
Trimethoprim
Loads g/day
40
30
20
10
0
27/10/2018
04/02/2019
15/05/2019
23/08/2019
01/12/2019


Keynsham
Trimethoprim
Loads g/day
5
4
3
2
1
0
03/08/2018
11/11/2018
19/02/2019
30/05/2019
07/09/2019
16/12/2019


Bath
Flucloxacilli
Loads g/day
60
40
20
0
27/10/2018
04/02/2019
15/05/2019
23/08/2019
01/12/2019


Keynsham
Flucloxacillin
Loads g/day
6
4
2
0
03/08/2018
11/11/2018
19/02/2019
30/05/2019
07/09/2019
16/12/2019

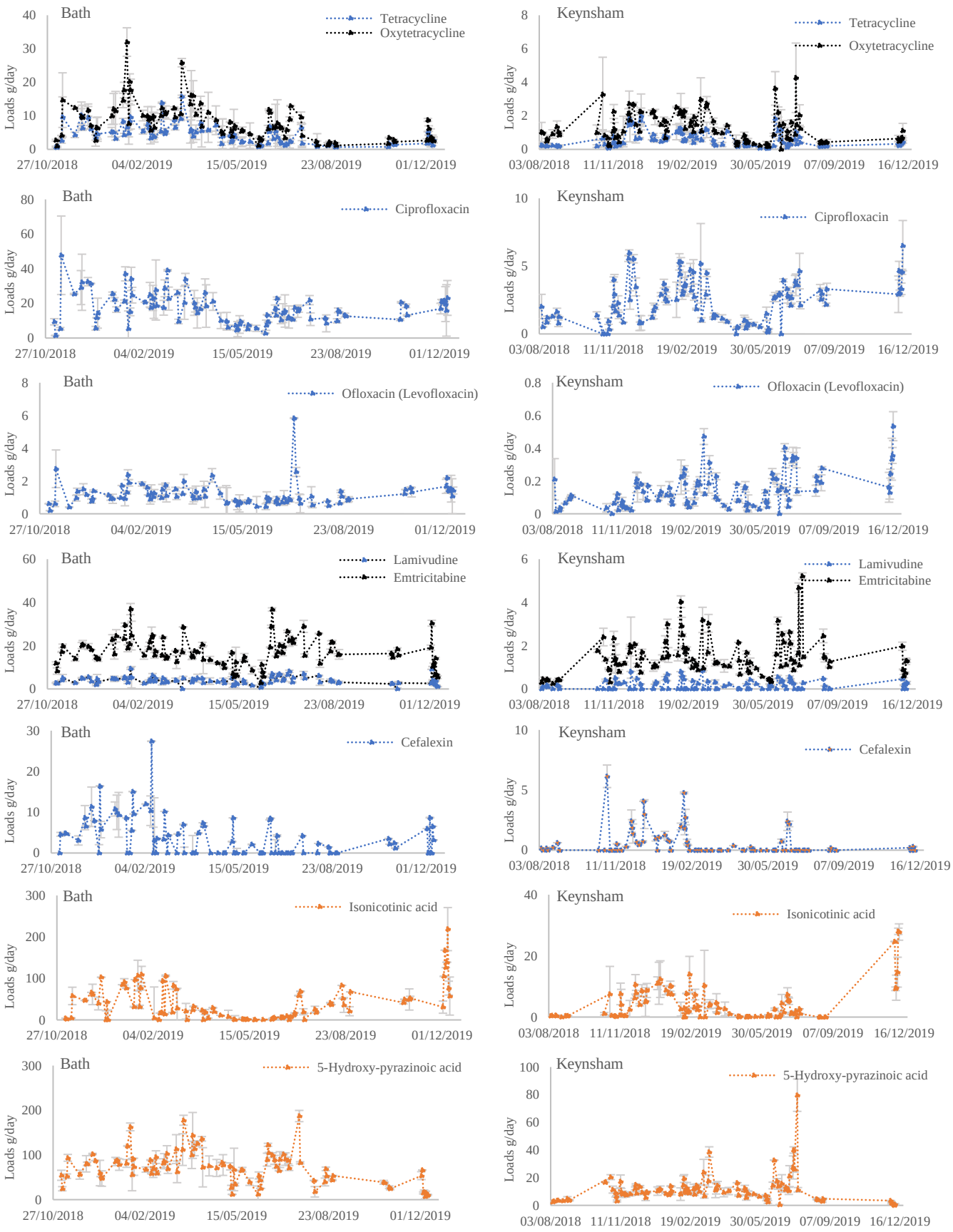


Figure 2. Scatter plots of average daily loads of AAs and metabolites in influent wastewater of Bath and Keynsham over the sampled period in 2018-2019

Previous work has been undertaken in the same catchment areas (Elder et al., 2021), whilst this study in 2015 investigated only a few AAs and a very short (one week) monitoring time, it allowed crossover comparison with this work (figure S3). Whilst lower daily averages were observed for SMX in Bath in 2015 and for CLR in Keynsham in 2018/19, results were comparable for the other AAs between the two sampling periods. For example, CIP 17.0 ± 8.9 g/day (2018/19) vs 10.2 ± 5.7 g/day (2015) in Bath, with Keynsham CIP levels averaging 2.5 ± 1.5 g/day (2018/19) vs 2.0 ± 1.6 g/day (2015).

3.2. Temporal variability of ARGs in Bath

3.2.1 DNA sequencing

3.2.1.1 Taxonomic profiling

Metagenomic sequencing was performed on two influent wastewater samples collected from Bath, one in November 2018 and other from March 2019. These two samples generated 12.5 million and 15.7 million paired end reads of average length of 247 and 245 base pairs respectively. After quality control and duplicate reads removal the samples contained 5,045,358 and 2,885,808 paired end reads of average length 239 and 232 respectively. Taxonomic profiling of each dataset revealed high bacterial diversity with a total of 509 bacterial species detected in November 2018 and 848 in March 2019. Taxonomic differences have been graphically represented (figure 3), however care must be taken drawing conclusions regarding differences in these samples due to the limited number of samples collected (n=1 each month). *Proteobacteria* was the dominant phyla in both samples (at 46% and 47% of total phyla detected for November and March respectively), with the next dominant reported as *Firmicutes*, at 25% in both samples. These are both frequently reported as the dominant phyla in influent wastewater (Lee et al., 2015; McLellan et al., 2010). It has been reported in several studies that horizontal transfer of resistance genes occurs in several bacterial phyla, including *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Hu et al., 2016). Furthermore, these phyla are known to be significant reservoirs of mobile ARGs and integrons (Hu et al., 2016; Huerta et al., 2013). The high density of bacteria in influent wastewater coupled with the chronic exposure to sublethal concentrations of AAs have led to WWTPs as hotspots for AMR (Karkman et al., 2018; Rizzo et al., 2013). Regarding potential pathogens of concern, the bacteria *Pseudomonas aeruginosa* was detected in March 2019. The carbapenem resistant *Pseudomonas aeruginosa,* is a pathogen that has been highlighted by WHO *as a bacterial species where there is a critical need to develop new AAs to treat infections (World Health Organisation, 2017).*

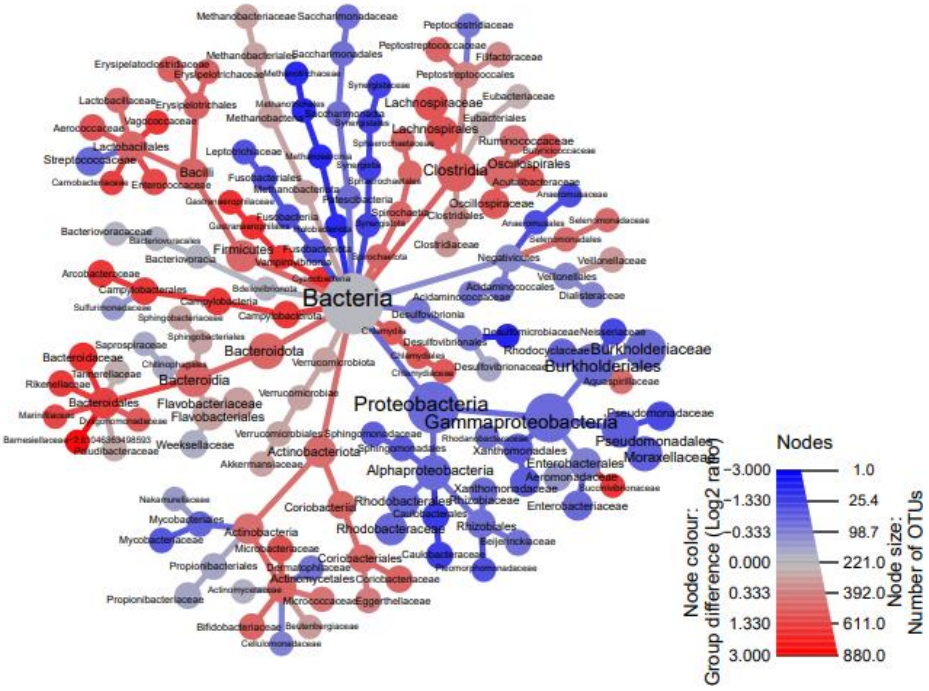


Figure 3. Taxonomical comparisons are shown between two metagenomic wastewater samples collected from Bath, one sample was collected in November 2018 and the other was collected in March 2019. Taxa shown as colours were found to be abundant at the corresponding sampling time points, while grey nodes indicate taxa that were found in similar proportions at each site. The depth of the colour indicates the log2 ratio of median abundances between the two groups. Red taxa represent higher abundance in November 2018 and blue taxa represent higher abundance in March 2019. The node size indicates the number of OTUs included.

3.2.1.2 Resistance gene profiling

In total, 46 ARGs were identified across the two wastewater samples, demonstrating a broad range of resistance across different AA classes (table S4). Of the 46 ARGs identified, the highest percentage of ARGs was predicted to confer resistance to aminoglycosides and tetracycline classes. With 29% and 17% of the total ARGs detected for November 2018 being associated with aminoglycosides and tetracyclines respectively, with 17% and 23% reported for March 2019 (figure 4). Other dominant ARGs were observed for macrolide resistance at 13% and 12% for Nov 2018 and March 2019 respectively. The resistance mechanisms of identified resistance genes in wastewater samples were also explored. The two samples gave very similar compositions, with the dominant resistance mechanisms demonstrated as antimicrobial inactivation at 40% and 46% for November 2018 and March 2019 respectively. This was followed by antimicrobial target protection at 28% and 19% for November 2018 and March 2019.

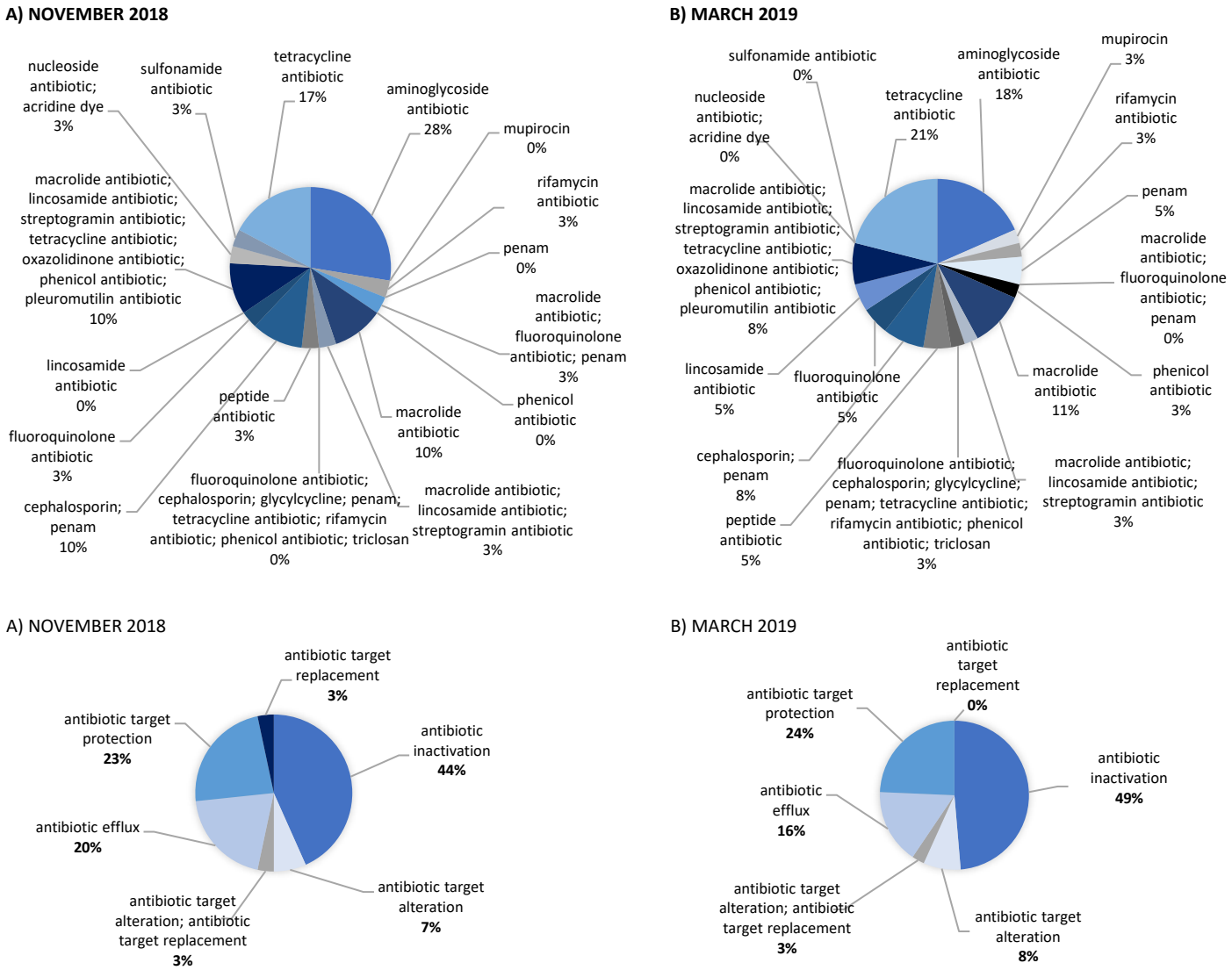


Figure 4. Composition of resistance gene class types present in influent wastewater November 2018 (A) and March 2019 (B), below composition of resistance gene mechanism types in November 2018 (C) and March 2019 (D)

3.2.2 Absolute concentrations of ARGs quantified with dPCR

Investigated ARGs were quantified in all wastewater samples extracted from Bath only (table S5). *ermB* was the highest prevalence in extracted samples, with an average concentration of 4.3E+09 ± 1.7E+09 copies/L across the sampling period respectively. The genes *intI1* and *sul1* were next prevalent and had similar concentration averages again at 5.8E+07 ± 2.1E+07 and 5.5E+07 ± 1.9E+07 copies/L. Regarding lowest prevalence, *qnrS* was the lowest detected gene, at an average concentration of 2.0E+06 ± 1.8E+06 copies/L. With regards to a previous study done in this catchment area in 2015 on three of the same ARGs studied, similar trends in concentrations were observed (*ermB* > *sul1* > *qnrS*) (Elder et al., 2021). Regarding concentration, higher values were observed during this study in 2019 (*ermB:* 1.8E+08 ± 1.5E+08, *sul1:* 5.4E+05 ± 3.1E+05 and *qnrS:* 2.3E+05 ± 10.0E+04 copies/L). Variations could be due to a number of reasons including changes in flows, changes in population size, and differences in extraction methodology.

3.2.3 Daily loads of AAs and ARGs

Flowrates were also taken into account to give gene loads (table S6). The overall distribution of ARG loads and the corresponding resistance AA classes were investigated over the studied period (figure 5). AAs from the sulphonamide class were the most prevalent, averaging 63 ± 24 g/day, with macrolides and lincosamides following at 26 ± 12 g/day. Quinolones AAs had the lowest prevalence in comparison at 20 ± 11 g/day in wastewater. The inclusion of AA metabolites within AA classes did not change this overall trend (sulphonamides > macrolides and lincosamides > quinolones), but macrolide and sulphonamides class averages did increase to 100 ± 39 g/day and 36 ± 15 g/day respectively. Quinolone metabolites were rarely detected, which may be because CIP and OFX are often excreted as parent compounds at 30-65% (Bergan et al., 1988) and 73% (Lode et al., 1987) respectively. Another factor could be the limited stability of quinolone metabolites in wastewater; it has previously been reported degradation between 29-62%, depending on the metabolite, after 24 hours at room temperature (Holton et al., 2022). In comparison, dmCLR and the acetyl sulphonamides reported higher stability in the same study. With dmCLR demonstrating little to no degradation in wastewater over a 24 hour period at room temperature, and sulphonamide metabolites exhibiting degradation between 10-30% over the same time period (Holton et al., 2022).

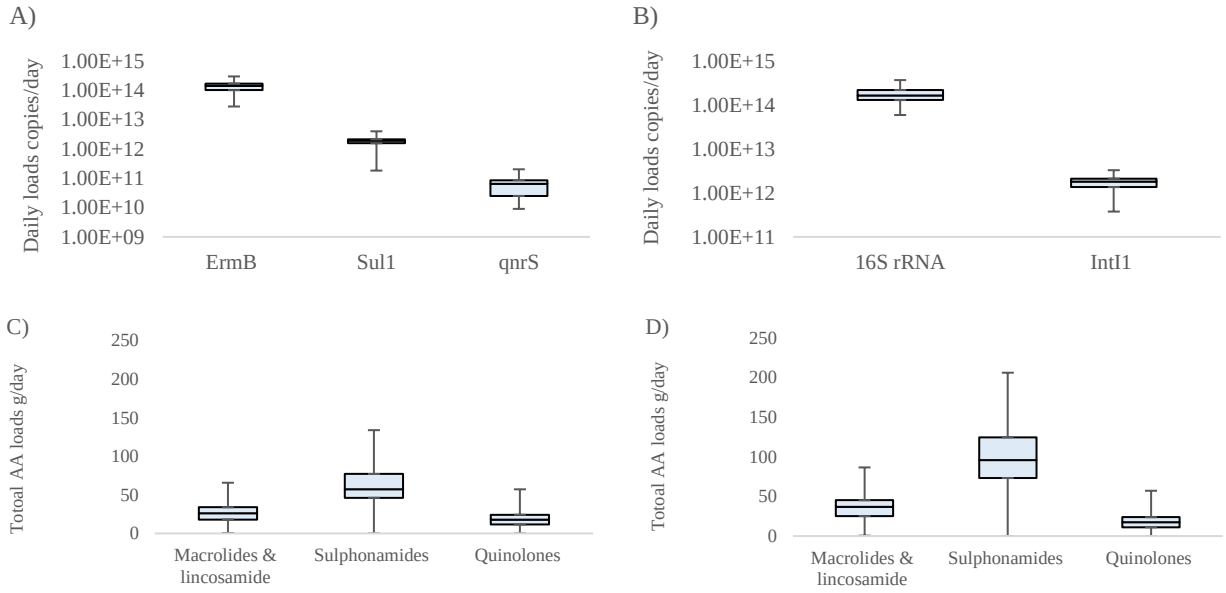


Figure 5. A) Box plots of absolute loads of ARGs (copies/day) in wastewater across the sampling period B) Box plots of absolute loads of *16S rRNA* and *intI1* in wastewater C) box plots of total cumulative loads of associated parent AAs (g/day) across the sampling period (metabolites have been removed) D) box plots of total cumulative loads AAs including metabolites. AAs and metabolites included in each class for C) and D) each class are detailed in table S1.

When investigating seasonal changes or ARGs in wastewater, *ermB*, *sul1* and *intI1* observed no statistically significant different loads in winter 2018/19 when compared to summer 2019 (table S7). Other studies have observed similar results with regards to ARGs in wastewater. One study investigating seasonal variation of *mecA* gene abundance in wastewater (which confers β-lactam resistance in methicillin resistant *Staphylococcus aureus* (MRSA)), reported variations over a year sampling but no obvious seasonal trend (Börjesson et al., 2009). Another study investigated 295 ARGs and mobile genetic elements, including resistance associated with tetracyclines, sulphonamides and macrolides, observed no significant seasonal variation of ARGs, except the absolute abundance of genes peaked during the spring (Zheng et al., 2020). In comparison, *qnrS* in this study was present in

significantly higher loads in summer than in winter (p=0.0018). Further work is required to understand this phenomenon.

Due to relatively stable ARG levels across 13 months monitoring time, limited correlations were observed between absolute loads of ARGs and total loads of associated AAs by class (figure 6). However, correlations between individual associated AAs and ARGs demonstrated some potential correlations where seasonal variability was reported (table S8). Statistically significant positive correlations were observed for *ermB* and the average monthly loads of the macrolides CLR and dmCLR (p=0.45, 0.48 and 0.58 for CLR, dmCLR r≤0.05). Other statistically significant correlations again showed weakly positive correlations between aSPY and *sul1* (p=0.28, r≤0.05) and OFX and *qnrS* (p=0.35, r≤0.05). A limitation here is that only a small number of ARGs were selected in this study, with one chosen representative ARG from each class. Expanding on the number of ARGs for each class would give a wider picture on overall resistance.

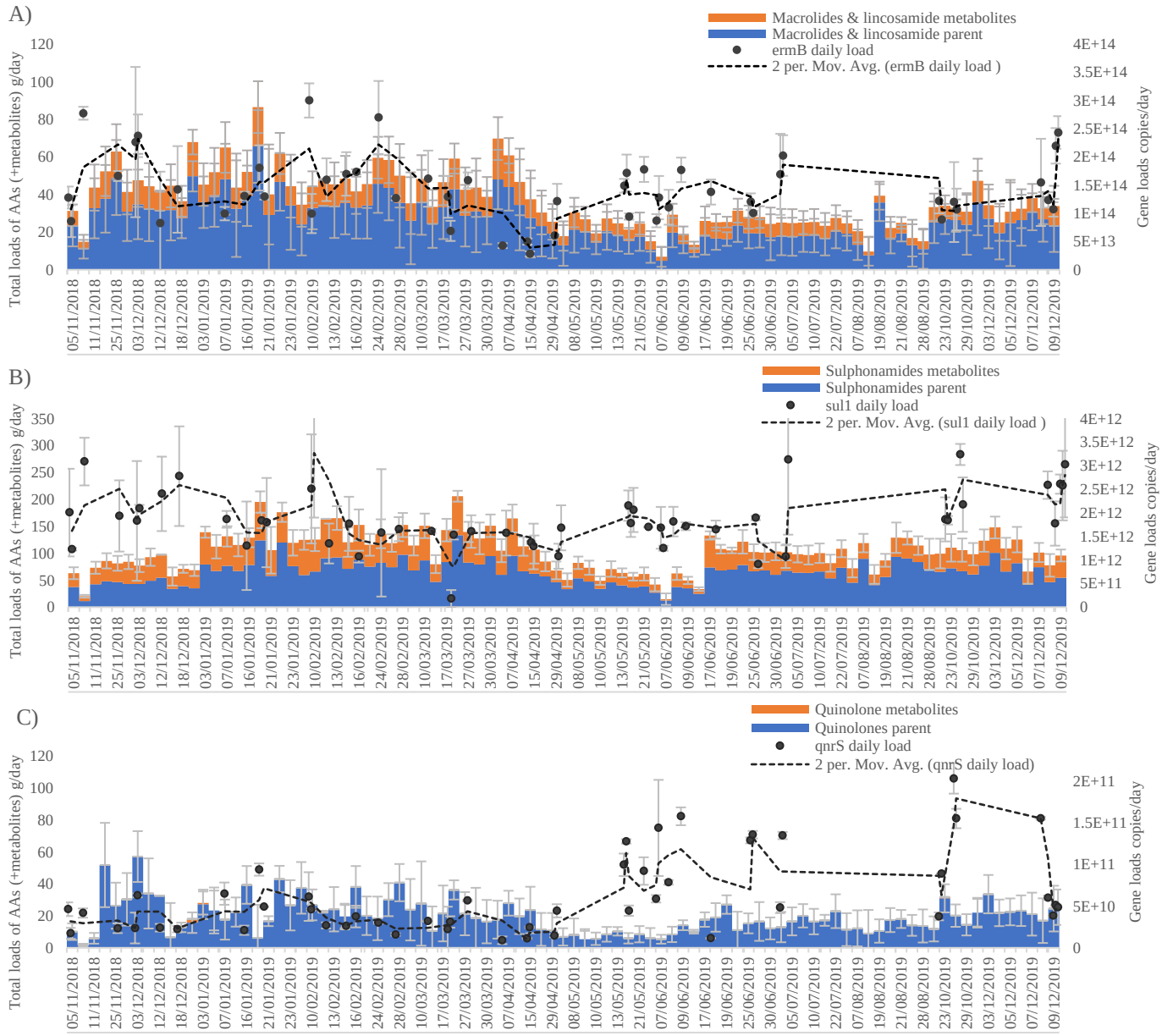


Figure 6. Daily loads of AAs by class and total loads of associated ARGs in influent wastewater in the studied period. Error bars represent standard deviation, n=2. A) macrolides and lincosamides AAs and *ermB* gene loads B) sulphonamide AAs and *sul1* gene loads C) quinolone AAs and *qnrS* gene loads.

Several studies have investigated relationships in environmental settings between certain AA classes and respective genes in wastewater (Huerta et al., 2013). For instance, correlations between abundance of TET genes and tetracycline levels (Li et al., 2015; Xu et al., 2015) and *sul* genes and sulphonamides (Gao et al., 2012). Regarding macrolides, Rodriguez-Mozaz *et al*. reported significant positive correlations between CIP and *qnrS*, OFX and *qnrS*, CLR and *ermB*, and SMX and *sul1* in wastewater streams, but no significant correlation between AZM and *ermB* (Rodriguez-Mozaz et al., 2015). Other studies however have observed weak correlations, or not statistically significant relationships, between TET genes and tetracyclines (Gao et al., 2012) and *sul* genes and sulphonamides (Xu et al., 2015).

AGR levels in this study (other than *qnrS*) did not exhibit seasonal variation. Statistical tests in this study have demonstrated that for several AAs, the levels do not change much (or at all), it is therefore not expected to observe significant seasonal changes in ARG levels. This longitudinal study focused on temporal observations of changes to daily AAs and ARGs loads. The population remains relatively constant (<±20%) as the city of Bath is studied across only a 13-month period. This is opposed to our previous studies which studied more locations with varying population sizes but focused on a shorter time period (Castrignanò et al., 2020; Elder et al., 2021). Elder *et al*. observed positive correlations between fluoroquinolones and *qnrS* loads between different locations ($r = 0.997$, $p < 0.004$) (Elder et al., 2021). The study also observed strong positive correlations between macrolide AAs and *ermB* ($r=0.928$, $p <0.0002$). However, it was highlighted that strong positive correlations were observed with both AA and gene loads with population size. It was theorised that correlations between AAs and gene loads are likely linked to population size as a key driving force. Furthermore in the case for *ermB*, it has been reported that abundance of this gene in influent wastewater could be heavily influenced by the presence of *ermB* in common gut bacteria (Pallares-Vega et al., 2021). Other studies have found that global variation between gene abundance strongly correlates with socio-economic, environmental, and health factors (Hendriksen et al., 2019), but these do not vary significantly between these two neighbouring sites.

In this study, <±20% population change might occur at certain times in Bath due to student population and visitors to the city. This change is within method uncertainties, and as such, might not lead to a measurable, statistically significant difference e.g. between seasons. The results presented here support our hypothesis that human population and its size, is a significant driver of AA and ARG levels in the environment. In conclusion, ARG levels show higher spatial inter-city rather than temporal intra-city variability which indicates their endemic, community driven nature rather than short term fluctuations in season driven AA usage.

3.2.4 Population and 16S normalised

To make fair comparisons between datasets and to aid in accounting for variabilities in the extraction protocol, gene loads can be normalised to *16S rRNA* to measure the estimated abundance of microorganisms present in the sample and to investigate possible selection occurring (table S6). Relative correlations of ARGs (normalised to *16S rRNA*) with associated AAs were also investigated (table S8 and S9). In general, normalising each of the gene targets to *16S rRNA* did not change the patterns of gene loads observed across samples. Gene loads were also normalised to the human population of Bath to calculate gene loads per capita; these were positively correlated to gene loads normalised by *16S rRNA* (figure 7). This observation is an important one. It indicates that the abundance of microorganisms is proportional to the size of human population (as already shown in our previous paper (Elder et al., 2021) and Kasprzyk-Hordern *et al.* (Kasprzyk-Hordern et al., 2022) where strong linear correlation was observed between *16S rRNA* and population size: $R^2 = 0.8786$, $r = 0.9373$ p value: 0.018657). Our data this further confirm that community size is a major driver of ARG levels in wastewater.

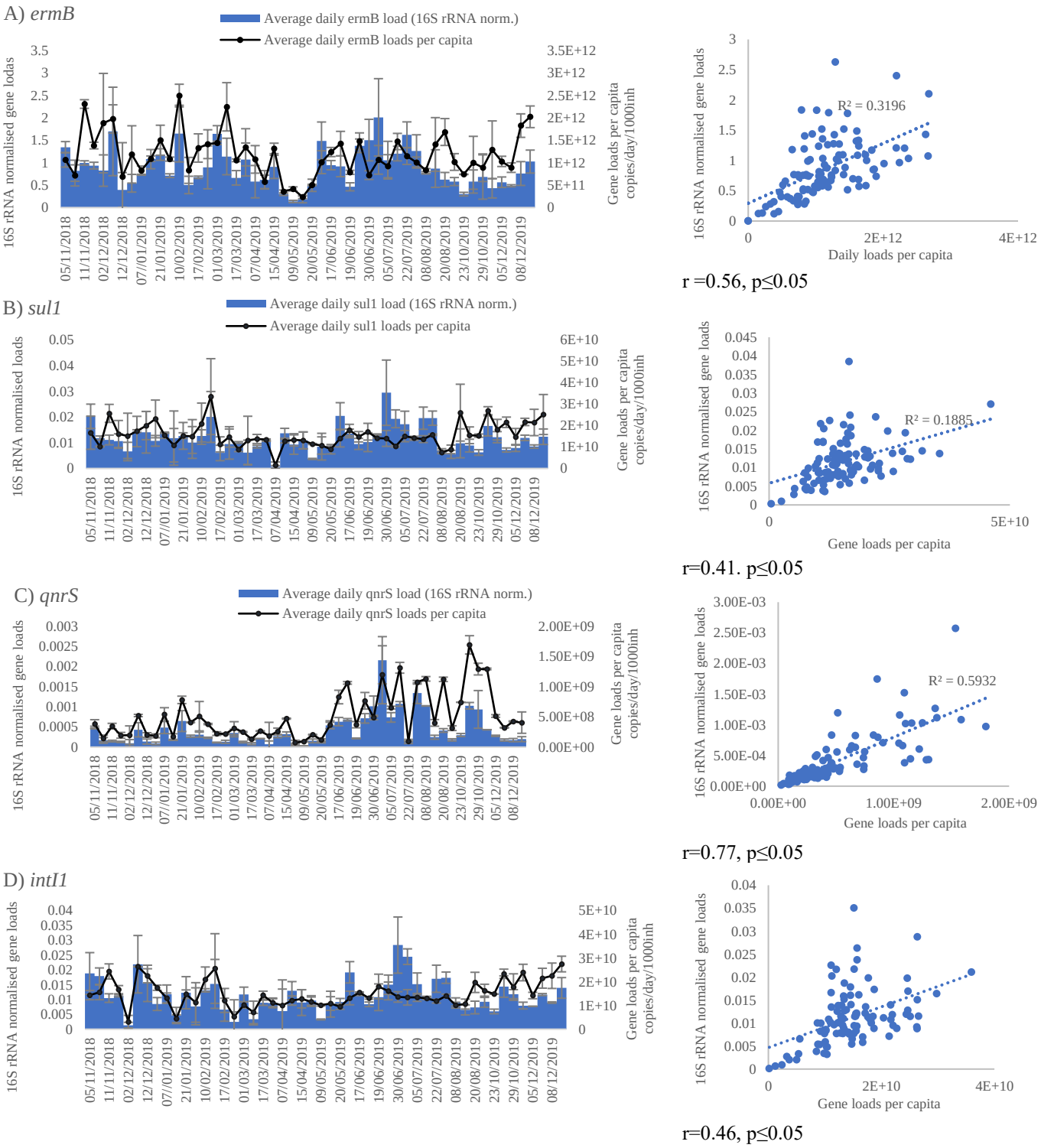


Figure 7. Average daily loads of ARGs normalised to *16S rRNA* and human population size (loads per capita)

3.2.5. *intI1* and ARGs

Relative abundance of *intI1*, the clinical class 1 integron-integrase gene, has been previously suggested as a suitable proxy for anthropogenic pollution (Gaze et al., 2011; Gillings et al., 2015). This has been attributed to its association to a diverse number of genes that confer resistance to AAs, metals and disinfectants, plus it can be found in a number of bacteria (pathogenic and non-pathogenic). The abundance of this gene can also change rapidly due to changes in the environment, as host cells have

rapid generation time and adapt to selective pressures of the environment. Recent work has also proposed that clinical *intI1* could be used to indicate the abundance of ARGs and to monitor the removal of ARGs in the wastewater treatment process (Zheng et al. 2020).

A common characteristic of *intI1* is its occurrence alongside sulphonamide resistance (M. Gillings et al. 2008). As a result, positive correlations are usually observed between abundances of *intI1* and *sul1* in wastewater (Makowska et al., 2016; Zieliński et al., 2021). Results in this study showed agreement with this, demonstrating strong positive correlations between absolute and relative wastewater loads of *intI1* and *sul1* ($r = 0.90$, $p = < 0.05$ absolute loads, $r = 0.90$, $p = <0.05$ relative loads). Correlations of *intI1* with other genes investigated were not observed (table S8 and S9).

3.3. Hospital input into AA and ARG levels in city wastewater

A range of AAs covering different classes were quantifiable in hospital effluent (table S10). Regarding ARGs, all seven targets were quantifiable in all samples (S11 and S12). The most prevalent ARG in hospital effluent was *ermB*, at 5.04E+09 ± 2.11E+09 copies/L; with *qnrS* having the lowest prevalence 2.96E+06 ± 2.92E+06 copies/L (figure 8). The two additional genes investigated in hospital effluent, *TetM* and *bla*-TEM, had similar abundances in general, at 8.71E+06 ± 5.05E+06 and 6.38E+06 ± 5.00E+06 copies/L respectively. Finally, the ARGs, *qnrS* and *tetM* both had low prevalence when compared to other studied genes and observed similar concentrations to each other in hospital effluent.

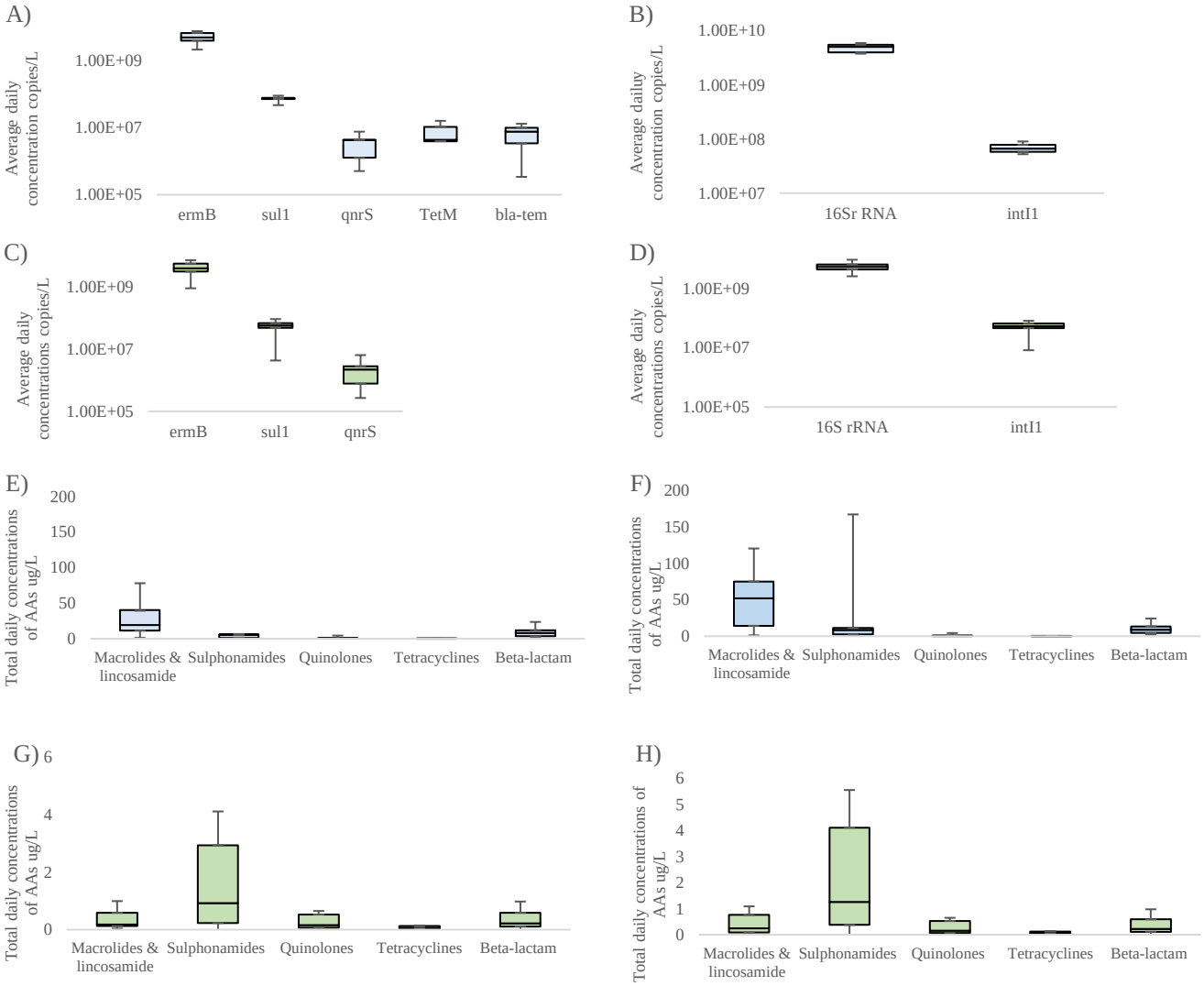


Figure 8. Box plot of concentrations in A) ARGs in hospital effluent; B) *16S rRNA* and *intI1* in hospital effluent; C) ARGs in community wastewater (Bath), August 2019; D) *16S rRNA* and *intI1* in community wastewater (Bath), August 2019; E) total AAs grouped by class with metabolites removed in hospital effluent; F) total AAs grouped by class with respective metabolites included in hospital effluent; G) total AAs grouped by class with metabolites removed in community wastewater (Bath) in August; and H) total AAs grouped by class with respective metabolites included in community wastewater (Bath) in August.

The overall abundances of AAs linked to the ARGs studied in hospital effluent vs community wastewater were also considered (figure 8). Concentrations of total AAs classes in hospital effluent were generally higher than community wastewater. Focusing on hospital effluent, the macrolides and lincosamides were measured at the highest abundance, with five-day averages at 50 ± 43 µg/L and 30 ± 24 µg/L, with and without metabolites respectively. Excluding metabolites, AAs from the beta-lactam class reported the next highest loads with five-day averages at 9 ± 8 µg/L, followed by those in the sulphonamide class at 3 ± 2 µg/L. When including metabolites, due to elevated levels of aSMX on the 06/08/2019 (147 ± 14 µg/L), sulphonamides observed the highest average after macrolides at 40 ± 67 µg/L with beta-lactams at 11 ± 7 µg/L. Quinolone and tetracycline AA were in the lowest abundance in hospital effluent giving five-day averages at 1.0 ± 1.6 µg/L and 0.02 ± 0.03 µg/L.

Interestingly, several AAs were present in hospital effluent that were not observed at all or with very low frequency in community wastewater, likely due to dilution of hospital wastewater with communal

discharge (table S12). Many AAs and metabolites were also observed in high concentrations. This is likely due to, with shorter sewage residence time, lower volume of flow and concentration of individuals at the source requiring AAs. LZD is an oxazolidinone antibiotic which is used in severe bacterial infections in the UK. It is often prescribed in hospital environments as it requires specialist supervision (National Institute for Health and Care Excellence, n.d.). It can be used to treat serious respiratory illness (pneumonia) and to treat skin and soft tissue infections caused by methicillin resistant *Staphylococcus aureus* (MRSA). It has also been identified by WHO as a recommended treatment of multi-resistant TB (World Health Organisation, 2019). Whilst LZD was not detected in community wastewater, three of the hospital effluent samples observed levels between 0.03 and 5.2 µg/L. AMX on the other hand is prescribed in both community and hospital settings and is a popular AA due to its effectiveness against both gram-negative and gram-positive infections. AMX was not detected in any samples in community wastewater but was quantifiable in all hospital effluent samples, ranging between 0.3 and 6.0 µg/L. The absence of AMX in community wastewater is likely due to lack of stability of the constrained beta-lactam ring (Hirte et al., 2016).

Fluoroquinolones are frequently found at high levels in hospital effluents, particularly in comparison to municipal wastewater (Rodriguez-Mozaz et al., 2015; Varela et al., 2014). Fluroquinolone AAs are used to treat a range of infections, from, combined with their stability and they are often excreted as parent compounds in urine (30-85%) (Novelli and Rosi, 2017), explains their high prevalence in environmental matrices. Regarding CIP, the average levels in hospital effluent for the five days were reported at 0.9 ± 1.6 µg/L, in comparison to the yearly average of CIP in community wastewater at 0.52 ± 0.24 µg/L. The highest concentrations of CIP were reported on the 06/08/2019 in hospital effluent at 3.9 ± 0.5 µg/L. Similar variable concentrations of CIP have been reported elsewhere in hospital effluents (Aydin et al., 2019; Varela et al., 2014; Verlicchi et al., 2012), with some studies reporting significantly higher quantities, ranging 101-236 µg/L (Diwan et al., 2010; Lindberg et al., 2004).

Other AAs in higher concentrations in hospital effluent (relative to community wastewater) included SMX, TMP, and MTZ. With SMX ranging from 0.22-.4.23 µg/L and TMP between 0.6-7.6 µg/L, these corresponding high levels are likely as SMX and TMP are often co-prescribed together. These are in range with other reports of hospital effluents; SMX has been reported between 0.15-373 µg/L (Aydin et al., 2019; Lindberg et al., 2004; Rodriguez-Mozaz et al., 2015; Verlicchi et al., 2012) and TMP at 0.14 and 7.6 µg/L (Lindberg et al., 2004; Rodriguez-Mozaz et al., 2015; Verlicchi et al., 2012). MTZ ranged from 10.2-17.1 µg/L in hospital effluent. High quantities of MTZ have also been reported in hospital streams in Sweden (Lindberg et al., 2004), Spain (Gómez et al., 2006) and Vietnam (Lien et al., 2016). High levels in hospital effluent were also noted with respective metabolites aSMX (0.7-146.6 µg/L) and hMTZ (6.8-37.6 µg/L). Whilst higher concentrations were often observed for hospital wastewater when compared with community wastewater, several AAs had the opposite trend. For example, TET and OTC had lower concentrations reported in hospital effluent when compared to community wastewater, with OTC at 0.05 ± 0.03 and 0.24 ± 0.16 µg/L, respectively; and TET at 0.03 ± 0.01 and 0.13 ± 0.08 µg/L. This could be attributed to the specific AA usage, TET and OTC are not typically associated with hospitals in the UK, being largely used to treat chlamydia and skin conditions, such as acne and rosacea.

Plotting sample composition by AA class for samples collected in August 2019 (figure 9) demonstrated a high percentage of macrolides in hospital effluent (43%) compared to community wastewater influent (12%). Interestingly a higher percentage composition of sulphonamides was observed for community wastewater (37%) versus hospital effluent (15%). A similar trend was noted for antiretrovirals, with AAs of this class making up 7% of community wastewater, compared to <1% of hospital effluent. By comparing percentage catchment prescription versus hospital prescriptions in August 2019 (figure 12), the percentage of sulphonamides prescribed in hospitals was significantly higher than in the wider catchment (57% versus 33% respectively). Prescriptions of macrolides however were lower in hospital prescribing (2%) in comparison to community prescribing (7%). Why the same composition patterns

were not reflected in wastewater is likely due to various reasons, including metabolism and degradation of AAs in wastewater, as well as patient compliance.

When comparing between community wastewater and hospital effluent in August 2019, comparable concentrations of resistant genes were observed (figure S4). A similar observation has been reported with Rodriguez-Mozaz *et al*., where the absolute concentration of genes *blaTEM, qnrS, ermB, sul1*, and *tetW* had comparable abundances between hospital and urban wastewater (Rodriguez-Mozaz et al., 2015). Furthermore, when investigating relationships between ARGs in hospital effluent, no clear relationship was established (table S13 and S14). This supports the theory that AMR levels are more of an endemic nature, developing over time in individual communities. This is also reflected in the low variability of AA prescribing.

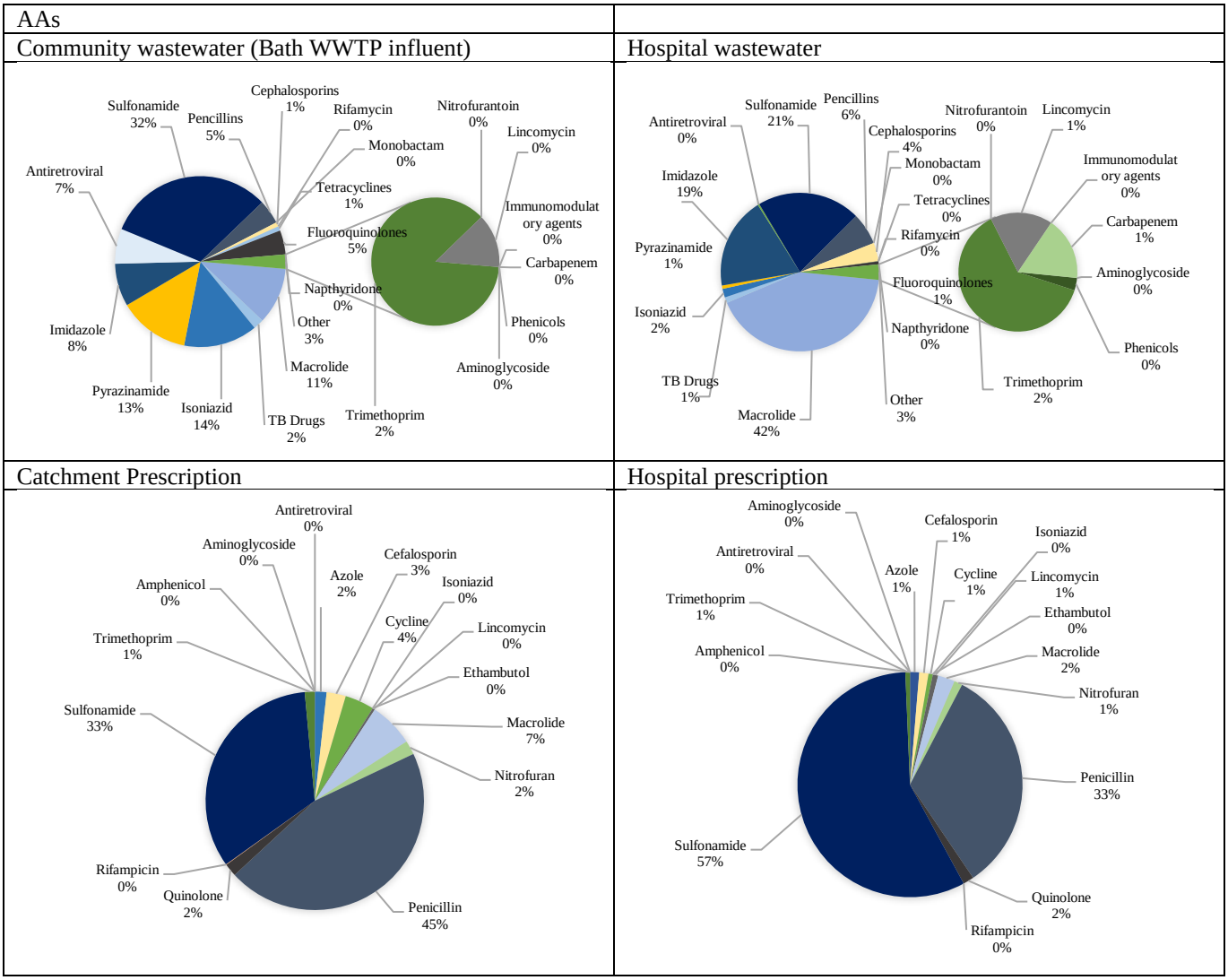


Figure 9. Comparisons in concentration composition of AA classes in community (A) and hospital (B) wastewater (n=1) in August 2019. C) Percentage comparison of prescriptions by AA class of RUH versus the rest of the catchment in August 2019.

3.4. Wastewater as a hotspot of AMR: measured vs predicted no effect concentrations

It is considered that the exposure of sublethal concentrations of AAs to bacterial communities present in wastewater streams could lead to selective pressures of ARGs and could influence the microbial ecology (Chow et al., 2021). However, this relationship between AAs and ARGs, particularly in aquatic environments (wastewater, surface waters etc) is not always clear. Predicted no effect concentrations (PNECs), have been considered to explore whether AA levels are high enough to influence ARG

abundance – they predict the concentration that a chemical, if exceeded, will likely cause adverse effects in an ecosystem. This can be used to investigate whether hospital effluent poses a higher risk, compared to community wastewater. PNECs often guide environmental risk assessments and, in the case of AMR, identify the risk of AAs in environmental matrices.

The PNEC table consists of two different values; (1) PNEC-Minimum inhibitory concentration (PNEC-MIC), published by Bengtsson-palme and Larsson (Bengtsson-Palme and Larsson, 2016), based on resistance promotion, and derived from EUCAST breakpoint data for AAs; (2) Environmental PNEC (PNEC-ENV), based on eco-toxicology, and intended to protect ecological systems. The lowest of the two PNEC values are used to regulate environmental AA levels. Hospitals have previously been highlighted as an area of concern. One study measuring particularly high AA burdens estimated 44% of AAs exceeded the PNEC values (Booth et al., 2020). This was in comparison to municipal wastewater, with 9% of AAs exceeding PNECs.

Average concentrations across the sampling period, for both hospital and community wastewater, have been compared with the PNEC values (Table 3). A handful of AAs were reported to have concentrations that fell below PNEC values for both hospital and community wastewater, including TET, OTC, and CHL. However, several AAs exceeded the PNEC in both communal and hospital wastewater, including CLR with average concentrations 0.47 ± 0.32 and 0.72 ± 0.31 μg/L in hospital and domestic wastewater respectively, above the PNEC of 0.08 μg/L. Similar observations were observed for CIP, with average concentrations 0.93 ± 1.66 and 0.52 ± 0.24 μg/L in hospital and domestic wastewater, again above the PNEC value of 0.064 μg/L. Both CIP and CLR have been previously reported to exceed the PNEC values, in a range of environmental matrices including hospital, municipal and surface waters (Booth et al., 2020; Hartmann et al., 1998). The levels exceeding PNEC values could have the potential to promote AMR.

Table 3. Average concentrations of AAs in hospital effluent (five days sampled) and Bath (community) wastewater over the year along with respective PNEC values

Bath	**Average Wastewater Concentration ug/L**		**PNEC ug/L**		
AA	**Hospital**	**Community**	**PNEC-MIC**	**PNEC-ENV**	**Lowest PNEC**
Chloramphenicol	0.18 ± 0.09	0.15 ± 0.17	8	N/A	8
Sulfamethoxazole	**1.47 ± 1.58**	0.46 ± 0.19	16	0.6	0.6
Azithromycin	**39.5 ± 41.56**	**0.31 ± 0.30**	0.25	0.02	0.02
Clarithromycin	**0.47 ± 0.32**	**0.72 ± 0.31**	0.25	0.08	0.08
Erythromycin	**2.20 ± 2.31**	0.06 ± 0.05	1	0.5	0.5
Ciprofloxacin	**0.93 ± 1.66**	**0.52 ± 0.24**	0.064	0.57	0.064
Ofloxacin	0.04 ± 0.05	0.04 ± 0.03	0.5 (levofloxacin)	10	0.5 (levofloxacin)
Amoxicillin	**11.13 ± 8.92**	-	0.25	N/A	0.25
Clindamycin	**0.27 ± 0.43**	0.04 ± 0.02	1	0.1	0.1
Metronidazole	**12.49 ± 3.40**	**0.23 ± 0.09**	0.125	N/A	0.125
Nitrofurantoin	**0.52 ± 0.51**	-	64	N/A	64
Oxytetracycline	0.05 ± 0.03	0.24 ± 0.16	0.5	18	0.5
Tetracycline	0.03 ± 0.01	0.13 ± 0.08	1	3.2	1
Ethambutol	0.07 ± 0.05	0.11 ± 0.12	2	N/A	2
Sulfadiazine	-	0.008 ± 0.007	N/A	13	13
Trimethoprim	**3.25 ± 2.76**	0.33 ± 0.12	0.5	100	0.5
Flucloxacillin	**5.66 ± 5.97**	**0.44 ± 0.23**			

* **Highlighted** values exceed the lowest PNEC value

Several AAs exceeded PNECs in hospital effluent, but often fell below in community wastewater (table S15). For example, AA concentrations in hospital and community wastewater respectively: SMX 1.47 ±1.58 and 0.46 ± 0.19 μg/L, PNEC of 0.6 μg/L; ERY 2.20 ± 2.31 vs 0.06 ± 0.05 μg/L, PNEC of 0.5 μg/L; and TMP 3.25 ± 2.76 and 0.33 ± 0.12 μg/L, PNEC at 0.5 μg/L. Thus for certain AAs, hospital effluents could pose a greater risk of selective pressures of ARGs. The hospital effluent contribution to Bath will likely contribute a small amount to the overall wastewater reaching the WWTP, however the unique environment that hospital effluents constitute (with concentrated AAs exceeding PNECs), highlights the importance of including hospitals in AMR surveillance.

However, previous work by Stanton *et al.* has observed that environmental concentrations of macrolide AAs (ERY, CLR, and AZM) do not positively select for resistance genes (Stanton et al., 2020). Instead, lowest observable effect concentrations in this study for macrolides were significantly higher than PNECs and the measured environmental concentrations. In this case, it was theorised that PNECs for macrolides could be underestimated when considering combined exposure effects. The same study also demonstrated for CIP, (whilst no significant selection of *qnrS* was observed), positive selection of *intI1* at environmentally relevant concentrations were demonstrated (> 7.8 and <15.6 μg/L). Due to this the authors theorised the likelihood that genes conferring resistance to different antimicrobials may also be co-selected by CIP, due to *intI1* association (e.g. *sul1* gene is frequently found on the class 1 integrons backbones). Results such as these could potentially indicate AA levels do not drive ARG levels in wastewater. This study has not only highlighted the need for compound specific assessment for selective potential of genes, but also that further research is essential for more informed AA and ARG regulation, for both environmental and public health purposes.

4. Conclusions

The manuscript presented results form a 13-month longitudinal study (with randomised sampling of 4 samples per week) aimed at providing insight into antimicrobial agents (AA) usage within two communities in the South West of the UK: Bath (120K inh city) and smaller Keynsham (30K inh town), and ARGs prevalence in community and hospital wastewaters with an overarching aim to test if there are correlations between AB usage and AGR prevalence. The key conclusions are as follows:

1. AA loads were higher in Bath than in Keynsham, corresponding to the larger population, indicating that AA usage is population size driven.
2. Several AAs in wastewater had higher loads in winter when compared to summer, including macrolide AAs and metabolites, aligning with increases in winter respiratory infections. In contrast, AAs such as sulfamethoxazole and sulfapyridine, stayed consistent over the study period.
3. As opposed to antibiotics, ARGs were found to be less variable, which indicates that fluctuations in antibiotic usage might either not directly affect ARG levels or this process spans beyond the 13-month monitoring period. However, it is important to note that weekly positive correlations between individual associated AAs and ARGs were observed where seasonal variability in AB use was reported: *ermB* and macrolides CLR and dmCLR ($p=0.45$, 0.48 and 0.58 for CLR, dmCLR $r\leq0.05$), aSPY and *sul1* ($p=0.28$, $r\leq0.05$) and OFX and *qnrS* ($p=0.35$, $r\leq0.05$).
4. Gene loads normalised to *16S rRNA* (gene load per microbe) were positively correlated to the gene loads normalised to the human population (gene load per capita), which indicates, yet again, that the abundance of microorganisms is proportional to the size of human population and that the community size is a major driver of ARG levels in wastewater.
5. ARG levels show higher spatial inter-city rather than temporal intra-city variability which indicates their endemic, community driven nature rather than impacts from short term fluctuations in season driven AB usage.

6. Comparison of hospital and community wastewater showed higher number of AAs and their metabolites, their frequency of occurrence and concentrations in hospital wastewater. This is likely due to, with shorter sewage residence time, lower volume of flow and concentration of individuals at the source requiring AAs when compared to community wastewater. Examples include: LZD (used only in severe bacterial infections) and AMX (widely used, also in community but with very low wastewater stability) that were found only in hospital wastewater. CIP, SMX, TMP, and MTZ, macrolides were found at much higher concentrations in hospital wastewater while TET and OTC, as well as antiretrovirals had an opposite trend as these AAs are used in communities to treat milder conditions. In contrast, comparable concentrations of resistant genes were observed in both community and hospital wastewater. This supports the hypothesis that AMR levels are more of an endemic nature, developing over time in individual communities.
7. Both hospital and community wastewater had AAs that exceeded PNEC values (e.g. CLR, CIP). In general, though, hospital effluents had a greater number of quantifiable AAs exceeding PNECs (e.g. SMX, ERY, TMP). Hospitals are therefore an important consideration in AMR surveillance as could be high risk areas for AMR.

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Credit authorship contribution statement

Natalie Sims: Conceptualisation, Methodology (experimental design, sampling, analysis of AAs with LCMS and analysis of ARGs with dPCR), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Andrew Kannan: Methodology (experimental design and sampling); Elizabeth Holton: Writing – original draft, Writing – review editing; Kishore Jagadeesan: Methodology (experimental design and sampling); Writing – review editing; Leonardos Mageiros: Methodology (sequencing), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Richard Standerwick: Methodology (conceptualisation, sampling, WWTP information) Writing – review editing; Project administration, Resources. Ruth Barden: Methodology (experimental design), Funding acquisition, resources, Writing – review editing; Ed Feil: Methodology (sequencing), Writing – review editing; Barbara Kasprzyk-Hordern: Conceptualisation, Methodology (experimental design, sampling and analysis), Writing-original draft, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources.

References

Amsden, G.W., 1996. Erythromycin, clarithromycin, and azithromycin: Are the differences real? Clin. Ther. https://doi.org/10.1016/S0149-2918(96)80179-2

Aydin, S., Aydin, M.E., Ulvi, A., Kilic, H., 2019. Antibiotics in hospital effluents: occurrence, contribution to urban wastewater, removal in a wastewater treatment plant, and environmental risk assessment. Environ. Sci. Pollut. Res. 26, 544–558. https://doi.org/10.1007/s11356-018-3563-0

Been, F., Rossi, L., Ort, C., Rudaz, S., Delémont, O., Esseiva, P., 2014. Population Normalization with Ammonium in Wastewater-Based Epidemiology: Application to Illicit Drug Monitoring. Environ. Sci. Technol. 48, 8162–8169. https://doi.org/10.1021/es5008388

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. https://doi.org/10.1016/j.envint.2015.10.015

Bergan, T., Dalhoff, A., Rohwedder, R., 1988. Pharmacokinetics of ciprofloxacin. Infection 16. https://doi.org/10.1007/BF01650500

Booth, A., Aga, D.S., Wester, A.L., 2020. Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. Environ. Int. https://doi.org/10.1016/j.envint.2020.105796

Börjesson, S., Melin, S., Matussek, A., Lindgren, P.E., 2009. A seasonal study of the mecA gene and Staphylococcus aureus including methicillin-resistant S. aureus in a municipal wastewater treatment plant. Water Res. 43, 925–932. https://doi.org/10.1016/j.watres.2008.11.036

Castrignanò, E., Yang, Z., Feil, E.J., Bade, R., Castiglioni, S., Causanilles, A., Gracia-Lor, E., Hernandez, F., Plósz, B.G., Ramin, P., Rousis, N.I., Ryu, Y., Thomas, K. V., de Voogt, P., Zuccato, E., Kasprzyk-Hordern, B., 2020. Enantiomeric profiling of quinolones and quinolones resistance gene qnrS in European wastewaters. Water Res. 175, 115653. https://doi.org/10.1016/j.watres.2020.115653

Chen, C., Kostakis, C., Gerber, J.P., Tscharke, B.J., Irvine, R.J., White, J.M., 2014. Towards finding a population biomarker for wastewater epidemiology studies. Sci. Total Environ. 487, 621–628. https://doi.org/10.1016/j.scitotenv.2013.11.075

Choi, P.M., Bowes, D.A., O'Brien, J.W., Li, J., Halden, R.U., Jiang, G., Thomas, K.V., Mueller, J.F., 2020. Do food and stress biomarkers work for wastewater-based epidemiology? A critical evaluation. Sci. Total Environ. 736, 139654. https://doi.org/10.1016/j.scitotenv.2020.139654

Chow, L.K.M., Ghaly, T.M., Gillings, M.R., 2021. A survey of sub-inhibitory concentrations of antibiotics in the environment. J. Environ. Sci. (China). https://doi.org/10.1016/j.jes.2020.05.030

Curtis, H.J., Walker, A.J., Mahtani, K.R., Goldacre, B., 2019. Time trends and geographical variation in prescribing of antibiotics in England 1998-2017. J. Antimicrob. Chemother. https://doi.org/10.1093/jac/dky377

Daughton, C.G., 2020. Wastewater surveillance for population-wide Covid-19: The present and future. Sci. Total Environ. 736, 139631. https://doi.org/10.1016/j.scitotenv.2020.139631

Daughton, C.G., 2018. Monitoring wastewater for assessing community health: Sewage Chemical-Information Mining (SCIM). Sci. Total Environ. 619–620, 748–764. https://doi.org/10.1016/j.scitotenv.2017.11.102

Daughton, C.G., 2001. Illicit drugs in municipal sewage: Proposed new nonintrusive tool to heighten public awareness of societal use of illicit-abused drugs and their potential for ecological consequences. ACS Symp. Ser.

Diwan, V., Tamhankar, A.J., Khandal, R.K., Sen, S., Aggarwal, M., Marothi, Y., Iyer, R. V., Sundblad-Tonderski, K., Stålsby- Lundborg, C., 2010. Antibiotics and antibiotic-resistant bacteria in waters associated with a hospital in Ujjain, India. BMC Public Health 10, 414. https://doi.org/10.1186/1471-2458-10-414

Elder, F.C.T., Proctor, K., Barden, R., Gaze, W.H., Snape, J., Feil, E.J., Kasprzyk-Hordern, B., 2021. Spatiotemporal profiling of antibiotics and resistance genes in a river catchment: Human population as the main driver of antibiotic and antibiotic resistance gene presence in the environment. Water Res. 203, 117533. https://doi.org/10.1016/j.watres.2021.117533

Galani, A., Alygizakis, N., Aalizadeh, R., Kastritis, E., Dimopoulos, M.A., Thomaidis, N.S., 2021. Patterns of pharmaceuticals use during the first wave of COVID-19 pandemic in Athens, Greece as revealed by wastewater-based epidemiology. Sci. Total Environ.

https://doi.org/10.1016/j.scitotenv.2021.149014

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. https://doi.org/10.1016/j.scitotenv.2012.01.061

Gaze, W.H., Zhang, L., Abdouslam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A.B.A., Wellington, E.M.H., 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J. 5, 1253–1261. https://doi.org/10.1038/ismej.2011.15

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

Gómez, M.J., Petrović, M., Fernández-Alba, A.R., Barceló, D., 2006. Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis in hospital effluent wastewaters. J. Chromatogr. A. https://doi.org/10.1016/j.chroma.2006.02.038

Guo, J., Li, J., Chen, H., Bond, P.L., Yuan, Z., 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. Water Res. https://doi.org/10.1016/j.watres.2017.07.002

Hartmann, A., Alder, A.C., Koller, T., Widmer, R.M., 1998. Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater . Environ. Toxicol. Chem. https://doi.org/10.1002/etc.5620170305

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T.L.C., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sopheak, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulibaly, K.J., Jergović, M., Hrenovic, J., Karpíšková, R., Villacis, J.E., Legesse, M., Eguale, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'Connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Avsejenko, J., Bērziņš, A., Bartkevics, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.J., Gemmell, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasyl, D., Bosevska, G., Kochubovski, M., Radu, C., Gassama, A., Radosavljevic, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Trkov, M., Esterhuyse, K., Keddy, K., Cerdà-Cuéllar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T. Van der, Kumburu, H.H., Sanneh, B., Bidjada, P., Njanpop-Lafourcade, B.M., Nikiema-Pessinaba, S.C., Levent, B., Meschke, J.S., Beck, N.K., Van, C.D., Phuc, N. Do, Tran, D.M.N., Kwenda, G., Tabo, D. adjim, Wester, A.L., Cuadros-Orellana, S., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. https://doi.org/10.1038/s41467-019-08853-3

Hirte, K., Seiwert, B., Schüürmann, G., Reemtsma, T., 2016. New hydrolysis products of the beta-lactam antibiotic amoxicillin, their pH-dependent formation and search in municipal wastewater. Water Res. https://doi.org/10.1016/j.watres.2015.11.028

Holton, E., Kasprzyk-Hordern, B., 2021. Multiresidue antibiotic-metabolite quantification method using ultra-performance liquid chromatography coupled with tandem mass spectrometry for

environmental and public exposure estimation. Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-021-03573-4

Holton, E., Sims, N., Jagadeesan, K., Standerwick, R., Kasprzyk-Hordern, B., 2022. Quantifying community-wide antimicrobials usage via wastewater-based epidemiology. https://doi.org/10.26434/CHEMRXIV-2022-PDSF4

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., Lin, I.Y.C., Wu, N., Weimer, B.C., Gao, G.F., Liu, Y., Zhu, B., 2016. The bacterial mobile resistome transfer network connecting the animal and human microbiomes. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.01802-16

Huerta, B., Marti, E., Gros, M., López, P., Pompêo, M., Armengol, J., Barceló, D., Balcázar, J.L., Rodríguez-Mozaz, S., Marcé, R., 2013. Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2013.03.071

Karkman, A., Do, T.T., Walsh, F., Virta, M.P.J., 2018. Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. https://doi.org/10.1016/j.tim.2017.09.005

Kasprzyk-Hordern, B., Proctor, K., Jagadeesan, K., Edler, F., Standerwick, R., Barden, R., 2022. Human population as a key driver of biochemical burden in an inter-city system: Implications for One Health concept. J. Hazard. Mater. 429, 127882. https://doi.org/10.1016/J.JHAZMAT.2021.127882

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: State of the knowledge and research needs. Sci. Total Environ. 739, 139076. https://doi.org/10.1016/j.scitotenv.2020.139076

Lanza, V.F., Baquero, F., Martínez, J.L., Ramos-Ruíz, R., González-Zorn, B., Andremont, A., Sánchez-Valenzuela, A., Ehrlich, S.D., Kennedy, S., Ruppé, E., van Schaik, W., Willems, R.J., de la Cruz, F., Coque, T.M., 2018. In-depth resistome analysis by targeted metagenomics. Microbiome. https://doi.org/10.1186/s40168-017-0387-y

Lee, S.H., Kang, H.J., Park, H.D., 2015. Influence of influent wastewater communities on temporal variation of activated sludge communities. Water Res. https://doi.org/10.1016/j.watres.2015.01.014

Li, J., Cheng, W., Xu, L., Strong, P.J., Chen, H., 2015. Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. Environ. Sci. Pollut. Res. 22, 4587–4596. https://doi.org/10.1007/s11356-014-3665-2

Lien, L.T.Q., Hoa, N.Q., Chuc, N.T.K., Thoa, N.T.M., Phuc, H.D., Diwan, V., Dat, N.T., Tamhankar, A.J., Lundborg, C.S., 2016. Antibiotics in wastewater of a rural and an urban hospital before and after wastewater treatment, and the relationship with antibiotic use-a one year study from Vietnam. Int. J. Environ. Res. Public Health. https://doi.org/10.3390/ijerph13060588

Lindberg, R., Jarnheimer, P.-Å., Olsen, B., Johansson, M., Tysklind, M., 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. Chemosphere 57, 1479–1488. https://doi.org/10.1016/j.chemosphere.2004.09.015

Lode, H., Hoffken, G., Olschewski, P., Sievers, B., Kirch, A., Borner, K., Koeppe, P., 1987. Pharmacokinetics of ofloxacin after parenteral and oral administration. Antimicrob. Agents Chemother. https://doi.org/10.1128/AAC.31.9.1338

Makowska, N., Koczura, R., Mokracka, J., 2016. Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. Chemosphere. https://doi.org/10.1016/j.chemosphere.2015.10.044

McLellan, S.L., Huse, S.M., Mueller-Spitz, S.R., Andreishcheva, E.N., Sogin, M.L., 2010. Diversity and population structure of sewage-derived microorganisms in wastewater treatment plant influent. Environ. Microbiol. https://doi.org/10.1111/j.1462-2920.2009.02075.x

Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.A.P., McManigal, B., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiù, S., Chowdhury, F., Cook, A.J., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N.P.J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S.J., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garrett, D., Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay, S.I., Holm, M., Hopkins, S., Iregbu, K.C., Jacobs, J., Jarovsky, D., Javanmardi, F., Khorana, M., Kissoon, N., Kobeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H.H., Lim, C., Limmathurotsakul, D., Loftus, M.J., Lunn, M., Ma, J., Mturi, N., Munera-Huertas, T., Musicha, P., Mussi-Pinhata, M.M., Nakamura, T., Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D., Obiero, C.W., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Peleg, A.Y., Perrone, C., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Riddell, A., Roberts, T., Robotham, J.V., Roca, A., Rudd, K.E., Russell, N., Schnall, J., Scott, J.A.G., Shivamallappa, M., Sifuentes-Osornio, J., Steenkeste, N., Stewardson, A.J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Turner, C., Turner, P., van Doorn, H.R., Velaphi, S., Vongpradith, A., Vu, H., Walsh, T., Waner, S., Wangrangsimakul, T., Wozniak, T., Zheng, P., Sartorius, B., Lopez, A.D., Stergachis, A., Moore, C., Dolecek, C., Naghavi, M., 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 0. https://doi.org/10.1016/S0140-6736(21)02724-0

National Institute for Health and Care Excellence, n.d. Linezolid [WWW Document]. URL https://bnf.nice.org.uk/drug/linezolid.html (accessed 2.2.22).

Novelli, A., Rosi, E., 2017. Pharmacological properties of oral antibiotics for the treatment of uncomplicated urinary tract infections. J. Chemother. https://doi.org/10.1080/1120009X.2017.1380357

Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2021. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. Water Res. https://doi.org/10.1016/j.watres.2020.116752

Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5, eaau9124. https://doi.org/10.1126/sciadv.aau9124

Petrovich, M.L., Zilberman, A., Kaplan, A., Eliraz, G.R., Wang, Y., Langenfeld, K., Duhaime, M., Wigginton, K., Poretsky, R., Avisar, D., Wells, G.F., 2020. Microbial and Viral Communities and Their Antibiotic Resistance Genes Throughout a Hospital Wastewater Treatment System. Front. Microbiol. https://doi.org/10.3389/fmicb.2020.00153

Polo, D., Quintela-Baluja, M., Corbishley, A., Jones, D.L., Singer, A.C., Graham, D.W., Romalde, J.L., 2020. Making waves: Wastewater-based epidemiology for COVID-19 – approaches and challenges for surveillance and prediction. Water Res. 186, 116404. https://doi.org/10.1016/j.watres.2020.116404

Price, R.H.M., Graham, C., Ramalingam, S., 2019. Association between viral seasonality and meteorological factors. Sci. Rep. https://doi.org/10.1038/s41598-018-37481-y

Riquelme, M. V, Garner, E., Gupta, S., Metch, J., Zhu, N., Blair, M.F., Arango-Argoty, G., Maile-Moskowitz, A., Li, A., Flach, C.-F., Aga, D.S., Nambi, I., Larsson, D.G.J., Bürgmann, H., Zhang, T., Pruden, A., Vikesland, P.J., 2021. Wastewater Based Epidemiology Enabled Surveillance of Antibiotic Resistance. medRxiv. https://doi.org/10.1101/2021.06.01.21258164

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Singh, R.R., Angeles, L.F., Butryn, D.M., Metch, J.W., Garner, E., Vikesland, P.J., Aga, D.S., 2019. Towards a harmonized method for the global reconnaissance of multi-class antimicrobials and other pharmaceuticals in wastewater and receiving surface waters. Environ. Int. https://doi.org/10.1016/j.envint.2019.01.025

Stanton, I.C., Murray, A.K., Zhang, L., Snape, J., Gaze, W.H., 2020. Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. Commun. Biol. https://doi.org/10.1038/s42003-020-01176-w

Varela, A.R., André, S., Nunes, O.C., Manaia, C.M., 2014. Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. Water Res. 54, 327–336. https://doi.org/10.1016/j.watres.2014.02.003

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci. Total Environ. 430, 109–118. https://doi.org/10.1016/j.scitotenv.2012.04.055

Wan, K., Zhang, M., Ye, C., Lin, W., Guo, L., Chen, S., Yu, X., 2019. Organic carbon: An overlooked factor that determines the antibiotic resistome in drinking water sand filter biofilm. Environ. Int. https://doi.org/10.1016/j.envint.2019.01.054

World Health Organisation, 2020. Antimicrobial resistance [WWW Document]. URL https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (accessed 9.15.21).

World Health Organisation, 2019. WHO consolidated guidelines on drug-resistant tuberculosis treatment.

World Health Organisation, 2018. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017-2018.

World Health Organisation, 2017. GLOBAL PRIORITY LIST OF ANTIBIOTIC-RESISTANT BACTERIA TO GUIDE RESEARCH, DISCOVERY, AND DEVELOPMENT OF NEW ANTIBIOTICS.

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X., Meng, W., 2015. Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. Chemosphere 119, 1379–1385. https://doi.org/10.1016/j.chemosphere.2014.02.040

Yuan, S.F., Liu, Z.H., Huang, R.P., Yin, H., Dang, Z., 2016. Levels of six antibiotics used in China estimated by means of wastewater-based epidemiology. Water Sci. Technol. https://doi.org/10.2166/wst.2015.526

Zhang, Y., Duan, L., Wang, B., Du, Y., Cagnetta, G., Huang, J., Blaney, L., Yu, G., 2019. Wastewater-based epidemiology in Beijing, China: Prevalence of antibiotic use in flu season and association of pharmaceuticals and personal care products with socioeconomic characteristics. Environ. Int. 125, 152–160. https://doi.org/10.1016/j.envint.2019.01.061

Zheng, W., Huyan, J., Tian, Z., Zhang, Y., Wen, X., 2020. Clinical class 1 integron-integrase gene – A promising indicator to monitor the abundance and elimination of antibiotic resistance genes in an urban wastewater treatment plant. Environ. Int. 135, 105372. https://doi.org/10.1016/j.envint.2019.105372

Zhou, Z.-C., Zheng, J., Wei, Y.-Y., Chen, T., Dahlgren, R.A., Shang, X., Chen, H., 2017. Antibiotic resistance genes in an urban river as impacted by bacterial community and physicochemical parameters. Environ. Sci. Pollut. Res. 24, 23753–23762. https://doi.org/10.1007/s11356-017-0032-0

Zieliński, W., Korzeniewska, E., Harnisz, M., Drzymała, J., Felis, E., Bajkacz, S., 2021. Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes – An epidemiological threat to workers and environment. Environ. Int. 156, 106641. https://doi.org/10.1016/j.envint.2021.106641

Zuccato, E., Chiabrando, C., Castiglioni, S., Bagnati, R., Fanelli, R., 2008. Estimating Community Drug Abuse by Wastewater Analysis. Environ. Health Perspect. 116, 1027–1032. https://doi.org/10.1289/ehp.11022

Supplementary Information

Community and hospital derived antimicrobials and resistance genes in a two-city: a one-year longitudinal study

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Water quality indicators (WQIs) methodology

Water quality indicators (WQIs) were analysed at Wessex Water. An Aquakem (Thermo Scientific) analyser was used for the quantitative measurement of water quality indicators, with the exception of COD. COD was analysed spectrophotometrically. Briefly, samples, either shaken or settled, were oxidised in tubes with chromic acid, a mixture of potassium dichromate, sulphuric acid in the presence of silver sulphate as a catalyst and mercuric ions to counteract interference from chloride. The sealed tubes are heated to 150 °C for three hours, cooled, and the degree of oxidation determined by spectrophotometry. The procedure is calibrated by processing a solution of potassium hydrogen phthalate as a standard material.

Al, Fe and Mn were analysed via inductively coupled plasma mass spectrometry (ICP/MS), firstly digestion of sample at 80 ± 5°C overnight in an acidic medium occurs to bring the metals into solution. Turbid samples are filtered by digestion before diluting and dosed with internal standard (Scandium, Gallium, Indium and Thallium). Al: Limits of quantification (LOQ), 0.01 mg/L, range: 0.01-2.00 mg/L. Fe: LOQ, 0.02 mg/L, range: 0.01-2.00 mg/L. Mn: LOQ, 0.001 mg/L, range: 0.001-0.500 mg/L.

Dissolved organic carbon (DOC) and total organic carbon (TOC). A SKALAR analyser was utilised for the analysis of dissolved organic carbon (DOC) and for total organic carbon (TOC). For TOC, samples are acidified to pH <3 to allow for the removal of all inorganic carbon prior to a digestion step. Digestion is achieved by pumping the digested mixture into a quartz mixing coil around a ultraviolet (UV) light source. Following this, dialysis is performed where a dialyzer separates the sample stream with the colour reagent stream by a CO_2 semi-permeable membrane. For dissolved organic carbon, an aliquot of samples are first filtered through a 0.4 μm GF/F Glass Microfiber Filters Diameter 47 mm. Limit of detection (LOD) is 0.4 mg/L with LOQ of 0.45 mg/L, range: 0-12 mg/L. Suspended solids: a known volume of sample is filtered through a previously washed, dried and weighed filter paper. After drying, the filter paper is re-weighed and the suspended solids deposited on the paper can be calculated. LOD, 5 mg/L if 200 mL of sample is filtered and 2 mg/L if 1000 mL of sample is filtered.

Biological oxygen demand (BOD): samples are diluted as necessary before incubating at 20 ± 0.5 °C in the dark for five days (117.5 ± 4.5 hrs). Following this, the sample is then seeded with bacteria of appropriate activity. The concentration of dissolved oxygen in the sample can then be determined both before and after the incubation period. The difference between these two readings can be used to calculate the BOD of the sample.

Phosphorus levels was determined via inductively couple plasma optical emission spectrometry (ICP-OES) using an Agilent 5110 system. Calibration range, 0-2 ppm.

Ammonia N: utilises ammonia reaction with sodium salicylate and hypochlorite ions, which are generated in situ by the alkaline hydrolysis of sodium dichloroisocyanurate. The absorbance of a blue product formed at pH 12.6 in the presence of sodium nitroprusside is measured spectrophotometrically at 660 nm and related to the ammonia concentration in the sample by means of a calibration curve (LOQ, 0.02 mg/L, range: 0.02-100 mg/L).

N total (TON): Nitrate is reduced to nitrite by hydrazine under alkaline conditions, using copper (II) ions as a catalyst. The total nitrite is then treated with sulphanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic conditions (in the presence of orthophosphoric acid). The absorbance of a characteristic pink azo – dye is measured spectrophotometrically at 540 nm and related to the total oxidised nitrogen concentration in the sample by means of a calibration curve (LOQ, 0.3 mg/L, range: 0.3-50 mg/L).

Nitrite: The diazotisation of sulphanilamide by nitrite in the presence of orthophosphoric acid, at pH 1.9, leads to the formation of an azo-dye with N-1-napthylethylenediamine. Its absorbance is then

measured at 540 nm and is related to the nitrite concentration by means of a calibration curve (LOQ, 0.03 mg/L, range: 0.03-10 mg/L).

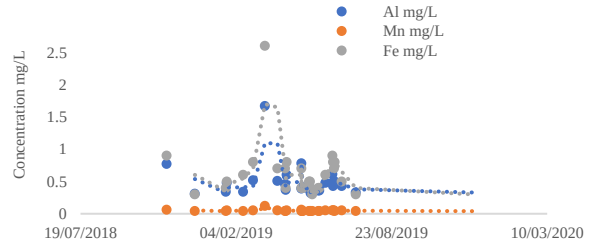
Nitrate: Nitrate is calculated using TON minus Nitrite. The calculation takes place after the samples have been analysed for both chemistries.

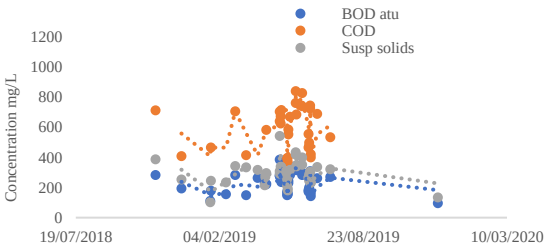
Ortophosphate: Orthophosphate ions react with a solution containing molybdic acid, ascorbic acid and antimony (II) ions in the presence of acid, to form a 12-molybdophosphoric acid. This is reduced in situ to a blue heteropoly compound (phosphomolybdenum blue) in which antimony is incorporated. The absorbance of the compound is measured spectrophotometrically at 880 nm and related to the orthophosphate concentration in the sample by means of a calibration curve. Soluble reactive phosphorus uses the same method as above, but the sample is filtered through a 0.45µm filter prior to analysing (LOQ, 0.06 mg/L, range 0.6-20 mg/L).

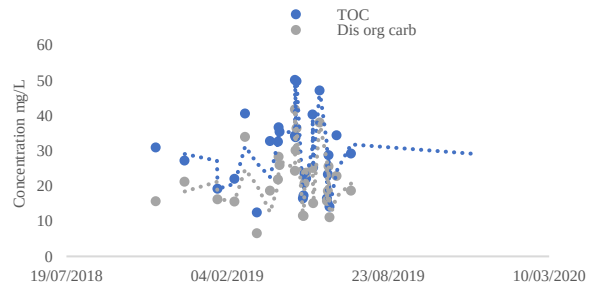
Chloride: Chloride ions were mixed with acid chloride colour reagent containing mercury (II) thiocyanate. The released thiocyanate ions then react in acid solution with iron (III) nitrate to give a reddish-brown coloured iron (III) thiocyanate complex. The resulting intensity of the stable colour produced is measured at a wavelength of 480 nm and is related to the chloride concentration by means of a calibration curve (LOQ 1 mg/L, range 1-1000 mg/L).

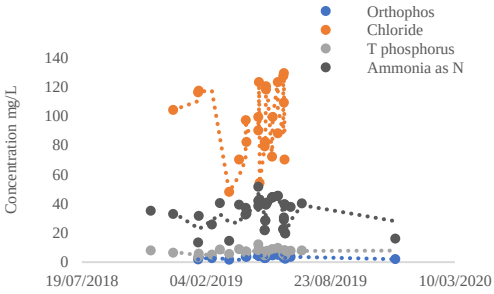
Results in studied sampling period:

Bath

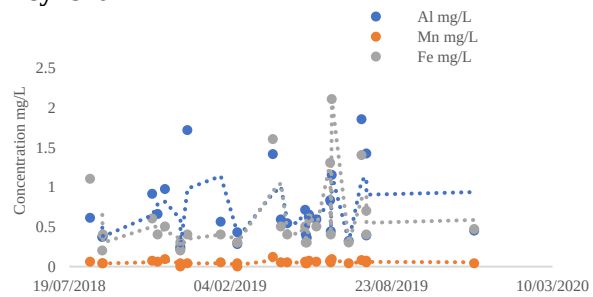


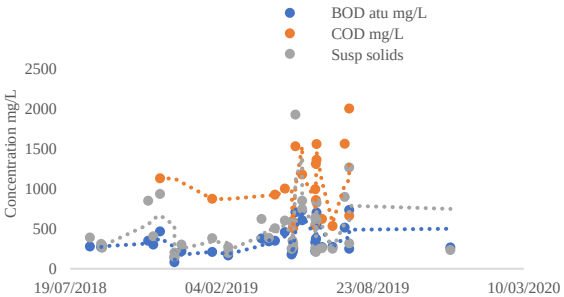






Keynsham





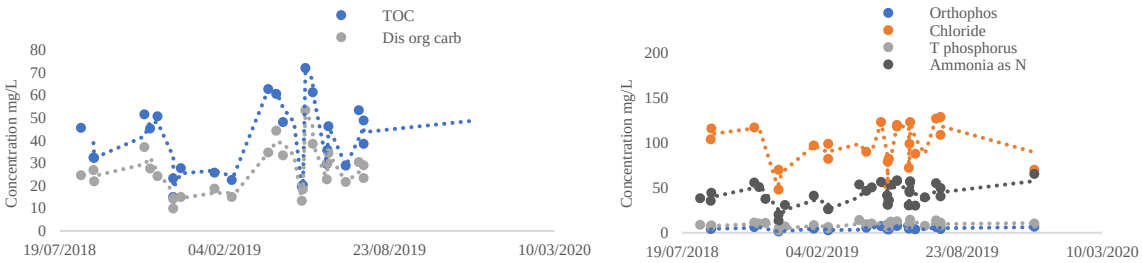


Figure 8. WQIs for Bath and Keynsham, plotted with two point moving averages trendlines.

Table S1: Chemical information of AA targets, ordered by class groupings, table taken from Holton and Kasprzyk-Hordern, 2021, https://rdcu.be/cxqhT

Grouping	Chemical	Abbrev	Class A	Class B	CAS No.	Salt form θ	Molec. Formula	M.I. mass	Supplier
Sulfonamide &	Sulfadiazine	SDZ	Sulfonamide	Parent	68-35-9		C10H10N4O2S	250.05	Sigma-Aldrich
Trimethoprim	Sulfapyridine	SPY	Sulfonamide	Parent	144-83-2		C11H11N3O2S	249.06	Sigma-Aldrich
	Sulfamethoxazole	SMX	Sulfonamide	Parent	723-46-6		C10H11N3O3S	253.05	Sigma-Aldrich
	Sulfasalazine	SLZ	Sulfonamides	Parent	599-79-1		C18H14N4O5S	398.07	Sigma-Aldrich
	Trimethoprim	TMP	Trimethoprim	Parent	738-70-5		C14H18N4O3	290.14	Sigma-Aldrich
	N-acetyl sulfadiazine	aSDZ	Sulfonamide	Metabolite	127-74-2		C12H12N4O3S	292.06	TRC
	N-acetyl sulfapyridine	aSPY	Sulfonamide	Metabolite	19077-98-6		C13H13N3O3S	291.07	TRC
	N-acetyl sulfamethoxazole	aSMX	Sulfonamide	Metabolite	21312-10-7		C12H13N3O4S	295.06	TRC
	4-hydroxy-trimethoprim	hTMP	Trimethoprim	Metabolite	112678-48-5		C14H18N4O4	306.13	TRC
Macrolide	Azithromycin	AZM	Macrolide	Parent	83905-01-5		C38H72N2O12	748.51	LCG
& Lincomycin	Erythromycin	ERY	Macrolide	Parent	114-07-8		C37H67NO13	733.46	Sigma-Aldrich
	Clarithromycin	CLR	Macrolide	Parent	81103-11-9		C38H69NO13	747.48	Sigma-Aldrich
	Clindamycin	CLI	Lincomycin	Parent	18323-44-9	Hydrochloride	C18H33ClN2O5S	424.18	Sigma-Aldrich
	N-desmethyl azithromycin	dmAZM	Macrolide	Metabolite	172617-84-4		C37H70N2O12	734.49	TRC
	N-desmethyl erythromycin A	dmERY	Macrolide	Metabolite	992-62-1		C36H65NO13	719.45	TRC
	N-desmethyl clarithromycin	dmCLR	Macrolide	Metabolite	101666-68-6		C37H67NO13	733.46	TRC
	N-desmethyl clindamycin	dmCLI	Lincomycin	Metabolite	22431-45-4		C17H31ClN2O5S	410.16	TRC
β-LACTAMS									
Penicillin	Amoxicillin	AMX	Penicillin	Parent	26787-78-0		C16H19N3O5S	365.10	Fluka
	Ampicillin	AMP	Penicillin	Parent	69-53-4	Trihydrate	C16H19N3O4S	349.11	Fluka
	Flucloxacillin	FLX	Penicillin	Parent	5250-39-5	Sodium	C19H17ClFN3O5S	453.06	Fluka
	Penicillin G	PenG	Penicillin	Parent	113-98-4	Sodium	C16H18N2O4S	334.10	Fluka
	Penicillin V	PenV	Penicillin	Parent	132-98-9	Potassium	C16H18N2O5S	350.09	Sigma-Aldrich
	Amoxicilloic acid	AMXa	Penicillin	Metabolite	42947-63-7 ¥	Trisodium salt	C16H21N3O6S	383.12	TRC
	Ampicilloic acid	AMPa	Penicillin	Metabolite	32746-94-4		C16H21N3O5S	367.12	TRC
	Penicilloic G acid	PenGa	Penicillin	Metabolite	11039-68-2		C9H14N2O5S	262.06	TRC
Cefalosporin	Cefalexin	LEX	Cefalosporin	Parent	23325-78-2	Monohydrate	C16H17N3O4S	347.09	Fluka

	Cefixime	CFM	Cefalosporin	Parent	79350-37-1	Trihydrate	C16H15N5O7S2	453.04	Fluka
	Ceftiofur	CTF	Cefalosporin	Parent	104010-37-9		C19H17N5O7S3	523.03	Fluka
	Ceftriaxone	CRO	Cefalosporin	Parent	104376-79-6	Disodium hemi(heptahydrate)	C18H18N8O7S3	554.05	Sigma-Aldrich
Monobactam	Aztreonam	ATM	Monobactam	Parent	78110-38-0		C13H17N5O8S2	435.05	TRC
Carbapenem	Imipenem	IPM	Carbapenem	Parent	64221-86-9		C12H17N3O4S	299.09	Sigma-Aldrich
	Meropenem	MEM	Carbapenem	Parent	119478-56-7	Trihydrate	C17H25N3O5S	383.15	Sigma-Aldrich
Quinolone	Besifloxacin	BSF	Quinolone	Parent	405165-61-9	Hydrochloride	C19H21ClFN3O3	393.13	MCE
	Ciprofloxacin	CIP	Quinolone	Parent/Metab.	85721-33-1		C17H18FN3O3	331.13	Fluka
	Danofloxacin	DFX	Quinolone	Parent	119478-55-6	Mesylate	C19H20FN3O3	357.15	LCG
	Enrofloxacin	ENR	Quinolone	Parent	93106-60-6		C19H22FN3O3	359.16	Sigma-Aldrich
	Flumequine	FLU	Quinolone	Parent	42835-25-6		C14H12FNO3	261.08	Fluka
	Gatifloxacin	GAT	Quinolone	Parent	112811-59-3		C19H22FN3O4	375.16	TRC
	Lomefloxacin	LOM	Quinolone	Parent	98079-52-8	Hydrochloride	C17H19F2N3O3	351.14	Sigma-Aldrich
	Moxifloxacin	MXF	Quinolone	Parent	268545-13-7	Hydrochloride	C21H24FN3O4	401.18	MCE
	Nadifloxacin	NAD	Quinolone	Parent	124858-35-1		C19H21FN2O4	360.15	MCE
	Nalidixic acid	NAL	Quinolone	Parent	389-08-2	Sodium	C12H12N2O3	232.08	Sigma-Aldrich
	Norfloxacin	NOR	Quinolone	Parent	70458-96-7		C16H18FN3O3	319.13	Sigma-Aldrich
	Ofloxacin (Levofloxacin) *	OFX	Quinolone	Parent	82419-36-1		C18H20FN3O4	361.14	Sigma-Aldrich
	Prulifloxacin	PFLX	Quinolone	Parent (prodrug)	123447-62-1		C21H20FN3O6S	461.11	Sigma-Aldrich
Grouping	**Chemical**	**Abbrev**	**Class A**	**Class B**	**CAS No.**	**Salt form θ**	**Molec. Formula**	**M.I. mass**	**Supplier**
	Sarafloxacin	SRF	Quinolone	Parent	91296-87-6	Hydrochloride	C20H17F2N3O3	385.12	Sigma-Aldrich
	Desethylene ciprofloxacin	deCIP	Quinolone	Metabolite	528851-31-2	Hydrochloride	C15H16FN3O3	305.12	TRC
	Hydroxy-norfloxacin	hNOR	Quinolone	Metabolite	109142-49-6		C16H18FN3O4	335.13	TRC
	Ofloxacin N-oxide	OFXo	Quinolone	Metabolite	104721-52-0	Acetic acid salt	C18H20FN3O5	377.14	TRC
	Desmethyl-ofloxacin	dmOFX	Quinolone	Metabolite	82419-52-1		C17H18FN3O4	347.13	TRC
	Ulifloxacin	UFX	Quinolone	Metabolite	112984-60-8		C16H16FN3O3S	349.09	TRC
TB (1st line)	Isoniazid	INH	Isoniazid	Parent	54-85-3		C6H7N3O	137.06	Sigma-Aldrich
	Pyrazinamide	PZA	Pyrazinamide	Parent	98-96-4		C5H5N3O	123.04	Sigma-Aldrich
	Ethambutol	EMB	Ethambutol	Parent	74-55-5	Dihydrochloride	C10H24N2O2	204.18	Sigma-Aldrich
	Rifampicin	RMP	Rifamycin	Parent	13292-46-1		C43H58N4O12	822.41	Sigma-Aldrich

	Rifabutin	RFB	Rifamycin	Parent	72559-06-9		C46H62N4O11	846.44	Sigma-Aldrich
	Isonicotinic acid	INa	Isoniazid	Metabolite	55-22-1		C6H5NO2	123.03	Sigma-Aldrich
	Acetyl-isoniazid	aINH	Isoniazid	Metabolite	1078-38-2		C8H9N3O2	179.07	Sigma-Aldrich
	5-Hydroxy-pyrazinoic acid	hPZA	Pyrazinamide	Metabolite	34604-60-9		C5H4N2O3	140.02	Sigma-Aldrich
	25-desacetyl rifampicin	daRMP	Rifamycin	Metabolite	16783-99-6		C41H56N4O11	780.39	Sigma-Aldrich
	25-O-desacetyl rifabutin	daRFB	Rifamycin	Metabolite	100324-63-8		C44H60N4O10	804.43	TRC
TB (MDR)	Capreomycin IA ≠	CAPIa	Aminoglycoside	Parent	1405-37-4	Sulfate	C25H44N14O8	668.35	TRC
	Capreomycin IB ≠	CAPIb	Aminoglycoside	Parent	1405-37-4	Sulfate	C25H44N14O7	652.35	TRC
	Gentamycin C1 ≠	GEN1	Aminoglycoside	Parent	1405-41-0	Sulfate salt hydrate	C21H43N5O7	477.32	Fluka
	Gentamycin C1a ≠	GEN1a	Aminoglycoside	Parent	1405-41-0	Sulfate salt hydrate	C19H39N5O7	449.28	Fluka
	Gentamycin C2 C2a C2b ≠	GEN2	Aminoglycoside	Parent	1405-41-0	Sulfate salt hydrate	C20H43N5O7	465.32	Fluka
	Kanamycin A ≠	KAN	Aminoglycoside	Parent	25389-94-0	Sulfate	C18H36N4O11	484.24	Sigma-Aldrich
	Streptomycin A ≠	STR	Aminoglycoside	Parent	3810-74-0	Sulfate	C21H39N7O12	581.27	Sigma-Aldrich
	D-cycloserine	DCS	Isoxazole	Parent/Metab.	68-41-7		C3H6N2O2	102.04	TRC
TB (other)	Delamanid	DMD	Nitroimidazole	Parent	681492-22-8		C25H25F3N4O6	534.17	Sigma-Aldrich
	Bedaquiline	BDQ	Diarylquinoline	Parent	843663-66-1		C32H31BrN2O2	554.16	Sigma-Aldrich
	Linezolid	LZD	Oxazolidinone	Parent	165800-03-3		C16H20FN3O4	337.14	Sigma-Aldrich
	Thalidomide	THAL	Thalidomide	Parent	50-35-1		C13H10N2O4	258.06	Sigma-Aldrich
OTHER									
Amphenicol	Chloramphenicol	CHL	Amphenicol	Parent	56-75-7		C11H12Cl2N2O5	322.01	Sigma-Aldrich
	Florfenicol	FLO	Amphenicol	Parent	73231-34-2		C12H14Cl2FNO4S	357.00	MCE
	2-Amino-1-(4-nitrophenyl)-1,3-propanediol	ANP	Amphenicol	Metabolite	2964-48-9		C9H12N2O4	212.08	Sigma-Aldrich
Cycline	Doxycycline	DOX	Cycline	Parent	24390-14-5	Hyclate	C22H24N2O8	444.15	Sigma-Aldrich
	Oxytetracycline	OTC	Cycline	Parent	2058-46-0	Hydrochloride	C22H24N2O9	460.15	TRC
	Tetracycline	TET	Cycline	Parent	64-75-5	Hydrochloride	C22H24N2O8	444.15	Sigma-Aldrich
Nitrofuran	Nitrofurantoin	NIT	Nitrofuran	Parent	67-20-9		C8H6N4O5	238.03	Sigma-Aldrich
	1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione	NPAHD	Nitrofuran	Metabolite	623145-57-3		C10H8N4O4	248.05	TRC
Azole	Metronidazole	MTZ	Azole	Parent	443-48-1		C6H9N3O3	171.06	Sigma-Aldrich

	Ketoconazole	KTC	Azole	Parent	65277-42-1	C26H28Cl2N4O4	530.15	Sigma-Aldrich
	Hydroxy-metronidazole	hMTZ	Azole	Metabolite	1215071-08-1	C6H9N3O4	187.06	TRC
	Deacetyl-ketoconazole	daKTC	Azole	Metabolite	67914-61-8	C24H26Cl2N4O3	488.14	TRC
Antiretroviral	Emtricitabine	FTC	ARV	Parent	143491-57-0	C8H10FN3O3S	247.04	TRC
	Lamivudine	3TC	ARV	Parent	134678-17-4	C8H11N3O3S	229.05	TRC

Monoisotopic mass (M.I. mass), multi-drug resistant (MDR), tuberculosis (TB), nucleoside reverse transcriptase inhibitor (NRTI)

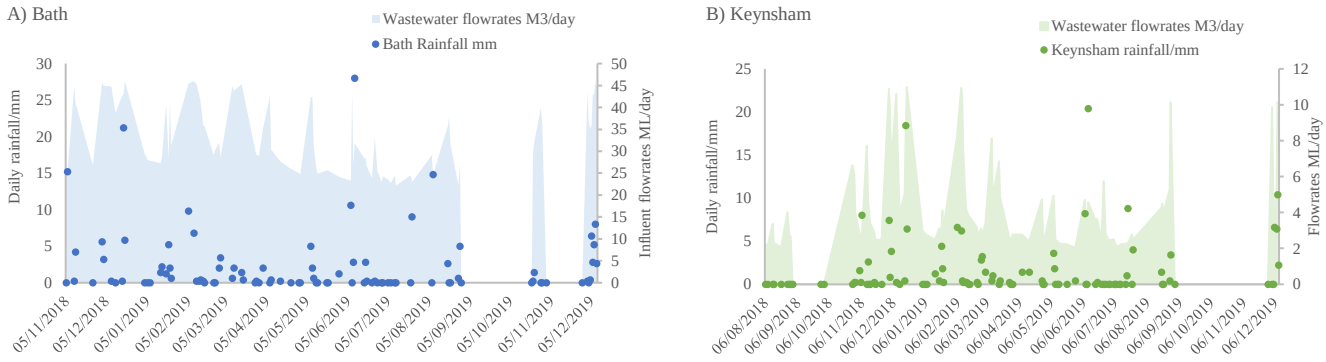
LC-MS method is not chiral (*); one standard used for all forms within the drug complex (≠); CAS for chiral free acid (¥); salt corrections considered in all calculations, i.e., analysis of the free base (θ)

Table S2. Internal standards used along with instrument and method detection limits (IDLs and MDLs) and instrument and method quantification limits (IQLs and MQLs) table adapted from Holton and Kasprzyk-Hordern, 2021, https://rdcu.be/cxqhT

			Mobile phase (ng L-1)		Influent wastewater (ng L-1)	
Class	**Abbrev**	**Internal Standard**	**IDL**	**IQL**	**MDL**	**MQL**
Sulfonamide &	SDZ	Sulfamethoxazole D4	0.015	0.050	0.230	0.76
Trimethoprim	SPY	Sulfamethoxazole D4	0.003	0.010	0.026	0.088
	SMX	Sulfamethoxazole D4	0.002	0.005	0.014	0.046
	SLZ	Sulfamethoxazole D4	1.500	5.000	5.130	17.09
	TMP	Trimethoprim D9	0.150	0.500	0.970	3.22
	aSDZ	Sulfamethoxazole D4	0.021	0.070	0.230	0.77
	aSPY	Sulfamethoxazole D4	0.167	0.556	1.350	4.51
	aSMX	Sulfamethoxazole D4	0.019	0.063	0.200	0.66
	hTMP	Trimethoprim D9	0.004	0.013	0.030	0.1
Macrolide	AZM	Clarithromycin D3	0.015	0.050	-	-
& Lincomycin	ERY	Clarithromycin D3	0.002	0.005	0.016	0.053
	CLR	Clarithromycin D3	0.002	0.005	0.014	0.046
	CLI	Flumequine 13C3	0.150	0.500	-	-
	dmAZM	Clarithromycin D3	0.375	1.250	-	-
	dmERY	Clarithromycin D3	0.002	0.007	0.020	0.07
	dmCLR	Clarithromycin D3	0.005	0.017	0.070	0.25
	dmCLI	Flumequine 13C3	0.002	0.005	-	-
β-LACTAMS	AMX	Sulfamethoxazole D4	1.500	5.000	-	-
Penicillin	AMP	Ampicillin D5	1.500	5.000	55.600	185
	FLX	Flumequine 13C3	0.150	0.500	1.630	5.45
	PenG	Penicillin G D7	0.150	0.500	5.470	18.2
	PenV	Penicillin G D7	3.000	10.000	81.300	271
	AMXa	Sulfamethoxazole D4	0.300	1.000	146.000	488
	AMPa	Ampicillin D5	0.150	0.500	31.800	106
	PenGa	Penicillin G D7	0.150	0.500	15.400	51.5
Cefalosporin	LEX	Trimethoprim D9	0.375	1.250	5.910	19.7
	CFM	Trimethoprim D9	1.500	5.000	13.200	44
	CTF	Flumequine 13C3	0.150	0.500	-	-
	CRO	Trimethoprim D9	7.500	25.000	48.400	161
Monobactam	ATM	Trimethoprim D9	0.300	1.000	-	-
Carbapenem	IPM	Metronidazole D4	1.500	5.000	-	-
	MEM	Trimethoprim D9	1.500	5.000	-	-
Quinolone	BSF	Flumequine 13C3	0.375	1.250	3.150	10.5
	CIP	Desmethyl-ofloxacin D8	0.150	0.500	1.130	3.77
	DFX	Desmethyl-ofloxacin D8	1.500	5.000	13.900	46.3
	ENR	Desmethyl-ofloxacin D8	0.030	0.100	0.250	0.82
	FLU	Flumequine 13C3	0.003	0.010	0.030	0.1
	GAT	Desmethyl-ofloxacin D8	0.003	0.010	0.030	0.09
	LOM	Desmethyl-ofloxacin D8	0.030	0.100	0.280	0.95
	MXF	Desmethyl-ofloxacin D8	0.375	1.250	3.040	10.1
	NAD	Flumequine 13C3	0.300	1.000	2.680	8.92

	NAL	Flumequine 13C3	0.003	0.010	0.030	0.11
	NOR	Desmethyl-ofloxacin D8	0.003	0.010	0.021	0.069
	OFX	Desmethyl-ofloxacin D8	0.030	0.100	0.410	1.37
	PFLX	Desmethyl-ofloxacin D8	0.300	1.000	1.780	5.95
	SRF	Desmethyl-ofloxacin D8	0.150	0.500	1.500	5.01
	deCIP	Desmethyl-ofloxacin D8	0.150	0.500	1.440	4.82
	hNOR	Desmethyl-ofloxacin D8	3.600	12.000	38.600	129
	OFXo	Desmethyl-ofloxacin D8	3.600	12.000	36.500	122
	dmOFX	Desmethyl-ofloxacin D8	0.150	0.500	1.340	4.45
	UFX	Desmethyl-ofloxacin D8	1.500	5.000	11.400	38.1
TB DRUGS	INH	Isoniazid D4	0.150	0.500	1.520	5.06
TB (1st line)	PZA	Metronidazole D4	1.500	5.000	-	-
	EMB	Metronidazole D4	0.003	0.010	0.130	0.42
	RMP	Rifabutin D7	0.375	1.250	-	-
	RFB	Rifabutin D7	0.150	0.500	1.680	5.62
	INa	Isoniazid D4	0.150	0.500	7.200	24
	aINH	Isoniazid D4	0.150	0.500	3.580	11.9
	hPZA	Metronidazole D4	0.030	0.100	-	-
	daRMP	Rifabutin D7	1.500	5.000	15.000	50
	daRFB	Rifabutin D7	0.030	0.100	0.330	1.11
TB (MDR)	CAPIa	Metronidazole D4	6.621	22.071	4528.000	15094
	CAPIb	Metronidazole D4	6.621	22.071	-	-
	GEN1	Metronidazole D4	2.820	9.400	-	-
	GEN1a	Metronidazole D4	2.070	6.900	-	-
	GEN2	Metronidazole D4	5.250	17.500	-	-
	KAN	Metronidazole D4	3.563	11.875	-	-
	STR	Metronidazole D4	12.000	40.000	2650.000	8835
	DCS	Metronidazole D4	0.150	0.500	-	-
TB (other)	DMD	Rifabutin D7	0.150	0.500	-	-
	BDQ	Rifabutin D7	1.500	5.000	-	-
	LZD	Chloramphenicol D5	0.030	0.100	0.270	0.89
	THAL	Trimethoprim D9	0.300	1.000	3.480	11.6
OTHER	CHL	Chloramphenicol D5	0.150	0.500	1.310	4.35
Amphenicol	FLO	Chloramphenicol D5	3.000	10.000	21.900	73.1
	ANP	Metronidazole D4	1.500	5.000	42.300	141
Cycline	DOX	Rifabutin D7	0.375	1.250	12.700	42.2
	OTC	Desmethyl-ofloxacin D8	0.300	1.000	6.350	21.2
	TET	Desmethyl-ofloxacin D8	0.150	0.500	1.700	5.66
Nitrofuran	NIT	Nitrofurantoin 13C3	0.300	1.000	3.460	11.54
	NPAHD	Chloramphenicol D5	0.030	0.100	0.350	1.18
Azole	MTZ	Metronidazole D4	0.030	0.100	0.270	0.91
	KTC	Flumequine 13C3	0.003	0.010	0.030	0.1
	hMTZ	Metronidazole D4	0.030	0.100	1.010	3.38
	daKTC	Flumequine 13C3	0.375	1.250	-	-
Antiviral	FTC	Metronidazole D4	0.150	0.500	1.570	5.24
	3TC	Metronidazole D4	0.300	1.000	9.510	31.7

Figure S1. Influent wastewater flow data (m3/day) and daily rainfall data (mm) for Bath and Keynsham over the days that were sampled only during the sampling period of 2018-2019.



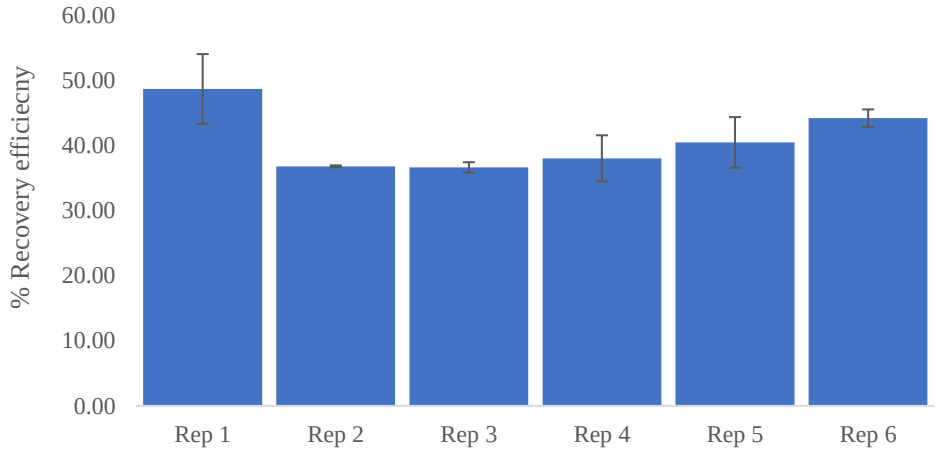


Figure S2: Recovery efficiency (%) of TaqMan™ Universal DNA Spike in Control from one wastewater sample extracted out six times (rep = replicates)

Table S3. p values for seasonal T-test results for AAs in Bath and Keynsham

	Saltford			Keynsham		
	Winter '18 vs Summer '19	**Winter '18 vs Spring '19**	**Spring '19 vs Summer '19**	**Winter '18 vs Summer '19**	**Winter '18 vs Spring '19**	**Spring '19 vs Summer '19**
CLR	**0.0000**	**0.0004**	**0.0017**	**0.0000**	**0.0328**	0.9634
dmCLR	**0.0000**	**0.0293**	**0.0017**	**0.0185**	0.2547	0.9684
ERY	**0.0007**	**0.0000**	**0.0000**	**0.0001**	0.1987	**0.0002**
dmERY	**0.0000**	0.0789	**0.0061**	-	-	-
INa	**0.0002**	**0.0000**	**0.0117**	**0.0000**	**0.0000**	0.8644
HPZA	0.9101	0.8481	0.9217	0.1570	0.3625	0.0874
MTZ	0.7254	**0.0012**	0.3003	**0.0019**	0.1314	0.4331
hMTZ	**0.0111**	**0.0000**	0.0889	**0.0053**	**0.0126**	0.9513
KTC	**0.0008**	**0.0365**	0.6252	**0.0047**	**0.0039**	0.5656
Lam	0.6512	**0.0028**	**0.0339**	**0.0236**	0.1215	0.7903
EMT	0.3880	**0.0001**	**0.0130**	0.9540	0.0570	0.1380
SPY	0.4669	0.1470	0.4605	0.7779	0.1261	0.0862
aSPY	**0.0000**	**0.0072**	0.0916	0.1446	0.0789	**0.0330**
SMX	0.5769	0.7892	0.4421	**0.0003**	0.1416	0.3101
aSMX	**0.0010**	0.1419	0.3336	0.0999	0.9581	0.1659
SLZ	0.0966	0.2900	**0.0080**	0.6975	0.0854	0.2104
FLX	0.1050	**0.0446**	0.6838	0.3324	**0.0112**	**0.0195**
Tet	**0.0000**	0.3600	**0.0004**	**0.0040**	**0.0003**	0.7901
OTC	**0.0000**	0.2852	**0.0011**	**0.0277**	**0.0014**	0.6613
CIP	**0.0000**	**0.0016**	0.5434	0.1224	**0.0001**	**0.0035**
OFX	0.3925	0.0644	0.8748	**0.0002**	0.2191	**0.0005**
TMP	**0.0003**	**0.0273**	0.1310	**0.0046**	0.1290	0.6980
Cli	**0.0005**	0.0728	0.3654	0.4820	**0.0039**	0.0841
dmCLI	0.9452	0.8982	0.8562	0.1524	**0.0002**	**0.0442**

*Statistically significant results are shown in **bold** ($p \leq 0.05$)

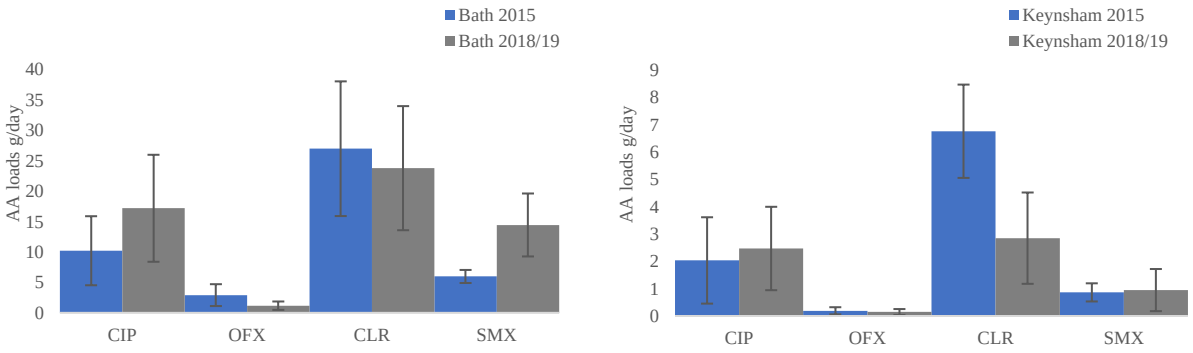


Figure S3. Comparison of averaged daily loads of AAs from 2015 (one week average) versus 2018/19 (12/13 months average for Bath and Keynsham respectively).

Table S4. ARG profiling from metagenomic sequencing data of influent wastewater sampled from Bath in November 2018 and March 2019. The % mapped donates the % coverage of each gene by the reads of each sample

					% mapped, only >80% shown	
Antibiotic Class	**Gene Family***	**Resistance Mechanism***	**Observed Pathogen****	**Gene**	**29/03/2019**	**13/11/2018**
Aminoglycoside antibiotic	ANT(2")	antibiotic inactivation	Pseudomonas aeruginosa	ANT(2")-Ia	100	100
	APH(2")	antibiotic inactivation	Enterococcus gallinarum	APH(2")-IIIa	100	
	APH(3")	antibiotic inactivation	Pseudomonas aeruginosa	APH(3")-Ib	100	100
	APH(3')	antibiotic inactivation	Campylobacter coli	APH(3')-IIIa	100	
	APH(6)	antibiotic inactivation	Pseudomonas aeruginosa	APH(6)-Id	100	100
	ANT(3")	antibiotic inactivation	Pseudomonas aeruginosa	aadA11		100
	ANT(3")	antibiotic inactivation	Acinetobacter lwoffii	aadA27	100	100
	ANT(3")	antibiotic inactivation	Pseudomonas aeruginosa	aadA6		100
	ANT(3")	antibiotic inactivation	Pseudomonas aeruginosa	aadA6/aadA10	81	95
	ANT(3")	antibiotic inactivation	Escherichia coli	aadA		82
Rifamycin antibiotic	rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	antibiotic target alteration; antibiotic target replacement	Bifidobacterium adolescentis	Bifidobacterium adolescentis rpoB conferring resistance to rifampicin	97	88
Penam	CARB beta-lactamase	antibiotic inactivation	Acinetobacter baumannii	CARB-10	96	
	RCP beta-lactamase	antibiotic inactivation	Rhodobacter capsulatus	RCP-1	100	
Mupirocin	antibiotic resistant isoleucyl-tRNA synthetase (ileS)	antibiotic target alteration	Bifidobacterium bifidum	Bifidobacteria intrinsic ileS conferring resistance to mupirocin	82	
Phenicol antibiotic	chloramphenicol acetyltransferase (CAT)	antibiotic inactivation	Campylobacter coli	Campylobacter coli chloramphenicol acetyltransferase	82	.
Macrolide antibiotic	macrolide esterase	antibiotic inactivation	Riemerella anatipestifer	EreD	100	
	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	Photobacterium damselae	mefC	100	98
	macrolide phosphotransferase (MPH)	antibiotic inactivation	uncultured bacterium	mphE	100	100
	macrolide phosphotransferase (MPH)	antibiotic inactivation	Photobacterium damselae	mphG	100	100
Peptide antibiotic	intrinsic colistin resistant phosphoethanolamine transferase	antibiotic target alteration	Moraxella osloensis	ICR-Mo	100	97
	undecaprenyl pyrophosphate related proteins	antibiotic target alteration	Escherichia coli	bacA	89	
Fluoroquinolone antibiotic	quinolone resistance protein (qnr)	antibiotic target protection	Salmonella enterica	QnrS2	88	

	small multidrug resistance (SMR) antibiotic efflux pump	antibiotic efflux	Vibrio cholerae	qacH	100	100
Lincosamide antibiotic	lincosamide nucleotidyltransferase (LNU)	antibiotic inactivation	Enterococcus faecium	lnuB	90	
	lincosamide nucleotidyltransferase (LNU)	antibiotic inactivation	Enterococcus faecalis	lnuG	88	
Sulfonamide antibiotic	sulfonamide resistant sul	antibiotic target replacement	Vibrio fluvialis	sul1		98
Tetracycline antibiotic	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	Acinetobacter sp.	tet(39)	100	100
	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	uncultured bacterium	tet(40)	97	
	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	Aeromonas salmonicida	tet(C)	100	80
	tetracycline-resistant ribosomal protection protein	antibiotic target protection	uncultured bacterium	tet(W/N/W)	86	
	tetracycline-resistant ribosomal protection protein	antibiotic target protection	Staphylococcus aureus	tetM	98	100
	tetracycline-resistant ribosomal protection protein	antibiotic target protection	Campylobacter jejuni	tetO	100	89
	tetracycline-resistant ribosomal protection protein	antibiotic target protection	Bacteroides fragilis	tetQ	98	
	tetracycline-resistant ribosomal protection protein	antibiotic target protection	Butyrivibrio fibrisolvens	tetW	100	100
Nucleoside antibiotic; acridine dye	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	Escherichia coli	mdtP		81
Multidrug resistance						
macrolide antibiotic; fluoroquinolone antibiotic; penam	resistance-nodulation-cell division (RND) antibiotic efflux pump	antibiotic efflux	Escherichia coli	CRP		91
macrolide antibiotic; lincosamide antibiotic; streptogramin antibiotic	Erm 23S ribosomal RNA methyltransferase	antibiotic target alteration	Enterococcus faecium	ErmB	100	100
macrolide antibiotic; lincosamide antibiotic; streptogramin antibiotic; tetracycline antibiotic; oxazolidinone antibiotic; phenicol antibiotic; pleuromutilin antibiotic	ABC-F ATP-binding cassette ribosomal protection protein	antibiotic target protection	Enterococcus faecalis	lsaE	100	100
macrolide antibiotic; lincosamide antibiotic; streptogramin antibiotic; tetracycline antibiotic; oxazolidinone antibiotic; phenicol antibiotic; pleuromutilin antibiotic	ABC-F ATP-binding cassette ribosomal protection protein	antibiotic target protection	Streptococcus pyogenes	mel	100	100
macrolide antibiotic; lincosamide antibiotic; streptogramin antibiotic; tetracycline antibiotic; oxazolidinone antibiotic; phenicol antibiotic; pleuromutilin antibiotic	ABC-F ATP-binding cassette ribosomal protection protein	antibiotic target protection	Acinetobacter baumannii	msrE	100	100
fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan	resistance-nodulation-cell division (RND) antibiotic efflux pump	antibiotic efflux	Escherichia coli	Escherichia coli acrA	85	
fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan	resistance-nodulation-cell division (RND) antibiotic efflux pump	antibiotic efflux	Escherichia coli	Escherichia coli acrA	85	
cephalosporin; penam	NPS beta-lactamase	antibiotic inactivation	Pseudomonas aeruginosa	NPS-1		86
cephalosporin; penam	OXA beta-lactamase	antibiotic inactivation	Acinetobacter johnsonii	OXA-211	92	84

cephalosporin; penam	OXA beta-lactamase	antibiotic inactivation	Acinetobacter johnsonii	OXA-333	98	
cephalosporin; penam	OXA beta-lactamase	antibiotic inactivation	Citrobacter freundii	OXA-101		83
cephalosporin; penam	OXA beta-lactamase	antibiotic inactivation	Pseudomonas aeruginosa	OXA-205	83	

*Drug class, gene family and resistance mechanisms are filds in CARD database and describe the corresponding AMR genes. **Observed pathogen is a known pathogen of public health importance that harbours that gene. This does not mean that this gene was found in this pathogen or couldn't be found elsewhere

Table S5. Absolute (not *16S rRNA* normalised) concentrations and loads of ARGs in wastewater in Bath

	16S rRNA				*ermB*				*sul1*				*IntI1*				*qnrS*			
Date	Copies/L	STD	Daily loads average copies/day	STD	Copies/L	STD	Daily loads average copies/day	STD	Copies/L	STD	Daily loads average copies/day	STD	Copies/L	STD	Daily loads average copies/day	STD	Copies/L	STD	Daily loads average copies/day	STD
05/11/18	3.49E+09	8.53E+08	9.61E+13	2.34E+13	4.66E+09	7.11E+08	1.28E+14	1.95E+13	7.30E+07	3.38E+07	2.01E+12	9.30E+11	6.27E+07	8.52E+06	1.72E+12	2.34E+11	1.69E+06	2.98E+05	4.64E+10	8.20E+09
06/11/18	4.01E+09	1.79E+08	1.02E+14	4.56E+12	3.38E+09	1.11E+09	8.62E+13	2.83E+13	4.83E+07	1.10E+06	1.23E+12	2.81E+10	7.32E+07	1.47E+07	1.87E+12	3.74E+11	6.82E+05	2.57E+05	1.74E+10	6.55E+09
11/11/18	6.19E+09	4.24E+07	2.79E+14	1.91E+12	6.16E+09	2.55E+08	2.78E+14	1.15E+13	6.86E+07	1.12E+07	3.09E+12	5.06E+11	6.49E+07	8.51E+06	2.92E+12	3.83E+11	9.27E+05	1.23E+05	4.17E+10	5.55E+09
25/11/18	6.65E+09	8.84E+08	1.78E+14	2.37E+13	6.20E+09	3.31E+08	1.66E+14	8.89E+12	7.22E+07	2.82E+07	1.94E+12	7.55E+11	7.46E+07	7.67E+06	2.00E+12	2.06E+11	8.76E+05	2.16E+05	2.35E+10	5.80E+09
02/12/18	5.77E+09	1.08E+09	2.64E+14	4.95E+13	4.96E+09	2.92E+09	2.26E+14	1.33E+14	4.00E+07	2.78E+07	1.83E+12	1.27E+12	8.26E+06	7.04E+06	3.77E+11	3.22E+11	5.20E+05	4.04E+05	2.38E+10	1.85E+10
03/12/18	3.57E+09	1.58E+09	1.60E+14	7.10E+13	5.29E+09	8.42E+08	2.38E+14	3.78E+13	4.67E+07	1.62E+06	2.10E+12	7.27E+10	7.06E+07	0.00E+00	3.17E+12	0.00E+00	1.40E+06	5.10E+04	6.29E+10	2.29E+09
12/12/18	4.78E+09	1.30E+09	1.85E+14	5.03E+13	2.14E+09	2.36E+09	8.28E+13	9.13E+13	6.21E+07	2.04E+07	2.40E+12	7.90E+11	6.98E+07	9.20E+06	2.70E+12	3.56E+11	6.23E+05	1.17E+05	2.41E+10	4.54E+09
19/12/18	5.89E+09	1.15E+09	2.53E+14	4.92E+13	3.31E+09	1.79E+09	1.43E+14	7.68E+13	6.46E+07	2.46E+07	2.78E+12	1.06E+12	4.88E+07	3.07E+06	2.10E+12	1.32E+11	5.23E+05	8.87E+04	2.25E+10	3.81E+09
07//01/19	5.13E+09	5.63E+08	1.43E+14	1.57E+13	3.58E+09	4.26E+08	9.95E+13	1.19E+13	6.70E+07	6.40E+06	1.86E+12	1.78E+11	5.68E+07	9.05E+06	1.58E+12	2.52E+11	2.34E+06	4.80E+05	6.50E+10	1.34E+10
15/01/19	4.56E+09	1.16E+09	1.24E+14	3.15E+13	4.80E+09	2.70E+08	1.31E+14	7.37E+12	4.78E+07	3.44E+07	1.30E+12	9.39E+11	1.95E+07	7.13E+06	5.32E+11	1.94E+11	7.73E+05	2.03E+05	2.11E+10	5.54E+09
21/01/19	5.49E+09	1.80E+09	1.55E+14	5.09E+13	6.39E+09	1.42E+09	1.81E+14	4.02E+13	6.50E+07	5.77E+06	1.84E+12	1.63E+11	6.27E+07	7.59E+06	1.77E+12	2.15E+11	3.32E+06	2.61E+05	9.40E+10	7.38E+09
23/01/19	5.84E+09	5.83E+08	1.83E+14	1.83E+13	4.15E+09	1.71E+08	1.30E+14	5.34E+12	5.73E+07	3.02E+07	1.79E+12	9.47E+11	4.32E+07	3.19E+07	1.35E+12	1.00E+12	1.57E+06	2.89E+03	4.92E+10	9.06E+07
10/02/19	4.18E+09	1.20E+09	1.92E+14	5.53E+13	6.53E+09	6.60E+08	3.00E+14	3.04E+13	5.45E+07	2.52E+07	2.51E+12	1.16E+12	5.47E+07	1.37E+07	2.52E+12	6.30E+11	1.34E+06	6.35E+05	6.15E+10	2.92E+10
11/02/19	4.58E+09	1.80E+08	1.99E+14	7.83E+12	2.29E+09	7.99E+08	9.97E+13	3.48E+13	9.26E+07	4.89E+07	4.03E+12	2.13E+12	7.06E+07	4.05E+07	3.07E+12	1.76E+12	1.06E+06	2.83E+04	4.60E+10	1.23E+09
17/02/19	6.59E+09	1.31E+09	2.35E+14	4.67E+13	4.47E+09	1.06E+09	1.60E+14	3.79E+13	3.78E+07	1.18E+07	1.35E+12	4.23E+11	4.07E+07	1.70E+07	1.45E+12	6.06E+11	7.53E+05	1.02E+05	2.69E+10	3.63E+09
25/02/19	6.84E+09	2.24E+09	2.07E+14	6.77E+13	5.61E+09	1.27E+09	1.70E+14	3.86E+13	5.84E+07	1.89E+07	1.77E+12	5.74E+11	2.07E+07	2.84E+07	6.28E+11	8.61E+11	8.68E+05	4.01E+04	2.63E+10	1.21E+09
01/03/19	3.71E+09	3.11E+08	1.06E+14	8.88E+12	6.07E+09	1.61E+08	1.74E+14	4.60E+12	3.75E+07	4.41E+06	1.07E+12	1.26E+11	4.30E+07	5.54E+06	1.23E+12	1.58E+11	1.31E+06	4.55E+05	3.76E+10	1.30E+10
10/03/19	5.40E+09	6.66E+08	2.42E+14	2.98E+13	6.03E+09	1.44E+09	2.70E+14	6.47E+13	3.52E+07	3.02E+07	1.58E+12	1.35E+12	1.90E+07	1.27E+07	8.50E+11	5.70E+11	6.70E+05	2.46E+04	3.00E+10	1.10E+09
17/03/19	4.32E+09	9.26E+08	1.96E+14	4.19E+13	2.80E+09	7.99E+07	1.27E+14	3.62E+12	3.65E+07	5.64E+06	1.66E+12	2.55E+11	3.81E+07	4.45E+06	1.72E+12	2.02E+11	3.53E+05	3.24E+04	1.60E+10	1.47E+09
29/03/19	5.22E+09	1.89E+08	1.52E+14	5.49E+12	5.57E+09	1.72E+09	1.62E+14	4.98E+13	5.57E+07	1.47E+06	1.62E+12	4.27E+10	4.62E+07	6.94E+06	1.34E+12	2.01E+11	1.10E+06	2.21E+04	3.20E+10	6.40E+08
07/04/19	7.50E+09	2.20E+09	3.19E+14	9.37E+13	3.04E+09	8.69E+08	1.29E+14	3.69E+13	4.32E+06	4.23E+06	1.83E+11	1.80E+11	2.84E+07	3.03E+07	1.20E+12	1.29E+12	5.25E+05	6.36E+05	2.23E+10	2.70E+10
08/04/19	3.71E+09	2.78E+08	1.13E+14	8.43E+12	2.27E+09	5.90E+08	6.90E+13	1.79E+13	5.06E+07	3.28E+06	1.53E+12	9.94E+10	4.78E+07	7.73E+06	1.45E+12	2.34E+11	1.02E+06	2.25E+05	3.10E+10	6.81E+09
15/04/19	6.50E+09	1.29E+09	1.80E+14	3.57E+13	5.74E+09	5.08E+08	1.59E+14	1.40E+13	5.80E+07	1.25E+07	1.60E+12	3.45E+11	5.55E+07	1.67E+07	1.54E+12	4.61E+11	2.05E+06	3.89E+04	5.67E+10	1.08E+09
29/04/19	5.52E+09	3.25E+07	1.44E+14	8.45E+11	1.65E+09	1.65E+08	4.28E+13	4.30E+12	6.05E+07	1.83E+07	1.57E+12	4.77E+11	5.36E+07	9.16E+06	1.39E+12	2.38E+11	3.49E+05	1.76E+05	9.07E+09	4.59E+09
09/05/19	8.77E+09	7.01E+08	3.71E+14	2.96E+13	1.19E+09	1.30E+08	5.01E+13	5.48E+12	3.25E+07	7.18E+05	1.37E+12	3.03E+10	2.88E+07	1.07E+06	1.22E+12	4.54E+10	2.69E+05	1.84E+04	1.14E+10	7.79E+08

10/05/19	5.30E+09	1.42E+09	1.65E+14	4.43E+13	9.06E+08	1.39E+08	2.83E+13	4.33E+12	4.13E+07	1.55E+07	1.29E+12	4.84E+11	4.16E+07	1.11E+07	1.30E+12	3.46E+11	7.89E+05	2.96E+04	2.46E+10	9.24E+08
20/05/19	4.91E+09	1.19E+09	1.26E+14	3.05E+13	2.36E+09	7.94E+07	6.05E+13	2.04E+12	4.21E+07	1.04E+07	1.08E+12	2.66E+11	4.43E+07	5.11E+06	1.14E+12	1.31E+11	5.65E+05	7.51E+04	1.45E+10	1.93E+09
21/05/19	3.20E+09	1.02E+08	8.21E+13	2.61E+12	4.75E+09	1.20E+09	1.22E+14	3.07E+13	6.57E+07	1.86E+07	1.68E+12	4.78E+11	6.16E+07	1.35E+07	1.58E+12	3.46E+11	1.73E+06	3.08E+05	4.44E+10	7.90E+09
17/06/19	5.56E+09	1.51E+08	1.58E+14	4.27E+12	5.25E+09	4.30E+08	1.49E+14	1.22E+13	7.59E+07	1.14E+07	2.15E+12	3.22E+11	6.55E+07	8.91E+05	1.86E+12	2.53E+10	3.51E+06	4.75E+05	9.96E+10	1.35E+10
18/06/19	6.87E+09	4.08E+08	1.96E+14	1.16E+13	6.02E+09	1.15E+09	1.72E+14	3.28E+13	6.25E+07	1.02E+07	1.78E+12	2.90E+11	5.51E+07	1.67E+06	1.57E+12	4.76E+10	4.48E+06	9.11E+04	1.28E+11	2.60E+09
19/06/19	7.77E+09	1.06E+09	2.07E+14	2.83E+13	3.56E+09	2.03E+08	9.45E+13	5.41E+12	7.76E+07	1.76E+07	2.06E+12	4.69E+11	8.13E+07	2.35E+07	2.16E+12	6.24E+11	1.68E+06	2.33E+05	4.46E+10	6.18E+09
25/06/19	3.90E+09	5.78E+07	1.29E+14	1.91E+12	5.38E+09	6.72E+08	1.78E+14	2.22E+13	5.13E+07	2.76E+05	1.70E+12	9.15E+09	5.85E+07	1.36E+07	1.94E+12	4.50E+11	2.78E+06	4.99E+05	9.21E+10	1.65E+10
30/06/19	2.58E+09	4.59E+08	5.94E+13	1.05E+13	3.78E+09	5.08E+08	8.70E+13	1.17E+13	7.32E+07	1.93E+07	1.68E+12	4.44E+11	7.11E+07	1.15E+07	1.63E+12	2.64E+11	2.56E+06	1.94E+05	5.89E+10	4.46E+09
01/07/19	2.69E+09	3.69E+08	6.54E+13	8.97E+12	5.26E+09	1.58E+09	1.28E+14	3.84E+13	5.14E+07	2.19E+06	1.25E+12	5.32E+10	6.63E+07	1.61E+07	1.61E+12	3.92E+11	5.92E+06	2.37E+06	1.44E+11	5.77E+10
05/07/19	4.56E+09	5.70E+08	1.07E+14	1.34E+13	4.71E+09	1.31E+09	1.11E+14	3.09E+13	7.70E+07	1.33E+07	1.81E+12	3.14E+11	6.82E+07	8.47E+06	1.61E+12	2.00E+11	3.33E+06	1.18E+05	7.86E+10	2.77E+09
10/07/19	6.01E+09	7.53E+07	1.47E+14	1.84E+12	7.24E+09	8.89E+08	1.77E+14	2.18E+13	7.01E+07	3.42E+06	1.72E+12	8.37E+10	6.37E+07	2.13E+06	1.56E+12	5.21E+10	6.45E+06	4.37E+05	1.58E+11	1.07E+10
22/07/19	3.56E+09	1.18E+09	8.73E+13	2.90E+13	5.62E+09	9.06E+08	1.38E+14	2.22E+13	6.71E+07	7.81E+06	1.65E+12	1.92E+11	5.78E+07	3.84E+06	1.42E+12	9.42E+10	4.70E+05	1.99E+04	1.15E+10	4.89E+08
07/08/19	4.28E+09	7.07E+08	9.77E+13	1.61E+13	5.29E+09	6.46E+08	1.21E+14	1.47E+13	8.29E+07	2.02E+06	1.89E+12	4.60E+10	7.37E+07	4.09E+06	1.68E+12	9.33E+10	5.65E+06	1.55E+05	1.29E+11	3.54E+09
08/08/19	5.64E+09	1.61E+08	1.33E+14	3.78E+12	4.27E+09	1.70E+08	1.01E+14	3.99E+12	3.87E+07	3.51E+06	9.10E+11	8.25E+10	5.17E+07	6.29E+06	1.22E+12	1.48E+11	5.77E+06	1.92E+05	1.36E+11	4.53E+09
19/08/19	7.18E+09	4.52E+08	1.97E+14	1.24E+13	6.17E+09	2.62E+09	1.69E+14	7.18E+13	3.91E+07	8.77E+06	1.07E+12	2.41E+11	4.63E+07	9.21E+06	1.27E+12	2.53E+11	1.76E+06	2.40E+05	4.82E+10	6.58E+09
20/08/19	7.93E+09	6.16E+08	3.27E+14	2.54E+13	4.91E+09	8.73E+08	2.03E+14	3.60E+13	7.58E+07	3.86E+07	3.13E+12	1.59E+12	5.74E+07	1.24E+07	2.37E+12	5.11E+11	3.26E+06	1.05E+05	1.35E+11	4.35E+09
22/10/19	7.09E+09	1.98E+08	2.10E+14	5.85E+12	4.13E+09	7.55E+08	1.22E+14	2.23E+13	6.29E+07	1.58E+07	1.86E+12	4.68E+11	6.56E+07	1.13E+07	1.94E+12	3.34E+11	1.28E+06	1.59E+05	3.78E+10	4.72E+09
23/10/19	9.35E+09	1.18E+09	2.99E+14	3.79E+13	2.78E+09	1.87E+07	8.91E+13	5.97E+11	5.76E+07	2.83E+06	1.84E+12	9.05E+10	5.56E+07	1.46E+05	1.78E+12	4.66E+09	2.79E+06	2.49E+03	8.92E+10	7.96E+07
28/10/19	4.98E+09	8.36E+08	1.99E+14	3.35E+13	3.00E+09	1.77E+09	1.20E+14	7.09E+13	8.10E+07	5.60E+06	3.24E+12	2.24E+11	7.01E+07	5.88E+06	2.80E+12	2.35E+11	5.08E+06	4.56E+05	2.03E+11	1.83E+10
29/10/19	5.13E+09	2.22E+09	1.86E+14	8.04E+13	2.95E+09	1.02E+09	1.07E+14	3.70E+13	6.01E+07	1.59E+07	2.18E+12	5.78E+11	5.89E+07	1.85E+07	2.13E+12	6.68E+11	4.28E+06	3.22E+05	1.55E+11	1.17E+10
02/12/19	8.24E+09	1.97E+07	3.60E+14	8.62E+11	3.55E+09	1.77E+09	1.55E+14	7.74E+13	5.93E+07	6.69E+06	2.59E+12	2.92E+11	6.56E+07	9.57E+06	2.87E+12	4.18E+11	3.56E+06	3.81E+04	1.55E+11	1.66E+09
05/12/19	6.15E+09	5.92E+08	2.21E+14	2.13E+13	3.45E+09	3.83E+08	1.24E+14	1.38E+13	4.94E+07	1.31E+07	1.77E+12	4.70E+11	4.74E+07	4.53E+06	1.70E+12	1.63E+11	1.67E+06	7.32E+04	6.00E+10	2.63E+09
07/12/19	5.14E+09	8.51E+08	2.21E+14	3.65E+13	2.50E+09	2.97E+08	1.07E+14	1.28E+13	6.08E+07	4.75E+06	2.61E+12	2.04E+11	6.01E+07	1.17E+07	2.58E+12	5.02E+11	8.98E+05	1.55E+04	3.86E+10	6.66E+08
08/12/19	6.50E+09	1.38E+09	3.00E+14	6.35E+13	4.76E+09	6.81E+08	2.20E+14	3.15E+13	5.58E+07	1.60E+07	2.58E+12	7.39E+11	5.85E+07	1.39E+07	2.70E+12	6.43E+11	1.11E+06	2.06E+04	5.11E+10	9.51E+08
09/12/19	5.52E+09	7.30E+08	2.41E+14	3.19E+13	5.57E+09	6.64E+08	2.43E+14	2.90E+13	6.93E+07	2.57E+07	3.03E+12	1.12E+12	7.55E+07	9.04E+06	3.30E+12	3.95E+11	1.12E+06	4.86E+05	4.87E+10	2.12E+10
Average:	**5.49E+09**	**7.94E+08**	**1.86E+14**	**2.76E+13**	**4.33E+09**	**8.56E+08**	**1.43E+14**	**2.94E+13**	**5.75E+07**	**1.40E+07**	**1.89E+12**	**4.88E+11**	**5.45E+07**	**1.07E+07**	**1.79E+12**	**3.65E+11**	**1.99E+06**	**2.41E+05**	**6.17E+10**	**7.60E+09**

Table S6. Relative loads of ARGs (*16S rRNA* normalised) and daily loads per capita of ARGs (absolute gene loads normalised to Bath city population size) in wastewater

	ermB				*sul1*				*IntI1*				*qnrS*			
Date	Relative daily loads copies/day	STD	Daily loads per capita copies/day	STD	Relative daily loads copies/day	STD	Daily loads per capita copies/day	STD	Relative daily loads copies/day	STD	Daily loads per capita copies/day	STD	Relative daily loads copies/day	STD	Daily loads per capita copies/day	STD
05/11/2018	1.35E+00	1.25E-01	1.07E+12	1.63E+11	2.03E-02	4.72E-03	1.67E+10	7.74E+09	1.88E-02	7.02E-03	1.43E+10	1.95E+09	4.86E-04	3.33E-05	3.86E+08	6.83E+07
06/11/2018	8.23E-01	2.35E-01	7.18E+11	2.36E+11	1.18E-02	8.02E-04	1.02E+10	2.34E+08	1.79E-02	2.79E-03	1.55E+10	3.11E+09	1.69E-04	7.05E-05	1.45E+08	5.45E+07
11/11/2018	9.97E-01	4.81E-02	2.31E+12	9.57E+10	1.11E-02	1.89E-03	2.57E+10	4.21E+09	1.05E-02	1.45E-03	2.43E+10	3.19E+09	1.50E-04	2.09E-05	3.48E+08	4.62E+07
25/11/2018	9.37E-01	7.47E-02	1.38E+12	7.40E+10	1.07E-02	2.82E-03	1.61E+10	6.29E+09	1.12E-02	3.40E-04	1.67E+10	1.71E+09	1.31E-04	1.51E-05	1.96E+08	4.83E+07
02/12/2018	8.26E-01	3.50E-01	1.89E+12	1.11E+12	6.60E-03	3.58E-03	1.52E+10	1.06E+10	1.34E-03	9.69E-04	3.14E+09	2.68E+09	9.85E-05	8.85E-05	1.98E+08	1.54E+08
03/12/2018	1.70E+00	9.88E-01	1.98E+12	3.15E+11	1.44E-02	5.91E-03	1.75E+10	6.05E+08	2.19E-02	9.69E-03	2.64E+10	0.00E+00	4.31E-04	1.76E-04	5.24E+08	1.91E+07
12/12/2018	3.95E-01	3.86E-01	6.89E+11	7.60E+11	1.41E-02	8.10E-03	2.00E+10	6.57E+09	1.54E-02	6.13E-03	2.25E+10	2.96E+09	1.39E-04	6.23E-05	2.01E+08	3.78E+07
19/12/2018	5.43E-01	1.97E-01	1.19E+12	6.39E+11	1.08E-02	2.08E-03	2.31E+10	8.80E+09	8.49E-03	2.17E-03	1.75E+10	1.10E+09	9.19E-05	3.29E-05	1.87E+08	3.17E+07
07//01/2019	7.33E-01	1.06E-02	8.29E+11	9.87E+10	1.38E-02	1.29E-04	1.55E+10	1.48E+09	1.18E-02	3.09E-03	1.32E+10	2.10E+09	4.88E-04	1.50E-04	5.41E+08	1.11E+08
15/01/2019	1.08E+00	2.15E-01	1.09E+12	6.13E+10	1.18E-02	1.05E-02	1.08E+10	7.82E+09	4.62E-03	2.74E-03	4.43E+09	1.62E+09	1.69E-04	1.56E-06	1.76E+08	4.61E+07
21/01/2019	1.18E+00	1.29E-01	1.50E+12	3.34E+11	1.27E-02	5.21E-03	1.53E+10	1.36E+09	1.23E-02	5.41E-03	1.48E+10	1.79E+09	6.47E-04	2.60E-04	7.82E+08	6.14E+07
23/01/2019	7.12E-01	4.20E-02	1.08E+12	4.45E+10	1.01E-02	6.19E-03	1.49E+10	7.88E+09	7.71E-03	6.24E-03	1.13E+10	8.33E+09	2.70E-04	2.75E-05	4.10E+08	7.54E+05
10/02/2019	1.65E+00	6.32E-01	2.50E+12	2.53E+11	1.27E-02	2.36E-03	2.09E+10	9.63E+09	1.31E-02	5.05E-04	2.10E+10	5.25E+09	3.11E-04	6.25E-05	5.12E+08	2.43E+08
11/02/2019	5.05E-01	1.94E-01	8.30E+11	2.89E+11	2.00E-02	9.90E-03	3.35E+10	1.77E+10	1.53E-02	8.25E-03	2.56E+10	1.47E+10	2.31E-04	1.53E-05	3.83E+08	1.02E+07
17/02/2019	6.76E-01	2.68E-02	1.33E+12	3.15E+11	6.04E-03	3.00E-03	1.12E+10	3.52E+09	6.57E-03	3.88E-03	1.21E+10	5.05E+09	1.15E-04	7.41E-06	2.24E+08	3.02E+07
25/02/2019	8.98E-01	4.80E-01	1.42E+12	3.21E+11	9.49E-03	5.87E-03	1.47E+10	4.78E+09	2.49E-03	3.34E-03	5.23E+09	7.17E+09	1.33E-04	3.76E-05	2.19E+08	1.01E+07
01/03/2019	1.65E+00	1.81E-01	1.45E+12	3.83E+10	1.01E-02	3.43E-04	8.93E+09	1.05E+09	1.17E-02	2.48E-03	1.02E+10	1.32E+09	3.50E-04	9.34E-05	3.13E+08	1.08E+08
10/03/2019	1.14E+00	4.08E-01	2.25E+12	5.39E+11	6.21E-03	4.81E-03	1.31E+10	1.12E+10	3.39E-03	1.94E-03	7.08E+09	4.75E+09	1.25E-04	2.00E-05	2.50E+08	9.19E+06
17/03/2019	6.65E-01	1.61E-01	1.06E+12	3.01E+10	8.51E-03	5.17E-04	1.38E+10	2.13E+09	8.90E-03	8.75E-04	1.44E+10	1.68E+09	8.44E-05	2.55E-05	1.33E+08	1.22E+07
29/03/2019	1.07E+00	3.68E-01	1.35E+12	4.15E+11	1.07E-02	1.04E-04	1.34E+10	3.56E+08	8.87E-03	1.65E-03	1.12E+10	1.67E+09	2.11E-04	3.44E-06	2.67E+08	5.33E+06
07/04/2019	5.79E-01	3.61E-01	1.08E+12	3.08E+11	6.38E-04	4.76E-04	1.53E+09	1.50E+09	6.14E-03	7.38E-03	1.00E+10	1.07E+10	7.35E-05	7.98E-05	1.86E+08	2.25E+08
08/04/2019	6.21E-01	2.06E-01	5.74E+11	1.49E+11	1.37E-02	1.91E-03	1.28E+10	8.28E+08	1.30E-02	3.05E-03	1.21E+10	1.95E+09	2.74E-04	4.00E-05	2.58E+08	5.67E+07
15/04/2019	9.08E-01	2.59E-01	1.32E+12	1.17E+11	9.30E-03	3.77E-03	1.34E+10	2.88E+09	8.98E-03	4.35E-03	1.28E+10	3.84E+09	3.21E-04	5.79E-05	4.72E+08	8.96E+06
29/04/2019	2.98E-01	2.82E-02	3.56E+11	3.58E+10	1.09E-02	3.26E-03	1.31E+10	3.97E+09	9.69E-03	1.60E-03	1.16E+10	1.98E+09	6.30E-05	3.16E-05	7.55E+07	3.82E+07
09/05/2019	1.36E-01	2.57E-02	4.17E+11	4.56E+10	3.71E-03	2.15E-04	1.14E+10	2.53E+08	3.29E-03	1.40E-04	1.01E+10	3.78E+08	3.09E-05	4.57E-06	9.47E+07	6.49E+06

10/05/2019	1.81E-01	7.46E-02	2.35E+11	3.60E+10	8.48E-03	5.20E-03	1.07E+10	4.03E+09	8.44E-03	4.36E-03	1.08E+10	2.88E+09	1.55E-04	4.71E-05	2.05E+08	7.69E+06
20/05/2019	4.97E-01	1.37E-01	5.04E+11	1.69E+10	8.59E-03	3.21E-05	9.00E+09	2.21E+09	9.18E-03	1.18E-03	9.46E+09	1.09E+09	1.17E-04	1.30E-05	1.21E+08	1.60E+07
21/05/2019	1.49E+00	4.21E-01	1.01E+12	2.55E+11	2.04E-02	5.17E-03	1.40E+10	3.98E+09	1.92E-02	3.60E-03	1.31E+10	2.88E+09	5.40E-04	7.90E-05	3.70E+08	6.57E+07
17/06/2019	9.46E-01	1.03E-01	1.24E+12	1.02E+11	1.36E-02	1.67E-03	1.79E+10	2.68E+09	1.18E-02	4.80E-04	1.55E+10	2.11E+08	6.33E-04	1.03E-04	8.29E+08	1.12E+08
18/06/2019	9.21E-01	2.32E-01	1.43E+12	2.73E+11	9.55E-03	2.14E-03	1.48E+10	2.42E+09	8.39E-03	7.73E-04	1.31E+10	3.96E+08	6.82E-04	2.84E-05	1.06E+09	2.16E+07
19/06/2019	4.64E-01	8.96E-02	7.87E+11	4.50E+10	1.02E-02	3.67E-03	1.72E+10	3.90E+09	1.08E-02	4.49E-03	1.80E+10	5.19E+09	2.16E-04	4.02E-07	3.71E+08	5.15E+07
25/06/2019	1.38E+00	1.52E-01	1.48E+12	1.85E+11	1.32E-02	2.66E-04	1.41E+10	7.62E+07	1.50E-02	3.27E-03	1.61E+10	3.74E+09	7.14E-04	1.18E-04	7.67E+08	1.37E+08
30/06/2019	1.51E+00	4.64E-01	7.24E+11	9.72E+10	2.95E-02	1.27E-02	1.40E+10	3.69E+09	2.83E-02	9.47E-03	1.36E+10	2.19E+09	1.01E-03	2.55E-04	4.90E+08	3.71E+07
01/07/2019	2.01E+00	8.63E-01	1.07E+12	3.20E+11	1.93E-02	3.46E-03	1.04E+10	4.43E+08	2.44E-02	2.63E-03	1.34E+10	3.26E+09	2.16E-03	5.85E-04	1.20E+09	4.80E+08
05/07/2019	1.06E+00	4.20E-01	9.23E+11	2.58E+11	1.72E-02	5.08E-03	1.51E+10	2.62E+09	1.52E-02	3.76E-03	1.34E+10	1.66E+09	7.39E-04	1.18E-04	6.54E+08	2.31E+07
10/07/2019	1.21E+00	1.63E-01	1.48E+12	1.81E+11	1.17E-02	7.16E-04	1.43E+10	6.97E+08	1.06E-02	4.87E-04	1.30E+10	4.34E+08	1.07E-03	5.93E-05	1.31E+09	8.91E+07
22/07/2019	1.63E+00	2.86E-01	1.15E+12	1.85E+11	1.96E-02	4.31E-03	1.37E+10	1.59E+09	1.70E-02	4.58E-03	1.18E+10	7.84E+08	1.41E-04	5.24E-05	9.60E+07	4.07E+06
07/08/2019	1.27E+00	3.60E-01	1.01E+12	1.23E+11	1.96E-02	2.76E-03	1.58E+10	3.83E+08	1.74E-02	1.91E-03	1.40E+10	7.77E+08	1.34E-03	2.57E-04	1.07E+09	2.95E+07
08/08/2019	7.58E-01	8.52E-03	8.37E+11	3.33E+10	6.87E-03	8.18E-04	7.58E+09	6.87E+08	9.19E-03	1.38E-03	1.01E+10	1.23E+09	1.02E-03	4.97E-06	1.13E+09	3.77E+07
19/08/2019	8.72E-01	4.19E-01	1.41E+12	5.98E+11	5.48E-03	1.56E-03	8.93E+09	2.00E+09	6.50E-03	1.69E-03	1.06E+10	2.10E+09	2.46E-04	4.88E-05	4.01E+08	5.48E+07
20/08/2019	6.25E-01	1.59E-01	1.69E+12	3.00E+11	9.78E-03	5.63E-03	2.61E+10	1.33E+10	7.32E-03	2.13E-03	1.97E+10	4.25E+09	4.14E-04	4.54E-05	1.12E+09	3.63E+07
22/10/2019	5.81E-01	9.03E-02	1.02E+12	1.86E+11	8.91E-03	2.48E-03	1.55E+10	3.89E+09	9.28E-03	1.85E-03	1.62E+10	2.78E+09	1.80E-04	2.75E-05	3.15E+08	3.93E+07
23/10/2019	3.00E-01	4.00E-02	7.42E+11	4.97E+09	6.22E-03	1.09E-03	1.53E+10	7.53E+08	5.99E-03	7.74E-04	1.48E+10	3.88E+07	3.00E-04	3.83E-05	7.42E+08	6.63E+05
28/10/2019	5.81E-01	2.58E-01	1.00E+12	5.90E+11	1.66E-02	3.91E-03	2.70E+10	1.87E+09	1.44E-02	3.60E-03	2.33E+10	1.96E+09	1.03E-03	8.10E-05	1.69E+09	1.52E+08
29/10/2019	6.82E-01	4.95E-01	8.90E+11	3.08E+11	1.22E-02	2.16E-03	1.81E+10	4.81E+09	1.18E-02	1.52E-03	1.78E+10	5.57E+09	9.36E-04	4.68E-04	1.29E+09	9.70E+07
02/12/2019	4.31E-01	2.16E-01	1.29E+12	6.44E+11	7.19E-03	7.94E-04	2.16E+10	2.43E+09	7.96E-03	1.14E-03	2.39E+10	3.48E+09	4.32E-04	3.59E-06	1.29E+09	1.39E+07
05/12/2019	5.66E-01	1.17E-01	1.03E+12	1.15E+11	7.97E-03	1.36E-03	1.48E+10	3.91E+09	7.71E-03	6.08E-06	1.42E+10	1.35E+09	2.81E-04	1.51E-05	5.15E+08	2.19E+07
07/12/2019	4.87E-01	2.28E-02	8.92E+11	1.06E+11	1.19E-02	1.05E-03	2.17E+10	1.70E+09	1.17E-02	3.46E-04	2.15E+10	4.18E+09	1.77E-04	2.63E-05	3.21E+08	5.54E+06
08/12/2019	7.60E-01	2.66E-01	1.83E+12	2.62E+11	8.52E-03	6.56E-04	2.15E+10	6.15E+09	8.98E-03	2.40E-04	2.25E+10	5.35E+09	1.75E-04	4.01E-05	4.26E+08	7.92E+06
09/12/2019	1.03E+00	2.56E-01	2.03E+12	2.41E+11	1.24E-02	3.02E-03	2.52E+10	9.34E+09	1.39E-02	3.48E-03	2.75E+10	3.29E+09	1.98E-04	6.19E-05	4.06E+08	1.77E+08
Average:	**8.86E-01**	**2.45E-01**	**1.19E+12**	**2.45E+11**	**1.17E-02**	**3.20E-03**	**1.57E+10**	**4.06E+09**	**1.13E-02**	**2.93E-03**	**1.49E+10**	**3.04E+09**	**4.10E-04**	**7.99E-05**	**5.14E+08**	**6.32E+07**

Table S7. Seasonal T-tests for ARGs studied in influent wastewater in Bath, comparing winter 2018/19 and summer 2019

Winter 18/19: Summer 19	**p values**	
	Relative (16S rRNA normalised) loads	**Absolute loads**
ermB	0.108	0.149
sul1	0.136	0.084
qnrS	**0.002**	**0.001**
intI1	0.067	0.385

*Statistically significant results (p≤0.05) shown in bold

Table S8: Pearson correlations coefficients of ARGs and AAs quantified in influent wastewater from Bath (every sample)

	16S rRNA	ermB	intI1	sul1	qnrS	ermB (16S normalised)	intI1 (16S normalised	sul1 (16S normalised	qnrS (16S normalised)
16S rRNA				**0.306**		**-0.873**	**-0.318**	**-0.281**	**-0.315**
ermB				**0.297**		**-0.280**			
intI1				**0.772**			**0.746**	**0.623**	
sul1	**0.306**	**0.297**	**0.772**			**-0.314**	**0.512**	**0.714**	
qnrS									**0.867**
ermB (16S normalised)	**-0.873**	**-0.280**		**-0.314**			**0.364**	**0.325**	**0.444**
intI1 (16S normalised	**-0.318**		**0.746**	**0.512**		**0.364**		**0.861**	**0.386**
sul1 (16S normalised	**-0.281**		**0.623**	**0.714**		**0.325**	**0.861**		**0.311**
qnrS (16S normalised)	**-0.315**				**0.867**	**0.444**	**0.386**	**0.311**	
ERY					**-0.308**				**-0.282**
CLR	**0.363**	**0.309**				**-0.394**			
dmERY	**0.705**	**0.458**		**0.338**		**-0.608**			
dmCLR		**0.282**							
INa						**-0.301**			
hPZA		**0.356**			**0.867**				**-0.292**
MTZ		**0.356**							
KTC		**0.349**							
hMTZ	**0.327**				**0.310**	**-0.338**			
3TC									
FTC									
SPY					**0.374**				
SMX									
SLZ					**0.311**				**0.287**
aSPY		**0.335**							
aSMX									
FLX									
AMXa	**0.380**	**0.535**	**0.422**	**0.365**		**-0.489**			
TET			**-0.319**		**-0.414**				**-0.344**
OTC			**-0.378**		**-0.322**				
CIP		**0.378**				**-0.279**			
OFX									
TMP									
NPAHD						**-0.349**			
CLI	**0.288**	**0.312**							
dmCLI									

*Statistically significant results (p≤0.05) only shown

Table S9: Pearson correlations coefficients of ARGs and AAs quantified in influent wastewater from Bath (using monthly averages)

	16S rRNA	ermB	intI1	sul1	qnrS	ermB (16S normalised)	intI1 (16S normalised	sul1 (16S normalised	qnrS (16S normalised)
16S rRNA			**0.637**	**0.595**		**-0.946**			**-0.387**
ermB			**0.404**	**0.543**			**0.504**	**0.574**	
intI1	**0.637**	**0.404**		**0.898**		**-0.518**	**0.766**	**0.650**	
sul1	**0.595**	**0.543**	**0.898**			**-0.568**	**0.756**	**0.820**	
qnrS									**0.929**
ermB (16S normalised)	**-0.946**		**-0.518**	**-0.568**					**0.488**
intI1 (16S normalised		**0.504**	**0.766**	**0.756**				**0.900**	
sul1 (16S normalised		**0.574**	**0.650**	**0.820**			**0.900**		
qnrS (16S normalised)	**-0.387**				**0.929**	**0.488**			
ERY					**-0.783**	**-0.387**			**-0.696**
CLR		**0.448**			**-0.447**	**-0.438**			**-0.478**
dmERY	**0.818**	**0.580**	**0.514**	**0.648**	**-0.524**	**-0.901**	**0.349**	**0.424**	**-0.650**
dmCLR		**0.479**			**-0.616**				**-0.548**
INa	**0.534**	**0.437**	**0.562**	**0.651**		**-0.521**	**0.307**	**0.383**	
hPZA	**-0.639**		**-0.715**	**-0.532**		**0.420**	**-0.406**		
MTZ		**0.555**		**0.330**		**-0.308**		**0.430**	
KTC	**-0.302**	**0.586**	**-0.376**						
hMTZ	**0.393**	**0.340**	**0.412**	**0.598**	**0.491**	**-0.505**		**0.328**	**0.307**
3TC	**-0.612**	**0.348**				**0.548**			**0.376**
FTC	**-0.506**	**0.367**			**0.387**	**0.351**		**0.290**	**0.497**
SPY					**0.482**		**-0.389**		**0.320**
SMX					**0.334**		**-0.322**		**0.283**
SLZ	**-0.537**				**0.323**	**0.520**			**0.523**
aSPY		**0.515**				**-0.375**		**0.295**	
aSMX		**0.425**							
FLX		**0.541**						**0.309**	
AMXa	**0.341**	**0.572**	**0.388**	**0.514**		**-0.469**		**0.295**	
TET		**0.414**	**-0.319**		**-0.728**				**-0.623**
OTC	**-0.351**	**0.319**	**-0.509**		**-0.534**				**-0.400**
CIP		**0.800**	**0.291**	**0.537**		**-0.408**	**0.339**	**0.568**	
OFX		**0.302**		**0.308**	**0.353**			**0.368**	**0.426**
TMP		**0.402**				**-0.289**			
NPAHD		**0.482**		**0.347**	**-0.411**	**-0.321**		**0.365**	**-0.385**
CLI		**0.429**		**0.342**		**-0.359**			
dmCLI	**-0.491**		**-0.604**	**-0.404**		**0.282**	**-0.586**	**-0.328**	

*Statistically significant results ($p \leq 0.05$) shown in **bold**

Table S10: Overall concentration of AAs in hospital effluent from a hospital that resides within the city of Bath's catchment area, collected in August 2019

Class	Abbrev	05/08/2019 Concentration ug/L	STD Dev	06/08/2019 Concentration ug/L	STD Dev	07/08/2019 Concentration ug/L	Std Dev	08/08/2019 Concentration ug/L	STD	09/08/2019 Concentration ug/L	STD	Weekly average Concentration ug/L	STD
Sulfonamide &	SDZ	-	-	-	-	-	-	-	-	-	-	-	-
Trimethoprim	SPY	0.49	0.03	2.05	0.15	4.11	0.49	0.24	0.06	0.20	0.02	1.42	1.69
	SMX	0.22	0.03	4.23	0.22	1.06	0.13	0.96	0.30	0.88	0.03	1.47	1.58
	SLZ	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	0.58	0.23	7.63	1.56	2.18	0.65	4.10	1.65	1.75	0.09	3.25	2.76
	aSDZ	-	-	-	-	-	-	0.02	-	-	-	0.02	-
	aSPY	0.26	0.03	3.21	0.24	2.81	0.32	0.30	0.07	0.16	0.01	1.35	1.53
	aSMX	0.72	0.29	146.58	13.93	2.15	0.18	2.73	0.77	0.92	0.22	30.62	64.83
	hTMP	-	-	0.01	0.00	-	-	0.00	0.00	0.00	-	0.01	0.00
Macrolide & Lincomycin	AZM	53.09	3.41	45.74	21.28	25.15	10.67	5.50	-	12.58	3.14	28.41	20.59
	ERY	0.67	0.12	6.10	1.34	0.58	0.10	2.47	1.56	1.15	0.29	2.20	2.31
	CLR	0.81	0.17	0.45	-	0.60	0.06	0.05	0.02	-	-	0.47	0.32
	CLI	3.30	0.18	0.17	0.04	0.68	0.42	0.08	0.02	0.10	0.02	0.87	1.38
	dmAZM	55.33	2.45	33.88	6.78	-	-	-	-	23.95	2.62	37.72	16.04
	dmERY	0.78	0.13	1.40	0.20	-	-	0.13	0.10	0.13	0.05	0.61	0.61
	dmCLR	0.14	-	-	-	0.18	0.07	0.02	0.00	0.06	-	0.10	0.07
	dmCLI	0.02	-	-	-	-	-	0.02	0.00	-	-	0.02	0.00
β-LACTAMS	AMX	5.28	0.31	6.16	2.22	0.32	0.15	0.61	0.43	6.18	5.19	3.71	2.98
Penicillin	AMP	-	-	-	-	-	-	-	-	-	-	-	-
	FLX	2.85	0.14	16.02	3.45	1.08	0.38	5.18	1.85	3.18	1.56	5.66	5.97
	PenG	-	-	-	-	-	-	-	-	-	-	-	-
	PenV	-	-	-	-	-	-	-	-	-	-	-	-
	AMXa	1.02	0.08	0.97	0.16	1.50	0.16	1.15	0.39	1.33	0.21	1.19	0.22
	AMPa	-	-	-	-	0.12	0.02	0.18	0.03	-	-	0.15	0.04
	PenGa	-	-	-	-	-	-	-	-	-	-	-	-

Cefalosporin	LEX	-	-	-	-	2.09	0.48	-	-	-	-	2.09	-
	CFM	0.47	-	-	-	-	-	-	-	-	-	0.47	-
	CTF	-	-	-	-	-	-	-	-	-	-	-	-
	CRO	-	-	6.14	0.77	1.33	0.31	3.47	1.90	-	-	3.65	2.41
Monobactam	ATM	-	-	-	-	-	-	-	-	-	-	-	-
Carbapenem	IPM	0.88	0.00	1.02	0.15	-	-	0.78	0.34	0.88	0.08	0.89	0.10
	MEM	-	-	-	-	-	-	-	-	-	-	-	-
Quinolone	BSF	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	0.11	0.04	3.89	0.49	0.29	0.06	0.22	0.07	0.13	0.05	0.93	1.66
	DFX	-	-	-	-	-	-	-	-	-	-	-	-
	ENR	-	-	-	-	-	-	-	-	-	-	-	-
	FLU	-	-	-	-	-	-	-	-	-	-	-	-
	GAT	-	-	-	-	-	-	-	-	-	-	-	-
	LOM	-	-	-	-	-	-	-	-	-	-	-	-
	MXF	-	-	-	-	-	-	-	-	-	-	-	-
	NAD	-	-	-	-	-	-	-	-	-	-	-	-
	NAL	-	-	-	-	-	-	-	-	-	-	-	-
	NOR	-	-	-	-	-	-	-	-	-	-	-	-
	OFX	0.11	0.00	0.01	0.01	-	-	0.03	0.03	0.03	0.02	0.04	0.05
	PFLX	-	-	-	-	-	-	-	-	-	-	-	-
	SRF	-	-	-	-	-	-	-	-	-	-	-	-
	deCIP	-	-	-	-	-	-	-	-	-	-	-	-
	hNOR	-	-	-	-	-	-	-	-	-	-	-	-
	OFXo	-	-	-	-	-	-	-	-	-	-	-	-
	dmOFX	-	-	-	-	-	-	-	-	-	-	-	-
	UFX	-	-	-	-	-	-	-	-	-	-	-	-
TB DRUGS	INH	-	-	-	-	-	-	-	-	-	-	-	-
TB (1st line)	PZA	-	-	-	-	0.06	0.01	-	-	0.11	0.00	0.09	0.04
	EMB	0.05	0.01	0.05	0.01	0.16	0.03	0.05	0.02	0.04	0.01	0.07	0.05
	RMP	-	-	-	-	-	-	-	-	-	-	-	-

	RFB	-	-	-	-	-	-	-	-	-	-	-	-
	INa	2.91	0.13	2.10	0.16	1.28	0.37	3.27	0.50	5.15	0.64	2.94	1.46
	aINH	-	-	-	-	-	-	-	-	-	-	-	-
	hPZA	0.72	0.18	1.68	0.07	0.70	-	-	-	-	-	1.04	0.56
	daRMP	-	-	-	-	-	-	-	-	-	-	-	-
	daRFB	-	-	-	-	-	-	-	-	-	-	-	-
TB (MDR)	CAPIa	-	-	-	-	-	-	-	-	-	-	-	-
	CAPIb	-	-	-	-	-	-	-	-	-	-	-	-
	GEN1	-	-	-	-	-	-	-	-	-	-	-	-
	GEN1a	-	-	-	-	-	-	-	-	-	-	-	-
	GEN2	-	-	-	-	-	-	-	-	-	-	-	-
	KAN	-	-	-	-	-	-	-	-	-	-	-	-
	STR	-	-	-	-	-	-	-	-	-	-	-	-
	DCS	0.02	0.01	0.02	0.00	0.01	0.00	0.01	0.01	0.01	-	0.01	0.01
TB (other)	DMD	-	-	-	-	-	-	-	-	-	-	-	-
	BDQ	-	-	-	-	-	-	-	-	-	-	-	-
	LZD	-	-	-	-	5.22	0.25	0.03	0.01	0.25	0.00	1.83	2.94
	THAL	-	-	-	-	-	-	-	-	-	-	-	-
OTHER	CHL	0.23	-	0.30	0.35	0.11	-	0.11	0.03	0.13	0.05	0.18	0.09
Amphenicol	FLO	-	-	-	-	-	-	-	-	-	-	-	-
	ANP	-	-	-	-	-	-	-	-	-	-	-	-
Cycline	DOX	-	-	-	-	-	-	-	-	-	-	-	-
	OTC	0.07	0.02	-	-	-	-	-	-	-	-	0.07	-
	TET	-	-	-	-	0.03	0.01	-	-	0.02	0.01	0.03	0.01
Nitrofuran	NIT	-	-	-	-	-	-	-	-	-	-	-	-
	NPAHD	-	-	-	-	-	-	-	-	-	-	-	-
Azole	MTZ	10.24	0.09	15.13	0.93	9.74	0.10	10.21	3.14	17.14	2.35	12.49	3.40
	KTC	-	-	-	-	-	-	-	-	-	-	-	-
	hMTZ	11.41	0.61	37.56	0.31	11.17	1.27	6.83	2.02	25.80	2.71	18.55	12.81

	daKTC	-	-	-	-	-	-	-	-	-	-	-	-
Antiviral	FTC	-	-	0.31	0.13	-	-	-	-	0.56	0.09	0.43	0.18
	3TC	-	-	-	-	0.10	-	-	-	0.12	-	0.11	0.01

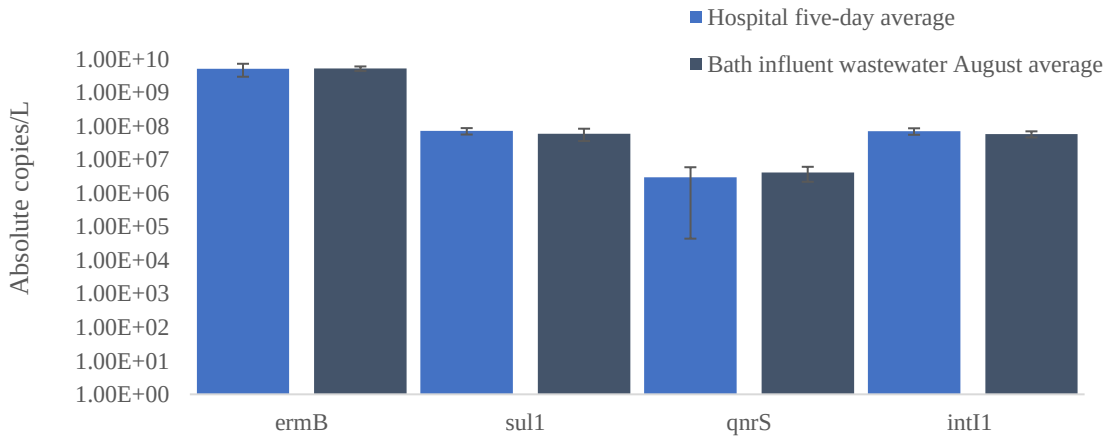


Figure S4. Comparison of absolute concentrations of ARGs in hospital effluent (sampled August 2019) and the averaged ARG concentration from Bath influent wastewater collected during August 2019.

Table S11: Absolute concentration of *16S rRNA* and ARGs in hospital effluent collected in August 2019

Gene	Associated Resistance	05/08/2019		06/08/2019		07/08/2019		08/08/2019		09/08/2019		Weekly Average	
		Copies/L	STD	Copies/L	STD	Copies/L	STD	Copies/L	STD	Copies/L	STD	copies/L	STD
16S rRNA	N/A	3979612000	587807261	5944449000	335602778	4451028750	682760741	3781230250	1533251100	5539616250	68685171	4739187250	957994515
ermB	Macrolide	3820612250	355052811	6456516500	2187016220	7306832750	2566327743	5540123750	302372648	2065687400	2343965783	5037954530	2105266876
sul1	Sulfonamide	87993801	10172295	74501406	273879	75533661	30616792	70360206	13240951	46025175	589960	70882850	15376002
qnrS	Quinolone	505327	80660	7514092	271095	4247888	59374	1250486	191179	1299921	150133	2963543	2919849
intI1	Anthropogenic pollution	90262935	23609612	52540257	7957145	78545166	15475049	57812370	53398	69860014	2396180	69804148	15303718
tetM	Tetracycline	3641760	463725	9941167	216685	3913100	598508	10350742	359487	15729617	861394	8715277	5054206
blaTEM	β-lactamase	340282	935	3343961	339632	5573404	128675	9724635	1857762	12893507	3600023	6375158	4998964

Table S12. Relative concentrations of ARGs in hospital effluent (normalised to *16S rRNA*) collected in August 2019

Gene	Associated Resistance	05/08/2019		06/08/2019		07/08/2019		08/08/2019		09/08/2019		Weekly Average	
		Copies/L	STD	Copies/L	STD	Copies/L	STD	Copies/L	STD	Copies/L	STD	Copies/L	STD
ermB	Macrolide	9.77E-01	2.34E-01	1.08E+00	3.07E-01	1.62E+00	3.29E-01	1.61E+00	7.34E-01	3.76E-01	4.28E-01	1.13E+00	5.16E-01
sul1	Sulfonamide	2.25E-02	5.89E-03	1.26E-02	6.63E-04	1.77E-02	9.59E-03	1.95E-02	4.41E-03	8.31E-03	2.10E-04	1.61E-02	5.68E-03
qnrS	Quinolone	1.27E-04	1.53E-06	1.27E-03	1.17E-04	9.65E-04	1.35E-04	3.72E-04	2.01E-04	2.35E-04	2.42E-05	5.93E-04	4.97E-04
intI1	Anthropogenic pollution	2.25E-02	2.61E-03	8.89E-03	1.84E-03	1.76E-02	7.79E-04	1.67E-02	6.77E-03	1.26E-02	3.65E-04	1.57E-02	5.15E-03
TetM	Tetracycline	9.16E-04	1.88E-05	1.68E-03	1.31E-04	8.79E-04	3.95E-07	2.96E-03	1.11E-03	2.84E-03	1.91E-04	1.85E-03	1.01E-03
blaTEM	β-lactamase	8.64E-05	1.25E-05	5.65E-04	8.90E-05	1.27E-03	2.24E-04	2.69E-03	6.01E-04	2.33E-03	6.79E-04	1.39E-03	1.12E-03

Table S13: Pearson correlations of ARGs concentrations in hospital effluent across five sampling days

	16Sr RNA	*ermB*	*intI1*	*sul1*	*qnrS*	*tetM*	*bla-tem*
16Sr RNA							
ermB	-0.11						
intI1	-0.47	-0.28					
sul1	-0.50	0.53	0.35				
qnrS	0.65	0.67	-0.54	0.13			
tetM	0.56	-0.54	-0.60	**-0.90**	-0.04		
bla-tem	0.17	-0.39	-0.39	**-0.92**	-0.28	0.81	

*Statistically significant results are shown in **bold** ($p \leq 0.05$)

Table S14: Pearson correlations of concentrations of ARGs normalised to 16S rRNA in hospital effluent across five sampling days

	ermB	*intI1*	*sul1*	*qnrS*	*tetM*	*bla-tem*
ermB						
intI1	0.28					
sul1	0.64	0.84				
qnrS	0.37	-0.60	-0.24			
tetM	-0.25	-0.43	-0.48	-0.32		
bla-tem	0.05	-0.22	-0.34	-0.28	0.86	

*Statistically significant results are shown in **bold** ($p \leq 0.05$)

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City's infectious disease treatment with antimicrobial agents – a longitudinal one year study of antimicrobials in two cities via wastewater-based epidemiology

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Abstract

Antimicrobial resistance (AMR) is one of the most significant global health threats. Inappropriate and over-usage of antimicrobial agents (AAs) is a major driver for AMR. Wastewater-based epidemiology (WBE) is a promising tool for monitoring AA usage in communities. Two urban catchment areas have been investigated in this study, one city and one small town in the Southwest of the UK over a 13-month period in 2018-2019. Per capita daily intake of 17 AAs and metabolites has been estimated and obtained estimates were triangulated with catchment specific AA prescription data to understand AA usage patterns (both seasons driven prescription and AA prescription compliance). Results have demonstrated positive correlations for all quantifiable parent AAs and metabolites in wastewater, and spatial variability in AA usage was observed even in neighbouring urban areas. WBE and catchment specific prescription data showed similar seasonal trends but with low correlation in intake. The reasons might be variable prescribing patterns, prescription/intake outside the studied catchment, and/or lack of patient compliance. WBE proved useful in differentiating between consumption vs topical usage and/or direct disposal of unused AA. WBE is superior to prescription data as it provides information on AAs prescribed outside of the monitoring catchment. However, data triangulation, of both prescription data and wastewater data, provides the most comprehensive approach to understanding AA usage in communities.

Keywords: Wastewater-based epidemiology (WBE), antimicrobials, antibiotics, antivirals, Antimicrobial resistance (AMR) prescription

1. Introduction

Antimicrobial resistance (AMR) is an ongoing and growing global health crisis, causing increases in mortality, higher risks of routine medical procedures, and increased economic burden (World Health Organisation, 2015). AMR surveillance is key to understand the extent of resistance, developing and monitoring effective intervention strategies as well as identifying new and emerging resistance threats. In response to growing resistance, the World Health Organisation (WHO) launched the Global Antimicrobial Resistance and Use Surveillance System (GLASS) in 2015, with the aim of establishing the first global collaborative effort to standardise AMR surveillance (WHO, 2015). Participation in the programme has grown from 729 surveillance sites in 22 countries in 2017, to 24 803 surveillance sites in 70 countries reported in 2019 (World Health Organisation, 2021). Whilst this shows positive trends, the high rates of resistance observed stress the importance of ongoing and harmonised global AMR surveillance. Furthermore, concerns and predictions have highlighted that the COVID-19 outbreak could further exacerbate AMR (Rezasoltani et al., 2020), and decreases in surveillance capacity could reduce the ability to provide AMR data during the pandemic (Tomczyk et al., 2021).

The ability to monitor antimicrobial agents (AAs) usage (as well as patterns of prescribing and patient prescription compliance) is critical for AMR surveillance. Whilst AA prescription data can provide valuable insight, several challenges exist to estimating true consumption: i) prescription data may not

be easily accessible for both primary and secondary care; ii) prescription data does not account for AAs that can be purchased over the counter or online; iii) just because an AA is prescribed, does not necessarily mean it has been consumed; and iv) consumption/excretion of AAs may happen in a different catchment to prescription. Compliance to AA is a well-regarded problem, with one study reporting over 1/3 of patients not completing the AA course (Kardas et al., 2005), or individuals reporting stopping treatment before course has finished due to feeling better or having side-effects (Pechere, 2000). Studies have shown that individuals not only admit to not finishing courses of treatment but they also admit stockpiling for self-prescribing at a later date (Kardas et al., 2005; Pechère, 2001).

Wastewater-based epidemiology (WBE) provides a multifaceted approach to community-wide public health monitoring and is a powerful tool for estimating near-real time monitoring of pharmaceutical consumption (Sims and Kasprzyk-Hordern, 2020). The concept is based primarily on the assumption that pharmaceuticals consumed within a community will be excreted either unchanged (parent) and/or as a mixture of metabolites into the sewage system. By measuring these pharmaceutical residues, otherwise known as biomarkers, in influent (untreated) wastewater, back-calculation can be applied to estimate consumption at the community level. WBE has been utilized to inform on a wide range of diverse public health, including illicit drugs (Castiglioni et al., 2006; Zuccato et al., 2005) pharmaceuticals (Kasprzyk-Hordern et al., 2008), tobacco (Castiglioni et al., 2015; Lai et al., 2017; Rodríguez-Álvarez et al., 2014) and alcohol (Boogaerts et al., 2016; Reid et al., 2011); as well as exposure to chemicals such as pesticides (Rousis et al., 2017), industrial chemicals (Been et al., 2017; Lopardo et al., 2019) or personal care products (Lopardo et al., 2018).

Due to the dynamic nature of AMR and the growing requirement for up-to-date and harmonised surveillance data, WBE could be an appropriate community-wide estimation tool for providing complimentary information to GLASS. Applying WBE in this context could not only allow monitoring of local to international trends of AAs, allowing spatial and temporal trends to be established; but could provide baselines for community AA usage and assess effectiveness of intervention strategies. Thereby, offering potential evidence and support for AMR policy decisions. The combined analysis of parent compound and metabolite in wastewater could also give information about community compliance to AAs as well as incidents of pharmaceuticals being directly disposed of into the sewage system (Kasprzyk-Hordern et al., 2021; Petrie et al., 2016).

A handful of studies have investigated back-calculation of biomarkers to compare with prescription rates at the community level (Baz-Lomba et al., 2016; Escolà Casas et al., 2021; van Nuijs et al., 2015; Zhang et al., 2019). One study focusing on four pharmaceuticals and respective metabolites of non-communicable diseases, investigated WBE to assess compliance to pharmaceuticals within a community (Riva et al., 2020). Estimating back-calculation of pharmaceuticals from wastewater and exploring elements of community compliance or incorrect disposal is not without its challenges. Several critical aspects have to be considered: i) establishing relationships between parent AAs and metabolites, ii) understanding human metabolism and establishment and use of metabolic transformation correction factors (CFs), iii) understanding stability of both AAs and their metabolites in wastewater, iv) appreciating all possible sources of AAs, e.g., veterinary usage. Prior to this study, a systematic review from our group has published a series of CFs for AAs (Holton et al., 2022) as well as a new PrAna tool was developed to monitor community prescriptions in selected catchment (Jagadeesan et al., 2022). In this study, validated CFs and catchment prescription data have been applied in a longitudinal one-year study of AA usage in two urban areas from the Southwest England: 120K city of Bath and 21K town Keynsham). The primary aims of this study were to:

1. Explore relationships between daily loads of AAs and their metabolites across a longitudinal study in two contrasting urban areas in Southwest England.

2. Understand spatiotemporal changes to AA/metabolite ratios to inform usage patterns of AAs (e.g. oral vs topical) as well as identify any direct disposal events.
3. Estimate AA intake in the studied catchments using WBE and compare with prescription data to understand prescription compliance and spatiotemporal prescription-AA consumption trends

2. Materials and Method

2.1 Target Analytes

For the study, 58 AAs and 26 metabolites were investigated using a method that has previously been developed within the group (Holton and Kasprzyk-Hordern, 2021) covering a broad range of AA classes (table 1), further chemical information on targets may be found in the supplementary information (table S1). Analytical standards and internal standards (deuterated labelled standards) were purchased from the following companies: Sigma-Aldrich (Gillingham, UK), TRC (Toronto, Canada), LGC (Middlesex, UK), or MCE (Cambridge, UK). All methanol (MeOH) used was HPLC-grade and formic acid (>95 % purity), were both obtained from Sigma-Aldrich. All glassware used in this project was deactivated using 5 % dimethylchlorosilane in toluene to avoid any loss of target analytes via adsorption. Regarding solid phase extraction (SPE), Oasis HLB (60 mg, 3 mL) cartridges were used for the extraction of target analytes, purchased from Waters (Manchester, UK). Polypropylene LC vials, along with Whatman GF/F 0.7 μm filters, were also obtained from Waters.

Table 1: AA targets investigated in this study, ordered by class groupings, table adapted from Holton and Kasprzyk-Hordern, 2021, https://rdcu.be/cxqhT

Grouping	Chemical	Abbrev
Sulphonamide &	Sulfadiazine	SDZ
Trimethoprim	Sulfapyridine	SPY
	Sulfamethoxazole	SMX
	Sulfasalazine	SLZ
	Trimethoprim	TMP
	N-acetyl sulfadiazine	aSDZ
	N-acetyl sulfapyridine	aSPY
	N-acetyl sulfamethoxazole	aSMX
	4-hydroxy-trimethoprim	hTMP
Macrolide	Azithromycin	AZM
& Lincosamide	Erythromycin	ERY
	Clarithromycin	CLR
	Clindamycin	CLI
	N-desmethyl azithromycin	dmAZM
	N-desmethyl erythromycin A	dmERY
	N-desmethyl clarithromycin	dmCLR
	N-desmethyl clindamycin	dmCLI
β-lactams		
Penicillin	Amoxicillin	AMX
	Ampicillin	AMP
	Flucloxacillin	FLX
	Penicillin G	PenG
	Penicillin V	PenV
	Amoxicilloic acid	AMXa

	Ampicilloic acid	AMPa
	Penicilloic G acid	PenGa
Cephalosporin	Cefalexin	LEX
	Cefixime	CFM
	Ceftiofur	CTF
	Ceftriaxone	CRO
Monobactam	Aztreonam	ATM
Carbapenem	Imipenem	IPM
	Meropenem	MEM
Quinolone	Besifloxacin	BSF
	Ciprofloxacin	CIP
	Danofloxacin	DFX
	Enrofloxacin	ENR
	Flumequine	FLU
	Gatifloxacin	GAT
	Lomefloxacin	LOM
	Moxifloxacin	MXF
	Nadifloxacin	NAD
	Nalidixic acid	NAL
	Norfloxacin	NOR
	Ofloxacin (Levofloxacin) *	OFX
	Prulifloxacin	PFLX
	Sarafloxacin	SRF
	Desethylene ciprofloxacin	deCIP
	Hydroxy-norfloxacin	hNOR
	Ofloxacin N-oxide	OFXo
	Desmethyl-ofloxacin	dmOFX
	Ulifloxacin	UFX
TB (1st line)	Isoniazid	INH
	Pyrazinamide	PZA
	Ethambutol	EMB
	Rifampicin	RMP
	Rifabutin	RFB
	Isonicotinic acid	INa
	Acetyl-isoniazid	aINH
	5-Hydroxy-pyrazinoic acid	hPZA
	25-desacetyl rifampicin	daRMP
	25-O-desacetyl rifabutin	daRFB
TB (MDR)	Capreomycin IA	CAPIa
	Capreomycin IB	CAPIb
	Gentamycin C1	GEN1
	Gentamycin C1a	GEN1a
	Gentamycin C2 C2a C2b	GEN2
	Kanamycin A	KAN
	Streptomycin A	STR
	D-cycloserine	DCS

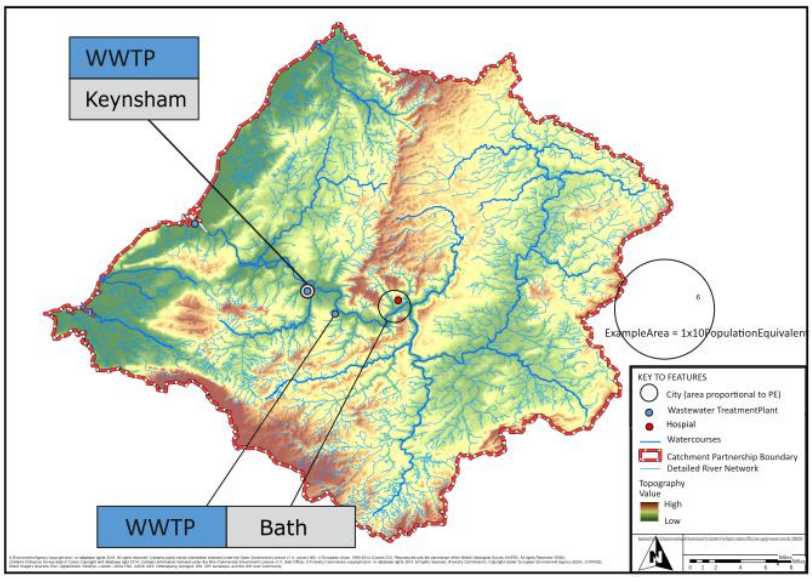
TB (other)	Delamanid	DMD
	Bedaquiline	BDQ
	Linezolid	LZD
	Thalidomide	THAL
OTHER		
Amphenicol	Chloramphenicol	CHL
	Florfenicol	FLO
	2-Amino-1-(4-nitrophenyl)-1,3-propanediol	ANP
Cycline	Doxycycline	DOX
	Oxytetracycline	OTC
	Tetracycline	TET
Nitrofuran	Nitrofurantoin	NIT
	1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione	NPAHD
Azole	Metronidazole	MTZ
	Ketoconazole	KTC
	Hydroxy-metronidazole	hMTZ
	Deacetyl-ketoconazole	daKTC
Antiretroviral	Emtricitabine	FTC
	Lamivudine	3TC

Multi-drug resistant (MDR), tuberculosis (TB), LC-MS method is not chiral (*)

2.2 Sampling sites and sample collection

Two wastewater treatment plants (WWTPs), corresponding to two catchment areas, were selected for this project. This study has previously been reported in Sims *et al.* (Sims et al., 2022). Both sites are within the Southwest of the UK and are a close distance from each other (figure 1). First, is the city of Bath with a population of 120,113 and second is a small town called Keynsham, population estimation of 21,247. Both sites have limited contribution from industry waste (<1 %). Bath, a UNESCO heritage world site, is popular with tourism, and has two Universities residing in its catchment area. Keynsham resides between Bath and the city of Bristol and so observes less tourism in general but will likely have a commuter input.

Influent (untreated) wastewater was collected using 24-hour composite flow proportional samplers for this project. Samples were collected every 15 minutes over the 24-hour period with composite samples combined, to create one 24-hour sample, before being transported on ice to the laboratory for sample processing (<1 hour). Wastewater samples were collected over a 13-month period between 2018-2019.



Site	Sewer residence time[a] (h)	Population served (population equivalents)	Industrial contributions to population equivalents	Mean flow ($m^3 d^{-1}$)
Bath	<0.5-9	120,113	1.2 %	33178
Keynsham	<0.5-2	21,247	0.1 %	3316

[a]under typical summer flows

Figure 1. Site information of studied WWTPs

2.3 Sample preparation and analysis

2.3.1 Sample Preparation

Influent wastewater samples that arrived at the lab were portioned into 50 mL samples and spiked with 50 ng of internal standard spiking mix (50 µL of a 1 µg mL^{-1} internal standard mix in MeOH). Samples were then frozen at this point, prior to the next steps. Samples, when fully defrosted, were filtered via GF/F filters before loading onto preconditioned Oasis HLB cartridges (2 mL of MeOH followed by 2 mL of miliq H_2O). Preconditioning of cartridges occurred at a rate of 1 mL min^{-1} and samples were loaded at a rate of 5 mL min^{-1}. Once loaded, samples were dried under vacuum for at least 30 minutes. Elution from SPE cartridges was achieved using 4 mL of MeOH at a rate of 1 mL min^{-1}. The extracts were then dried under N_2 using a TurboVap evaporator (Caliper, UK) at 40 °C. Reconstitution into polypropylene vials occurred with 500 µL of 80:20 H_2O: MeOH. Samples were then stored at -18 °C until analysis.

2.3.2 Liquid Chromatography

Full method development and analytical procedures have been previously described (Holton and Kasprzyk-Hordern, 2021). Internal standard assignment and instrument and method performance may be found in the supplementary information (table S2). Briefly, liquid chromatography-mass spectrometry was achieved using a Waters, Acquity UPLC system coupled to a XEVO triple quadrupole mass spectrometer. A reverse phase BEH C18 column (50 x 2.1 mm x 1.7 µm) was used. Mobile phase A consisted of 95:5 H_2O:MeOH with 0.1 % formic acid and mobile phase B was 100 % MeOH. The total method is 19 minutes long. An injection volume was 20 µL and flow rate was at 0.2 mL min^{-1}. Starting conditions were 0 % B (1 min), followed by 8.5 min gradient to 40 % B, 3.5 min gradient to 100 % B, 3 min hold, before dropping back to 0 % B.

2.3.3 Mass Spectrometry

As previously mentioned, a XEVO triple quadrupole mass spectrometer was used. This method was in ESI positive mode. The source desolvation temperature was at 400 °C. Regarding gas flows, the cone gas was at 100 L h^{-1} and the desolvation gas was at 1000 L h^{-1}. Argon was used as the collision gas and nitrogen for the nebulising and desolvation gas. Full breakdown on specific compounds transitions and collision cell energies may be found in Holton *et al.* (Holton and Kasprzyk-Hordern, 2021).

2.4 AA Intake calculations and correction factors

2.4.1 Correction factors

Correction factors (CFs) (table 2) have been previously calculated in a systematic pharmacokinetic review by Holton. *et al*. (Holton et al., 2022). To acheieve representative results, the number of observations performed in each study was used for weighting calculated CF values. These CFs are utilised to calculate drug intake in a community, PNDI, by using the amount of AA and/or metabolites detected in wastewater and correcting for human excretion percentage. SPY has been included in this study but it is of note that this AA is no longer prescribed for use in humans in the UK. It is however, a major vetrinary pharmaceutical. SPY and aSPY are both major metabolites for SLZ, which is prescribed for humans in the UK as an anti-inflammatory.

Table 2. Target AA CFs, adapted from Holton *et al.* (Holton et al., 2022)

Metabolite	Abbrev.	CF
Sulfamethoxazole	SMX	6.57
Acetyl sulfamethoxazole	aSMX	1.96
Trimethoprim	TMP	1.58
Hydroxy trimethoprim	hTMP	24.77
Sulfadiazine	SDZ	2.68
Acetyl sulfadiazine	aSDZ	3.88
Sulfasalazine	SLZ	6.25
Sulfapyridine	SPY [SLZ]	11.96
Acetyl sulfapyridine	aSPY [SLZ]	3.62
Sulfapyridine	SPY	4.67
Acetyl sulfapyridine	aSPY [SPY]	2.91
Metronidazole	MTZ	3.98
Hydroxy metronidazole	hMTZ	3.66
Clindamycin	CLI	9.07
Desmethyl clindamycin	dmCLI	19.62
Clarithromycin	CLR	2.92
Desmethyl clarithromycin	dmCLR	18.20
Ciprofloxacin	CIP	1.99
Desethylene ciprofloxacin	deCIP	77.08
Ofloxacin	OFX	1.25
Desmethyl ofloxacin	dmOFX	29.23
N-oxide ofloxacin	oOFX	95.76
Norfloxacin	NOR	2.33
Hydroxy norfloxacin	hNOR	1904.57
Lamivudine	3TC	1.45

Emtricitabine	FTC	1.29
Oxytetracycline	OTC	4.43
Tetracycline	TET	1.55
Doxycycline	DOX	1.22
Nitrofurantoin	NIT	2.90
1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione	NPAHD	-

2.4.2 AA Intake calculations and population normalisation

Daily mass loads (DLs, g day^{-1}) of AAs were calculated to account for variable wastewater flows. This was done by multiplying the total concentrations of AAs (mg L^{-1}) in wastewater over the 24-hour period by daily wastewater flow rates (L day^{-1})

$$DLs_{Analyte}\ (g\ day^{-1}) = Concentration_{Analyte}\ (g\ L^{-1}) * daily\ wastewater\ flow\ (L\ day^{-1})$$

DLs (g day^{-1}) of AAs could then be further normalised to population size of the two catchment areas studied in this project to give population normalised daily loads, PNDLs (mg 1000inh^{-1} day^{-1}). This allows for the fair comparison between different sized communities.

$$PNDL_{Analyte}\ (mg\ 1000inh^{-1}\ day^{-1}) = \frac{Daily\ mass\ load\ of\ AA\ (mg\ day^{-1})}{Population\ size\ of\ WWTP} * 1000$$

To calculate population normalised daily intakes, PNDI (mg 1000inh^{-1} day^{-1}), correction factors (CFs) were taken into account using:

$$PNDI_{AA}\ (mg\ 1000inh^{-1}\ day^{-1}) = PNDL_{Analyte}\ (mg\ 1000inh^{-1}\ day^{-1}) * CF_{Analyte})$$

Where CF were calculated using:

$$CF = \frac{\frac{MW_{AA}}{MW_{Analyte}}}{\%\ Excretion_{Analyte}} * 100$$

Where MW_{AA} and $MW_{Analyte}$ are the molecular weights of the parent AA and the molecular weights of the analyte (either parent compound of respective metabolite). CFs were previously calculated in Holton. *et al*. (Holton et al., 2022). The % $Excretion_{Analyte}$ is the percentage of the target analyte (parent or metabolite) excreted in urine or faeces.

2.5 Prescription tool

An R package, PrAna (Jagadeesan et al., 2022), was utilised to extract total prescription quantities of the pharmaceuticals for the WWTP catchments for the study period 2018 and 2019. This R package generates list of general practices (GPs) and the total prescription quantity of pharmaceuticals inside each WWTP catchment, specific to a study period. This R package uses England-level monthly prescription datasets and GP information (such as GP name, address, and postcode), provided by NHS Digital.

2.6 Population equivalent estimation

A population equivalent (PE-NHS) for the each WWTP catchment was estimated, by the number of people registered in the general practices (GPs) located inside each WWTP catchment zone. Individual GP information, such as postcode and number of people registered, were obtained from NHS Digital (https://digital.nhs.uk/). WWTPs catchment maps were provided by Wessex Water. Briefly, an R (Ihaka and Gentleman, 1996) package was used, PrAna (Jagadeesan et al., 2022) and WWTPs catchment maps

to identify the GPs located inside each WWTPs catchments. The number of patients registered for these GPs were then calculated using R, an open-source software for statistical computing and graphics.

Water utility estimates (table 3) were used to calculate population equivalents in wastewater (PE-WW). The resident population estimate was calculated by multiplying the occupancy rate with the number of properties residing in the designated catchment area. Multi-occupancy buildings were also taken into consideration, including care-homes and university halls. Other additional inputs into the wastewater stream were also considered (e.g. commercial or industrial waste) by using certain water quality indicators (WQIs). For example, industrial PE was calculated via the supply flow to commercial properties and using estimates of 60 g biological oxygen demand (BOD) per capita per day. Chemical oxygen demand (COD) was used to calculate input from tankard waste, by observing the amount of COD present in the known volume of waste (assumption that there was 120 g of COD per capita per day).

Table 3. Populations equivalents used in the study (2018/19), wastewater (above) and NHS data (below)

Wessex Water Population Data	**PE-WW**			
City/Town served	**Bath**		**Keynsham**	
Year	**2018**	**2019**	**2018**	**2019**
Domestic-Billed Properties	43,611	45,274	8,028	9,144
Average Household Size	2.23	2.25	2.23	2.25
Resident Population Estimate	97,253	101,866	17,902	20,572
Adjustment for Care Homes	1,411	1,411	315	315
Adjustment for Universities Adjustment for Schools/Colleges	5,800 800	5,800 800	0 0	0 0
Non-Resident Population	7,250	7,250	123	123
Commercial PE	2,026	2,006	222	222
Trade Effluent PE	1,139	980	0	13
Tankered Waste PE	0	0	0	0
Total PE served by WWTP	115,679	**120,113**	18,562	21,247

PE-NHS			
Bath		Keynsham	
2018	2019	2018	2019
116,030 (SD 770)	118,598(SD 248)	24,640 (SD 284)	25,272(SD 214)

2.7 Statistical analysis

Pearson correlation coefficients were used to explore the potential relationships between parent compounds/metabolites and catchment prescription data. P values were calculated, via paired sample T tests, to investigate statistical significance in longitudinal trends in AAs and metabolites. Statistical significance in all tests was defined as $p \leq 0.05$.

3. Results and Discussion

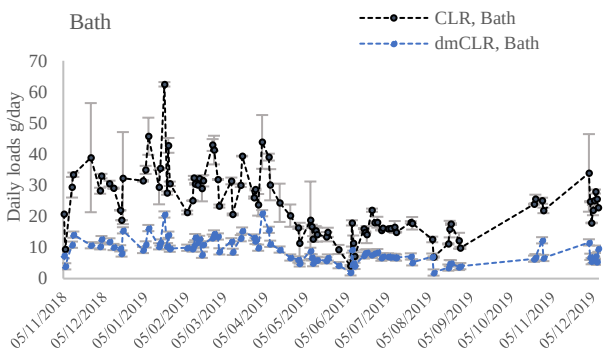
3.1. Abundance of AAs in a 13-months intercity longitudinal study

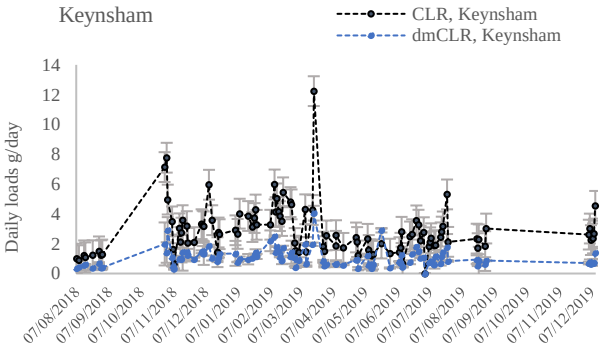
A total number of 58 parent AAs and 26 metabolites were tested in two contrasting catchments (>100K inhabitants in the city of Bath and <20K inhabitants in the town Keynsham) over 13 months. A diverse range of AAs (17 parent AAs and 8 metabolites) was quantified in the wastewater of both communities. AA average loads were, as expected, higher in Bath than in Keynsham (on average 83 ± 24% higher) as AA loads are population size driven. Full breakdowns of loads of all studied AAs can be found in

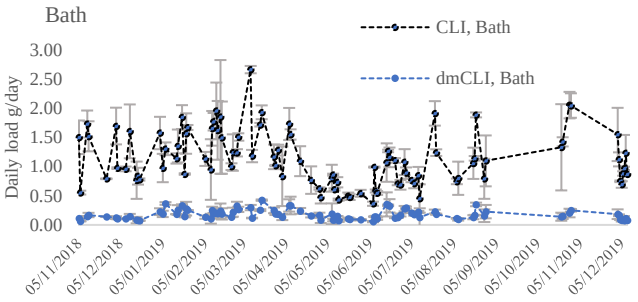
Sims *et al.*(Sims et al., 2022). Seasonal variability of a handful of AAs across different classes was also observed, including MTZ, SLZ, LEX, CIP, and CLR reporting higher loads in winter versus summer. Other AAs reported low temporal variability during the studied period, including the sulphonamides SMX and SPY. A common observation was that the total prescribed AA mass in winter was higher than in summer - particularly for the macrolide, penicillins and cephalosporin classes (Curtis et al., 2019).

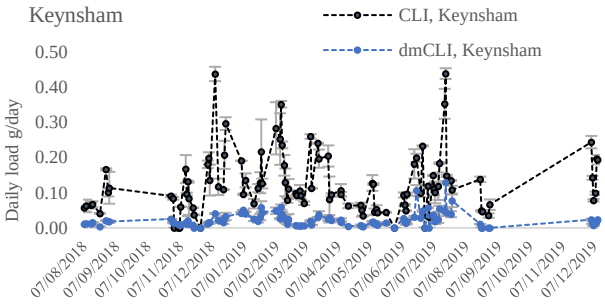
3.2 Correlations of AA/Metabolites in wastewater

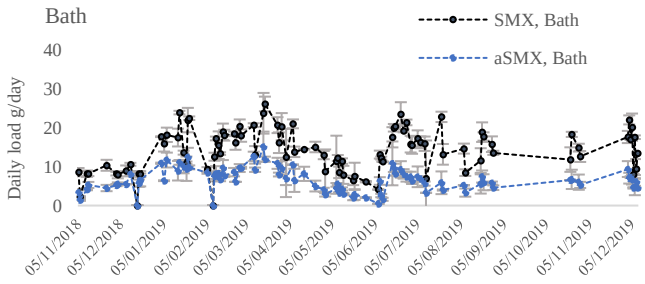
The relationship between parent compounds and metabolites have been investigated further (figure 2), including calculating ratios for individual sampling points for the two catchment areas. Applying correlation statistics between AAs and associated metabolites showed mostly strong correlations in mass loads for both Bath and Keynsham (table 4). CLR and its major metabolite dmCLR demonstrated the strongest correlations ($r=0.9$, $p\leq0.05$ and $r=0.8$, $p\leq0.05$ for Bath and Keynsham, respectively). Furthermore, correlations between AAs that are prescribed as a combination were also observed. For example, SMX and TMP are commonly prescribed together in the UK as a combination known as co-trimoxazole (National Institute for Health and Care Excellence, n.d.). This is due to the different mechanisms of action and combined ability to tackle infections to reduce emerging AA resistance. Positive correlations were observed between SMX and TMP (r value 0.5 and 0.6 for Bath and Keynsham respectively, both p values ≤0.05), aSMX and TMP (r value 0.6 for both Bath and Keynsham, p values ≤0.05). Furthermore, co-trimoxazole is one-part TMP to five parts SMX. On average, whilst loads of SMX in wastewater were higher in general than TMP, the ratio between loads of SMX:TMP were 1.41 ± 0.50 and 2.83 ± 2.41 for Bath and Keynsham, respectively. Reasoning why the ratio between the two is lower than expected could be due to differing metabolism patterns of SMX and TMP, and that TMP can be prescribed by itself.

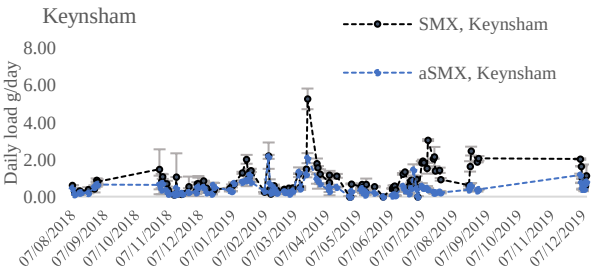

Bath
CLR, Bath
dmCLR, Bath
Daily loads g/day

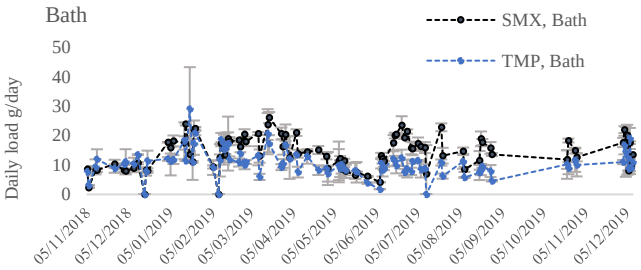

Keynsham
CLR, Keynsham
dmCLR, Keynsham
Daily loads g/day

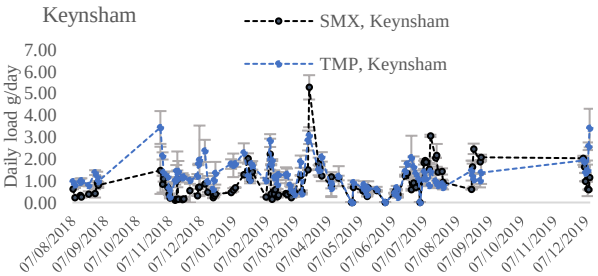

Bath
CLI, Bath
dmCLI, Bath
Daily load g/day

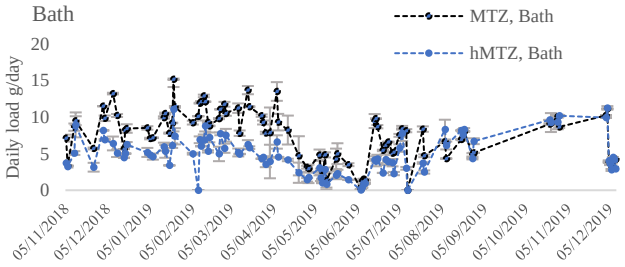

Keynsham
CLI, Keynsham
dmCLI, Keynsham
Daily load g/day

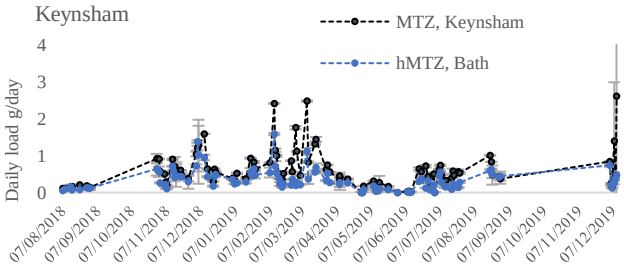

Bath
SMX, Bath
aSMX, Bath
Daily load g/day


Keynsham
SMX, Keynsham
aSMX, Keynsham
Daily load g/day


Bath
SMX, Bath
TMP, Bath
Daily load g/day


Keynsham
SMX, Keynsham
TMP, Keynsham
Daily load g/day


Bath
MTZ, Bath
hMTZ, Bath
Daily load g/day


Keynsham
MTZ, Keynsham
hMTZ, Bath
Daily load g/day

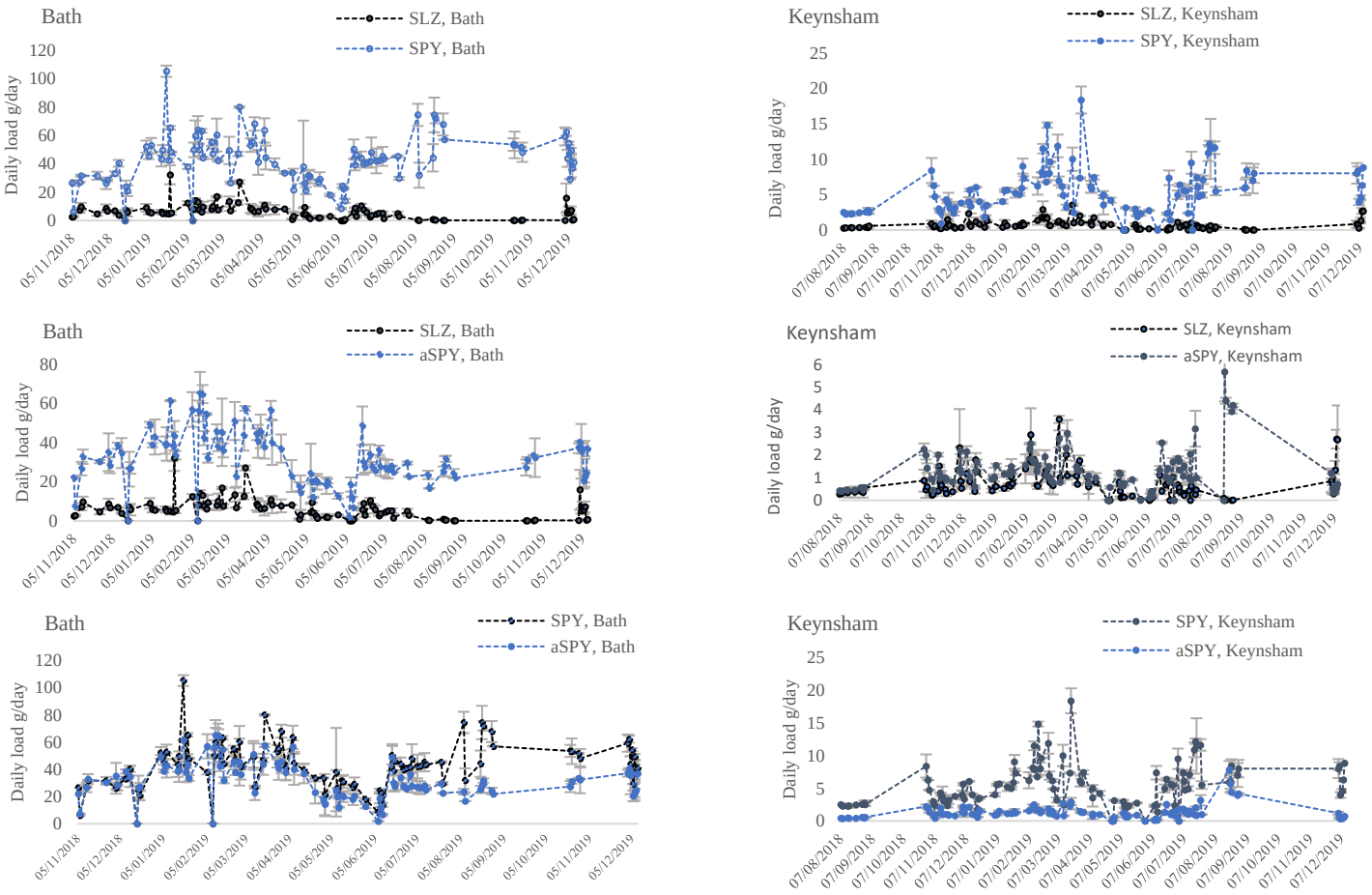


Figure 2. Daily loads (g/day) of parent AAs and respective metabolites in influent wastewater in Bath and Keynsham

Table 4. Pearson correlation coefficients (r value) of parent compound and respective metabolite, all correlations were statistically significant ($p \leq 0.05$)

Parent AA + metabolite	Bath		Keynsham	
	r value	Average ratio AA/metabolite	*r* value	Average ratio AA/metabolite
CLR + dmCLR	0.90	2.74 ± 0.63	0.79	2.81 ± 0.90
MTZ + hMTZ	0.71	1.70 ± 0.68	0.75	2.07 ± 1.21
SPY + aSPY	0.70	1.45 ± 0.53	0.44	5.00 ± 3.29
SLZ + SPY	0.49	0.34 ± 0.22	0.39	0.39 ± 0.23
SLZ + aSPY	0.55	0.46 ± 0.33	0.40	1.90 ± 1.53
SMX + aSMX	0.76	2.40 ± 1.23	0.60	2.52 ± 1.96
CLI + dmCLI	0.60	17.05 ± 6.01	0.91	7.20 ± 3.67
SMX + TMP*	0.51	1.44 ± 0.52	0.42	0.82 ± 0.57

*not parent/metabolite, but two AAs frequently co-prescribed; ± standard deviation

3.3 Catchment comparison of AA/metabolite ratios to verify consumption vs topical application

The comparison of ratios between AA/metabolites in the two sites was investigated (figure 3). Ratios between parent and metabolites will be important for determining consumption of AAs, however many AAs can be prescribed topically as creams or gels. Full breakdown of formulations of prescription data for both sites have been presented (table S3), Due to the presence of outliers, medians were also calculated. In general, median values were close to that of the average, demonstrating normal distribution (table 5). The ratio of CLR/dmCLR in Bath and Keynsham had concordant results, of 2.74

± 0.63 and 2.81 ± 0.90 respectively. This is potentially attributed to both CLR and dmCLR having demonstrated good stability in wastewater, with minimal degradation over a 24-hour period at room temperature (Holton et al., 2022).

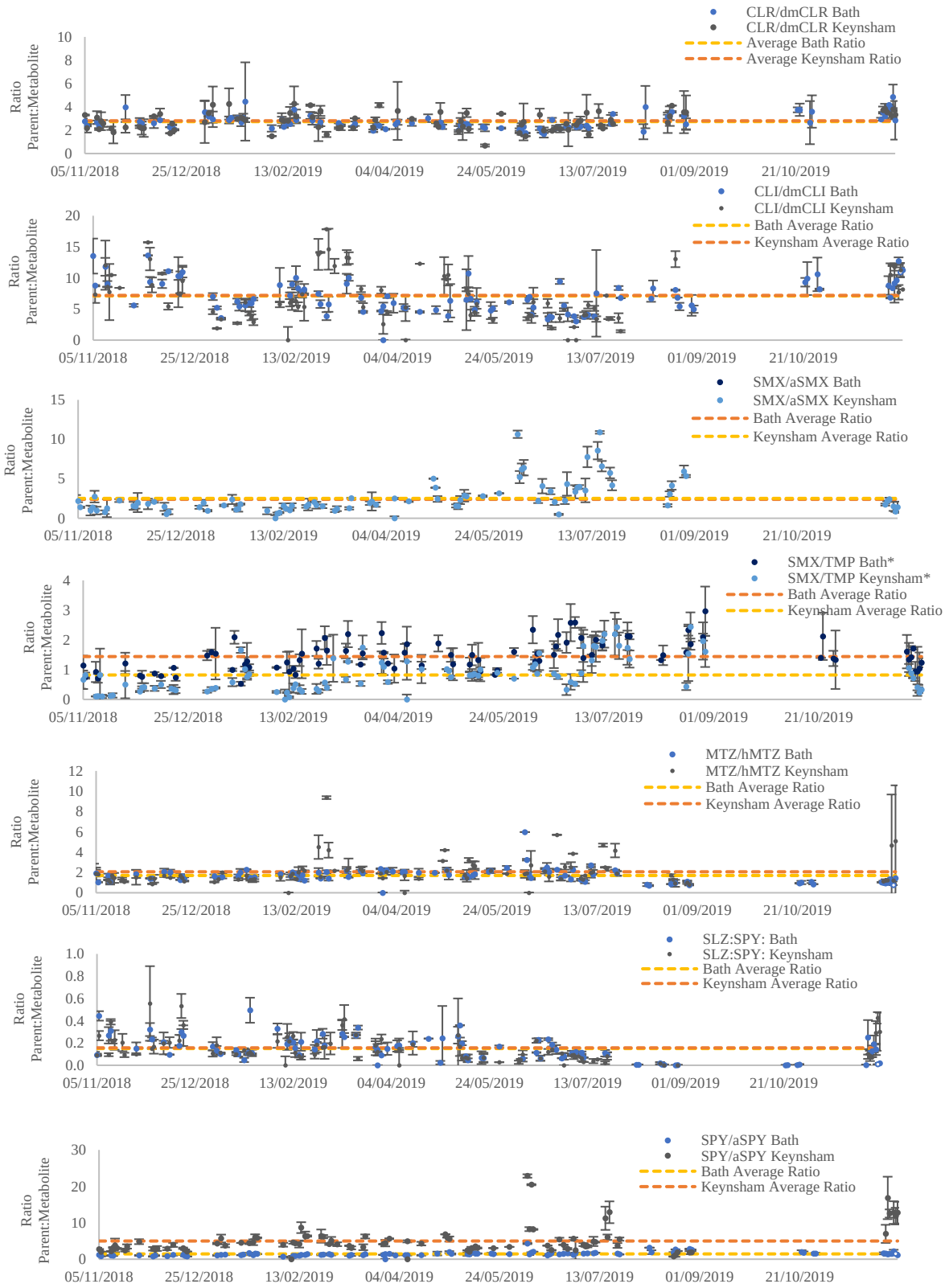


Figure 3. Comparison of ratios and the average ratio across the sampling period of AA/metabolite in Bath and Keynsham

Ratios between SLZ/SPY were consistent in both Bath and Keynsham, at 0.15 ± 0.11 and 0.16 ± 0.11 respectively. SPY and aSPY are both active, major metabolites of SLZ consumption (a prescribed anti-inflammatory). Ratios of SLZ/aSPY and SPY/aSPY did observe statistical differences between the two catchment sites, with Keynsham having higher ratios in both (figure 3). This corresponds to lower levels of aSPY in Keynsham (PNDLs Bath:268 ± 109 mg^{-1} 1000inh day^{-1} vs Keynsham: 65 ± 53 mg^{-1} 1000inh day^{-1}). This could be attributed to stability. Whilst SPY was reportedly stable in wastewater at room temperature (24 hour: 93.0 ± 7.6%), aSPY demonstrated a drop to 75.4 ± 6.0% after 24 hours (Holton et al., 2022). SLZ observed similar stability to aSPY, reporting a drop to 73.4 ± 5.4% after 24 hours.

These dissimilarities observed in ratio, between the two catchment sites, could be attributed to the differences in sewage systems. Resident times for Bath vary between <0.5-9 hours, and for Keynsham between <0.5-2 hours. Bath also has a larger sewage system with an anoxic transport pipe that carries most of the community wastewater to the WWTP. Further studies are needed to better understand potential biodegradation within the sewage system. SLZ had similar PNDLs (Bath: 50.0 ± 44.3 Keynsham: 38.8 ± 32.4 g/day). It is worth mentioning that SLZ is prescribed purely in tablet form and is not utilised topically in the UK (National Institute for Health and Care Excellence, n.d.). Complexities will arise for these compounds; as, whilst SPY is no longer prescribed for human use, it is still used in veterinary practices. The Southwest of England, where both catchments reside, has a strong agricultural presence. Hence, SPY and aSPY could be present in influent wastewater because of run-off, rather than from consumption of SLZ at the community level.

The ratio between CLI and its metabolite dmCLI was higher than the other AAs/metabolites observed in this study at 7.10 ± 2.51 and 7.20 ± 3.67 for Bath and Keynsham, respectively. A previous study has demonstrated a lack of stability for these compounds, with degradation of over 80% reported for both CLI and dmCLI in wastewater after 24 hours (Holton et al., 2022). As degradation was similar for both CLI and dmCLI, it might be theorised that the ratios should have been lower. Stability could be an important factor here, as the standard deviations are large. However, CLI creams can be applied topically, as a first-line acne treatment and for the treatment of bacterial vaginosis. When investigating the breakdown between oral versus topical prescriptions, CLI was predominately prescribed in topical form (figure S1). Bath reported an average of 0.11 kg/month of oral prescriptions versus 0.052 kg/month of topical prescriptions. Keynsham reported 0.096 kg/month of oral versus 0.044 kg/month of topical prescriptions. Whilst, in general, the monthly oral prescriptions outweighed topical, November and December 2019 in both catchment sites observed similar prescribing for both (~0.059 kg/month for both Bath and Keynsham). This could therefore also cause higher levels of parent compound in comparison to the metabolite, hence could also push this ratio up. Whilst contribution from topical AAs could be low, understanding the breakdown of prescription data (topical versus oral) is still important when back calculating consumption at the community level. For other AA/metabolites that have previously demonstrated good stability in wastewater, e.g. CLR and dmCLR, concordance in the ratios between the two catchment sites is observed. The importance of biomarker stability is therefore highlighted again, especially for comparing between different communities. Different catchment areas will likely have unique conditions in the sewage pipes which could be facilitating faster degradation or transformation of either parent or metabolite. Again, more studies on fate of AAs and metabolites from source to WWTP could provide invaluable insight to these potential processes. However, by comparing the ratios of stable biomarkers between different catchment areas, WBE has promising potential to provide highly accurate data and comparisons.

Table 4. Ratio averages and medians of Bath and Keynsham for AA/metabolites in wastewater

	Bath			Keynsham			Statistical difference	Urinary excretion ratio *(parent/metabolite)*
AA/metabolite ratio	**Average**	**STD**	**Median**	**Average**	**STD**	**Median**	***p* value**	
CLR/dmCLR	2.74	0.63	2.59	2.81	0.90	2.81	1.43E-01	6.12
CLI/dmCLI	7.10	2.51	6.65	7.20	3.67	6.01	4.57E-01	2.09
SMX/aSMX	2.40	1.23	2.20	2.52	1.96	1.92	3.47E-01	0.35
SMX/TMP*	1.44	0.52	1.38	0.82	0.57	0.68	**<0.01**	N/A
MTZ/hMTZ	1.70	0.68	1.67	2.07	1.21	1.71	**<0.01**	1.03
SLZ/SPY	0.15	0.11	0.14	0.16	0.11	0.12	3.88E-01	1.20
SLZ/aSPY	0.18	0.13	0.18	0.74	0.75	0.60	**<0.01**	0.42
SPY/aSPY	1.45	0.53	1.38	5.00	3.29	4.17	**<0.01**	0.73

Statistically significant values are in **bold.**

3.3.1 Potential direct disposal events

Exploring ratios between parent and metabolites in wastewater can give insight into potential disposal events into the sewage system. Regarding MTZ/hMTZ, the ratios for Keynsham were slightly higher than for Bath, and whilst comparable, the difference was statistically significant ($p \leq 0.05$). The averaged ratio for MTZ/hMTZ in Bath was 1.70 ± 0.68, and for Keynsham it was 2.07 ± 1.27. Comparing these ratios to the urinary ratio-excretion (parent/metabolite), MTZ/hMTZ had the closest match, with a ratio of 1.03 (table 4). Both parent and metabolite exhibited similar levels of degradation by ~40% in wastewater over 24 hours (Holton et al., 2022). Interestingly, on the 28/02/2019 in Keynsham, MTZ/hMTZ had a ratio of 9.37 ± 0.14. The parent MTZ levels observed were 82.6 ± 2.2 mg $1000inh^{-1}$ day^{-1} with hMTZ levels recorded only at 8.82 ± 0.36 mg $1000inh^{-1}$ day^{-1}. Whilst this was not the highest MTZ level recorded over the sampling period, it was still above average (average PNDL, MTZ: 28.4 ± 23.4 mg $1000inh^{-1}$ day^{-1}). Several days measured ~100 mg $1000inh^{-1}$ day^{-1} for MTZ, but these days observed corresponding high levels of hMTZ (e.g. 09/02/2019, MTZ: 113.51 ± 0.21 mg $1000inh^{-1}$ day^{-1}, hMTZ: 74.5 ± 1.45 mg $1000inh^{-1}$ day^{-1}, ratio 1.52 ± 0.03). This suggest an event of potential direct disposal. However, MTZ is used to treat a range of infections in the UK including of the skin, rosacea and other conditions such as bacterial vaginosis. Of note is that MTZ can be prescribed as a gel or a cream, as well as tablets. The type of MTZ, the dose and treatment time will vary on the type and severity of infection. Some infections can be treated with a single dose tablet, with the standard length of treatment for oral tablets tends to be 5-7 days with people usually feeling better within a few days. Rosacea requires a longer treatment time, using the cream twice a day for 2 months. Treatments applied topically will result in significantly different metabolism in the body. Hence, the proportion of MTZ prescriptions in gel or tablet form could impact observed ratios in wastewater. However, MTZ in both Keynsham and Bath was predominantly prescribed in oral form in comparison to topical form during the sampling period (figure S1). With Bath reporting on average 0.45 kg/month of tablets and capsules prescribed, versus 0.016 kg/month of creams and gels: and Keynsham reporting on average 0.39 kg/month of tablets capsules, versus 0.013 kg/month of creams and gel. Topical treatments of AAs tend to contain a lower dose than tablet or capsule form and are applied more frequently, so potential run off into the sewage system (e.g. from showering) may result in a low and consistent contribution to the overall AA level observed in wastewater.

Previous work done in the catchment area has demonstrated several potential disposal events of pharmaceuticals, including fluoxetine (Petrie et al., 2016); and carbamazepine, propranolol and diltiazem (Kasprzyk-Hordern et al., 2021). It has previously been reported that incorrect disposal practices of pharmaceuticals is a global issue, where unused pharmaceuticals are disposed of into sinks, toilets, or rubbish bins (Tong et al., 2011). antimicrobialswere likely disposed of down the sewage

system. However, due to the nature of AA prescribing (intermittent rather than continuous) as well as variable oral, intravenous or topical applications, it is more difficult to pinpoint all the disposal events. Education and awareness of the environmental risks of incorrect disposal is essential to ensure unused AAs do not unnecessarily end up in the sewar system.

3.4 Population normalised AAs, temporal and spatial differences – data triangulation with catchment demographics and prescribing patterns

To understand spatial and temporal variability of AA usage in the two catchment areas, AA loads in wastewater were normalised to population size (PNDLs) to allow a representative comparison (appendix 1). When comparing overall distribution of AA and metabolite loads per capita between Bath and Keynsham, similar usage of AAs in the two communities can be observed (figure S2). When investigating annual trends with monthly averages (figure S3), higher population normalised loads of AAs could be observed for Bath in general all year round when compared to Keynsham. For example, monthly averages of SMX were found consistently higher in Bath in comparison to Keynsham throughout the sampling period by 60 ± 17% (with overall year averages 121 ± 43 vs 45 ± 36 mg/1000inh/day respectively). Unsurprisingly, aSMX followed a similar trend with population normalised loads as its parent compound, with ~60 % higher each month in Bath when compared to Keynsham. Similar results of consistent higher loads per capita in Bath were observed for MTZ and the two antiviral drugs 3TC and FTC (51 ± 22, 54 ± 20, and 53 ± 9 % higher each month in Bath vs Keynsham, respectively).

A less consistent inter-city trend was observed for CLR. Over the winter months, monthly averages of PNDls of CLR were significantly higher in Bath when compared to Keynsham ($p \leq 0.05$). Between December 2018-February 2019, CLR averaged 273 ± 72 mg $1000inh^{-1}$ day^{-1} in Bath vs 176 ±51 mg $1000inh^{-1}$ day^{-1} in Keynsham (77% difference). In contrast, in summer, PNDLs were much lower, and comparable between the two sites: with CLR averaging 121 ± 35 and 102 mg $1000inh^{-1}$ day^{-1} in Bath and Keynsham, respectively (19% difference). Whilst it is known that macrolide prescriptions often peak in winter, more macrolide AAs were prescribed per person in winter in Bath than Keynsham. Respiratory infections do tend to follow predictable fluctuations, with colder months leading to rapid spreading of infections, due to individuals spending increased time in enclosed spaces indoors (Price et al., 2019). Furthermore, the spread of infections occurs much quicker in urbanised areas, such as cities, due to higher population densities and an increased amount of shared air space (Alirol et al., 2011). Potentially, the reason why Bath had more CLR prescribed per person in winter could be due to more rapid spreading of viral infections in a city versus a town.

It has also been highlighted that spatial differences in AA prescribing do occur. Higher AA prescribing rates have been associated with a higher proportion of patients over 65 and under 18, larger population sizes, ruralness, and deprivation (Curtis et al., 2019; Devine et al., 2021; Thomson et al., 2020). Comparing age demographics between the two sites (figure S4), Keynsham and Bath had a similar percentage of younger children (0-9 years old) at 10%. Regarding the older population, Keynsham had a slightly higher proportion in the 65-95+ age bracket, at 24%, with Bath reporting 16%. Perhaps unsurprisingly, Bath had a higher proportion of younger adults (20-34) around 29%, with Keynsham at 18%. This is in line with the strong university presence in Bath, with two universities residing in the catchment area.

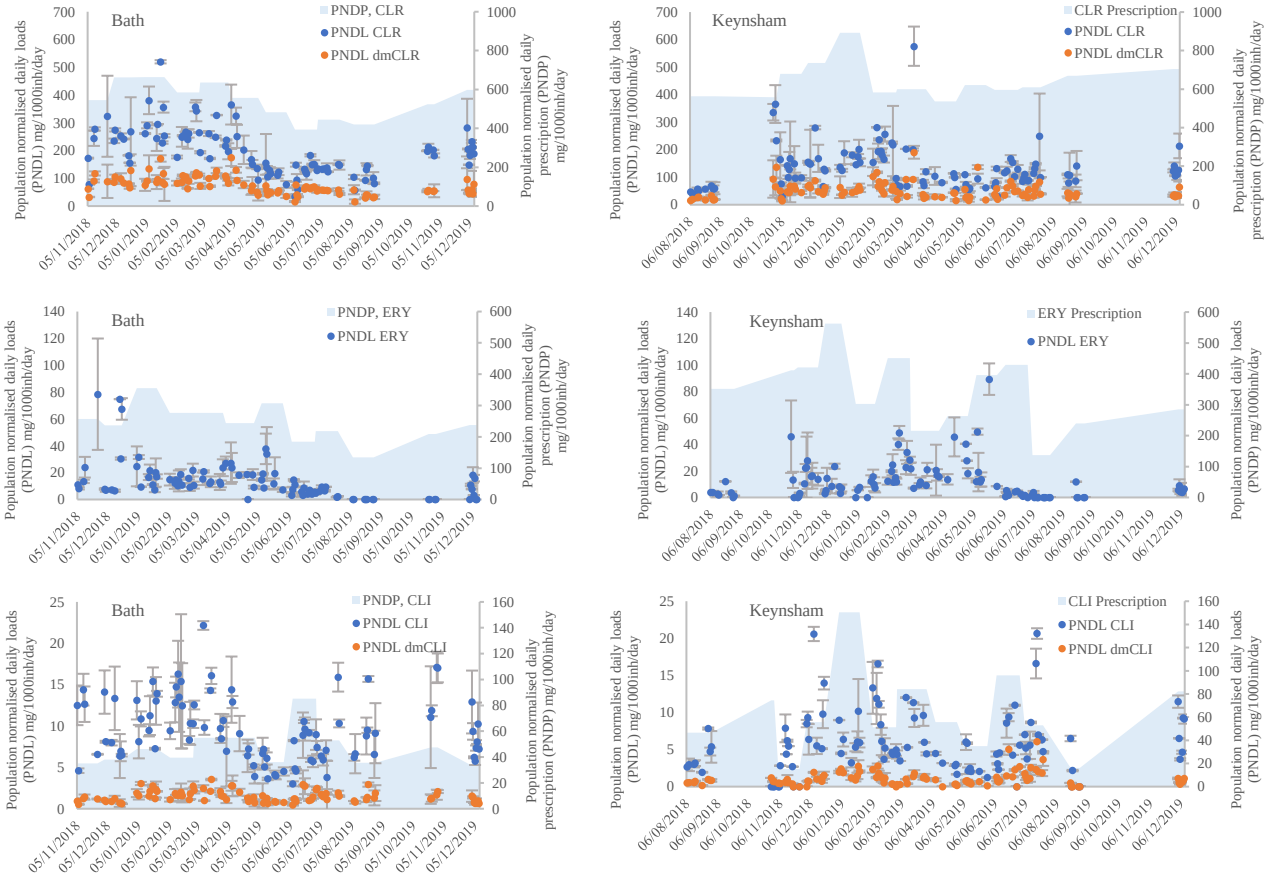
Regarding spatial trends, whilst seasonality of certain diseases will influence AA usage, fluctuating populations will also play a role here. Bath will have population fluctuations due to tourism and an active student population (equating to an estimated 7,250 and 5,800, respectively, additional to the calculated resident PE (table 2)). Whilst university students will typically be away over summer months (June-September), this is considered peak season for tourism. It is estimated that populations fluctuations in Bath will be around ±20%. Considering the high uncertainties associated with

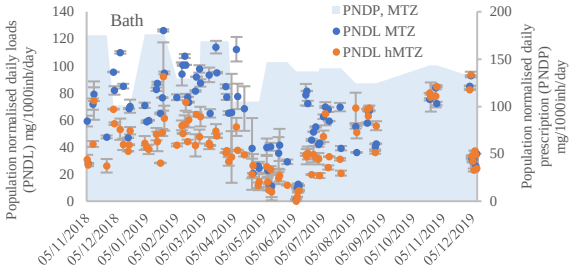
wastewater sampling, it is unlikely the effects of a variable population at this scale will have a visible and notable impact upon AA levels.

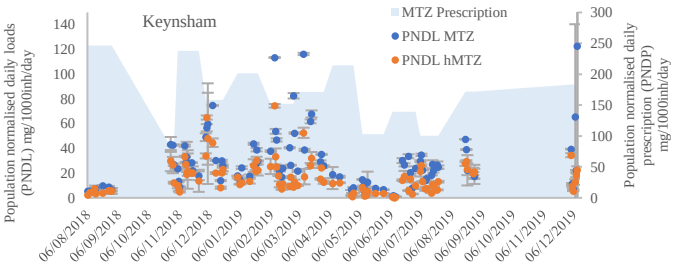
Whilst Keynsham has a significantly reduced non-residential input in comparison to Bath, there will be fluctuations in the population from those commuting. Keynsham is in close proximity to two cities (Bristol and Bath), resulting in additional contribution to respective WWTPs. Population fluctuations due to commuting however, will be occurring all year round so Keynsham is likely considered a more stable population in comparison to Bath.

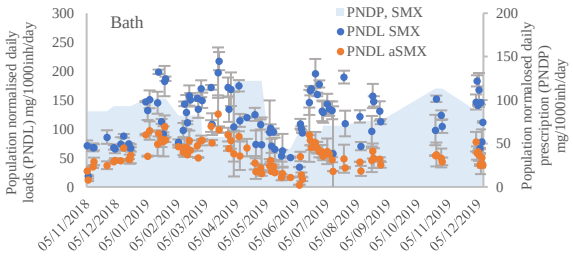
3.5 Comparison of WBE to prescription data – patient compliance

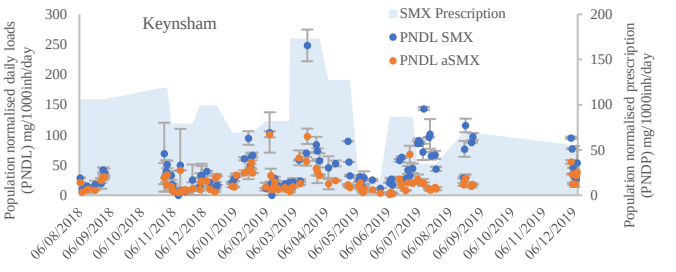
Catchment prescription data for both Bath and Keynsham was also compared with PNDLs in wastewater (figure 4). Interestingly for Keynsham, TET and OFX had months where these were not prescribed by GPs in the catchment area, but were still quantifiable in wastewater. Looking at average prescribing lengths of these AAs, TET can be prescribed long-term for the treatment of acne and rosacea, both requiring longer treatment times of minimum 12 weeks for acne and 6-12 weeks for rosacea. OFX is used in the treatment of different infections including taken orally for the treatment of urinary tract infections (UTIs), and lower respiratory infections but also can be found in eye drops for the treatment of certain infections. OFX tends to be prescribed in much shorter lengths in comparison to TET (days rather than months). Their detection in wastewater during those periods could be attributed to many reasons, including individuals registered to GPs outside the catchment area, delayed prescriptions (in the case of UTIs) or potential self-prescribing. This does highlight the complexity of estimating community usage from wastewater, but also how using prescription data at face value does not always necessarily give the reflection of what is being actually used in the community.

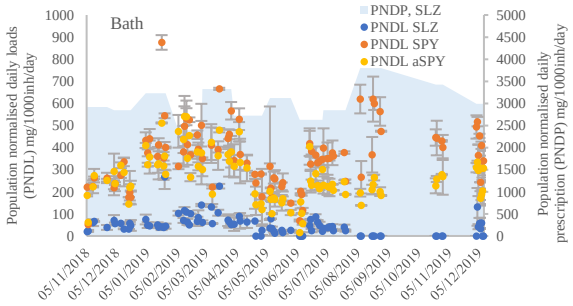


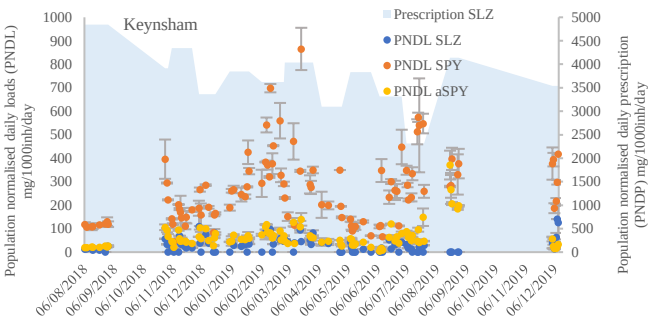

Bath
PNDP, MTZ
PNDL MTZ
PNDL hMTZ
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day


Keynsham
MTZ Prescription
PNDL MTZ
PNDL hMTZ
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day


Bath
PNDP, SMX
PNDL SMX
PNDL aSMX
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day


Keynsham
SMX Prescription
PNDL SMX
PNDL aSMX
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised prescription (PNDP) mg/1000inh/day


Bath
PNDP, SLZ
PNDL SLZ
PNDL SPY
PNDL aSPY
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day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Prescription SLZ
PNDL SLZ
PNDL SPY
PNDL aSPY
Population normalised daily loads (PNDL) mg/1000inh/day
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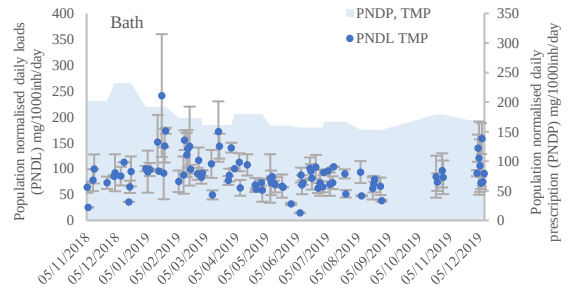
Bath
PNDP, TMP
PNDL TMP
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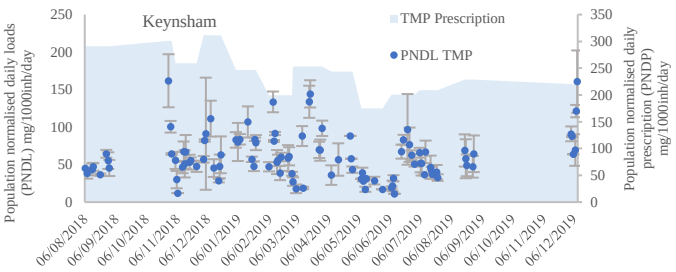
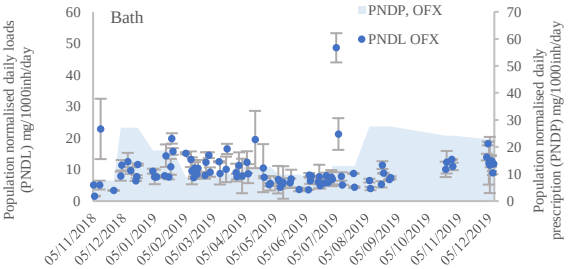
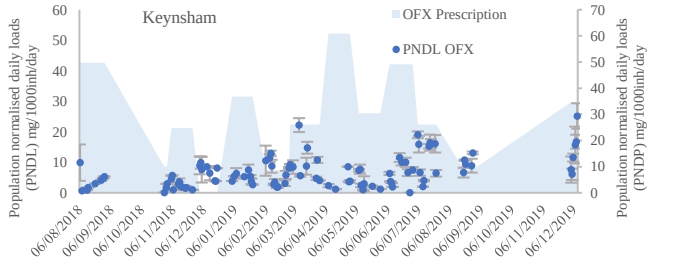
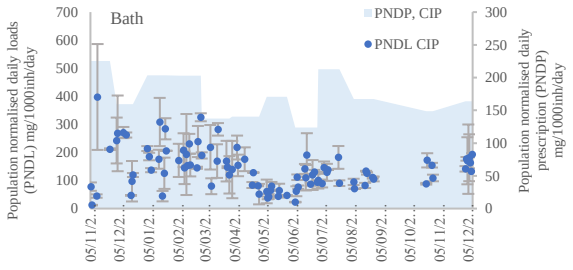
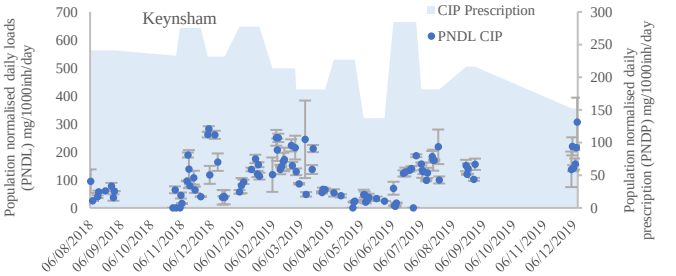
Population normalised daily prescription (PNDP) mg/1000inh/day


Keynsham
TMP Prescription
PNDL TMP
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day


Bath
PNDP, OFX
PNDL OFX
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day


Keynsham
OFX Prescription
PNDL OFX
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily loads (PNDP) mg/1000inh/day


Bath
PNDP, CIP
PNDL CIP
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day


Keynsham
CIP Prescription
PNDL CIP
Population normalised daily loads (PNDL) mg/1000inh/day
Population normalised daily prescription (PNDP) mg/1000inh/day

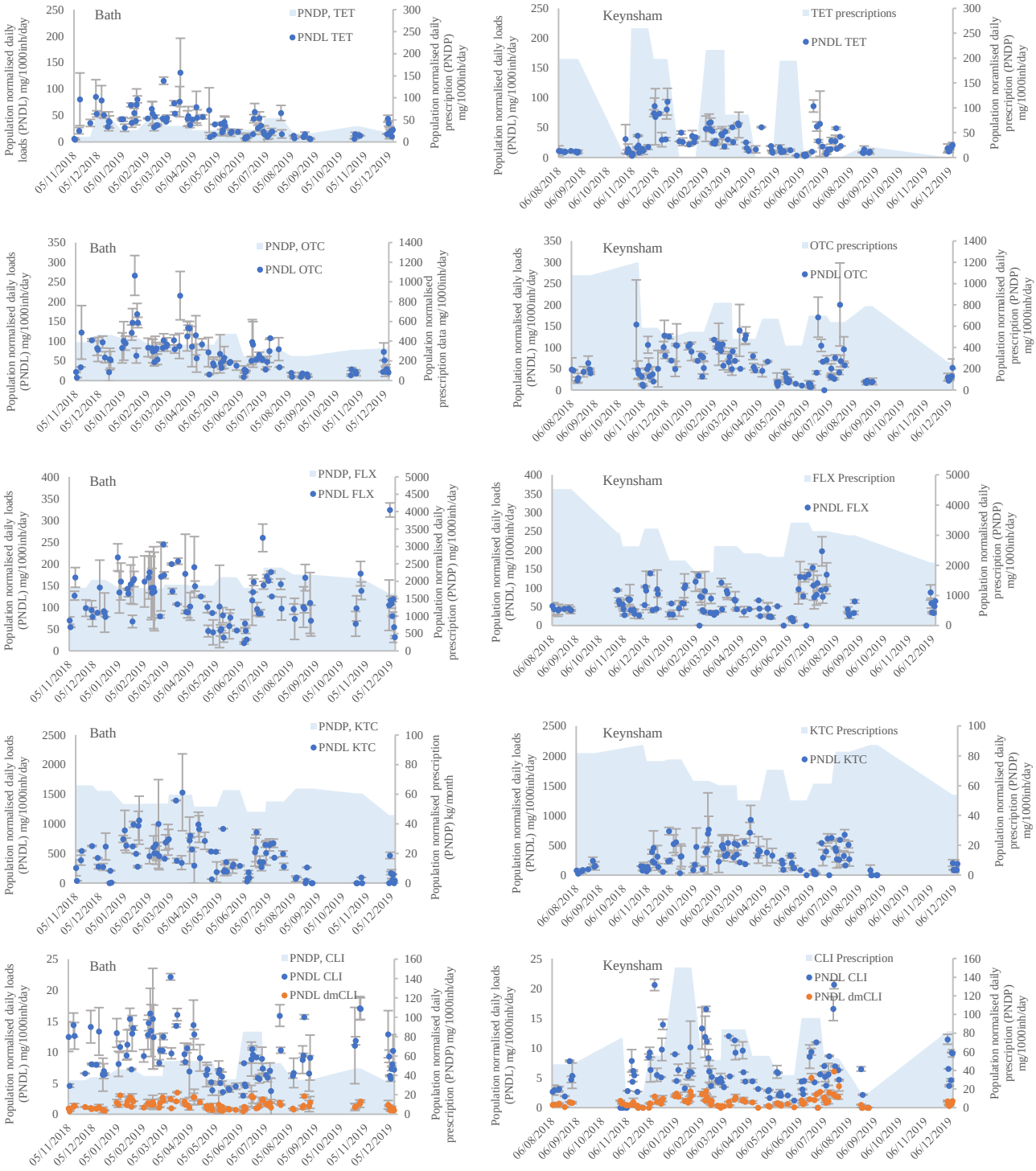


Figure 4. Comparison of PNDLs (mg/1000inh/day) to the monthly catchment prescription data that has been normalised to the population size (mg/1000inh/day).

To further investigate relationships between AAs in wastewater and prescribing patterns, correlations between average monthly loads in wastewater with the abundance of AAs prescribed in each catchment per month were investigated. CLR in Bath had strong positive correlation (r value 0.90, $p \leq 0.05$) between wastewater loads and prescription data. As previously mentioned, CLR has demonstrated strong seasonal changes, with prescription rates being higher in winter months when compared to summer (figure 4). However, whilst catchment prescribing rates for CLR peaked in Keynsham during winter, correlations between prescription and loads were low with no statistical significance (r value 0.05, p

value ≥0.05). Keynsham is a commuter town, so reasoning for this could be partially explained due to fluctuating populations with individuals being prescribed AAs in the catchment area but working outside the catchment area in the cities in close proximity.

Table 5 Correlations of average monthly loads of parent AAs to average monthly catchment prescribing

	Bath	**Keynsham r value**
AA	**r value**	
CLR	**0.90**	-0.05
dmCLR	**0.83**	-0.13
ERY	0.28	0,22
MTZ	0.11	-0.16
hMTZ	-0.01	-0.14
FLX	-0.07	0.05
SMX	0.02	0.17
aSMX	0.10	0.52
TMP	0.13	0.51
SLZ	-0.19	-0.07
SDZ	-0.08	-
CIP	0.23	-0.30
OFX	0.21	-0.10
CLI	-0.05	0.21
dmCLI	0.53	0.34
TET	0.35	0.19
OTC	0.49	0.23
KTC	-0.16	-0.31

Statistically significant values are in **bold.**

Weaker correlations between wastewater and prescription data could be partially due to the nature of AA prescribing, with shorter courses for some AAs, some lasting days with others lasting months. Whilst prescribing data is an invaluable source of information for identifying prescribing patterns and trends, it does not necessarily give a true representation of how AAs are being used in the community. Items prescribed by a GP could be consumed, stock-piled or disposed of. Furthermore, AA compliance could be a further issue, as previously mentioned one survey identified 1/3 of patients not fully complying to AA treatment with 1/4 admitting to stockpiling of AAs to use at a later date (Kardas et al., 2005). For AAs that are not stable, and as a result are rarely observed in wastewater (e.g. penicillins), prescription data can give valuable insight.

Notably, two antivirals FTC and 3TC were monitored in influent wastewater of both sites, however prescriptions of these were not found in the catchment region. It could be that these antivirals were prescribed outside the catchment region or could be from an unknown source. A similar observation was observed for several tuberculosis (TB) drugs, including metabolites hPZA and INa. The usual course of treatment is two antibiotics (isoniazid and rifampicin) for six months, along with additional two antibiotics (pyrazinamide and ethambutol) for the first two months of the six-month period (NHS, n.d.). These are often prescribed in combination due to challenges of multi-drug resistance with many TB infections. Of note is that there are no drugs licensed in the UK for treating animals, and so presence in community wastewater likely originates from human sources (Animal and Plant Health Agency, 2017). Further work is required to fully understand this phenomenon.

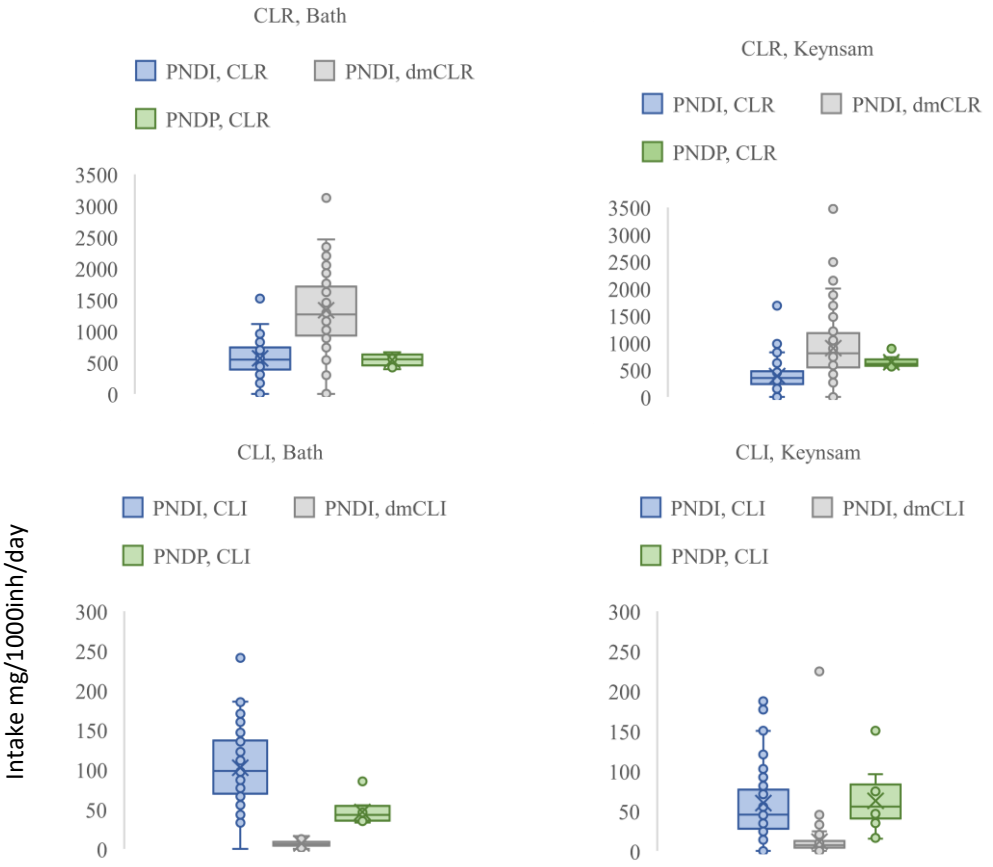
3.6 AA Intake calculations of AAs

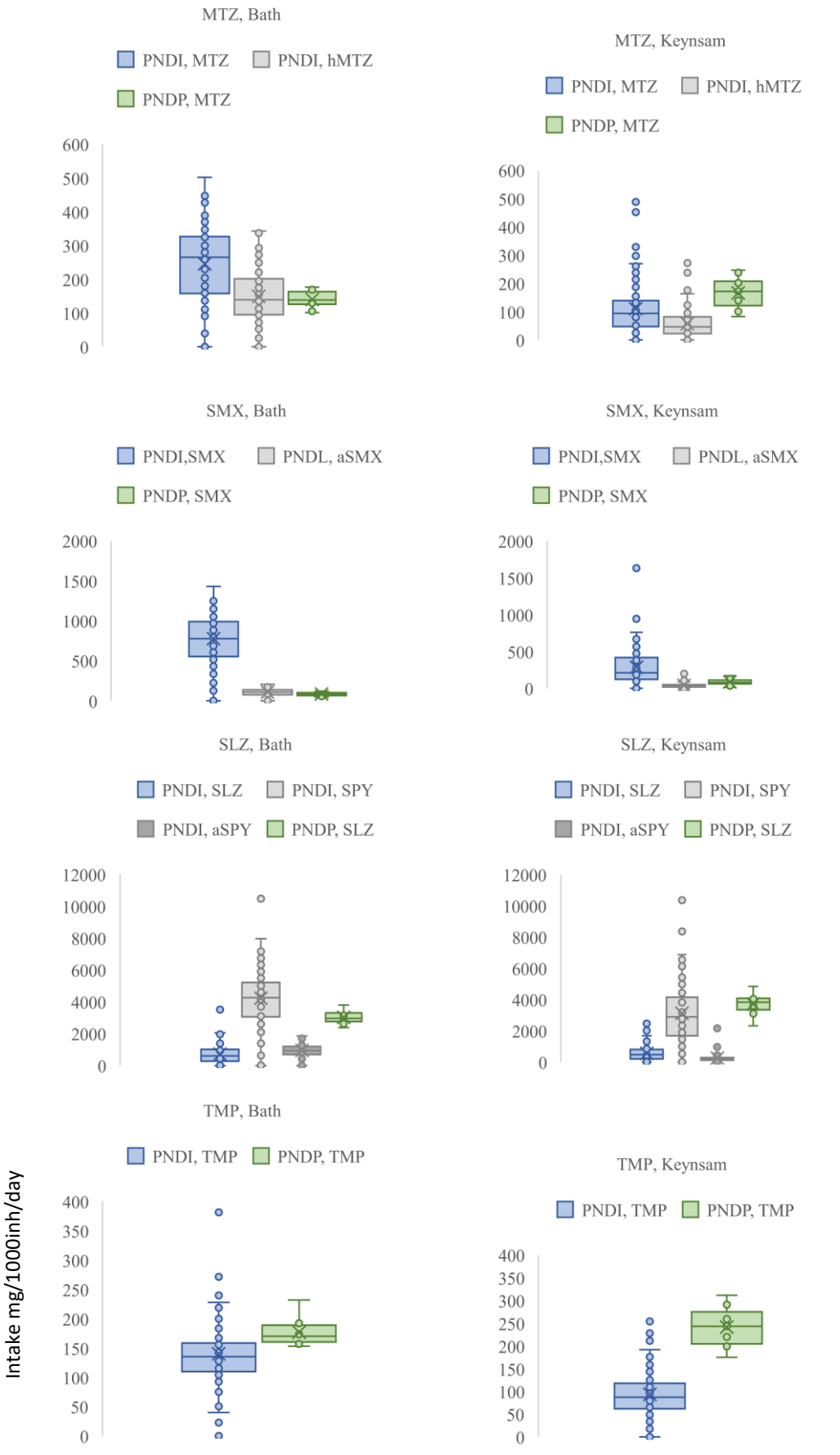
Population normalised daily prescription (PNDPs mg/day/1000inh) were calculated from the total monthly mass of the drug prescribed; these were compared against the population normalised daily intake (PNDIs). As previously mentioned these were calculated in a prior comprehensive literature review investigating the pharmacokinetic data of selected AAs to generate CFs (Holton et al., 2022). The calculated CF values were used to back calculate the mass of drug consumed at the community level, using the amount of AA or respective metabolite quantified in wastewater and the proportion that

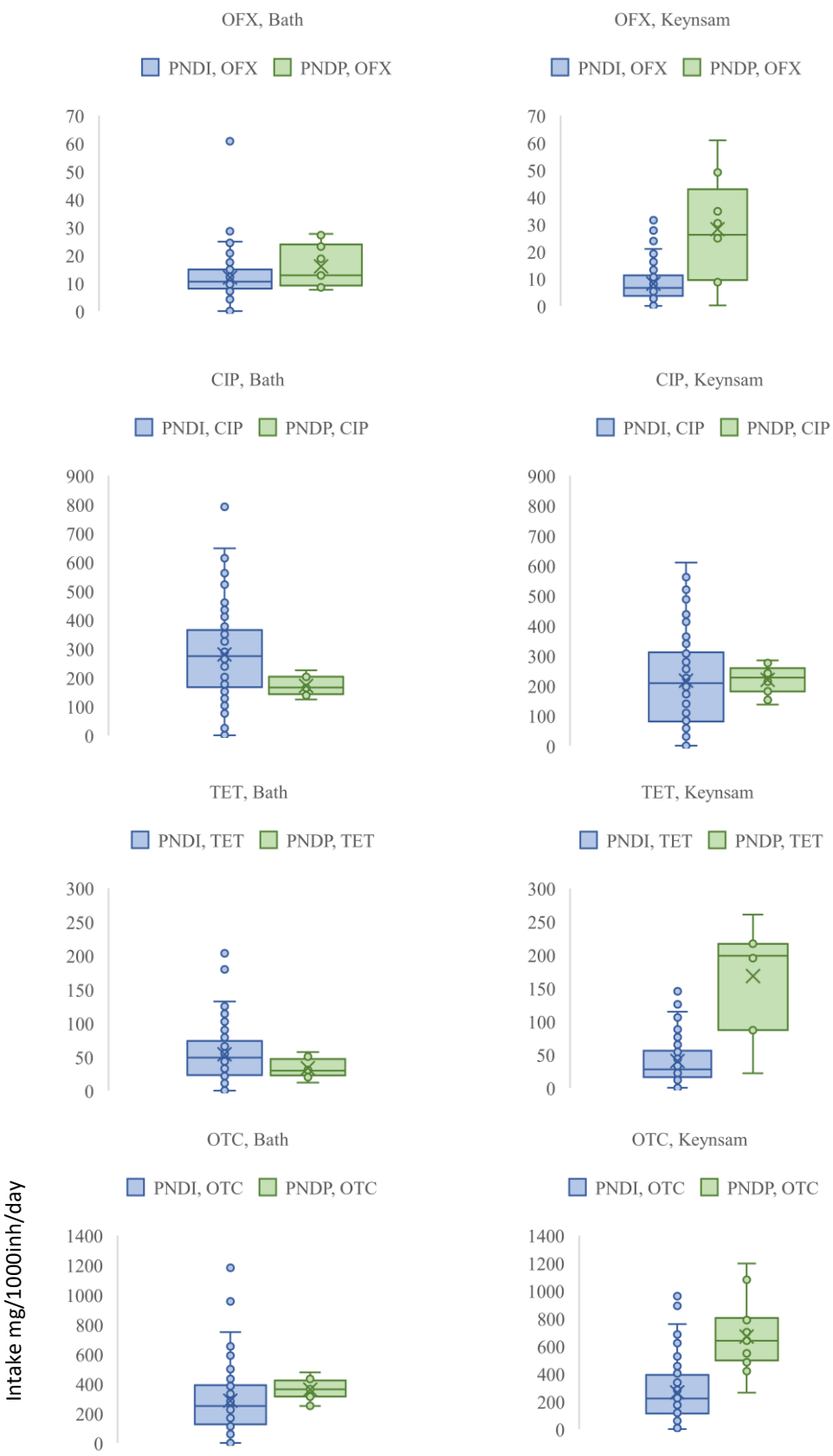
target analyte is excreted via urine or faeces (figure 5). The macrolide CLR gave good agreement between the PNDI and PNDP in Bath (PNDI: 577 ± 189 mg/day/1000inh, PNDP: 543 ± 92 mg/day/1000inh). However, the PNDP values were higher in general for CLR Keynsham (PNDI: 427 ± 183, PNDP: 642 ± 96 mg/day/1000inh). The metabolite dmCLR did not give this same closeness being much higher than its parent compound after correction (PNDI for Bath: 1347 ± 402 mg/day/1000inh, PNDI for Keynsham: 922 ± 323 mg/day/1000inh).

Notably, PNDIs for several AAs were in good agreement between the two sites but the PNDP was different (table S4). Agreement between PNDIs between different locations could indicate that WBE provides a better estimate of community AA usage than prescriptions alone. This is because WBE can account for the potential problems with prescriptions. As mentioned earlier, drugs prescribed may not have been used, delayed prescription, stored or disposed of, and some treatments may last many months (e.g. AAs prescribed for acne treatment). Patients of GP surgeries may also live outside the studied wastewater catchment area, so use/disposal of those pharmaceuticals not captured in the studied wastewater data. Vice versa some individuals may be consuming pharmaceuticals that were prescribed elsewhere within the studied catchment area so would show in wastewater data and not the catchment prescription data. Also, whilst some laboratory stability studies in wastewater have been investigated (Holton et al., 2022), it is recognised that wastewater from different sites could provide very different matrices and the environment within the sewage system could be very different.

For several AAs, the PNDPs were higher in Keynsham than in Bath (e.g. CLR, OFX, TET). Again, this could be for many reasons, including variation in prescription and dispensing locations, bulk prescription of antibiotics and Keynsham has relatively higher population of > 40 aged people. A key difference could be the socioeconomic indices, further work in this area is needed to explore this phenomenon.




MTZ, Bath
PNDI, MTZ
PNDI, hMTZ
PNDP, MTZ
600
500
400
300
200
100
0
MTZ, Keynsam
PNDI, MTZ
PNDI, hMTZ
PNDP, MTZ
600
500
400
300
200
100
0
SMX, Bath
PNDI,SMX
PNDL, aSMX
PNDP, SMX
2000
1500
1000
500
0
SMX, Keynsam
PNDI,SMX
PNDL, aSMX
PNDP, SMX
2000
1500
1000
500
0
SLZ, Bath
PNDI, SLZ
PNDI, SPY
PNDI, aSPY
PNDP, SLZ
12000
10000
8000
6000
4000
2000
0
SLZ, Keynsam
PNDI, SLZ
PNDI, SPY
PNDI, aSPY
PNDP, SLZ
12000
10000
8000
6000
4000
2000
0
TMP, Bath
PNDI, TMP
PNDP, TMP
400
350
300
250
200
150
100
50
0
TMP, Keynsam
PNDI, TMP
PNDP, TMP
400
350
300
250
200
150
100
50
0
Intake mg/1000inh/day


OFX, Bath
PNDI, OFX
PNDP, OFX
70
60
50
40
30
20
10
0
OFX, Keynsam
PNDI, OFX
PNDP, OFX
70
60
50
40
30
20
10
0
CIP, Bath
PNDI, CIP
PNDP, CIP
900
800
700
600
500
400
300
200
100
0
CIP, Keynsam
PNDI, CIP
PNDP, CIP
900
800
700
600
500
400
300
200
100
0
TET, Bath
PNDI, TET
PNDP, TET
300
250
200
150
100
50
0
TET, Keynsam
PNDI, TET
PNDP, TET
300
250
200
150
100
50
0
OTC, Bath
PNDI, OTC
PNDP, OTC
1400
1200
1000
800
600
400
200
0
OTC, Keynsam
PNDI, OTC
PNDP, OTC
1400
1200
1000
800
600
400
200
0
Intake mg/1000inh/day

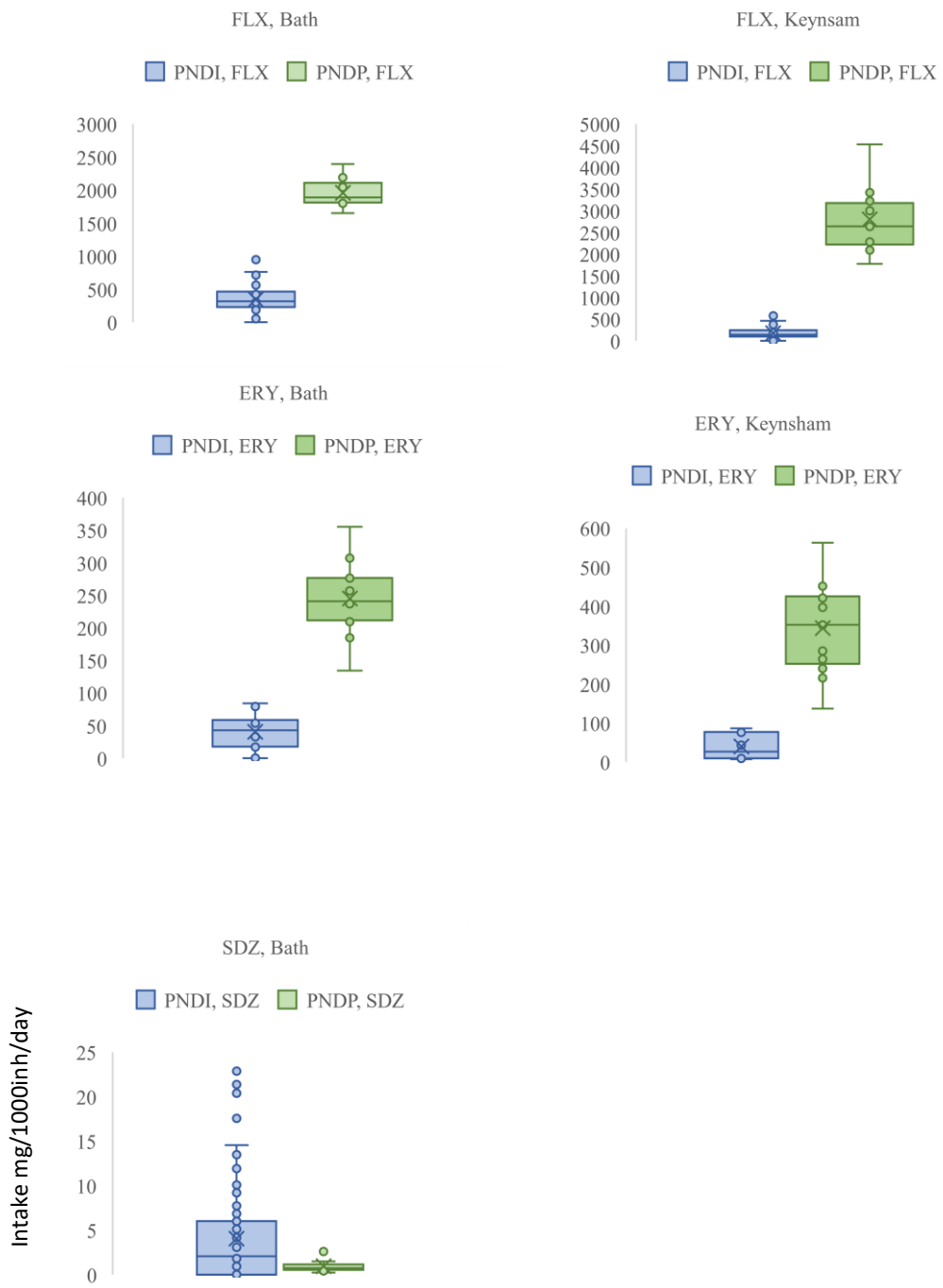


Figure 5. Box plot comparisons of AA PNDIs (n= 92 Bath, n=102 Keynsham) with the monthly averages of PNDPs (n= 12 Bath, n= 13 Keynsham). Breakdown of the monthly averages may be found in figure S5.

4. Conclusions

AMR is of growing concern/will continue to be a problem. It is well recognised that surveillance is critical to tackling AMR. This study investigated AA usage in two different catchment areas, and results have demonstrated similarities in trends of AA usage in both Bath and Keynsham across the sampling period. They have also indicated, even with their close proximity, that more AA is consumed per person in Bath than in Keynsham. A similar observation has been identified by previous work in the group

done within the catchment area, identifying human population size as a key driver of AAs in the environment (Elder et al., 2021). These results demonstrate promising potential for data triangulation of wastewater data and prescription data to provide a comprehensive approach to AA usage in communities. This provides invaluable AMR surveillance data complimentary to surveillance done in clinics and the environment, critical all aspects are covered in a one health aspect. Further work is needed in more communities to explore this concept further. In locations where prescription rates are challenging to access, unknown or where AAs may be obtained easily over the counter, there is clear potential for WBE to provide data in AA usage.

This study aimed to further build upon knowledge in the catchment area and results have demonstrated some key findings:

- Parent AAs and metabolites all observed positive correlations in community wastewater
- Spatial variability in AA usage was observed, even in neighbouring urban areas
- WBE and prescription data showed similar seasonal trends but with low correlation in intake. The reasons might be variable prescribing patterns and/or lack of patient compliance Application of CFs to several AAs to back-calculate intake observed good agreement with estimated intake from the prescription data, closer matches were observed in the catchment with the larger population size (Bath)
- WBE proved useful in differentiating between consumption vs topical usage and/or direct disposal of unused AA
- WBE is superior to prescription data as it provides information on AAs prescribed outside of the monitoring catchment
- Data triangulation (WBE, prescription, and demographics data) provides the most comprehensive approach to AA usage in any given catchment

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Credit authorship contribution statement

Natalie Sims: Conceptualisation, Methodology (sampling, sample preparation, LCMS analysis, back-calculations), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Elizabeth Holton: Methodology (CFs), Writing – review editing; Kishore Jagadeesan: Methodology – PrAna analysis, Writing – review editing; Richard Standerwick: Methodology (conceptualisation, sampling, WWTP information) Writing – review editing; Project administration, Resources. Ruth Barden: Funding acquisition, resources, Writing – review editing; Barbara Kasprzyk-Hordern: Conceptualisation, Methodology (experimental design), Writing-original draft, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources.

References

Alirol, E., Getaz, L., Stoll, B., Chappuis, F., Loutan, L., 2011. Urbanisation and infectious diseases in a globalised world. Lancet Infect. Dis. https://doi.org/10.1016/S1473-3099(10)70223-1

Animal and Plant Health Agency, 2017. Bovine tuberculosis in domestic pets.

Baz-Lomba, J.A., Salvatore, S., Gracia-Lor, E., Bade, R., Castiglioni, S., Castrignanò, E., Causanilles, A., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.-K., van Nuijs, A., Ort, C., Plósz, B.G., Ramin, P., Reid, M., Rousis, N.I., Ryu, Y., de Voogt, P., Bramness, J., Thomas, K., 2016. Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in

wastewater with sale, seizure and consumption data for 8 European cities. BMC Public Health 16, 1035. https://doi.org/10.1186/s12889-016-3686-5

Been, F., Bastiaensen, M., Lai, F.Y., van Nuijs, A.L.N., Covaci, A., 2017. Liquid Chromatography–Tandem Mass Spectrometry Analysis of Biomarkers of Exposure to Phosphorus Flame Retardants in Wastewater to Monitor Community-Wide Exposure. Anal. Chem. 89, 10045–10053. https://doi.org/10.1021/acs.analchem.7b02705

Boogaerts, T., Covaci, A., Kinyua, J., Neels, H., van Nuijs, A.L.N., 2016. Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach. Drug Alcohol Depend. 160, 170–176. https://doi.org/10.1016/j.drugalcdep.2016.01.002

Castiglioni, S., Senta, I., Borsotti, A., Davoli, E., Zuccato, E., 2015. A novel approach for monitoring tobacco use in local communities by wastewater analysis. Tob. Control 24, 38–42. https://doi.org/10.1136/tobaccocontrol-2014-051553

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2006. Identification and Measurement of Illicit Drugs and Their Metabolites in Urban Wastewater by Liquid Chromatography−Tandem Mass Spectrometry. Anal. Chem. 78, 8421–8429. https://doi.org/10.1021/ac061095b

Curtis, H.J., Walker, A.J., Mahtani, K.R., Goldacre, B., 2019. Time trends and geographical variation in prescribing of antibiotics in England 1998-2017. J. Antimicrob. Chemother. https://doi.org/10.1093/jac/dky377

Devine, P., O'kane, M., Bucholc, M., 2021. Trends, Variation, and Factors Influencing Antibiotic Prescribing: A Longitudinal Study in Primary Care Using a Multilevel Modelling Approach. Antibiot. 2022, Vol. 11, Page 17 11, 17. https://doi.org/10.3390/ANTIBIOTICS11010017

Elder, F.C.T., Proctor, K., Barden, R., Gaze, W.H., Snape, J., Feil, E.J., Kasprzyk-Hordern, B., 2021. Spatiotemporal profiling of antibiotics and resistance genes in a river catchment: Human population as the main driver of antibiotic and antibiotic resistance gene presence in the environment. Water Res. 203, 117533. https://doi.org/10.1016/j.watres.2021.117533

Escolà Casas, M., Schröter, N.S., Zammit, I., Castaño-Trias, M., Rodriguez-Mozaz, S., Gago-Ferrero, P., Corominas, L., 2021. Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution. Environ. Int. 150, 106404. https://doi.org/10.1016/j.envint.2021.106404

Holton, E., Kasprzyk-Hordern, B., 2021. Multiresidue antibiotic-metabolite quantification method using ultra-performance liquid chromatography coupled with tandem mass spectrometry for environmental and public exposure estimation. Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-021-03573-4

Holton, E., Sims, N., Jagadeesan, K., Standerwick, R., Kasprzyk-Hordern, B., 2022. Quantifying community-wide antimicrobials usage via wastewater-based epidemiology. https://doi.org/10.26434/CHEMRXIV-2022-PDSF4

Ihaka, R., Gentleman, R., 1996. R: A Language for Data Analysis and Graphics. J. Comput. Graph. Stat. https://doi.org/10.1080/10618600.1996.10474713

Jagadeesan, K.K., Grant, J., Griffin, S., Barden, R., Kasprzyk-Hordern, B., 2022. PrAna: an R package to calculate and visualize England NHS primary care prescribing data. BMC Med. Inform. Decis. Mak. 22. https://doi.org/10.1186/S12911-021-01727-Z

Kardas, P., Devine, S., Golembesky, A., Roberts, C., 2005. A systematic review and meta-analysis of misuse of antibiotic therapies in the community. Int. J. Antimicrob. Agents 26, 106–113. https://doi.org/10.1016/j.ijantimicag.2005.04.017

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. Multiresidue methods for the analysis of

pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography–electrospray tandem mass spectrometry. Anal. Bioanal. Chem. 391, 1293–1308. https://doi.org/10.1007/s00216-008-1854-x

Kasprzyk-Hordern, B., Proctor, K., Jagadeesan, K., Watkins, S., Standerwick, R., Barden, R., Barnett, J., 2021. Diagnosing Down-the-Drain Disposal of Unused Pharmaceuticals at a River Catchment Level: Unrecognized Sources of Environmental Contamination That Require Nontechnological Solutions. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.1c01274

Lai, F.Y., Been, F., Covaci, A., van Nuijs, A.L.N., 2017. Novel Wastewater-Based Epidemiology Approach Based on Liquid Chromatography–Tandem Mass Spectrometry for Assessing Population Exposure to Tobacco-Specific Toxicants and Carcinogens. Anal. Chem. 89, 9268–9278. https://doi.org/10.1021/acs.analchem.7b02052

Li, D., Yang, M., Hu, J., Zhang, Y., Chang, H., Jin, F., 2008. Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. Water Res. https://doi.org/10.1016/j.watres.2007.07.016

Lopardo, L., Adams, D., Cummins, A., Kasprzyk-Hordern, B., 2018. Verifying community-wide exposure to endocrine disruptors in personal care products – In quest for metabolic biomarkers of exposure via in vitro studies and wastewater-based epidemiology. Water Res. 143, 117–126. https://doi.org/10.1016/j.watres.2018.06.028

Lopardo, L., Petrie, B., Proctor, K., Youdan, J., Barden, R., Kasprzyk-Hordern, B., 2019. Estimation of community-wide exposure to bisphenol A via water fingerprinting. Environ. Int. 125, 1–8. https://doi.org/10.1016/j.envint.2018.12.048

National Institute for Health and Care Excellence, n.d. CO-TRIMOXAZOLE [WWW Document]. URL https://bnf.nice.org.uk/drug/co-trimoxazole.html

National Institute for Health and Care Excellence, n.d. SULFASALAZINE [WWW Document]. URL https://bnf.nice.org.uk/drug/sulfasalazine.html

NHS, n.d. Treatment Tuberculosis (TB) [WWW Document]. URL https://www.nhs.uk/conditions/tuberculosis-tb/treatment/

Pechere, J.C., 2000. Parameters important in short antibiotic courses. J. Int. Med. Res.

Pechère, J.C., 2001. Patients' interviews and misuse of antibiotics, in: Clinical Infectious Diseases. https://doi.org/10.1086/321844

Petrie, B., Youdan, J., Barden, R., Kasprzyk-Hordern, B., 2016. New Framework To Diagnose the Direct Disposal of Prescribed Drugs in Wastewater - A Case Study of the Antidepressant Fluoxetine. Environ. Sci. Technol. 50, 3781–3789. https://doi.org/10.1021/acs.est.6b00291

Price, R.H.M., Graham, C., Ramalingam, S., 2019. Association between viral seasonality and meteorological factors. Sci. Rep. https://doi.org/10.1038/s41598-018-37481-y

Reid, M.J., Langford, K.H., Mørland, J., Thomas, K. V., 2011. Analysis and Interpretation of Specific Ethanol Metabolites, Ethyl Sulfate, and Ethyl Glucuronide in Sewage Effluent for the Quantitative Measurement of Regional Alcohol Consumption. Alcohol. Clin. Exp. Res. 35, no-no. https://doi.org/10.1111/j.1530-0277.2011.01505.x

Rezasoltani, S., Yadegar, A., Hatami, B., Asadzadeh Aghdaei, H., Zali, M.R., 2020. Antimicrobial Resistance as a Hidden Menace Lurking Behind the COVID-19 Outbreak: The Global Impacts of Too Much Hygiene on AMR. Front. Microbiol. https://doi.org/10.3389/fmicb.2020.590683

Riva, F., Castiglioni, S., Pacciani, C., Zuccato, E., 2020. Testing urban wastewater to assess compliance with prescription data through wastewater-based epidemiology: First case study in

Italy. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2020.139741

Rodríguez-Álvarez, T., Rodil, R., Rico, M., Cela, R., Quintana, J.B., 2014. Assessment of local tobacco consumption by liquid chromatography-tandem mass spectrometry sewage analysis of nicotine and its metabolites, cotinine and trans-3′-hydroxycotinine, after enzymatic deconjugation. Anal. Chem. 86, 10274–10281. https://doi.org/10.1021/ac503330c

Rousis, N.I., Gracia-Lor, E., Zuccato, E., Bade, R., Baz-Lomba, J.A., Castrignanò, E., Causanilles, A., Covaci, A., de Voogt, P., Hernàndez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.-K., Plósz, B.G., Ramin, P., Ryu, Y., Thomas, K. V., van Nuijs, A., Yang, Z., Castiglioni, S., 2017. Wastewater-based epidemiology to assess pan-European pesticide exposure. Water Res. 121, 270–279. https://doi.org/10.1016/j.watres.2017.05.044

Sims, N., Kannan, A., Holton, E., Jagadeesan, K., Mageiros, L., Standerwick, R., Craft, T., Barden, R., Feil, E.J., Kasprzyk-Hordern, B., 2022. Wastewater-Based Epidemiology for monitoring community derived antimicrobials and resistance genes: a one-year longitudinal study. https://doi.org/10.26434/chemrxiv-2022-7cl0h

Sims, N., Kasprzyk-Hordern, B., 2020. Future perspectives of wastewater-based epidemiology: Monitoring infectious disease spread and resistance to the community level. Environ. Int. 139. https://doi.org/10.1016/j.envint.2020.105689

Thomson, K., Berry, R., Robinson, T., Brown, H., Bambra, C., Todd, A., 2020. An examination of trends in antibiotic prescribing in primary care and the association with area-level deprivation in England. BMC Public Health. https://doi.org/10.1186/s12889-020-09227-x

Tomczyk, S., Taylor, A., Brown, A., De Kraker, M.E.A., El-Saed, A., Alshamrani, M., Hendriksen, R.S., Jacob, M., Löfmark, S., Perovic, O., Shetty, N., Sievert, D., Smith, R., Stelling, J., Thakur, S., Vietor, A.C., Eckmanns, T., 2021. Impact of the COVID-19 pandemic on the surveillance, prevention and control of antimicrobial resistance: A global survey. J. Antimicrob. Chemother. https://doi.org/10.1093/jac/dkab300

Tong, A.Y.C., Peake, B.M., Braund, R., 2011. Disposal practices for unused medications around the world. Environ. Int. https://doi.org/10.1016/j.envint.2010.10.002

van Nuijs, A.L.N., Covaci, A., Beyers, H., Bervoets, L., Blust, R., Verpooten, G., Neels, H., Jorens, P.G., 2015. Do concentrations of pharmaceuticals in sewage reflect prescription figures? Environ. Sci. Pollut. Res. 22, 9110–9118. https://doi.org/10.1007/s11356-014-4066-2

WHO, 2015. Global Antimicrobial Resistance and Use Surveillance System (GLASS) [WWW Document]. URL https://www.who.int/initiatives/glass

World Health Organisation, 2021. Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report.

World Health Organisation, 2015. Global action plan on antimicrobial resistance, World Health Organisation. https://doi.org/ISBN 978 92 4 150976 3

Zhang, Y., Duan, L., Wang, B., Du, Y., Cagnetta, G., Huang, J., Blaney, L., Yu, G., 2019. Wastewater-based epidemiology in Beijing, China: Prevalence of antibiotic use in flu season and association of pharmaceuticals and personal care products with socioeconomic characteristics. Environ. Int. 125, 152–160. https://doi.org/10.1016/j.envint.2019.01.061

Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S., Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. Environ. Heal. 4, 14. https://doi.org/10.1186/1476-069X-4-14

City's infectious disease treatment with antimicrobial agents – a longitudinal one year study of antimicrobials in two cities via wastewater-based epidemiology

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Table S1: Chemical information of AA targets, ordered by class groupings, table taken from Holton and Kasprzyk-Hordern, 2021, https://rdcu.be/cxqhT

Grouping	Chemical	Abbrev	Class A	Class B	CAS No.	Salt form θ	Molec. Formula	M.I. mass	Supplier
Sulfonamide &	Sulfadiazine	SDZ	Sulfonamide	Parent	68-35-9		C10H10N4O2S	250.05	Sigma-Aldrich
Trimethoprim	Sulfapyridine	SPY	Sulfonamide	Parent	144-83-2		C11H11N3O2S	249.06	Sigma-Aldrich
	Sulfamethoxazole	SMX	Sulfonamide	Parent	723-46-6		C10H11N3O3S	253.05	Sigma-Aldrich
	Sulfasalazine	SLZ	Sulfonamides	Parent	599-79-1		C18H14N4O5S	398.07	Sigma-Aldrich
	Trimethoprim	TMP	Trimethoprim	Parent	738-70-5		C14H18N4O3	290.14	Sigma-Aldrich
	N-acetyl sulfadiazine	aSDZ	Sulfonamide	Metabolite	127-74-2		C12H12N4O3S	292.06	TRC
	N-acetyl sulfapyridine	aSPY	Sulfonamide	Metabolite	19077-98-6		C13H13N3O3S	291.07	TRC
	N-acetyl sulfamethoxazole	aSMX	Sulfonamide	Metabolite	21312-10-7		C12H13N3O4S	295.06	TRC
	4-hydroxy-trimethoprim	hTMP	Trimethoprim	Metabolite	112678-48-5		C14H18N4O4	306.13	TRC
Macrolide	Azithromycin	AZM	Macrolide	Parent	83905-01-5		C38H72N2O12	748.51	LCG
& Lincomycin	Erythromycin	ERY	Macrolide	Parent	114-07-8		C37H67NO13	733.46	Sigma-Aldrich
	Clarithromycin	CLR	Macrolide	Parent	81103-11-9		C38H69NO13	747.48	Sigma-Aldrich
	Clindamycin	CLI	Lincomycin	Parent	18323-44-9	Hydrochloride	C18H33ClN2O5S	424.18	Sigma-Aldrich
	N-desmethyl azithromycin	dmAZM	Macrolide	Metabolite	172617-84-4		C37H70N2O12	734.49	TRC
	N-desmethyl erythromycin A	dmERY	Macrolide	Metabolite	992-62-1		C36H65NO13	719.45	TRC
	N-desmethyl clarithromycin	dmCLR	Macrolide	Metabolite	101666-68-6		C37H67NO13	733.46	TRC
	N-desmethyl clindamycin	dmCLI	Lincomycin	Metabolite	22431-45-4		C17H31ClN2O5S	410.16	TRC
β-LACTAMS									
Penicillin	Amoxicillin	AMX	Penicillin	Parent	26787-78-0		C16H19N3O5S	365.10	Fluka
	Ampicillin	AMP	Penicillin	Parent	69-53-4	Trihydrate	C16H19N3O4S	349.11	Fluka
	Flucloxacillin	FLX	Penicillin	Parent	5250-39-5	Sodium	C19H17ClFN3O5S	453.06	Fluka
	Penicillin G	PenG	Penicillin	Parent	113-98-4	Sodium	C16H18N2O4S	334.10	Fluka
	Penicillin V	PenV	Penicillin	Parent	132-98-9	Potassium	C16H18N2O5S	350.09	Sigma-Aldrich
	Amoxicilloic acid	AMXa	Penicillin	Metabolite	42947-63-7 ¥	Trisodium salt	C16H21N3O6S	383.12	TRC
	Ampicilloic acid	AMPa	Penicillin	Metabolite	32746-94-4		C16H21N3O5S	367.12	TRC
	Penicilloic G acid	PenGa	Penicillin	Metabolite	11039-68-2		C9H14N2O5S	262.06	TRC
Cefalosporin	Cefalexin	LEX	Cefalosporin	Parent	23325-78-2	Monohydrate	C16H17N3O4S	347.09	Fluka

	Cefixime	CFM	Cefalosporin	Parent	79350-37-1	Trihydrate	C16H15N5O7S2	453.04	Fluka
	Ceftiofur	CTF	Cefalosporin	Parent	104010-37-9		C19H17N5O7S3	523.03	Fluka
	Ceftriaxone	CRO	Cefalosporin	Parent	104376-79-6	Disodium hemi(heptahydrate)	C18H18N8O7S3	554.05	Sigma-Aldrich
Monobactam	Aztreonam	ATM	Monobactam	Parent	78110-38-0		C13H17N5O8S2	435.05	TRC
Carbapenem	Imipenem	IPM	Carbapenem	Parent	64221-86-9		C12H17N3O4S	299.09	Sigma-Aldrich
	Meropenem	MEM	Carbapenem	Parent	119478-56-7	Trihydrate	C17H25N3O5S	383.15	Sigma-Aldrich
Quinolone	Besifloxacin	BSF	Quinolone	Parent	405165-61-9	Hydrochloride	C19H21ClFN3O3	393.13	MCE
	Ciprofloxacin	CIP	Quinolone	Parent/Metab.	85721-33-1		C17H18FN3O3	331.13	Fluka
	Danofloxacin	DFX	Quinolone	Parent	119478-55-6	Mesylate	C19H20FN3O3	357.15	LCG
	Enrofloxacin	ENR	Quinolone	Parent	93106-60-6		C19H22FN3O3	359.16	Sigma-Aldrich
	Flumequine	FLU	Quinolone	Parent	42835-25-6		C14H12FNO3	261.08	Fluka
	Gatifloxacin	GAT	Quinolone	Parent	112811-59-3		C19H22FN3O4	375.16	TRC
	Lomefloxacin	LOM	Quinolone	Parent	98079-52-8	Hydrochloride	C17H19F2N3O3	351.14	Sigma-Aldrich
	Moxifloxacin	MXF	Quinolone	Parent	268545-13-7	Hydrochloride	C21H24FN3O4	401.18	MCE
	Nadifloxacin	NAD	Quinolone	Parent	124858-35-1		C19H21FN2O4	360.15	MCE
	Nalidixic acid	NAL	Quinolone	Parent	389-08-2	Sodium	C12H12N2O3	232.08	Sigma-Aldrich
	Norfloxacin	NOR	Quinolone	Parent	70458-96-7		C16H18FN3O3	319.13	Sigma-Aldrich
	Ofloxacin (Levofloxacin) *	OFX	Quinolone	Parent	82419-36-1		C18H20FN3O4	361.14	Sigma-Aldrich
	Prulifloxacin	PFLX	Quinolone	Parent (prodrug)	123447-62-1		C21H20FN3O6S	461.11	Sigma-Aldrich
Grouping	**Chemical**	**Abbrev**	**Class A**	**Class B**	**CAS No.**	**Salt form θ**	**Molec. Formula**	**M.I. mass**	**Supplier**
	Sarafloxacin	SRF	Quinolone	Parent	91296-87-6	Hydrochloride	C20H17F2N3O3	385.12	Sigma-Aldrich
	Desethylene ciprofloxacin	deCIP	Quinolone	Metabolite	528851-31-2	Hydrochloride	C15H16FN3O3	305.12	TRC
	Hydroxy-norfloxacin	hNOR	Quinolone	Metabolite	109142-49-6		C16H18FN3O4	335.13	TRC
	Ofloxacin N-oxide	OFXo	Quinolone	Metabolite	104721-52-0	Acetic acid salt	C18H20FN3O5	377.14	TRC
	Desmethyl-ofloxacin	dmOFX	Quinolone	Metabolite	82419-52-1		C17H18FN3O4	347.13	TRC
	Ulifloxacin	UFX	Quinolone	Metabolite	112984-60-8		C16H16FN3O3S	349.09	TRC
TB (1st line)	Isoniazid	INH	Isoniazid	Parent	54-85-3		C6H7N3O	137.06	Sigma-Aldrich
	Pyrazinamide	PZA	Pyrazinamide	Parent	98-96-4		C5H5N3O	123.04	Sigma-Aldrich
	Ethambutol	EMB	Ethambutol	Parent	74-55-5	Dihydrochloride	C10H24N2O2	204.18	Sigma-Aldrich
	Rifampicin	RMP	Rifamycin	Parent	13292-46-1		C43H58N4O12	822.41	Sigma-Aldrich

	Rifabutin	RFB	Rifamycin	Parent	72559-06-9		C46H62N4O11	846.44	Sigma-Aldrich
	Isonicotinic acid	INa	Isoniazid	Metabolite	55-22-1		C6H5NO2	123.03	Sigma-Aldrich
	Acetyl-isoniazid	aINH	Isoniazid	Metabolite	1078-38-2		C8H9N3O2	179.07	Sigma-Aldrich
	5-Hydroxy-pyrazinoic acid	hPZA	Pyrazinamide	Metabolite	34604-60-9		C5H4N2O3	140.02	Sigma-Aldrich
	25-desacetyl rifampicin	daRMP	Rifamycin	Metabolite	16783-99-6		C41H56N4O11	780.39	Sigma-Aldrich
	25-O-desacetyl rifabutin	daRFB	Rifamycin	Metabolite	100324-63-8		C44H60N4O10	804.43	TRC
TB (MDR)	Capreomycin IA ≠	CAPIa	Aminoglycoside	Parent	1405-37-4	Sulfate	C25H44N14O8	668.35	TRC
	Capreomycin IB ≠	CAPIb	Aminoglycoside	Parent	1405-37-4	Sulfate	C25H44N14O7	652.35	TRC
	Gentamycin C1 ≠	GEN1	Aminoglycoside	Parent	1405-41-0	Sulfate salt hydrate	C21H43N5O7	477.32	Fluka
	Gentamycin C1a ≠	GEN1a	Aminoglycoside	Parent	1405-41-0	Sulfate salt hydrate	C19H39N5O7	449.28	Fluka
	Gentamycin C2 C2a C2b ≠	GEN2	Aminoglycoside	Parent	1405-41-0	Sulfate salt hydrate	C20H43N5O7	465.32	Fluka
	Kanamycin A ≠	KAN	Aminoglycoside	Parent	25389-94-0	Sulfate	C18H36N4O11	484.24	Sigma-Aldrich
	Streptomycin A ≠	STR	Aminoglycoside	Parent	3810-74-0	Sulfate	C21H39N7O12	581.27	Sigma-Aldrich
	D-cycloserine	DCS	Isoxazole	Parent/Metab.	68-41-7		C3H6N2O2	102.04	TRC
TB (other)	Delamanid	DMD	Nitroimidazole	Parent	681492-22-8		C25H25F3N4O6	534.17	Sigma-Aldrich
	Bedaquiline	BDQ	Diarylquinoline	Parent	843663-66-1		C32H31BrN2O2	554.16	Sigma-Aldrich
	Linezolid	LZD	Oxazolidinone	Parent	165800-03-3		C16H20FN3O4	337.14	Sigma-Aldrich
	Thalidomide	THAL	Thalidomide	Parent	50-35-1		C13H10N2O4	258.06	Sigma-Aldrich
OTHER									
Amphenicol	Chloramphenicol	CHL	Amphenicol	Parent	56-75-7		C11H12Cl2N2O5	322.01	Sigma-Aldrich
	Florfenicol	FLO	Amphenicol	Parent	73231-34-2		C12H14Cl2FNO4S	357.00	MCE
	2-Amino-1-(4-nitrophenyl)-1,3-propanediol	ANP	Amphenicol	Metabolite	2964-48-9		C9H12N2O4	212.08	Sigma-Aldrich
Cycline	Doxycycline	DOX	Cycline	Parent	24390-14-5	Hyclate	C22H24N2O8	444.15	Sigma-Aldrich
	Oxytetracycline	OTC	Cycline	Parent	2058-46-0	Hydrochloride	C22H24N2O9	460.15	TRC
	Tetracycline	TET	Cycline	Parent	64-75-5	Hydrochloride	C22H24N2O8	444.15	Sigma-Aldrich
Nitrofuran	Nitrofurantoin	NIT	Nitrofuran	Parent	67-20-9		C8H6N4O5	238.03	Sigma-Aldrich
	1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione	NPAHD	Nitrofuran	Metabolite	623145-57-3		C10H8N4O4	248.05	TRC
Azole	Metronidazole	MTZ	Azole	Parent	443-48-1		C6H9N3O3	171.06	Sigma-Aldrich

	Ketoconazole	KTC	Azole	Parent	65277-42-1	C26H28Cl2N4O4	530.15	Sigma-Aldrich
	Hydroxy-metronidazole	hMTZ	Azole	Metabolite	1215071-08-1	C6H9N3O4	187.06	TRC
	Deacetyl-ketoconazole	daKTC	Azole	Metabolite	67914-61-8	C24H26Cl2N4O3	488.14	TRC
Antiretroviral	Emtricitabine	FTC	ARV	Parent	143491-57-0	C8H10FN3O3S	247.04	TRC
	Lamivudine	3TC	ARV	Parent	134678-17-4	C8H11N3O3S	229.05	TRC

Monoisotopic mass (M.I. mass), multi-drug resistant (MDR), tuberculosis (TB), nucleoside reverse transcriptase inhibitor (NRTI)

LC-MS method is not chiral (*); one standard used for all forms within the drug complex (≠); CAS for chiral free acid (¥); salt corrections considered in all calculations, i.e., analysis of the free base (θ)

Table S2. Internal standards used along with instrument and method detection limits (IDLs and MDLs) and instrument and method quantification limits (IQLs and MQLs) table adapted from Holton and Kasprzyk-Hordern, 2021, https://rdcu.be/cxqhT

			Mobile phase (ng L-1)		Influent wastewater (ng L-1)	
Class	**Abbrev**	**Internal Standard**	**IDL**	**IQL**	**MDL**	**MQL**
Sulfonamide &	SDZ	Sulfamethoxazole D4	0.015	0.050	0.230	0.76
Trimethoprim	SPY	Sulfamethoxazole D4	0.003	0.010	0.026	0.088
	SMX	Sulfamethoxazole D4	0.002	0.005	0.014	0.046
	SLZ	Sulfamethoxazole D4	1.500	5.000	5.130	17.09
	TMP	Trimethoprim D9	0.150	0.500	0.970	3.22
	aSDZ	Sulfamethoxazole D4	0.021	0.070	0.230	0.77
	aSPY	Sulfamethoxazole D4	0.167	0.556	1.350	4.51
	aSMX	Sulfamethoxazole D4	0.019	0.063	0.200	0.66
	hTMP	Trimethoprim D9	0.004	0.013	0.030	0.1
Macrolide	AZM	Clarithromycin D3	0.015	0.050	-	-
& Lincomycin	ERY	Clarithromycin D3	0.002	0.005	0.016	0.053
	CLR	Clarithromycin D3	0.002	0.005	0.014	0.046
	CLI	Flumequine 13C3	0.150	0.500	-	-
	dmAZM	Clarithromycin D3	0.375	1.250	-	-
	dmERY	Clarithromycin D3	0.002	0.007	0.020	0.07
	dmCLR	Clarithromycin D3	0.005	0.017	0.070	0.25
	dmCLI	Flumequine 13C3	0.002	0.005	-	-
β-LACTAMS	AMX	Sulfamethoxazole D4	1.500	5.000	-	-
Penicillin	AMP	Ampicillin D5	1.500	5.000	55.600	185
	FLX	Flumequine 13C3	0.150	0.500	1.630	5.45
	PenG	Penicillin G D7	0.150	0.500	5.470	18.2
	PenV	Penicillin G D7	3.000	10.000	81.300	271
	AMXa	Sulfamethoxazole D4	0.300	1.000	146.000	488
	AMPa	Ampicillin D5	0.150	0.500	31.800	106
	PenGa	Penicillin G D7	0.150	0.500	15.400	51.5
Cefalosporin	LEX	Trimethoprim D9	0.375	1.250	5.910	19.7
	CFM	Trimethoprim D9	1.500	5.000	13.200	44
	CTF	Flumequine 13C3	0.150	0.500	-	-
	CRO	Trimethoprim D9	7.500	25.000	48.400	161
Monobactam	ATM	Trimethoprim D9	0.300	1.000	-	-
Carbapenem	IPM	Metronidazole D4	1.500	5.000	-	-
	MEM	Trimethoprim D9	1.500	5.000	-	-
Quinolone	BSF	Flumequine 13C3	0.375	1.250	3.150	10.5
	CIP	Desmethyl-ofloxacin D8	0.150	0.500	1.130	3.77
	DFX	Desmethyl-ofloxacin D8	1.500	5.000	13.900	46.3
	ENR	Desmethyl-ofloxacin D8	0.030	0.100	0.250	0.82
	FLU	Flumequine 13C3	0.003	0.010	0.030	0.1
	GAT	Desmethyl-ofloxacin D8	0.003	0.010	0.030	0.09
	LOM	Desmethyl-ofloxacin D8	0.030	0.100	0.280	0.95
	MXF	Desmethyl-ofloxacin D8	0.375	1.250	3.040	10.1
	NAD	Flumequine 13C3	0.300	1.000	2.680	8.92

	NAL	Flumequine 13C3	0.003	0.010	0.030	0.11
	NOR	Desmethyl-ofloxacin D8	0.003	0.010	0.021	0.069
	OFX	Desmethyl-ofloxacin D8	0.030	0.100	0.410	1.37
	PFLX	Desmethyl-ofloxacin D8	0.300	1.000	1.780	5.95
	SRF	Desmethyl-ofloxacin D8	0.150	0.500	1.500	5.01
	deCIP	Desmethyl-ofloxacin D8	0.150	0.500	1.440	4.82
	hNOR	Desmethyl-ofloxacin D8	3.600	12.000	38.600	129
	OFXo	Desmethyl-ofloxacin D8	3.600	12.000	36.500	122
	dmOFX	Desmethyl-ofloxacin D8	0.150	0.500	1.340	4.45
	UFX	Desmethyl-ofloxacin D8	1.500	5.000	11.400	38.1
TB DRUGS	INH	Isoniazid D4	0.150	0.500	1.520	5.06
TB (1st line)	PZA	Metronidazole D4	1.500	5.000	-	-
	EMB	Metronidazole D4	0.003	0.010	0.130	0.42
	RMP	Rifabutin D7	0.375	1.250	-	-
	RFB	Rifabutin D7	0.150	0.500	1.680	5.62
	INa	Isoniazid D4	0.150	0.500	7.200	24
	aINH	Isoniazid D4	0.150	0.500	3.580	11.9
	hPZA	Metronidazole D4	0.030	0.100	-	-
	daRMP	Rifabutin D7	1.500	5.000	15.000	50
	daRFB	Rifabutin D7	0.030	0.100	0.330	1.11
TB (MDR)	CAPIa	Metronidazole D4	6.621	22.071	4528.000	15094
	CAPIb	Metronidazole D4	6.621	22.071	-	-
	GEN1	Metronidazole D4	2.820	9.400	-	-
	GEN1a	Metronidazole D4	2.070	6.900	-	-
	GEN2	Metronidazole D4	5.250	17.500	-	-
	KAN	Metronidazole D4	3.563	11.875	-	-
	STR	Metronidazole D4	12.000	40.000	2650.000	8835
	DCS	Metronidazole D4	0.150	0.500	-	-
TB (other)	DMD	Rifabutin D7	0.150	0.500	-	-
	BDQ	Rifabutin D7	1.500	5.000	-	-
	LZD	Chloramphenicol D5	0.030	0.100	0.270	0.89
	THAL	Trimethoprim D9	0.300	1.000	3.480	11.6
OTHER	CHL	Chloramphenicol D5	0.150	0.500	1.310	4.35
Amphenicol	FLO	Chloramphenicol D5	3.000	10.000	21.900	73.1
	ANP	Metronidazole D4	1.500	5.000	42.300	141
Cycline	DOX	Rifabutin D7	0.375	1.250	12.700	42.2
	OTC	Desmethyl-ofloxacin D8	0.300	1.000	6.350	21.2
	TET	Desmethyl-ofloxacin D8	0.150	0.500	1.700	5.66
Nitrofuran	NIT	Nitrofurantoin 13C3	0.300	1.000	3.460	11.54
	NPAHD	Chloramphenicol D5	0.030	0.100	0.350	1.18
Azole	MTZ	Metronidazole D4	0.030	0.100	0.270	0.91
	KTC	Flumequine 13C3	0.003	0.010	0.030	0.1
	hMTZ	Metronidazole D4	0.030	0.100	1.010	3.38
	daKTC	Flumequine 13C3	0.375	1.250	-	-
Antiviral	FTC	Metronidazole D4	0.150	0.500	1.570	5.24
	3TC	Metronidazole D4	0.300	1.000	9.510	31.7

Table S3. Formulation data of prescribed AAs investigated in this study during the sampling period for Bath and Keynsham

Bath													
AA	**Formulation**	**Nov-18**	**Dec-18**	**Jan-19**	**Feb-19**	**Mar-19**	**Apr-19**	**May-19**	**Jun-19**	**Jul-19**	**Aug-19**	**Oct-19**	**Dec-19**
amoxicillin trihydrate	Capsule	5.82	7.5	9.02	7.12	5.83	5.94	5.01	4.45	3.98	3.91	6.53	7.83
amoxicillin trihydrate	Oral suspension	1.16	1.64	1.33	1.06	0.969	0.9	0.728	0.803	0.778	0.68	1.01	1.78
amoxicillin trihydrate	Powder	0	0.003	0.027	0	0.009	0.003	0	0.006	0.09	0	0.024	0.018
amoxicillin trihydrate	Tablet	2.07	2.24	2.21	1.89	1.68	1.69	1.61	1.36	1.26	1.4	1.89	1.68
azithromycin	Oral suspension	0.012	0.0024	0.0024	0.009	0.0186	0.0066	0.0072	0.0048	0.0036	0.0198	0.0048	0.0036
azithromycin	Tablet	0.256	0.32	0.371	0.232	0.277	0.247	0.269	0.242	0.245	0.266	0.227	0.241
azithromycin dihydrate	Capsule	0.0403	0.0315	0.0278	0.0345	0.0463	0.0343	0.0373	0.0195	0.036	0.0358	0.036	0.041
cefalexin	Capsule	0.5	0.499	0.474	0.476	0.59	0.472	0.659	0.389	0.514	0.667	0.678	0.613
cefalexin	Oral suspension	0.152	0.223	0.065	0.09	0.0725	0.08	0.108	0.103	0.085	0.0775	0.04	0.0575
cefalexin	Tablet	0.0725	0.12	0.185	0.201	0.211	0.263	0.27	0.305	0.286	0.3	0.261	0.259
chloramphenicol	Eye drops	0.00605	0.0069	0.0081	0.0069	0.0067	0.006	0.00445	0.00395	0.0049	0.0043	0.00495	0.00595
chloramphenicol	Eye ointment	0.00164	0.0016	0.00224	0.0022	0.00176	0.0022	0.00244	0.00192	0.00276	0.0028	0.0024	0.00296
chloramphenicol	Ear drops	0.0005	0	0	0	0	0	0	0	0	0	0	0.001
ciprofloxacin	Oral suspension	0	0	0.015	0	0.01	0	0	0	0	0.01	0.045	0.04
ciprofloxacin hydrochloride	Eye drops	0	0.000045	0.000045	0.00003	0.000045	0.000015	0	0.00003	0.00006	0.00003	0.000015	0.000105
ciprofloxacin hydrochloride	Tablet	0.793	0.561	0.728	0.74	0.492	0.513	0.624	0.453	0.776	0.602	0.499	0.558
ciprofloxacin hydrochloride	Ear drops	0	0	0	0.000075	0.000015	0.000135	0	0	0	0.000045	0.00003	0.000015
clarithromycin	Oral suspension	0.116	0.114	0.165	0.229	0.184	0.109	0.0858	0.0788	0.103	0.0613	0.124	0.13
clarithromycin	Tablet	1.75	2.16	2.2	1.82	1.97	1.85	1.65	1.28	1.49	1.42	1.75	1.97
clarithromycin	Modified-release tablet	0	0	0	0	0	0.007	0	0	0	0	0.01	0.005
clindamycin hydrochloride	Capsule	0.0582	0.078	0.113	0.0768	0.138	0.146	0.0603	0.26	0.14	0.0708	0.107	0.0582
clindamycin phosphate	Cream	0.0064	0.004	0.0064	0.0056	0.0072	0.0064	0.0072	0.0072	0.0024	0.0048	0.0064	0.0088
clindamycin phosphate	Gel	0.0489	0.0417	0.0396	0.0564	0.0462	0.0405	0.0504	0.0336	0.0429	0.0426	0.0498	0.0501
clindamycin phosphate	Liquid	0.0097	0.0102	0.009	0.0054	0.009	0.0078	0.0135	0.0108	0.0075	0.0119	0.0102	0.0042
erythromycin	Gastro-resistant tablet	0.827	0.775	0.957	0.776	0.846	0.754	0.886	0.585	0.595	0.439	0.586	0.805
erythromycin	Gel	0.0024	0.0006	0.0042	0.0042	0.003	0.0012	0.0018	0.0006	0.0012	0.003	0.003	0

erythromycin	Liquid	0.0612	0.0572	0.0718	0.0624	0.064	0.0614	0.0658	0.0694	0.0492	0.0498	0.0342	0.0498
erythromycin	Gastro-resistant capsule	0	0	0	0	0	0.007	0	0	0.007	0	0	0
erythromycin ethyl succinate	Oral suspension	0.019	0.015	0.055	0.03	0.0175	0.0175	0.015	0.0125	0.0075	0.03	0.03	0.059
erythromycin ethyl succinate	Tablet	0	0	0	0	0	0	0	0.028	0	0	0	0
erythromycin stearate	Tablet	0.127	0.144	0.0823	0.084	0.143	0.056	0.05	0.176	0.056	0.106	0.112	0.056
flucloxacillin sodium	Capsule	5.86	6.57	5.98	5.84	6.28	6.32	7.18	5.92	7.67	7.15	6.93	5.49
flucloxacillin sodium	Oral solution	0.258	0.188	0.418	0.258	0.233	0.248	0.265	0.293	0.37	0.323	0.248	0.253
flucloxacillin sodium	Powder for solution for injection	0	0	0	0	0	0	0	0	0	0.025	0	0
ketoconazole	Cream	0.0066	0.0054	0.012	0.0018	0.0066	0.0036	0.006	0.0078	0.012	0.0048	0.0072	0.0066
ketoconazole	NA	0.223	0.214	0.182	0.192	0.209	0.182	0.223	0.166	0.19	0.228	0.214	0.161
levofloxacin hemihydrate	Eye drops	0.00015	0.00015	0.000175	0.00015	0.00005	0.000025	0	0	0	0.000025	0	0
levofloxacin hemihydrate	Tablet	0	0	0	0.007	0	0	0	0	0.0035	0	0	0
metronidazole	Cream	0.00615	0.00698	0.00585	0.00675	0.00615	0.0075	0.00945	0.00668	0.00585	0.0057	0.00863	0.00765
metronidazole	Gel	0.0057	0.00668	0.00563	0.00559	0.00728	0.00709	0.00589	0.00773	0.00469	0.00608	0.00521	0.00626
metronidazole	Tablet	0.547	0.33	0.593	0.456	0.55	0.327	0.483	0.444	0.423	0.394	0.469	0.438
metronidazole	Vaginal gel	0.0024	0.0036	0.0012	0.0033	0.0039	0.0015	0.0036	0.003	0.0033	0.003	0.0024	0.003
metronidazole	Suppository	0	0	0	0	0.02	0	0	0	0	0	0	0
metronidazole benzoate	Oral suspension	0	0.02	0.008	0.004	0	0.008	0.008	0.016	0	0.008	0.004	0
ofloxacin	Eye drops	0.000255	0.00018	0.000195	0.00009	0.00003	0.000105	0.00024	0.000225	0.00021	0.000255	0.00009	0.00015
ofloxacin	Tablet	0.0264	0.0728	0.068	0.0324	0.0372	0.0464	0.0224	0.0192	0.0468	0.0892	0.084	0.0784
oxytetracycline calcium	Cream	0.0027	0	0.0027	0.0045	0.0108	0	0.0135	0.0054	0.0162	0.0153	0.0207	0.0423
oxytetracycline dihydrate	Tablet	1.37	1.63	1.58	1.35	1.31	1.33	1.72	0.907	1.15	0.899	1.12	1.15
oxytetracycline hydrochloride	Ointment	0	0	0	0	0	0	0.0009	0.0009	0	0	0	0
rifampicin	Capsule	0.0378	0.0336	0.0336	0.0486	0.042	0.0546	0.0546	0.0168	0.0546	0.0294	0.0378	0.0168
sulfadiazine silver	Cream	0.0032	0.002	0.0008	0.0043	0.004	0.0055	0.0015	0.002	0.0025	0.0025	0.0095	0.003
sulfamethoxazole	Tablet	0.265	0.308	0.331	0.281	0.202	0.418	0.134	0.215	0.25	0.259	0.362	0.296
sulfamethoxazole	Oral suspension	0	0	0	0	0.008	0.008	0.004	0	0	0.008	0.016	0.008
sulfasalazine	Gastro-resistant tablet	5.71	4.34	5.93	3.55	5.37	4.9	5.71	4.62	3.91	6.55	6.75	4.57
sulfasalazine	Tablet	3.57	4.91	4.4	3.51	4.78	4.23	4.4	3.77	5.14	5.33	4.6	5.08
tetracycline hydrochloride	Tablet	0.042	0.2	0.1	0.184	0.133	0.098	0.112	0.102	0.198	0.072	0.13	0.077

trimethoprim	Oral suspension	0.0501	0.0463	0.0455	0.0351	0.035	0.042	0.0275	0.0296	0.0243	0.0287	0.0316	0.0364
trimethoprim	Tablet	0.586	0.736	0.619	0.575	0.536	0.588	0.53	0.517	0.557	0.507	0.588	0.528

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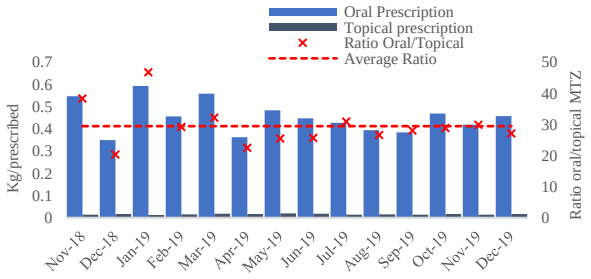
AA	Formulation	Aug-18	Nov-18	Dec-18	Jan-19	Feb-19	Mar-19	Apr-19	May-19	Jun-19	Jul-19	Aug-19	Dec-19
amoxicillin trihydrate	Capsule	0.925	1.45	1.83	1.6	0.921	1.07	0.929	0.719	0.638	0.505	0.505	1.21
amoxicillin trihydrate	Oral suspension	0.158	0.368	0.47	0.3	0.235	0.284	0.271	0.103	0.08	0.0734	0.097	0.273
amoxicillin trihydrate	Tablet	0.409	0.535	0.545	0.276	0.28	0.319	0.392	0.34	0.323	0.348	0.362	0.193
amoxicillin trihydrate	Powder	0	0	0	0	0	0	0	0	0	0	0	0
azithromycin	Tablet	0.0673	0.066	0.107	0.0725	0.0368	0.0793	0.0608	0.077	0.0415	0.0353	0.0788	0.0508
azithromycin	Oral suspension	0.0156	0.0138	0.018	0	0.0072	0.0024	0.0108	0	0.006	0	0.006	0
azithromycin dihydrate	Capsule	0.0255	0.0315	0.0253	0.0075	0.0113	0.009	0.0128	0.00975	0.006	0.0128	0.006	0.0128
cefalexin	Capsule	0.129	0.114	0.0705	0.0585	0.122	0.0305	0.057	0.0705	0.045	0.0395	0.057	0.088
cefalexin	Oral suspension	0.01	0.0075	0.0025	0.02	0	0.03	0.0175	0.0075	0.0075	0.0125	0.0125	0.01
cefalexin	Tablet	0.0648	0.042	0.035	0.0105	0.0105	0	0.007	0.00125	0.007	0	0	0
ceftriaxone sodium	Powder for solution for injection	0	0	0	0	0	0	0	0	0.004	0	0	0
chloramphenicol	Eye drops	0.00085	0.00085	0.00115	0.001	0.00095	0.00055	0.00105	0.0004	0.00095	0.0004	0.0007	0.0006
chloramphenicol	Eye ointment	0.00052	0.00068	0.00064	0.00024	0.0006	0.0008	0.00032	0.00056	0.00068	0.00032	0.00048	0.00068
ciprofloxacin	Oral suspension	0.01	0.005	0	0	0	0	0	0	0	0.01	0	0
ciprofloxacin hydrochloride	Eye drops	0	0.000015	0.000015	0.00003	0.000015	0	0	0	0.000015	0	0	0.000075
ciprofloxacin hydrochloride	Tablet	0.126	0.151	0.131	0.097	0.073	0.065	0.0715	0.06	0.089	0.06	0.0805	0.0478
ciprofloxacin hydrochloride	Ear drops	0	0	0	0	0	0	0.000015	0	0.000015	0	0	0
clarithromycin	Oral suspension	0.0193	0.0175	0.028	0.0175	0.0105	0.0228	0.014	0.0193	0.0105	0.0228	0.021	0.0193
clarithromycin	Tablet	0.299	0.366	0.388	0.342	0.257	0.259	0.204	0.257	0.241	0.218	0.245	0.262
clarithromycin	Modified-release tablet	0	0	0	0	0	0	0	0	0.007	0	0	0
clindamycin hydrochloride	Capsule	0.0126	0	0.024	0.0654	0.0204	0.0336	0	0.012	0.0312	0.0036	0	0
clindamycin phosphate	Cream	0.0016	0	0	0.0016	0.0016	0	0.0024	0.0016	0	0.0008	0	0.0024
clindamycin phosphate	Gel	0.009	0.0048	0.0057	0.0057	0.0048	0.0042	0.003	0.0048	0.0033	0.006	0.0057	0.0054

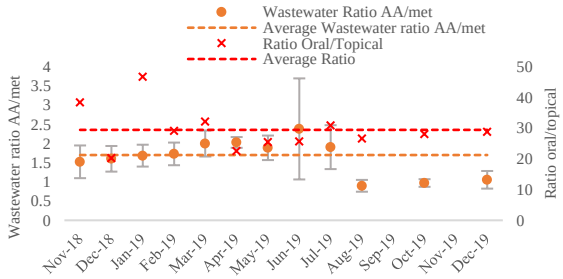
clindamycin phosphate	Liquid	0.003	0.0039	0.0018	0.0015	0.0021	0.0021	0.0018	0.0015	0.0009	0.0006	0.0018	0.0006
erythromycin	Gastro-resistant tablet	0.189	0.229	0.308	0.091	0.098	0.07	0.042	0.154	0.204	0.049	0.084	0.0595
erythromycin	Liquid	0.006	0.008	0.0084	0.006	0.0072	0.0096	0.014	0	0.0108	0.0036	0.0024	0.0056
erythromycin	Gel	0.0036	0.0006	0.0012	0	0.003	0.0036	0	0	0.0024	0.0006	0.0012	0.0012
erythromycin ethyl succinate	Oral suspension	0.0075	0.01	0.01	0	0.025	0.005	0.005	0.005	0	0	0	0.005
erythromycin ethyl succinate	Tablet	2.43	1.43	1.79	0	0	0	0	0	0	0	0	0.028
flucloxacillin sodium	Capsule	2.43	1.43	1.79	0.775	0.797	1.04	0.828	0.888	1.27	1.27	1.07	0.796
flucloxacillin sodium	Oral solution	0.13	0.055	0.0325	0.0825	0.035	0.0375	0.0175	0.0425	0.0575	0.025	0.065	0.025
ketoconazole	Cream	0.0006	0.0024	0.0006	0.0012	0	0	0.0018	0.0024	0.0012	0.0012	0	0.0006
ketoconazole	NA	0.0456	0.0408	0.0432	0.0192	0.0192	0.012	0.0288	0.012	0.0288	0.0312	0.0288	0.0216
levofloxacin hemihydrate	Tablet	0	0	0	0	0.014	0	0	0.007	0	0	0	0
levofloxacin hemihydrate	Eye drops	0	0.000025	0	0	0	0	0	0.000025	0	0	0	0
metronidazole	Cream	0.00255	0.0012	0.00113	0.00173	0.000675	0.00075	0.00135	0.000975	0.00158	0.00143	0.00135	0.000225
metronidazole	Gel	0.00188	0.00173	0.00188	0.00045	0.0021	0.00225	0.00293	0.00199	0.0003	0.0003	0.0021	0.000525
metronidazole	Tablet	0.134	0.131	0.086	0.0705	0.0354	0.0752	0.132	0.0404	0.0748	0.0384	0.0756	0.0708
metronidazole	Vaginal gel	0.0003	0.0006	0.0006	0.0003	0.0003	0.0009	0	0.0009	0.0003	0.0009	0.0015	0.0003
metronidazole benzoate	Oral suspension	0	0.004	0.012	0	0	0	0	0	0	0	0	0
ofloxacin	Eye drops	0.000105	0.000015	0.00009	0.00003	0	0	0.00006	0.000015	0.000075	0.000045	0.00006	0.000015
ofloxacin	Tablet	0.028	0.014	0	0.0124	0	0.0112	0.0392	0.0084	0.0316	0.0168	0	0.0056
oxytetracycline calcium	Cream	0.0018	0	0	0	0.0009	0.0027	0.0018	0.0036	0	0	0.0009	0.0018
oxytetracycline dihydrate	Tablet	0.607	0.33	0.288	0.228	0.319	0.14	0.214	0.126	0.284	0.189	0.256	0.085
oxytetracycline hydrochloride	Ointment	0	0	0	0	0	0	0	0.0009	0	0	0	0
sulfadiazine silver	Cream	0	0	0	0	0	0	0	0	0	0	0	0
sulfamethoxazole	Tablet	0.056	0.0448	0.056	0.0224	0.0224	0.0896	0.0824	0	0	0	0.0112	0.0136
sulfamethoxazole	Oral suspension	0.004	0	0	0	0.008	0	0	0	0	0	0	0
sulfasalazine	Gastro-resistant tablet	1.92	1.53	1.37	1.02	1.31	0.966	0.854	0.742	0.952	0.488	1.3	1.05
sulfasalazine	Tablet	0.812	0.921	0.532	0.532	0.252	0.364	0.448	0.7	0.476	0.308	0.448	0.336
sulfasalazine	Oral suspension	0	0	0	0	0	0	0	0	0	0	0	0

tetracycline hydrochloride	Tablet	0.112	0.147	0.112	0	0	0.028	0	0	0	0	0	0
trimethoprim	Oral suspension	0.0128	0.0114	0.0092	0.0037	0.0036	0.008	0.0033	0.005	0.003	0.008	0.008	0.0075
trimethoprim	Tablet	0.151	0.135	0.167	0.0708	0.0605	0.0682	0.0996	0.0606	0.0454	0.0535	0.0873	0.0651

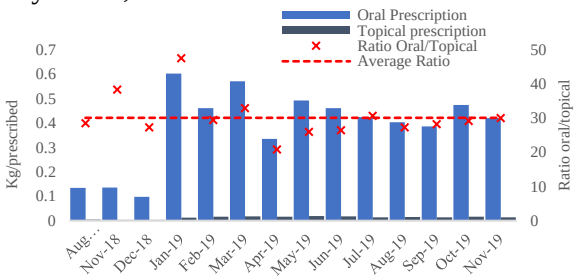
Figure S1. Monthly ratios of oral versus topical prescriptions of MTZ and CLI with comparisons to ratios of loads of MTZ/hMTZ and CLI/dmCLI in wastewater during the studied period

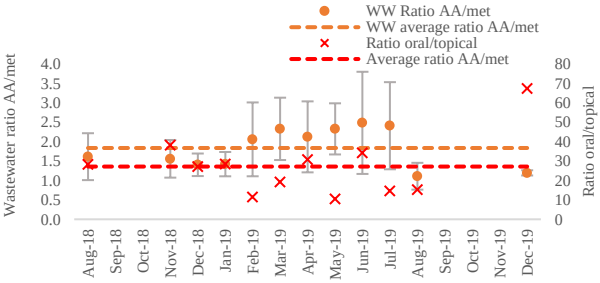
Bath, MTZ



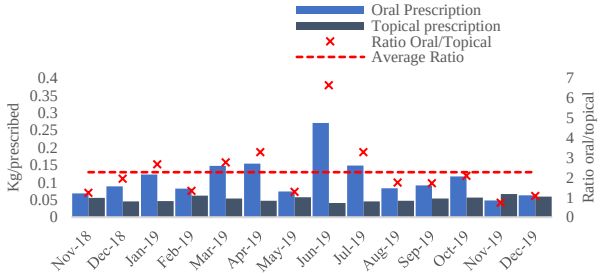


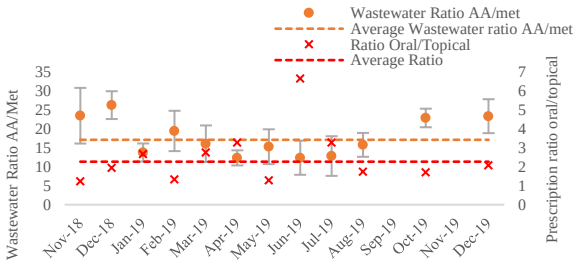
Keynsham, MTZ



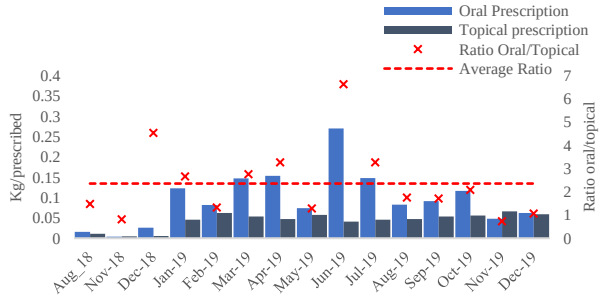


Bath, CLI





Keynsham, CLI



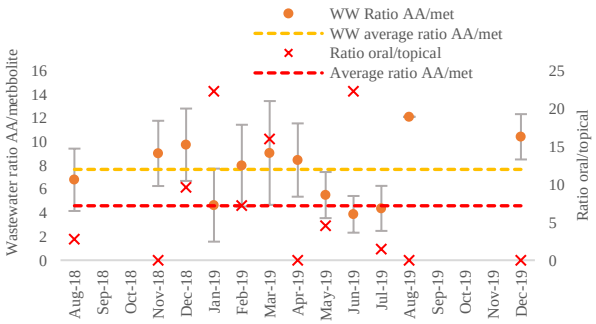


Table S4. Complete breakdown of PNDIs and PNDPs for AAs studied

	CLR		dmCLR			CLI		dmCLI			MTZ		hMTZ			SMX		aSMX			TMP			SLZ
Bath	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**
November '18	640	281	1430	585	546	110	47	5	2	35	227	81	147	74	175	411	168	61	24	87	107	43	202	330
December '18	673	130	1698	367	663	98	36	4	1	38	317	81	184	39	101	476	51	99	16	93	129	39	232	365
January '19	919	269	1904	571	664	124	30	9	3	46	319	84	182	67	176	986	231	165	29	102	209	81	192	919
February '19	769	151	1762	312	585	134	29	8	2	39	358	47	196	36	130	847	199	130	19	82	172	46	173	1081
March '19	678	143	1761	360	636	141	49	9	3	55	339	65	160	38	169	1084	226	178	37	58	172	64	161	1061
April '19	644	289	1623	882	557	91	43	8	4	55	235	126	108	55	105	770	201	102	42	122	132	36	180	486
May '19	351	68	939	210	482	56	15	4	1	36	116	49	58	25	147	489	131	56	20	44	107	25	160	394
June '19	352	126	1041	341	394	79	27	7	4	85	167	116	80	53	137	887	309	107	56	65	119	39	157	896
July '19	402	27	1024	105	445	90	41	8	2	53	234	50	126	60	140	896	172	92	24	71	130	29	167	1608
August '19	299	87	653	239	419	97	36	7	3	36	208	50	215	45	124	786	196	88	21	76	103	29	153	299
October '19	586	40	1219	432	524	155	35	7	2	47	306	20	292	13	143	788	159	100	10	113	134	15	179	523
December '19	608	114	1106	336	599	91	26	4	2	33	182	107	164	100	132	856	270	103	27	88	169	48	167	616
Average	**577**	**189**	**1347**	**402**	**543**	**106**	**28**	**7**	**2**	**47**	**251**	**77**	**159**	**63**	**140**	**773**	**209**	**107**	**36**	**83**	**140**	**33**	**177**	**715**

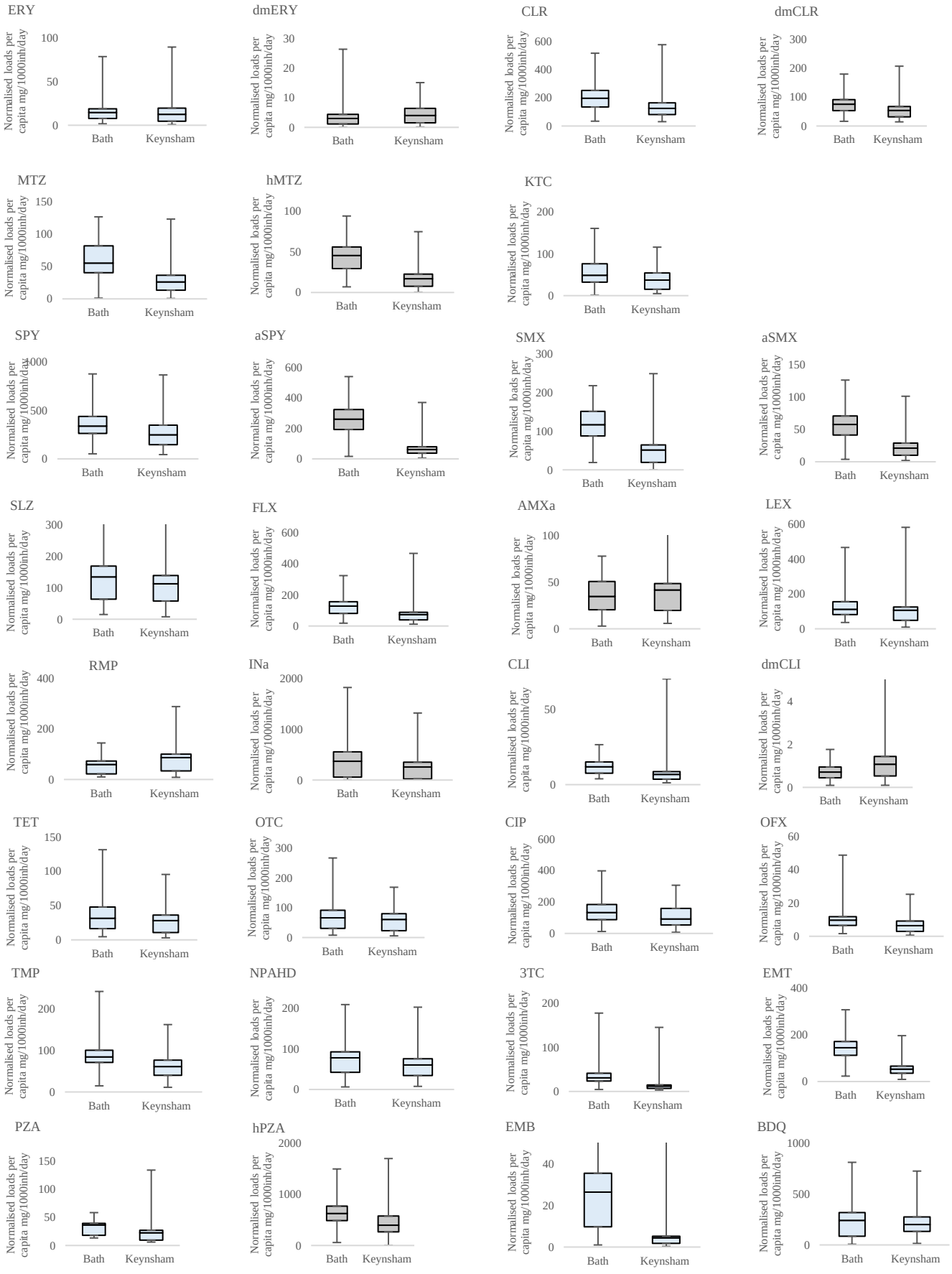
	SLZ			OFX			CIP			TET			OTC			FLX			ERY			SDZ		
Bath	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**
November '18	330	146	2919	10	11	8	296	315	225	46	48	12	257	227	391	303	134	1816	79	86	257	2	2	1
December '18	365	161	2848	12	3	27	372	190	159	83	33	57	283	110	463	255	89	2041	84	88	237	2	2	1
January '19	919	971	3227	14	6	19	372	161	203	80	28	27	587	269	433	432	113	1819	53	24	355	5	4	0
February '19	1081	282	2388	14	3	9	395	110	203	79	38	50	342	70	371	463	120	1799	42	12	277	10	9	1
March '19	1061	570	3322	13	4	10	342	122	138	98	48	36	530	193	363	431	141	1900	44	14	277	10	7	1
April '19	486	258	2723	12	6	13	257	118	140	68	27	27	320	122	363	333	139	1872	60	18	244	2	2	2
May '19	394	283	3122	7	1	10	110	29	171	39	12	31	214	50	475	180	68	2116	56	33	307	5	2	0
June '19	896	511	2626	8	2	8	207	88	124	38	27	28	224	125	250	290	200	1811	20	10	185	3	2	1
July '19	1608	399	2845	18	20	13	249	68	212	32	24	54	270	125	318	434	84	2392	17	9	218	2	1	1
August '19	299	206	3799	9	3	28	205	45	167	14	5	20	62	19	250	298	95	2191	-	-	134	9	3	1

October '19	523	422	3424	15	2	24	261	77	149	18	6	36	95	29	313	347	146	2087	-	-	209	5	4	3
December '19	616	275	2990	16	3	23	332	41	164	35	18	21	151	83	327	343	260	1647	33	18	238	5	5	1
Average	**715**	**401**	**3019**	**12**	**3**	**16**	**283**	**85**	**171**	**52**	**28**	**33**	**278**	**156**	**360**	**342**	**85**	**1958**	**49**	**22**	**245**	**5**	**3**	**1**

	CLR		**dmCLR**			**CLI**		**dmCLI**			**MTZ**		**hMTZ**			**SMX**		**aSMX**			**TMP**		
Keynsham	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**
August '18	162	25	413	111	564	36	17	6	2	46	29	6	18	6	246	152	78	34	20	106	74	14	291
October '18	910	203	1637	400	558	37	2	8	3	74	150	37	84	35	83	351	98	53	17	119	172	78	301
November '18	338	127	852	316	679	39	19	5	2	15	98	39	65	33	238	111	98	19	10	79	77	26	259
December '18	448	175	1115	279	736	90	47	10	4	56	169	82	120	68	159	153	71	37	16	99	104	43	311
January '19	478	79	955	187	892	57	21	16	6	150	107	41	70	26	201	342	161	68	27	69	123	28	246
February '19	586	151	1303	429	582	74	40	11	8	51	177	118	80	71	152	164	176	44	53	82	103	44	199
March '19	475	480	1134	1010	599	64	32	9	5	84	199	118	83	49	172	474	466	82	55	173	117	74	253
April '19	281	69	576	187	536	30	10	4	3	56	52	31	26	20	215	364	124	39	10	128	93	27	243
May '19	216	60	815	776	621	31	18	6	1	35	36	15	16	9	103	153	52	22	12	17	43	13	175
June '19	324	121	919	355	597	112	187	37	67	96	59	54	28	25	140	235	103	34	39	87	82	47	201
July '19	358	137	902	342	609	88	55	21	13	53	97	27	43	24	100	554	169	33	12	35	74	18	209
August '19	308	71	654	145	670	28	18	5	-	16	116	53	89	16	172	534	215	41	11	69	91	15	229
December '19	400	116	707	239	704	68	27	7	3	82	178	174	63	38	184	360	176	65	27	56	156	57	220
Average	**406**	**114**	**922**	**257**	**642**	**58**	**47**	**11**	**18**	**63**	**113**	**49**	**60**	**20**	**166**	**304**	**106**	**44**	**16**	**86**	**101**	**22**	**241**

	SLZ			**OFX**			**CIP**			**TET**			**OTC**			**FLX**			**ERY**		
Keynsham	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**	**PNDI**	**STD**	**PNDP**
August '18	671	194	4844	5	4	50	100	51	241	16	2	198	190	56	1079	127	12	4531	9	7	352
October '18	388	205	3918	3	1	10	100	40	233	28	18	0	355	286	1198	211	56	3085	86	67	412
November '18	254	127	4345	3	2	25	155	113	276	22	15	260	181	123	585	138	50	2634	43	26	421
December '18	352	229	3362	9	3	0	297	216	232	104	37	198	425	121	510	239	97	3222	27	20	563
January '19	522	247	3848	6	2	37	231	78	277	48	10	0	340	113	548	178	84	2159	25	15	303
February '19	1013	436	3624	8	5	9	369	91	214	60	22	217	398	103	820	229	125	1776	76	47	451

March '19	901	689	4037	12	7	26	231	142	181	54	26	87	384	157	482	187	85	2641	43	19	215
April '19	588	207	3098	5	3	61	80	31	227	30	25	0	162	92	671	105	20	2398	77	41	264
May '19	157	122	3834	4	4	30	68	21	137	16	6	195	99	43	419	103	39	2277	85	88	396
June '19	607	319	3318	8	4	49	180	133	284	41	45	0	247	243	700	207	156	3413	9	5	429
July '19	915	508	2315	14	8	26	306	80	181	41	30	0	362	294	639	299	147	3132	8	5	137
August '19	708	842	4138	12	3	9	266	45	216	15	4	22	87	8	788	116	43	3000	10	13	240
December '19	589	392	3605	17	9	35	391	129	152	24	7	0	143	47	264	161	60	2094	17	5	285


ERY
dmERY
CLR
dmCLR
MTZ
hMTZ
KTC
SPY
aSPY
SMX
aSMX
SLZ
FLX
AMXa
LEX
RMP
INa
CLI
dmCLI
TET
OTC
CIP
OFX
TMP
NPAHD
3TC
EMT
PZA
hPZA
EMB
BDQ
Normalised loads per capita mg/1000inh/day
Bath
Keynsham

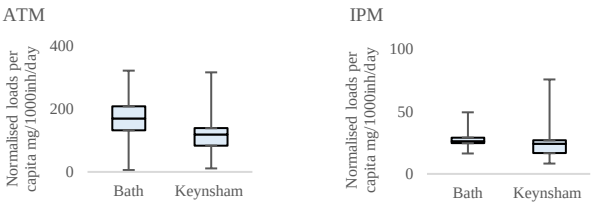
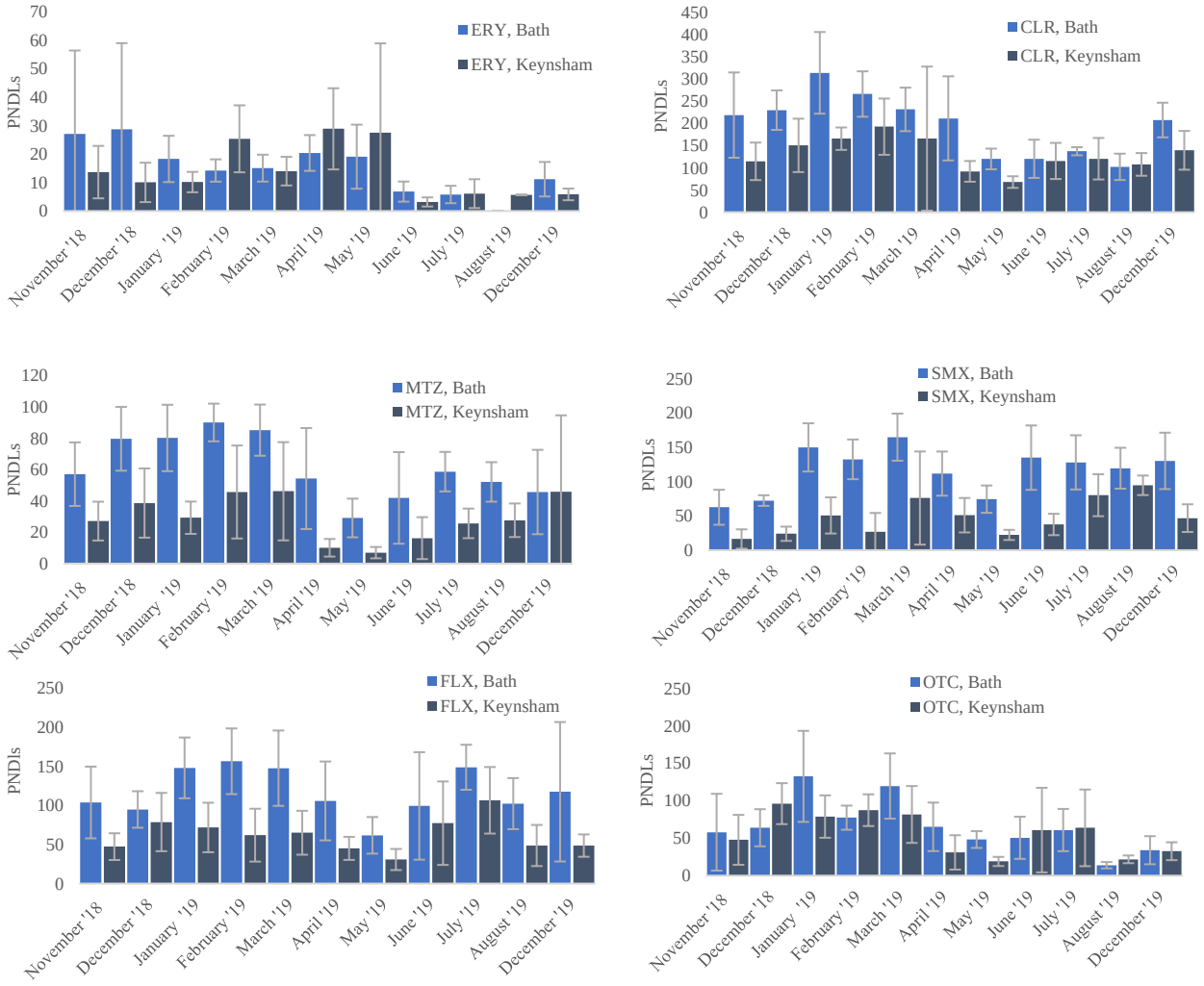
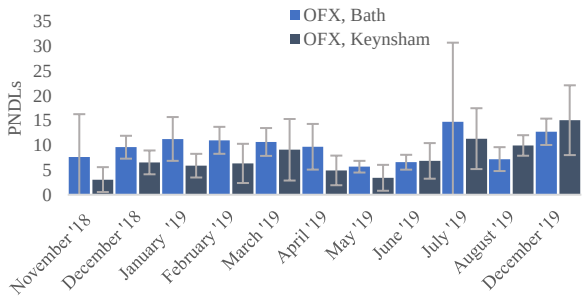
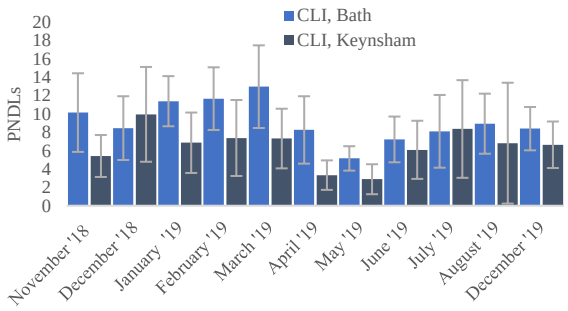
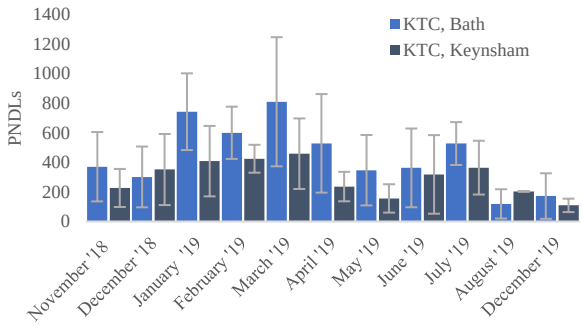


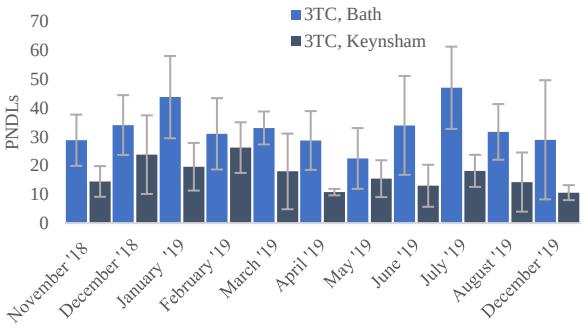
Figure S2. Comparison of population normalised loads (PNDLs) of AAs and respective metabolites between Bath and Keynsham

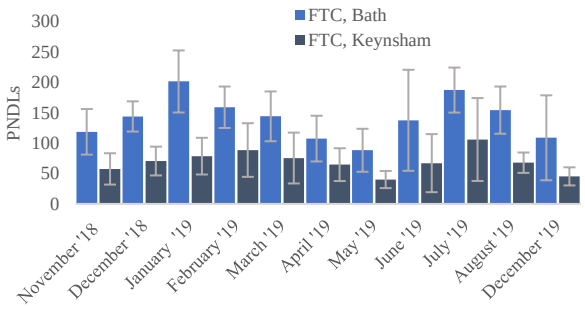


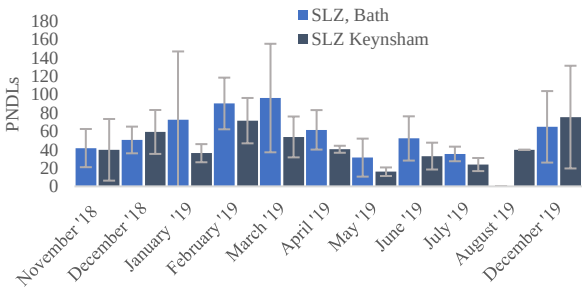

OFX, Bath
OFX, Keynsham
PNDLs
35
30
25
20
15
10
5
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

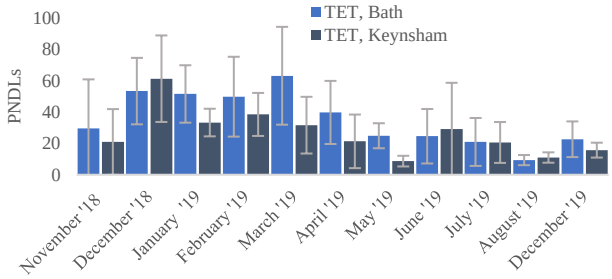

CLI, Bath
CLI, Keynsham
PNDLs
20
18
16
14
12
10
8
6
4
2
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

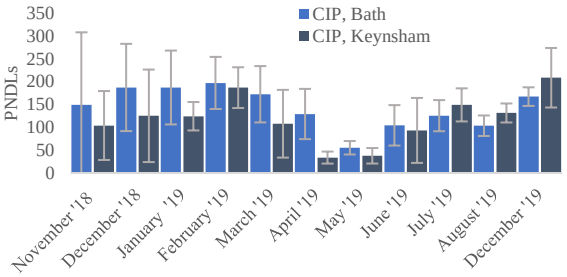

KTC, Bath
KTC, Keynsham
PNDLs
1400
1200
1000
800
600
400
200
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

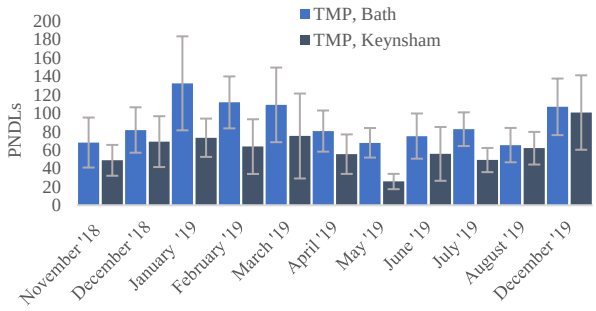

3TC, Bath
3TC, Keynsham
PNDLs
70
60
50
40
30
20
10
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


FTC, Bath
FTC, Keynsham
PNDLs
300
250
200
150
100
50
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


SLZ, Bath
SLZ Keynsham
PNDLs
180
160
140
120
100
80
60
40
20
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


TET, Bath
TET, Keynsham
PNDLs
100
80
60
40
20
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


CIP, Bath
CIP, Keynsham
PNDLs
350
300
250
200
150
100
50
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


TMP, Bath
TMP, Keynsham
PNDLs
200
180
160
140
120
100
80
60
40
20
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

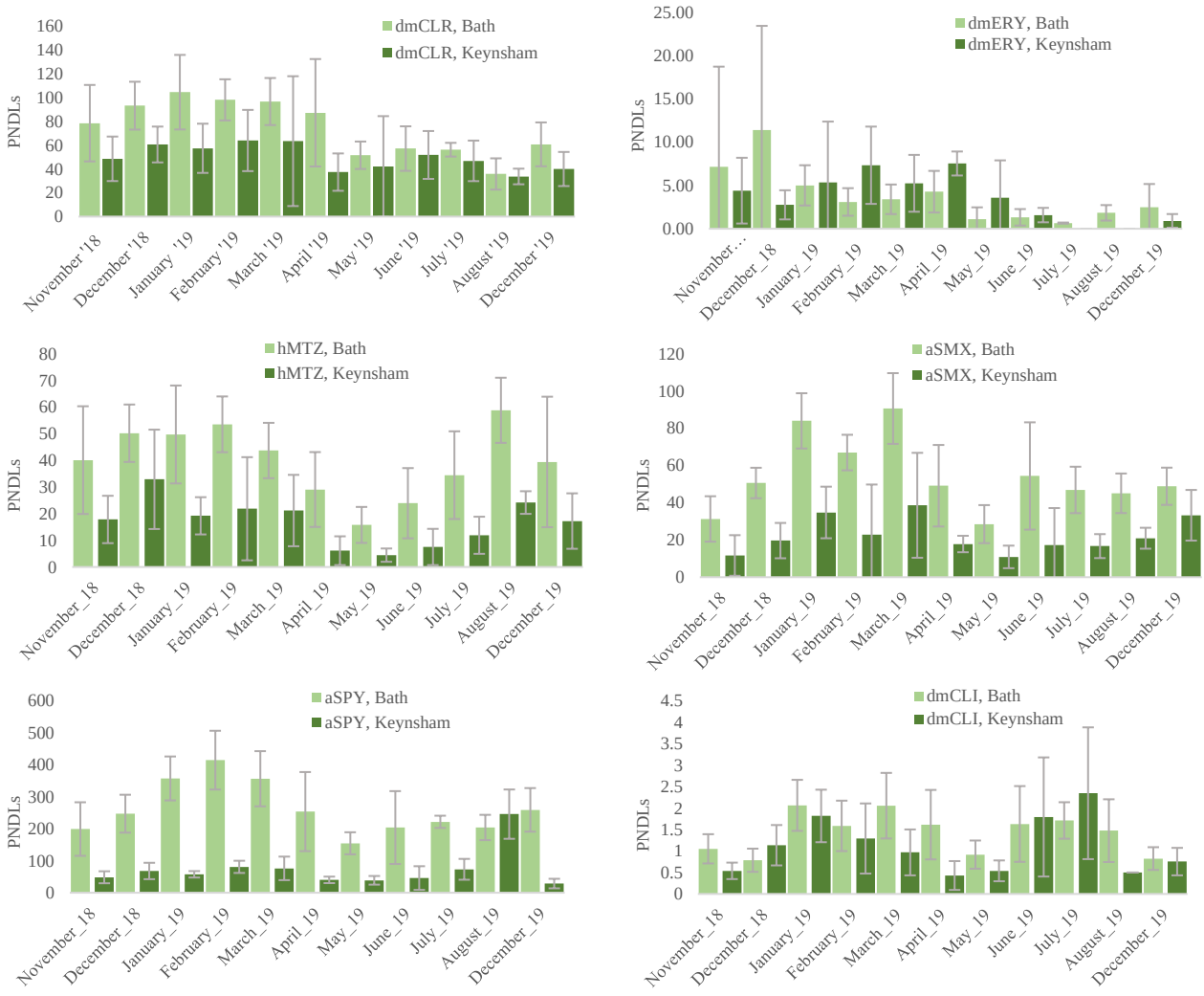


Figure S3. Comparison of PNDLs of AAs and respective metabolites in wastewater for Bath and Keynsham (only comparing months where both sites were sampled)

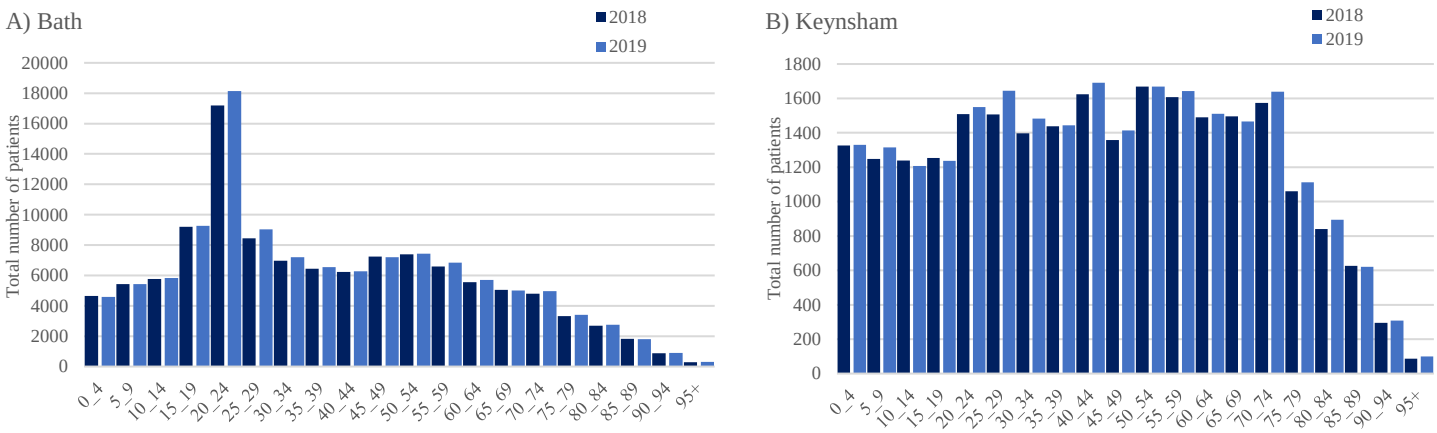
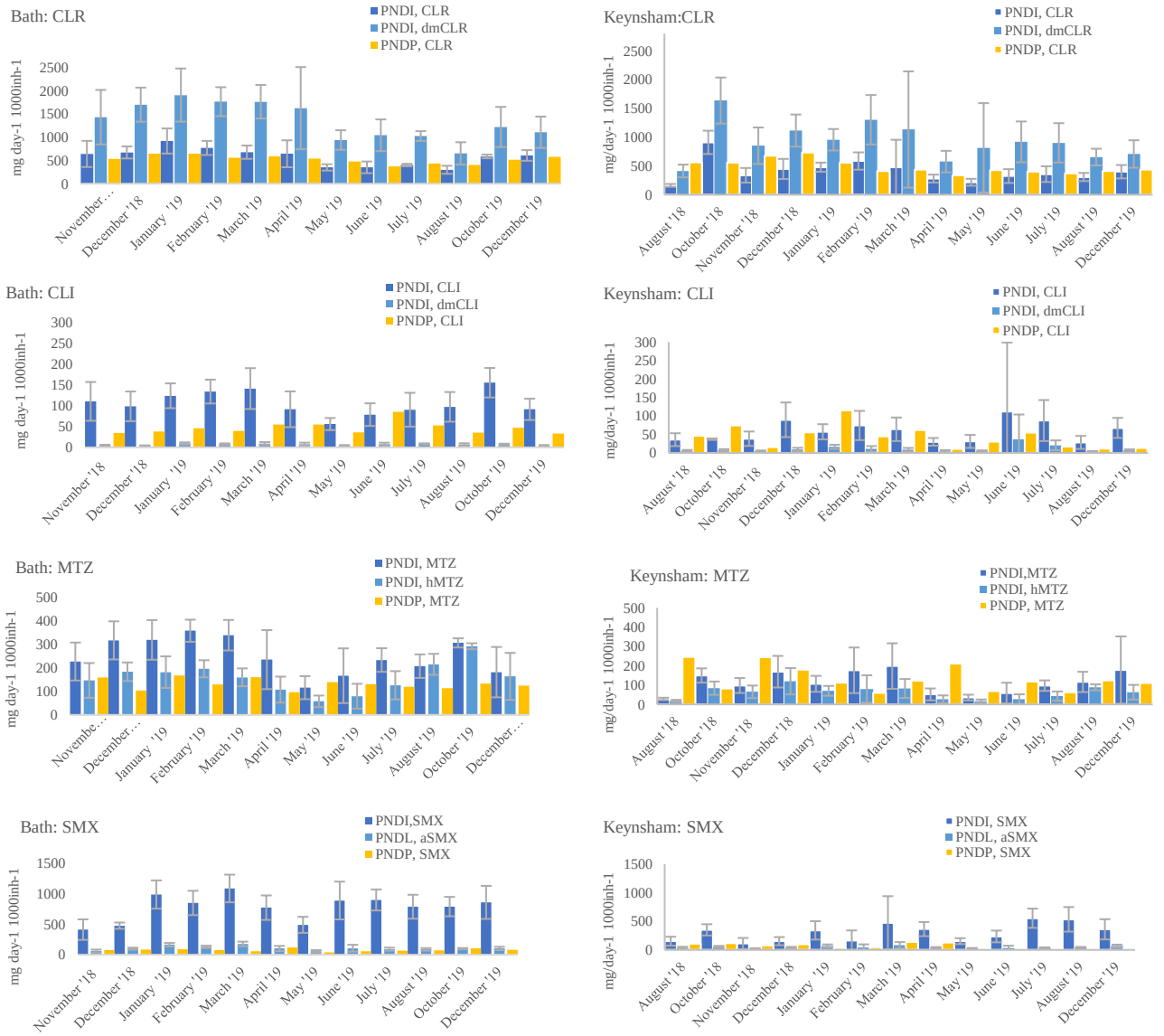
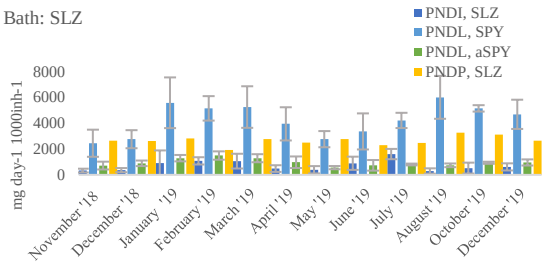
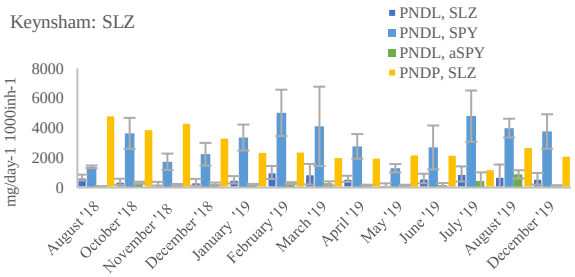
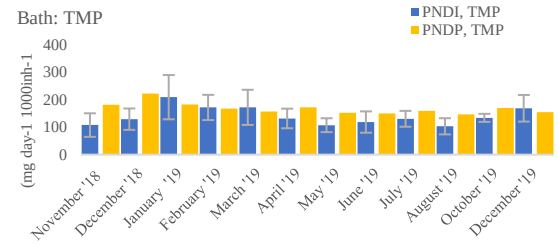


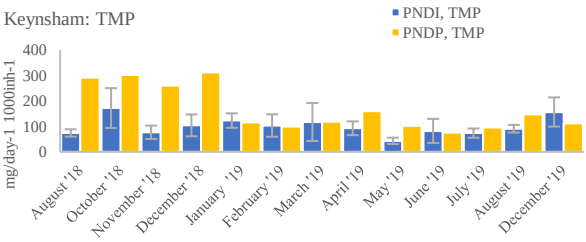
Figure S4. Age demographics patients at GP surgeries within the catchment areas of Bath and Keynsham for 2018 and 2019

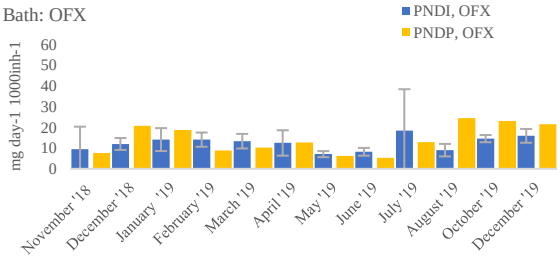


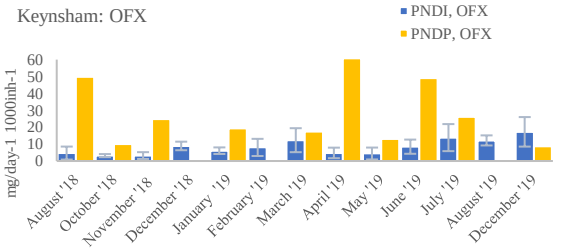

Bath: SLZ
PNDI, SLZ
PNDL, SPY
PNDL, aSPY
PNDP, SLZ
mg day-1 1000inh-1
8000
6000
4000
2000
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
October '19
December '19

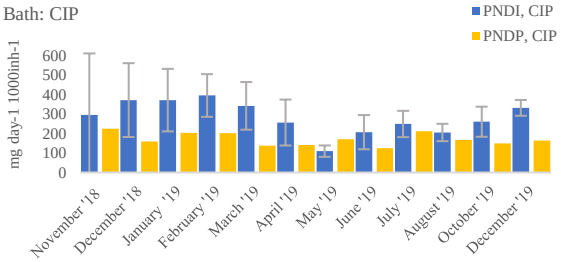

Keynsham: SLZ
PNDL, SLZ
PNDL, SPY
PNDL, aSPY
PNDP, SLZ
mg/day-1 1000inh-1
8000
6000
4000
2000
0
August '18
October '18
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

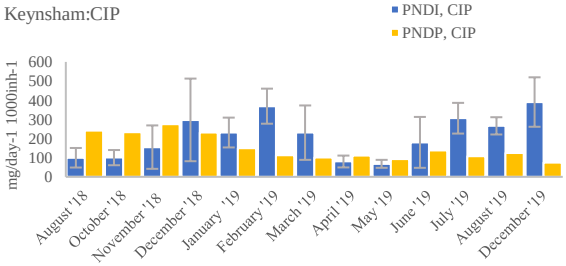

Bath: TMP
PNDI, TMP
PNDP, TMP
(mg day-1 1000inh-1
400
300
200
100
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
October '19
December '19

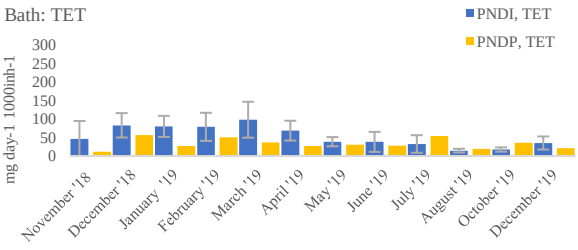

Keynsham: TMP
PNDI, TMP
PNDP, TMP
mg/day-1 1000inh-1
400
300
200
100
0
August '18
October '18
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

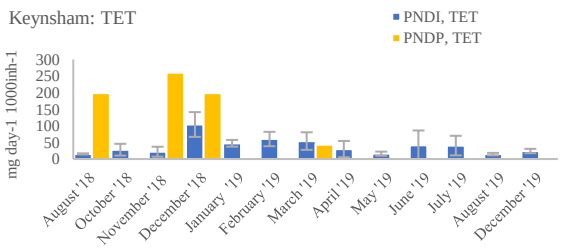

Bath: OFX
PNDI, OFX
PNDP, OFX
mg day-1 1000inh-1
60
50
40
30
20
10
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
October '19
December '19

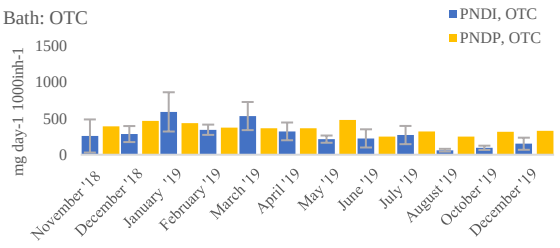

Keynsham: OFX
PNDI, OFX
PNDP, OFX
mg/day-1 1000inh-1
60
50
40
30
20
10
0
August '18
October '18
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

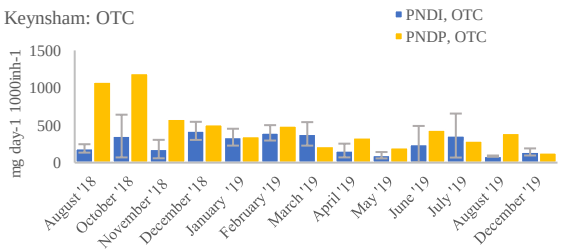

Bath: CIP
PNDI, CIP
PNDP, CIP
mg day-1 1000inh-1
600
500
400
300
200
100
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
October '19
December '19


Keynsham:CIP
PNDI, CIP
PNDP, CIP
mg/day-1 1000inh-1
600
500
400
300
200
100
0
August '18
October '18
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


Bath: TET
PNDI, TET
PNDP, TET
mg day-1 1000inh-1
300
250
200
150
100
50
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
October '19
December '19


Keynsham: TET
PNDI, TET
PNDP, TET
mg day-1 1000inh-1
300
250
200
150
100
50
0
August '18
October '18
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19


Bath: OTC
PNDI, OTC
PNDP, OTC
mg day-1 1000inh-1
1500
1000
500
0
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
October '19
December '19


Keynsham: OTC
PNDI, OTC
PNDP, OTC
mg day-1 1000inh-1
1500
1000
500
0
August '18
October '18
November '18
December '18
January '19
February '19
March '19
April '19
May '19
June '19
July '19
August '19
December '19

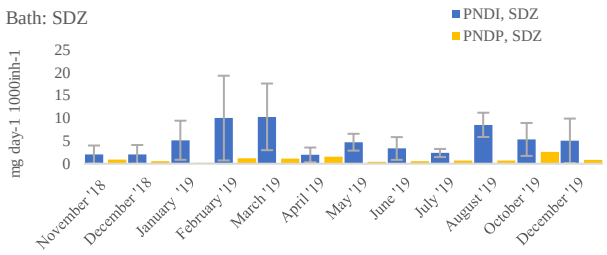


Figure S5. Average monthly comparison of PNDPs and PNDLs for AAs and metabolites in both Bath and Keynsham.

This declaration concerns the article entitled:									
In-situ Multi-Mode Extraction (iMME) sampler for a wide-scope multi-residue analysis of > 100 chemicals of emerging concern (CEC) and antimicrobial resistance genes in the aqueous environment in urban areas and remote (off-the-grid) locations									
Publication status (tick one)									
Draft manuscript	**X**	**Submitted**		**In review**		**Accepted**		**Published**	
Publication details (reference)									
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In-situ Multi-Mode Extraction (iMME) sampler for a wide-scope multi-residue analysis of over 100 chemicals of emerging concern (CEC) and antimicrobial resistance genes in the aqueous environment in urban areas and remote (off-the-grid) locations

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Abstract

Chemical pollution (including chemicals of emerging concern – CECs) continues to gain increasing attention as a global threat to human health and the environment, with numerous reports on the adverse and sometimes devasting effects upon ecosystems the presence of these chemicals can have. Whilst many studies have investigated presence of CECs in aquatic environments, these studies have been focused on higher income countries, leaving significant knowledge gaps for many low-middle income countries. This study proposes a new integrated powerless, in-situ multi-mode extraction (iMME) sampler for the analysis of chemicals (105 chemicals of emerging concern, CECs) and biological (5 genes) markers in water in contrasting settings: an urbanized Avon River in the UK and remote Olifants River in Kruger National Park in South Africa. The overarching goal was to develop a sampling device that maintains integrity of a diverse range of analytes via analyte immobilization using polymeric and glass fibre materials, without access to power supply or cold chain for sample transportation. Chemical analysis was achieved using an ultra-performance liquid chromatography coupled with tandem mass spectrometry. Several mobile CECs showed low stability in river water, at room temperature and typical 24h sampling/transport time. It is therefore recommended that, in the absence of cooling or analyte immobilization options, environmental water samples are spiked with internal standards on site, immediately after collection. iMME has proven effective in immobilization, concentration and increased stability of CECs at room temperature (and at least 7 days storage) allowing for sample collection at remote locations. The results from the River Avon and Olifants River sampling indicate that the pristine environment of Olifants catchment is largely unaffected by CECs common in the urbanized River Avon in the UK with a few exceptions: lifestyle chemicals (e.g., caffeine, nicotine and their metabolites), paracetamol and UV filters due to tourism and carbamazepine due to its persistent nature. iMME equipped with an additional gene extraction capability provides an exciting new opportunity of comprehensive biochemical profiling of aqueous samples with one powerless in-situ device. Further work is required to provide full integration of the device and comprehensive assessment of performance in both chemicals and biological targets.

Keywords: Chemical pollution, rivers, sampling, stability

1. Introduction

Chemicals of emerging concerns (CECs) including pharmaceuticals, personal care products and pesticides have caused increasing concern for the freshwater environment over the past decades. They are found ubiquitously due to widespread usage in society and can have undesirable effects such as persistence and activity at low concentrations (Petrie et al., 2015a). The presence of CECs in surface waters can result in adverse effects in aquatic ecosystems, due to endocrine disrupting properties and other negative ecotoxicological effects (Brodin et al., 2013; Kidd et al., 2007; Schultz et al., 2011). Further concerns arise from low level exposure of antimicrobial agents (AA) which can contribute to antimicrobial resistance (AMR) (Marti et al., 2014). Whilst CEC levels are often low and sublethal for existing aquatic organisms (pg/L-ng L-1), chronic exposure to complex mixtures of CECs are

challenging to assess (Schwarzenbach, 2006). Furthermore, pharmaceuticals can either be excreted unchanged (as the parent compound) or as human metabolites (derivative structures). Some metabolites are also produced via degradation or transformation of parent compounds in wastewater treatment plants (WWTPs) or in water environments. Metabolites can have equal or higher toxicity than the parent compound (Bedner and MacCrehan, 2006; Neuwoehner et al., 2009) and many metabolites are understudied in the environment, potentially due to costs or availability of analytical grade standards or they simply may not be known (Petrie et al., 2015a).

As many CECs are poorly removed by WWTPs (Kasprzyk-Hordern et al., 2008), the analysis of CECs in freshwater systems is critical for investigating their environmental fate and exposure to aquatic ecosystems. CEC concentrations in surface waters can highlight potential spots for concern or identify CECs that may need further investigation. In the European Union alone, there are over 100,000 chemicals approved for use (European Chemicals Agency, 2008). Whilst the advancement of analytical tools such as high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) has allowed for the analysis of a wide range of CECs in water (Fatta et al., 2007), prioritizing which chemicals to focus on is therefore a challenge.

The analysis of chemicals such as CECs is of critical importance to protect natural environments and public health. Large numbers of CECs have been investigated in surface waters across the world, however many of these studies have focused on a catchment approach (Bagnis et al., 2020; Hossain et al., 2018; K'oreje et al., 2016; Kasprzyk-Hordern et al., 2009; Kolpin et al., 2004; Loos et al., 2007; Metcalfe et al., 2010; Peng et al., 2008; Vieno et al., 2005; Williams et al., 2019; Wood et al., 2017). Several nationwide studies of CECs in multiple rivers have been conducted (Batt et al., 2016; Guruge et al., 2019; Scott et al., 2014; Yao et al., 2018). In comparison, international campaigns from multiple geographic locations are far less common, with only a handful in literature (Table S1). Unfortunately, multi-target, trace analysis of chemically different CECs in environmental waters requires highly sophisticated instrumentation and well-trained specialists. As a result, there is very little published literature on the presence and fate of CECs in many regions and countries worldwide. Under representation has been reported for some Asian, African and South American countries (aus der Beek et al., 2016). Hence there is no incentive to influence regulations on national and global scales. One of the key issues lies in current inability to collect and transport water samples over long distances without compromising sample integrity during transport from the sampling point to the analyzing lab, often based in different, cities, countries and continents. Therefore, there is an urgent need to develop in-situ sample preparation approaches that will allow for rapid stabilization of analytes immediately after sample collection and during transport (up to week or longer).

Currently there are no standardized procedures for collecting environmental samples from surface waters (Hughes et al., 2013). As a result, sampling protocols can vary significantly between studies as observed by table S1. These differences have included: (i) the shipping and storage temperature of samples; (ii) duration of transport, (ii) addition of internal standard (IS); (iii) type of containers used (PP or PE plastic versus glass); (iv) pre-extraction filtration. All these different factors can contribute to analyte stability during sampling, resulting in challenges when making comparisons between studies and temporal trends.

It should be noted the majority of studies detailed in table 1 have included a stability experiment, an element which can be missing from multinational studies (Batt et al., 2016; Guruge et al., 2019; Scott et al., 2014). In these reports, all have claimed that analyte losses in sample storage and transport are negligible. However, it is critical to assess whether these stability experiments are fit for purpose. For Wilkinson *et al.* the stability study was conducted via spiking analytes into LC-MS water and monitoring at different temperatures; sampling at days two and seven. The freezing of environmental samples before shipping in this study was not included in the stability experiment. Hence, the freeze/thaw cycle, a potential route for analyte loss, was not accounted for. It has already been

highlighted in the literature there can be a loss of certain compounds after freezing (Baker and Kasprzyk-Hordern, 2011; Fedorova et al., 2014). Whilst it has been reported that there are compounds that are stable, such as carbamazepine and trimethoprim, others such as doxycycline and risperidone experience declining concentrations when frozen at -18 °C. Furthermore, this proposal by Wilkinson *et al.* for international sampling is to cover multiple matrices (surface water, effluent and influent wastewater). The importance of undertaking a matrix-specific stability study is further highlighted by the fact that some compounds will have varying stability in the matrix in question. In the case for fluoroquinolones, enrofloxacin has shown to be still present after the freeze thaw cycle in an effluent wastewater sample, but absent in an influent wastewater sample (Fedorova et al., 2014).

McLachlen *et al* also opted to study analyte stability in Milli-Q-water at room temperature over a period of three weeks. The surface water samples collected, however, are shipped as liquids (unfrozen) at 4 °C. Whilst it has been reported short term that it may be beneficial to store samples at 4 °C in comparison to -18 °C (Fedorova et al., 2014), in environmental samples there is a risk that microbiological action could potentially degrade or transform analytes in question (Castiglioni et al., 2011). This is an important consideration if shipping takes several days to reach the analysis lab. Samples may be kept for a prolonged time (e.g. 1-2 weeks) at 4 °C before spiking of internal standards and/or before the extraction protocols take place (McLachlan et al., 2007). Whilst the matrix and microbiological communities will vary between temporal and spatial graphic location, a stability study on the matrix in question should be done.

There are several similarities between these international studies. They all employ grab sampling techniques for surface water samples and all focus on chemical concentrations in the aqueous phase. Yet sorption to sediments is recognized to be important in CECs in freshwater systems. Another similarity is that all the samples are spiked after transportation to the lab and not on site, and therefore will not account for any analyte losses during transport. Two of the studies filter before spiking with internal standards, which could result in unaccounted losses of a fraction of the target analytes (Singh et al., 2019; Wilkinson et al., 2019). Furthermore, only one of the other studies has opted to transport samples on solid phase extraction (SPE) cartridges (R. R. Singh et al., 2019). The other studies transport samples as liquids, and none have specified if the temperature of the samples were recorded on arrival.

This paper proposes a new in-situ multi-mode extraction (iMME) utilizing polymeric and glass fibre filters for multiresidue analysis of 105 CECs. The CECs in two contrasting rivers were selected, the largely urbanized River Avon (UK) and remotely positioned Olifants River in Kruger National Park (South Africa). The objective being to overcome limitations of analyte stability during sampling and transport. This is of importance in the international context and to enable sampling in remote locations where there is no access to the cold chain. This approach is also cost effective as it allows for the extraction of >100 compounds (and potentially many more) within one extraction protocol. An important consideration with regards to antimicrobial resistance (AMR) is the analysis of chemical antimicrobial agents (AAs) but also the analysis of biological targets, such as antimicrobial resistance genes (ARGs). Monitoring environmental levels of ARGs is important for monitoring spread of resistance and the scale of the problem (Larsson et al., 2018). Furthermore, low levels of particular AAs in an environment may be identified as a low concern, however low levels of certain AAs could be as a result of high levels of resistance in the bacterial communities that are present. The River Avon was therefore also investigated for six ARGs using the iMME sampler to link to AAs reported in this study, and to explore the potential of on-site sampling for both chemicals and genes with this sampler.

2. Experimental

2.1. Materials: Chemical Targets

A total of 105 CECs were investigated in this study, these were split into two groups: A) general pharmaceutical, including personal care products B) AAs, spanning a broad range of classes (table 1). Methods used in this study were previously developed and established as discussed in Petrie *et al.* (Petrie et al., 2015b) and Holton *et al.* (Holton and Kasprzyk-Hordern, 2021). CEC analytes and internal standards (IS) were ordered from Sigma-Aldrich, LGC standards, TRC or MCE. All standards were purchased as 1.0 or 0.1 mg/mL solutions or in powdered forms, those in powdered forms were prepared at concentrations of 1 mg/mL in the appropriate solvents. All standards were stored in the dark at -20 °C. Methanol (MeOH) was purchased from Sigma-Aldrich and was HPLC grade, the water was of 18.2 MΩ quality.

Mobile phase buffers included formic acid (HCOOH, >95 %), ammonium acetate (NH_4OAc), acetic acid (CH_3COOH) and ammonium fluoride (NH_4F) were purchased from either Merck or Fischer Scientific. All glassware was deactivated by rinsing once with 5 % dimethylchlorosilane (DMDCS), twice with toluene and three times with MeOH. This was to avoid any losses of basic compounds on the -OH sites present on the glass surface. Oasis HLB SPE cartridges (60 mg, 3 mL) were purchased from Waters. The filters (Whatman GF/F 0.7-µm and GF/D) and polyproplene LC vials were also purchased from Waters.

Table 1. Chemical and biological targets investigated in this project

CEC group / Analytical method	Compound class/Associated resistance	Compound
Group A	Parabens	Methylparaben
		Propylparaben
		Butylparaben
	UV Filters	1-benzophenone
		2-benzophenone
		3-benzophenone
		4-benzophenone
	Plasticizer	Bisphenol A
	Steroid Estrogen	E1
		E2
		EE2
	NSAIDs	Ketoprofen
		Ibuprofen
		Naproxen
	Hypertension	Valsartan
		Irbesartan
		Lisinopril
	Stimulants and metabolites	Amphetamine
		Methamphetamine
		MDMA
		MDPV
		Cocaine
		Anhydroecgonine methyl ester

		Benzoylecgonine
		Cocaethylene
	Analgesics and metabolites	Methadone
		Acetaminophen
		EDDP
		Codeine
		Norcodeine
		Dihydrocodeine
		N-desmethyltramadol
		O-desmethyltramadol
	Opioid metabolite	6-acetylmorphine
	Human Indicators	Cotinine
		Caffeine
		Nicotine
		1,7 dimethylxanthine
	Anti-epileptic	Carbamazepine
		Carbamazepine 10, 11-epoxide
		10,11-dihydro-10-hydroxycarbmazepine
	Anaesthetic and metabolite	Ketamine
		Norketamine
	Antidepressants	Venlafaxine
		Citalopram
		Desmethylcitalopram
	Antipsychotic	Quetiapine
	Lipid regulator	Bezafibrate
	Diabetes	Metformin
		Gliclazide
	Beta blocker	Atenolol
		Metoprolol
	Histamine H_2 receptor antagonist	Cimetidine
	Calcium channel blocker	Diltiazem
	Anticancer	Azathioprine
		Ifosfamide
	Antihistamine	Fexofenadine
Group B	Sulphonamide & trimethoprim	Sulfadiazine
		Sulfapyridine
		Sulfamethoxazole
		Sulfasalazine
		Trimethoprim
		N-acetyl sulfadiazine
		N-acetyl sulfapyridine
		N-acetyl sulfamethoxazole
		4-hydroxy-trimethoprim
	Macrolide & lincosamide	Erythromycin
		Clarithromycin

		Clindamycin
		N-desmethyl erythromycin
		N-desmethyl clarithromycin
		N-desmethyl clindamycin
	β-lactams	Flucloxacillin
	Cephalosporins	Cefalexin
		Cefixime
		Ceftiofur
	Quinolone	Norfloxacin
		Ciprofloxacin
		Lomefloxacin
		Danofloxacin
		Enrofloxacin
		Nadifloxacin
		Ofloxacin (Levofloxacin)
		Gatifloxacin
		Sarafloxacin
		Moxifloxacin
		Flumequine
		Nalidixic acid
		Desethylene ciprofloxacin
		Hydroxy-norfloxacin
		Desmethyl-ofloxacin
		Ofloxacin N-oxide
	TB drugs (1st line)	Rifampicin
		Rifabutin
		25-desacetyl rifampicin
		25-O-desacetyl rifabutin
	TB other	Linezolid
	Amphenicol	Florfenicol
	Nitrofurantoin	Nitrofurantoin
		NPAHD
	Azole	Metronidazole
		Hydroxy-metronidazole
	Antiretroviral	Lamivudine
		Emtricitabine
Group C	Macrolide resistance	*ermB*
	Sulphonamide resistance	*sul1*
	Quinolone resistance	*qnrS*
	Beta-lactamase resistance	*bla-CTX*
	Beta-lactamase resistance	*bla-TEM*
	Potential marker of anthropogenic pollution	*intI1*
	Normalisation of genes to the bacterial population	*16S rRNA*

NPAHD: 1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione

2.2 Materials: Biological Targets

Antimicrobial resistant genes (ARGs, group C, Table 1) were also included in this study to explore an integrated approach towards risk assessment. As a range of AAs were explored in this project, an additional three river samples were collected to investigate several ARG targets via digital PCR (dPCR). All PCR reagents were purchased from Thermo Fischer. The chosen ARG targets were *ermB, sul1, intI1, qnrS, bla-CTX* and *bla-TEM.* Additionally, *16S rRNA* was analysed for information about bacterial population and a TaqMan™ Universal DNA Spike in Control (Thermo Fisher) was spiked into the lysis step of the DNA extraction kit (10 µL) to assess extraction efficiency of the kit. To filter river water on site for DNA extraction, Whatman® plastic filter holders (47 mm) were purchased along with sterilised cellulose filter papers (0.2 µm, Merck). Holders and filter papers were autoclaved in-house following manufacture instructions.

2.2. Sample collection

2.2.1 Extraction of Group A and B chemicals from river water samples

River water samples were portioned into 100 mL samples. After immediate spiking of the IS mix, samples were then immediately processed on-site using the iMME sampler (figure 1). The spiked river water samples were loaded into 50 mL syringes and pushed gently through the iMME sampler consisting of GF/D (2.7 µm) and GF/F (0.7 µm) filters and the conditioned Oasis HLB cartridge (conditioned using 1 mL of MeOH followed by 1 mL of H_2O) at approximately 3 mL min^{-1}. After the river sample had been filtered, air was pumped through via the syringe several times to purge any remaining water. Cartridges were then transported back to the lab at the University of Bath, UK (from <1hrs in the UK to 2 days for samples collected in South Africa), for further processing.

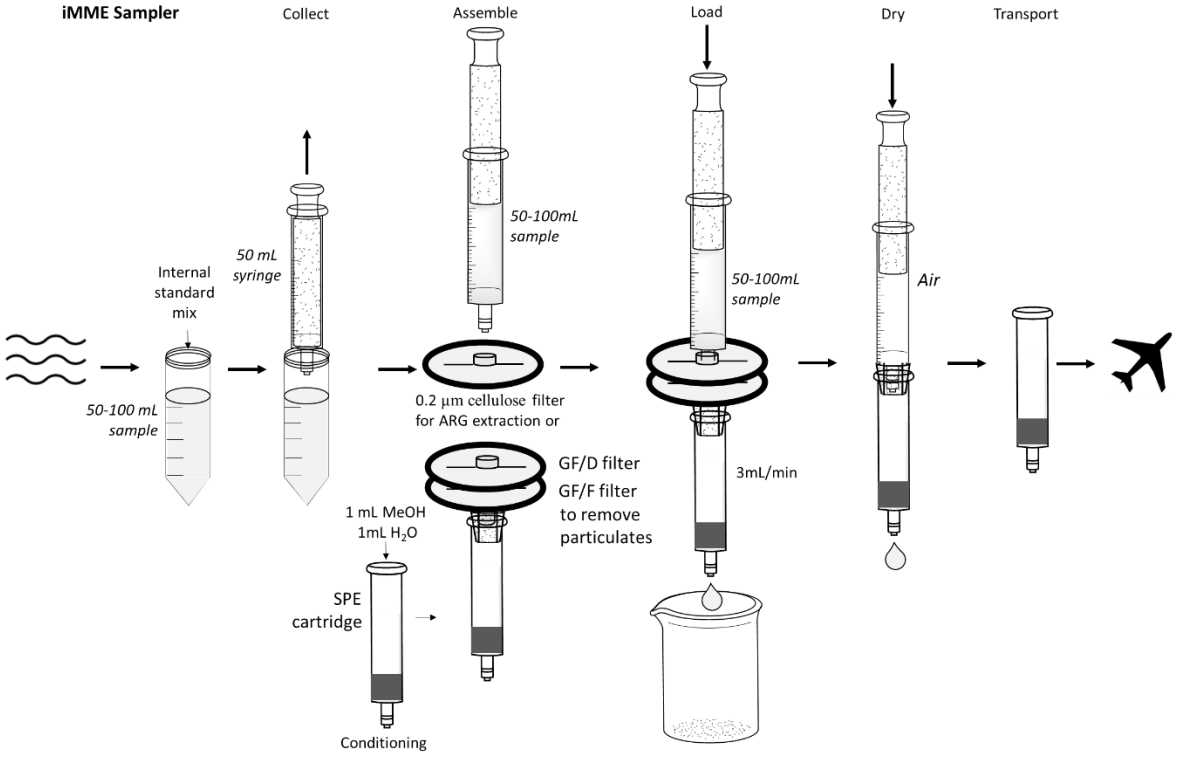


Figure 1. Schematic of the process of using the iMME sampler for river samples.

Analyte elution was then performed at the University of Bath (UK) following the protocols found in Petrie *et al.* and Holton *et al.*, with 4 mL of MeOH at a flow rate of 1 mL min^{-1} into silanised glass vials (Holton and Kasprzyk-Hordern, 2021; Petrie et al., 2015b). The extracts were then dried under a flow

of nitrogen using a TurboVap evaporator (Caliper, UK, 40 °C, N_2, <5 psi). Reconstitution was performed using 500 µL 80:20 H_2O:MeOH before being transferred into polypropylene vials ready for analysis.

2.2.1 Extraction of group C (ARGs) from river water samples

Alongside samples collected for chemical analysis in the UK, three additional biological river water sample replicates (100 mL) were filtered via 50 mL syringes through sterilized filter holder units containing 0.2 µm cellulose filter papers (Whatman, UK). Filter papers were kept in the housing unit and transported back to the lab. DNA was then extracted directly from the filter paper using FastDNA SPIN Kit for Soil (MP Bio, UK). The amount of extracted DNA was determined using a Qubit 4 Fluorometer (Thermo Scientific, UK). DNA was kept at -20 °C before further analysis.

2.2.2 Environmental Applications

To validate the sampler, grab samples were collected from a medium-sized river in the South-West of England (SWE), downstream from the city of Bath, as well as from Olifants River in Kruger National Park (South Africa) (figure S1). Samples for the UK were collected during June 2019 and samples of South Africa were collected during February 2018. An effort was made to collect samples from the middle of the stream. Samples were spiked and extracted on site before being further processed in the lab. UK samples were tested for all three groups of analytes and SA samples were tested only for group A.

2.3. Liquid chromatography mass spectrometry

Liquid chromatography was performed using a Waters Acquity UPLC system (Waters, UK). To validate for a broad range of compounds, three chromatographic methods have been applied. Sample preparation was the same for all methods, but respective analytical methods did vary (figure 2). Analysis for group A (covering a broad range of pharmaceuticals), consists of two LC-MS/MS methods (ESI + and -). For group B focusing on AAs, one LC-MS/MS method has been developed (ESI+).

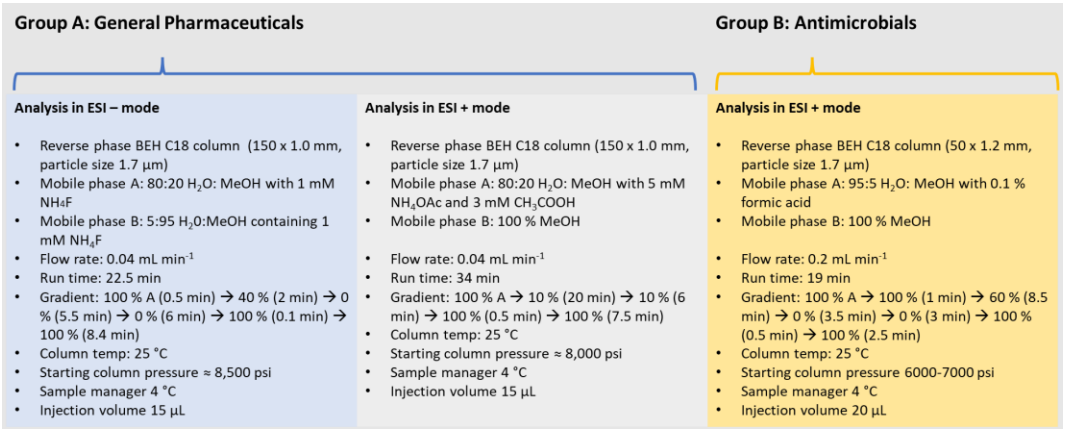


Figure 2. Overview of the UPLC-MS/MS methods used in river water.

2.4. LC-TQD Instrument performance

Full instrument and method validation have been previously detailed (Holton and Kasprzyk-Hordern, 2021; Petrie et al., 2015b). Linearity has been established by triplicate injections of a 17-point calibration curve, ranging in concentrations from 0.01-1000 ng mL^{-1}. Inter- and intra- day accuracy and precision was performed via triplicate injections of varying concentration within a 24-hour period and across three consecutive days. The instrument limit of detection (IDL) was determined at the concentration that gave a signal to noise (S/N) ≥ 3, with the instrument limit of quantification (IQL) determined at the concentration that gave a S/N ≥ 10 (table S2).

2.5. iMME -LC-TQD Performance

SPE recoveries of target analytes were determined by spiking river water (50 mL) at initial concentrations of 10, 100 and 500 ng L-1 before sample processing via the iMME filter method. Method quantification and method detection limits (MQLs and MDLs respectively) were calculated using the following equation:

$$MQL = \frac{IQL}{Rec\ x\ CF}$$

Where IQL is the instrument limit of quantification, Rec is the SPE recovery of the target analyte in river water and the CF is the SPE concentration factor. MDLs are calculated using the same formula but using IDLs instead. Method inter- and intra- day accuracy and precision were established by injecting matrix river QCs (10, 100 and 500 ng L^{-1}) in triplicate over three consecutive days.

2.6 Analysis of ARGs – dPCR

ARG analysis was performed using a dPCR set up, utilising the QuantStudio® 3D Digital PCR System (Thermo Scientific, UK). The reaction mix was made up according to manufacturer instructions and consisted of QuantStudio® 3D Digital PCR Master Mix, TaqMan™ primers with MGB probes, sterile water, and the DNA sample (diluted when appropriate). This mixture was then portioned onto dPCR chip wells and sealed before the thermo cycling reaction took place.

The first step of the PCR thermo cycling conditions was the temperature was ramped to 95 °C and held for 10 min. It was then lowered to 60 °C for 2 min before increasing to 98 °C for 30 s. To allow for efficient gene amplification, this cycle between 60 °C and 98 °C was repeated 40 times. Temperature was then lowered to 60 °C and held for 2 min, before cooling to room temperature. After allowing 30 mins for chips to equilibrate to room temperature, each chip was processed and read utilising the QuantStudio 3D Digital PCR system chip reader. For ARG analysis, the AnalysisSuite™ software was used for quantification of the target gene and any DNA dilutions were considered here. The DNA extracted from each biological replicate was run in triplicate for each gene investigated.

2.6. CEC Stability Studies

2.6.1. CEC stability over 24 hours in river water

The stability of target CECs in river samples at room temperature over 24 hours was investigated. This was achieved by spiking triplicate samples of river water (50 mL) with analytes at initial concentrations of 100 ng L-1 and 500 ng /L before being left covered for 24 hours. Samples were then spiked with 50 ng of IS solution before being processed via the iMME sampler.

2.6.2. Weeklong stability after iMME sampling

To investigate the stability of target CECs on SPE cartridge, river water was aliquoted and spiked with 100 ng L^{-1} of analyte solution. The spiked river water was then processed onto cartridge via the iMME sampler. Day 0 spiked was with 50 µL of IS at same time of analyte spiking. All proceeding samples were processed onto SPE cartridges and kept at respective conditions for the determined number of days. When samples were eluted, 50 ng of IS was added. Time points were processed in duplicate. Day 0, 1, 2, 3, 5, and 7 and storage at two different temperatures: room temperature and freezer temperature (21 °C and -18 °C).

3. Results and discussion

3.1 iMME-LC-TQD Performance

LC-TQD performance results are available in the supplementary information (table S2). iMME -LC-TQD performance and SPE recoveries for the CECs studied are gathered in figure 3. Good recoveries were observed generally (93 ± 32%); particularly compounds from the illicit drugs classes (~116%) and the betablockers (~110%). Regarding AAs, parent analytes from the sulfonamides and macrolides generally had good recoveries from river water samples (87% and 97%, respectively). The metabolite recoveries for sulfonamides and macrolides AAs did vary; with some performing well, like desmethyl-erythromycin (108%), and others observing lower recoveries, such as acetyl-sulfamethoxazole (47 %).

For several CECs, SPE recoveries could not be calculated at 10 ng L^{-1} spiked river water samples (e.g., amphetamine, 1,7 dimethylxanthine, and rifamycin); and several CECs reported poor recoveries at 10 ng L^{-1} but good at the higher concentrations of 100 and 500 ng L^{-1} (table S3). Several CECs demonstrated consistently poor recoveries from river water across all three spiked concentrations, these included 4-hydroxy trimethoprim (11%) and ceftiofur (22%). Oasis HLB cartridges are often used in multi-residue analytical methods due to the ability to extract a broad and diverse range of CECs. However, it is recognized that not all CECs will be recovered or perform well, and optimization for specific CECs could be better achieved. The method performance for studied CECs was also investigated (table 2). In general, both methods (for Group A and B compounds) achieved good sensitivity for the CECs in river water matrices (MDLs <0.1 ng L^{-1}) and in line with previous results (Holton and Kasprzyk-Hordern, 2021; Petrie et al., 2015b).

For CECs from group A, the lowest MDLs were observed for benzoylecgonine at 0.0533 ng L-1 (MQL = 0.1777 ng L-1) and cocaine at 0.0649 ng L-1 (MQL=0.2164 ng L-1). The higher MDL for this group was observed for the antihistamine cimetidine, with MDLs reported at 10 ng L-1 (MQL=34 ng L-1). Regarding group B, the lowest MDLs were observed for erythromycin at 0.0051 ng L-1 (MQL at 0.0169 ng L-1) and sulfamethoxazole with an MDL of 0.0163 ng L-1 (MQL of 0.0543 ng L-1). Higher MDLs were observed for the quinolone metabolites: hydroxy-norfloxacin at 108.7 ng L-1 and desmethyl-ofloxacin at 31.8 ng L-1.

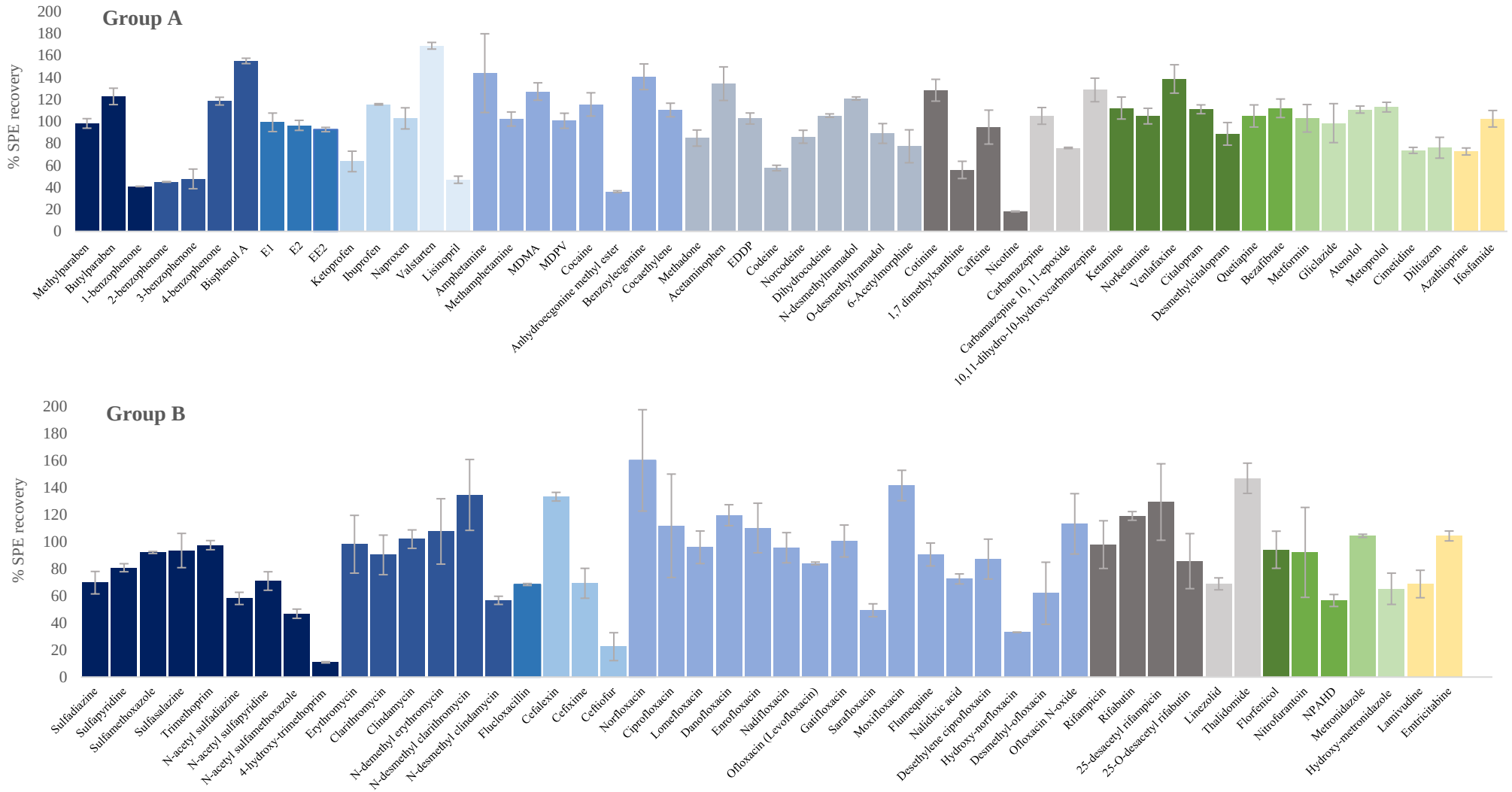


Figure 3. iMME -LC-TQD Performance data showing SPE recoveries of studied CECs spiked at 100 and 500 ng L-1 in collected river water samples, processed straight away after spiking. SPE recoveries (%) here was taken from averaging percentage recovery from 100 and 500 ng L-1 spiked.

Table 2. SPE recoveries, method performance limits, and matrix inter- and intra-day accuracy of target CECs in river water spiked at 10, 100, and 500 ng L^{-1}, respectively. Compounds organised by compound class

CEC Group	Compound	Compound Class	SPE Recoveries (%)[†]	MDL ng L-1	MQL ng L-1	Intra-day matrix performance[§]		Inter-day matrix performance[§]	
						Precision %	Accuracy %	Precision %	Accuracy %
Group A	Methylparaben	Parabens	98	0.102	0.611	4	95	5	100
	Propylparaben		121	0.33	0.989	18	104	23	109
	Butylparaben		123	0.122	0.407	5	79	6	79
	1-benzophenone	UV Filters	41	1.83	6.11	10	162	13	160
	2-benzophenone		45	0.666	2.22	4	154	7	153
	3-benzophenone		48	0.157	0.523	25	159	40	160
	4-benzophenone		119	2.62	8.52	5	81	8	80
	Bisphenol A*	Plasticizer	155	0.193	0.645	49	80	34	79
	E1	Steroid Estrogen	99	1.51	5.04	4	96	5	97
	E2		96	3.11	10.4	10	90	9	90
	EE2		93	3.24	10.8	4	110	12	107
	Ketoprofen	NSAIDs	64	1.73	8.48	5	105	148	141
	Ibuprofen		116	0.0865	0.432	3	94	5	94
	Naproxen		103	1.46	4.86	16	96	34	66
	Valsartan	Hypertension	169	8.88	29.6	5	38	13	29
	Irbesartan		114	0.263	0.875	3	77	15	85
	Lisinopril		47	6.39	21.3	5	100	5	101
	Amphetamine[†]	Stimulants and metabolites	144	0.521	1.74	3	58	4	56
	Methamphetamine		102	0.147	0.489	1	95	3	94
	MDMA		127	0.118	0.393	3	66	4	66
	MDPV		101	0.149	0.497	3	94	5	94
	Cocaine		116	0.0649	0.216	2	77	2	77
	Anhydroecgonine methyl ester		36	2.08	6.92	4	149	6	150
	Benzoylecgonine		141	0.0533	0.178	2	52	3	51
	Cocaethylene		110	0.0679	0.226	3	81	4	81
	Methadone	Analgesics and metabolites	85	0.0883	0.294	6	110	7	108
	Acetaminophen		134	0.558	1.86	5	84	4	84
	EDDP		103	0.073	0.243	4	92	4	94
	Codeine		58	1.73	8.67	5	138	6	135
	Norcodeine		86	3.71	12.4	5	96	6	97
	Dihydrocodeine		105	0.712	2.37	3	85	2	85
	N-desmethyltramadol		121	0.0827	0.414	4	57	8	57
	O-desmethyltramadol		89	3.37	11.2	3	104	5	102
	6-acetylmorphine[†]	Opioid metabolite	77	0.387	1.29	6	125	5	123
	Cotinine	Human Indicators	128	0.0779	0.389	2	111	5	109
	Caffeine		95	1.05	5.27	4	110	9	107
	Nicotine		18	8.27	27.6	23	169	18	171
	1,7 dimethylxanthine[†]		56	5.36	17.9	7	145	10	144
	Carbamazepine	Anti-epileptic	105	0.143	0.476	7	89	7	88
	Carbamazepine 10, 11-epoxide		76	0.395	1.32	4	122	7	120

	10,11-dihydro-10-hydroxycarbmazepine		129	0.388	3.88	8	65	10	62
	Ketamine	Anaesthetic and metabolite	112	0.134	0.446	4	84	4	82
	Norketamine		105	0.0715	0.238	3	86	5	88
	Venlafaxine	Antidepressants	139	0.0721	0.288	6	43	10	39
	Citalopram		111	0.45	4.5	6	92	6	91
	Desmethylcitalopram		89	0.169	0.563	7	103	8	106
	Quetiapine	Antipsychotic	105	0.0714	0.238	3	91	3	91
	Bezafibrate	Lipid regulator	112	0.268	0.893	4	57	4	60
	Metformin	Diabetes	103	0.146	0.486	16	91	16	91
	Gliclazide		99	0.0761	0.254	4	82	7	86
	Atenolol	Beta blocker	111	0.677	2.26	3	86	5	87
	Metoprolol		113	1.33	4.42	2	87	5	85
	Cimetidine	Histamine H_2 receptor antagonist	74	10.2	33.9	3	110	7	108
	Diltiazem	Calcium channel blocker	76	0.394	1.31	3	120	5	120
	Azathioprine	Anticancer	73	0.413	1.38	6	125	4	124
	Ifosfamide		102	0.0976	0.488	0	189	4	190
	Fexofenadine	Antihistamine	52	1.45	4.82	2	87	5	85
Group B	Sulfadiazine	Sulphonamide & trimethoprim	70	0.215	0.716	7	70	31	62
	Sulfapyridine		81	0.0371	0.124	6	96	9	88
	Sulfamethoxazole		92	0.0163	0.0543	8	97	6	91
	Sulfasalazine		94	16	53.4	18	94	15	115
	Trimethoprim		98	1.54	5.13	12	102	4	98
	N-acetyl sulfadiazine		58	0.361	1.2	11	56	3	58
	N-acetyl sulfapyridine		71	2.25	7.82	8	74	7	81
	N-acetyl sulfamethoxazole		47	0.405	1.35	9	57	8	58
	4-hydroxy-trimethoprim		11	0.349	1.16	10	11	2	11
	Erythromycin	Macrolide & lincosamide	98	0.0153	0.0509	14	114	11	104
	Clarithromycin		90	0.0166	0.0554	13	100	9	109
	Clindamycin		102	1.47	4.9	6	107	4	112
	N-desmethyl erythromycin		108	0.0205	0.0683	20	111	12	100
	N-desmethyl clarithromycin		135	0.0371	0.124	15	150	11	167
	N-desmethyl clindamycin		57	0.0264	0.0881	12	58	3	60
	Flucloxacillin	β-Lactams	68	2.19	7.3	8	71	10	74
	Cefalexin	Cephalosporins	133	2.81	9.37	25	122	152	74
	Cefixime		69	21.6	72.1	16	69	64	240
	Ceftiofur		22	6.67	22.2	15	50	5	51
	Norfloxacin*	Quinolone	160	0.0187	0.0624	15	160	11	178
	Ciprofloxacin		112	1.34	4.47	22	131	39	127
	Lomefloxacin		96	0.313	1.04	21	97	3	99
	Danofloxacin		120	12.5	41.8	9	120	7	116
	Enrofloxacin		110	0.272	0.907	18	111	11	131
	Nadifloxacin		96	3.14	10.5	13	128	14	156
	Ofloxacin (Levofloxacin)		84	0.357	1.19	11	85	6	91
	Gatifloxacin		101	0.0298	0.0994	20	97	14	104
	Sarafloxacin		49	3.04	10.1	12	49	60	75
	Moxifloxacin†		142	2.65	8.82	25	142	6	147

Flumequine		91	0.0331	0.11	10	102	4	98
Nalidixic acid		73	0.0413	0.138	10	74	3	75
Desethylene ciprofloxacin		87	1.72	5.73	40	236	88	234
Hydroxy-norfloxacin		33	109	362	8	33	14	39
Desmethyl-ofloxacin		62	2.42	8.07	8	73	42	104
Ofloxacin N-oxide		113	31.8	106	10	113	5	118
Rifampicin[†]	TB drugs (1st line)	98	3.83	12.8	16	98	27	114
Rifabutin		119	1.26	4.2	26	140	14	139
25-desacetyl rifampicin[†]		129	26.2	87.3	16	129	12	148
25-O-desacetyl rifabutin		86	11.6	38.6	32	99	16	106
Linezolid	TB other	69	0.35	1.17	9	74	12	65
Florfenicol	Amphenicol	94	2.04	6.8	5	94	3	92
Nitrofurantoin	Nitrofurantoin	92	31.9	106	16	92	13	87
NPAHD		57	3.25	10.8	13	59	8	55
Metronidazole	Azole	104	0.53	1.77	6	114	4	111
Hydroxy-metronidazole		65	0.287	0.957	6	65	32	71
Lamivudine	Antiretroviral	69	0.46	1.53	7	69	76	170
Emtricitabine		104	4.36	14.5	12	125	10	139

[†] Based on two concentrations (100 and 500 ng L-1), [§] Based on three concentrations (10, 100, and 500 ng L-1) *semi-quantitative

3.2 CEC stability over 24 hours in river water

CECs demonstrated variable stability in UK river water over 24 hour. A full breakdown of results may be found in the supplementary information (table S4). In general CECs from group A demonstrated better overall stability in comparison to group B (overall averages, 0 ± 21% vs -2 ± 41% degradation respectively). In group A, good stabilities were observed for all CECs studied from the beta-blockers and antiepileptic classes (-7 ± 4%). Regarding group B, whilst good stabilities were observed for AAs, including sulfamethoxazole, sulfapyridine and trimethoprim (5 ± 3%). Poorer stability was observed for methyl paraben and ofloxacin (figure 4). Ofloxacin observed ~50% degradation in river water over 24-hours, yet minimal degradation was observed for 24-hour stability study on cartridge (~12%). Similar results for the antiviral, emtricitabine, were also observed, with ~50% degradation in river water but only observing ~-20% on cartridge.

The inclusion of metabolites in this study showed some interesting results. Cocaine appeared to degrade by ~50%, whilst its major metabolite benzoylecgonine increased over the 24-hour period by ~60% - potentially indicating a transformation process. When comparing both cocaine and benzoylecgonine stability, samples processed onto cartridge demonstrated minimal changes (~10% reduction, for both). Other studies have also observed variable stability of cocaine and potential transformation to benzoylecgonine in both river water and wastewaters (Castiglioni et al., 2006; Gheorghe et al., 2008). This study indicates that processing onto cartridge after sample collection could be beneficial for many CECs and reduce potential degradation. However, due to logistical limitations, this study did not investigate the stability of CECs in SA river water, which had different biochemical characteristics and may have had different patterns of stability. In summary, CECs showed different levels of stability during the 24h study, suggesting that transport to a laboratory without a cold-chain might be detrimental to sample integrity. Therefore, there is a need for on-site analyte stabilization procedures, such as presented in this paper, via the iMME sampler methodology.

3.3 Weeklong stability after iMME sampling

In general, most compounds demonstrated good stability on cartridge, at room temperature (21 °C) over the 7 days (figure 4). With overall averages -2 ± 22% and 13 ± 24% degradation for groups A and B

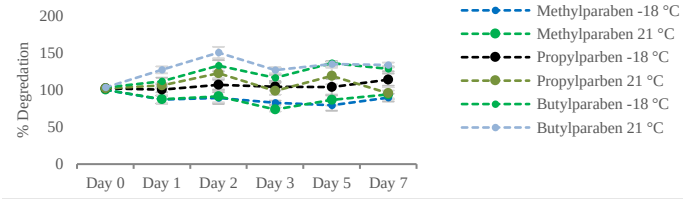
respectively. SPE cartridges stored in a freezer (-18 °C) were also investigated for comparison. Again, good performance of CECs was observed with <1 ± 16% and 8 ± 18% overall average for degradation for group A and B respectively. Full breakdown tables may be found in the supplementary information (tables SI5 and SI6). Many CECs however were stable under both storage conditions, indicating a very good performance of iMME when stored at room temperature. A few CECs displayed better stability when stored in colder temperatures; for example, amphetamine reported minimal degradation at day 7 when stored in the freezer (8 ± 6% degradation) compared to 21 °C (64 ± 2%). 2-benzophenone exhibited similar levels of degradation at both temperatures, at day 7, reporting ~30% degradation. Conversely, ketoprofen appeared to be less stable at freezer temperatures on cartridge, reporting 44 ± 16% degradation at day 7 vs the 11 ± 9% degradation stored at room temperature.

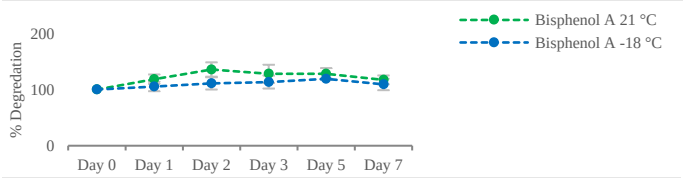
Regarding AAs, in general good stability was reported across AAs stored on cartridge at room temperature and at -18 °C (figure 5). A few AAs, however, exhibited greater stability stored at -18 °C. For example, emtricitabine reported 13 ± 8% degradation (-18 °C) vs 40 ± 7% (21 °C) at 7 days. Another was norfloxacin, which reported -10 ± 24% (-18 °C) at day 7 vs 44 ± 26% (21 °C) at the same time point. A couple of quinolone metabolites also reported similar, with desethylene ciprofloxacin 14 ± 22% vs 56 ± 24% and ofloxacin N-oxide 5 ± 15% vs 39 ± 20%. Cefalexin also reported greater stability at -18 °C vs 21 °C, with day 7 observing <1 ± 25% and 57 ± 10. Flucloxacillin exhibited similar levels of degradation at both room and freezer temperatures, 37 ± 11% vs 37 ± 17%. Ceftiofur also exhibited some degradation at room and freeze temperature, with ~59% after 7 days. Conversely rifabutin appeared to demonstrate greater stability at room temperature vs freezer, at 39 ± 5% (-18) vs -3 ± 16% respectively.

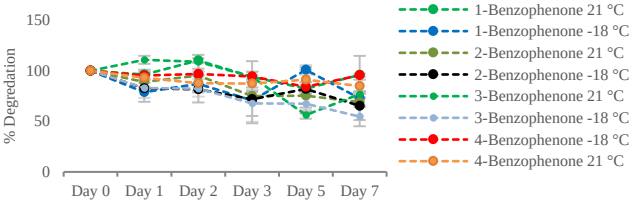
Results here have demonstrated that whilst storing SPE cartridge at freezer temperatures provides the best stability, storing cartridges at room temperature still provided stability of at least ~20% for the majority of CECs studied; especially in comparison to stability of CECs in aqueous phase, under the same conditions. For example, ofloxacin observed no degradation when stored at room temperature on cartridge over the 24 hours, yet over the same time period and temperature in river water reported ~50% degradation (table S4). Despite the indication for potential transformation of cocaine to benzoylecgonine in river water after 24 hours (±50%), both cocaine and benzoylecgonine stored on cartridge at room temperature reported minimal degradation in comparison between 10-20% (table S6).

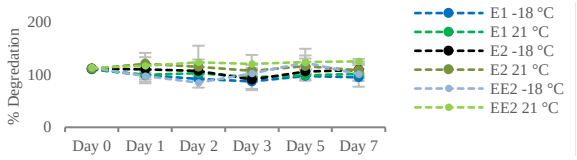
Therefore, it appears that for many CECs, processing onto SPE cartridges after sample collection is best practice. This indicates that CECs in general appear more stable on sorbent then in aqueous form. This is in agreement with Singh *et al.*, who reported that shipping internationally on SPE cartridges (kept cold) provided the best analyte stability (R. R. Singh et al., 2019). Whilst deuterated analogue internal standards account for degradation in transport and storage, it is not practical in large multi-residue methods for every CEC to have its own deuterated internal standard. Whilst internal standards tend to be assigned across class, assuming similar chemical properties, results from this study do indicate that stability can vary across compounds of a class. Therefore, spiking of internal standards and processing onto cartridge as soon as samples are collected is recommended as greater stability has been observed when CECs are immobilized onto sorbent.

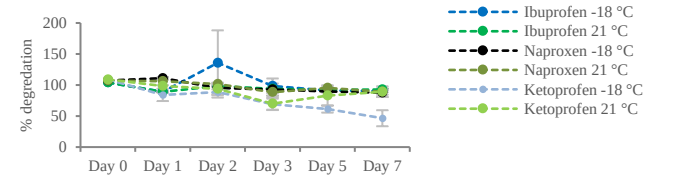
Group A

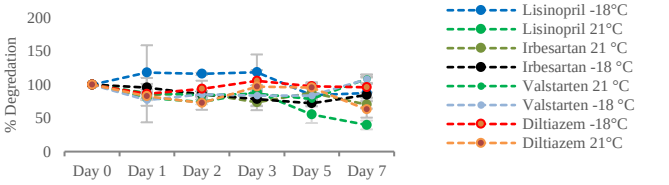

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Methylparaben -18 °C
Methylparaben 21 °C
Propylparben -18 °C
Propylparben 21 °C
Butylparaben -18 °C
Butylparaben 21 °C

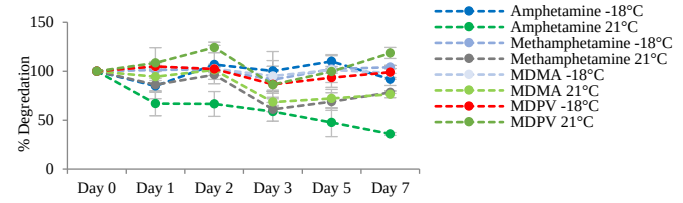

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Bisphenol A 21 °C
Bisphenol A -18 °C

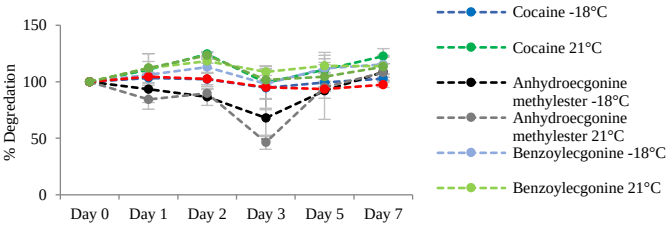

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
1-Benzophenone 21 °C
1-Benzophenone -18 °C
2-Benzophenone 21 °C
2-Benzophenone -18 °C
3-Benzophenone 21 °C
3-Benzophenone -18 °C
4-Benzophenone -18 °C
4-Benzophenone 21 °C

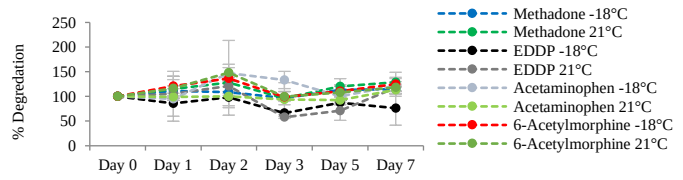

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
E1 -18 °C
E1 21 °C
E2 -18 °C
E2 21 °C
EE2 -18 °C
EE2 21 °C

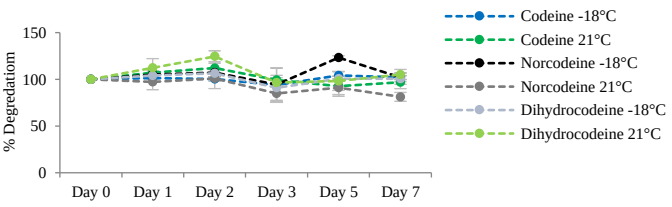

% degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Ibuprofen -18 °C
Ibuprofen 21 °C
Naproxen -18 °C
Naproxen 21 °C
Ketoprofen -18 °C
Ketoprofen 21 °C

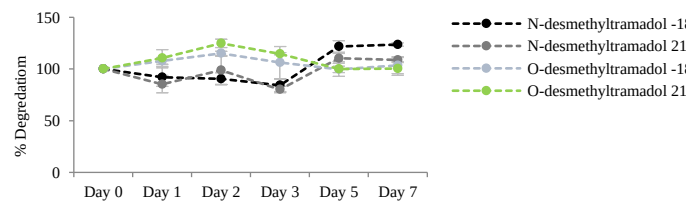

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Lisinopril -18°C
Lisinopril 21°C
Irbesartan 21 °C
Irbesartan -18 °C
Valstarten 21 °C
Valstarten -18 °C
Diltiazem -18°C
Diltiazem 21°C

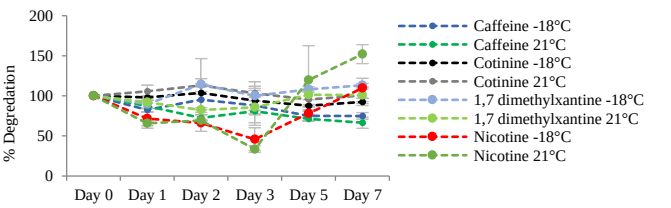

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Amphetamine -18°C
Amphetamine 21°C
Methamphetamine -18°C
Methamphetamine 21°C
MDMA -18°C
MDMA 21°C
MDPV -18°C
MDPV 21°C

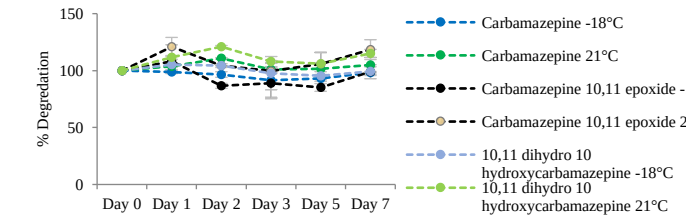

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Cocaine -18°C
Cocaine 21°C
Anhydroecgonine methylester -18°C
Anhydroecgonine methylester 21°C
Benzoylecgonine -18°C
Benzoylecgonine 21°C

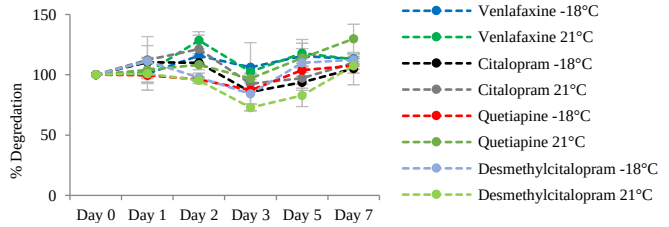

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Methadone -18°C
Methadone 21°C
EDDP -18°C
EDDP 21°C
Acetaminophen -18°C
Acetaminophen 21°C
6-Acetylmorphine -18°C
6-Acetylmorphine 21°C

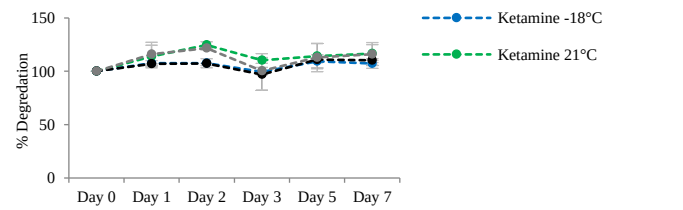

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Codeine -18°C
Codeine 21°C
Norcodeine -18°C
Norcodeine 21°C
Dihydrocodeine -18°C
Dihydrocodeine 21°C

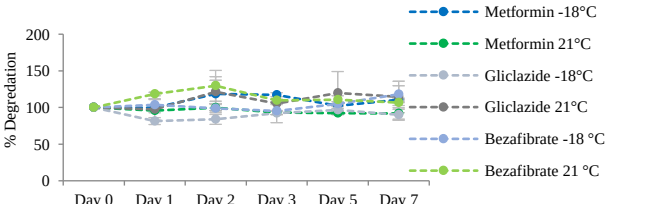

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
N-desmethyltramadol -18°C
N-desmethyltramadol 21°C
O-desmethyltramadol -18°C
O-desmethyltramadol 21°C

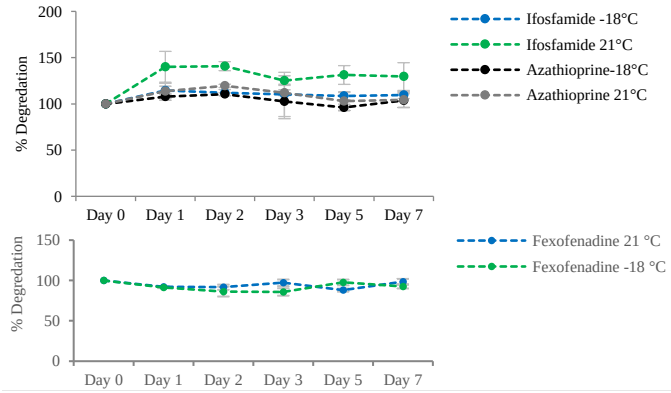

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Caffeine -18°C
Caffeine 21°C
Cotinine -18°C
Cotinine 21°C
1,7 dimethylxantine -18°C
1,7 dimethylxantine 21°C
Nicotine -18°C
Nicotine 21°C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Carbamazepine -18°C
Carbamazepine 21°C
Carbamazepine 10,11 epoxide -18°C
Carbamazepine 10,11 epoxide 21°C
10,11 dihydro 10 hydroxycarbamazepine -18°C
10,11 dihydro 10 hydroxycarbamazepine 21°C

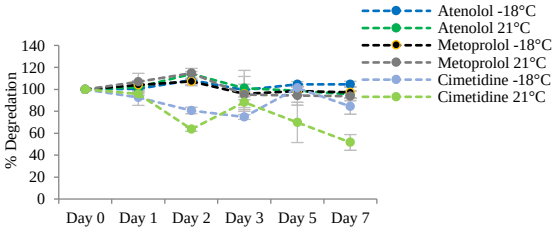

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Venlafaxine -18°C
Venlafaxine 21°C
Citalopram -18°C
Citalopram 21°C
Quetiapine -18°C
Quetiapine 21°C
Desmethylcitalopram -18°C
Desmethylcitalopram 21°C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Ketamine -18°C
Ketamine 21°C

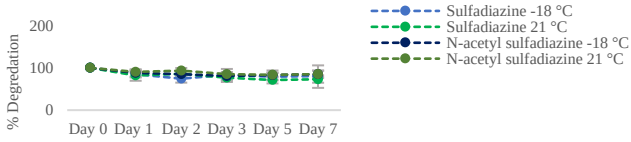

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Metformin -18°C
Metformin 21°C
Gliclazide -18°C
Gliclazide 21°C
Bezafibrate -18 °C
Bezafibrate 21 °C

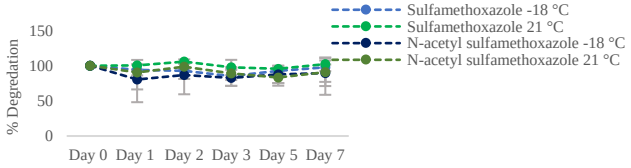

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Ifosfamide -18°C
Ifosfamide 21°C
Azathioprine-18°C
Azathioprine 21°C

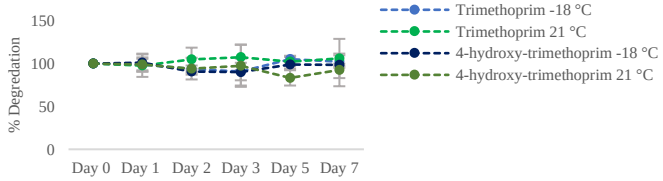
% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Fexofenadine 21 °C
Fexofenadine -18 °C

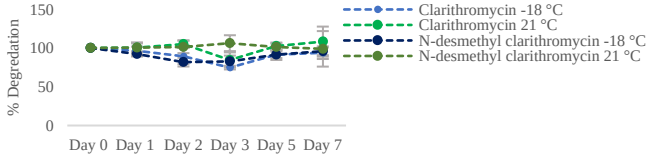

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Atenolol -18°C
Atenolol 21°C
Metoprolol -18°C
Metoprolol 21°C
Cimetidine -18°C
Cimetidine 21°C

Group B


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Sulfadiazine -18 °C
Sulfadiazine 21 °C
N-acetyl sulfadiazine -18 °C
N-acetyl sulfadiazine 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Sulfamethoxazole -18 °C
Sulfamethoxazole 21 °C
N-acetyl sulfamethoxazole -18 °C
N-acetyl sulfamethoxazole 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Trimethoprim -18 °C
Trimethoprim 21 °C
4-hydroxy-trimethoprim -18 °C
4-hydroxy-trimethoprim 21 °C


% Degredation
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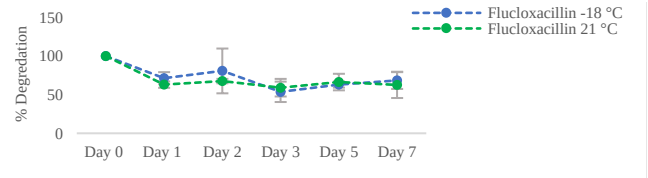
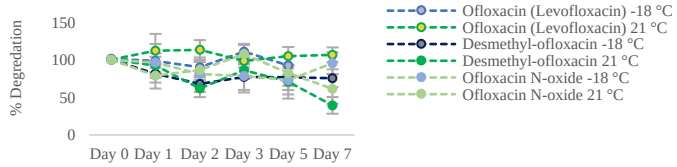
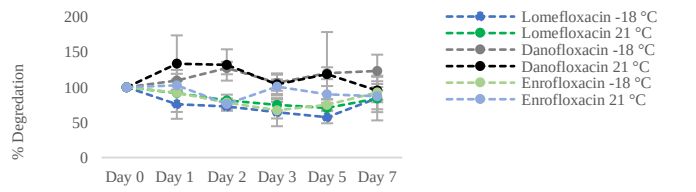
Clarithromycin 21 °C
N-desmethyl clarithromycin -18 °C
N-desmethyl clarithromycin 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Flucloxacillin -18 °C
Flucloxacillin 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Ofloxacin (Levofloxacin) -18 °C
Ofloxacin (Levofloxacin) 21 °C
Desmethyl-ofloxacin -18 °C
Desmethyl-ofloxacin 21 °C
Ofloxacin N-oxide -18 °C
Ofloxacin N-oxide 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
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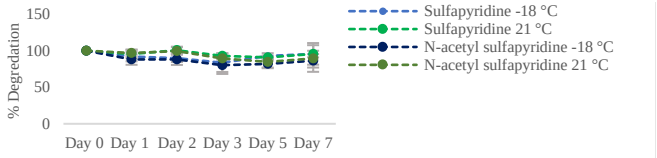
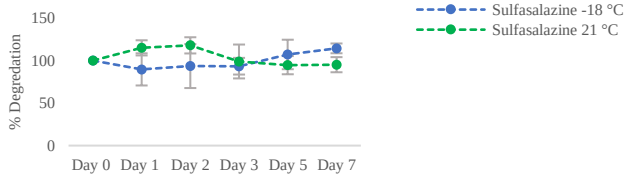
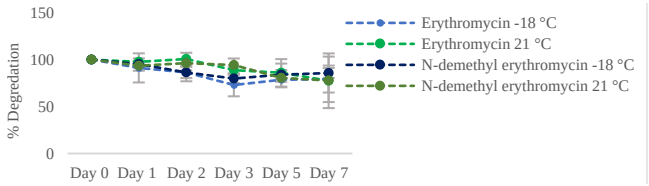
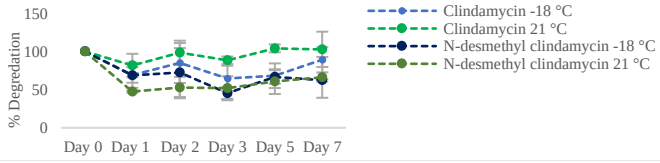
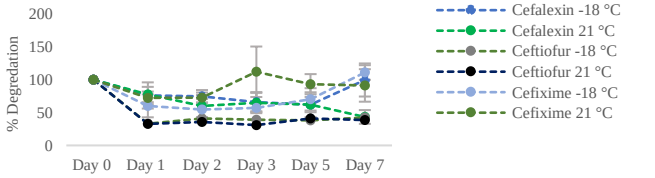
Lomefloxacin 21 °C
Danofloxacin -18 °C
Danofloxacin 21 °C
Enrofloxacin -18 °C
Enrofloxacin 21 °C

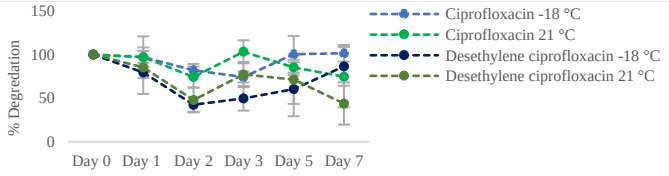

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Sulfapyridine -18 °C
Sulfapyridine 21 °C
N-acetyl sulfapyridine -18 °C
N-acetyl sulfapyridine 21 °C

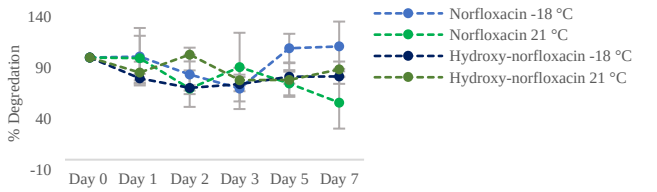

% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Sulfasalazine -18 °C
Sulfasalazine 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Erythromycin -18 °C
Erythromycin 21 °C
N-demethyl erythromycin -18 °C
N-demethyl erythromycin 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Clindamycin -18 °C
Clindamycin 21 °C
N-desmethyl clindamycin -18 °C
N-desmethyl clindamycin 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Cefalexin -18 °C
Cefalexin 21 °C
Ceftiofur -18 °C
Ceftiofur 21 °C
Cefixime -18 °C
Cefixime 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Ciprofloxacin -18 °C
Ciprofloxacin 21 °C
Desethylene ciprofloxacin -18 °C
Desethylene ciprofloxacin 21 °C


% Degredation
Day 0 Day 1 Day 2 Day 3 Day 5 Day 7
Norfloxacin -18 °C
Norfloxacin 21 °C
Hydroxy-norfloxacin -18 °C
Hydroxy-norfloxacin 21 °C

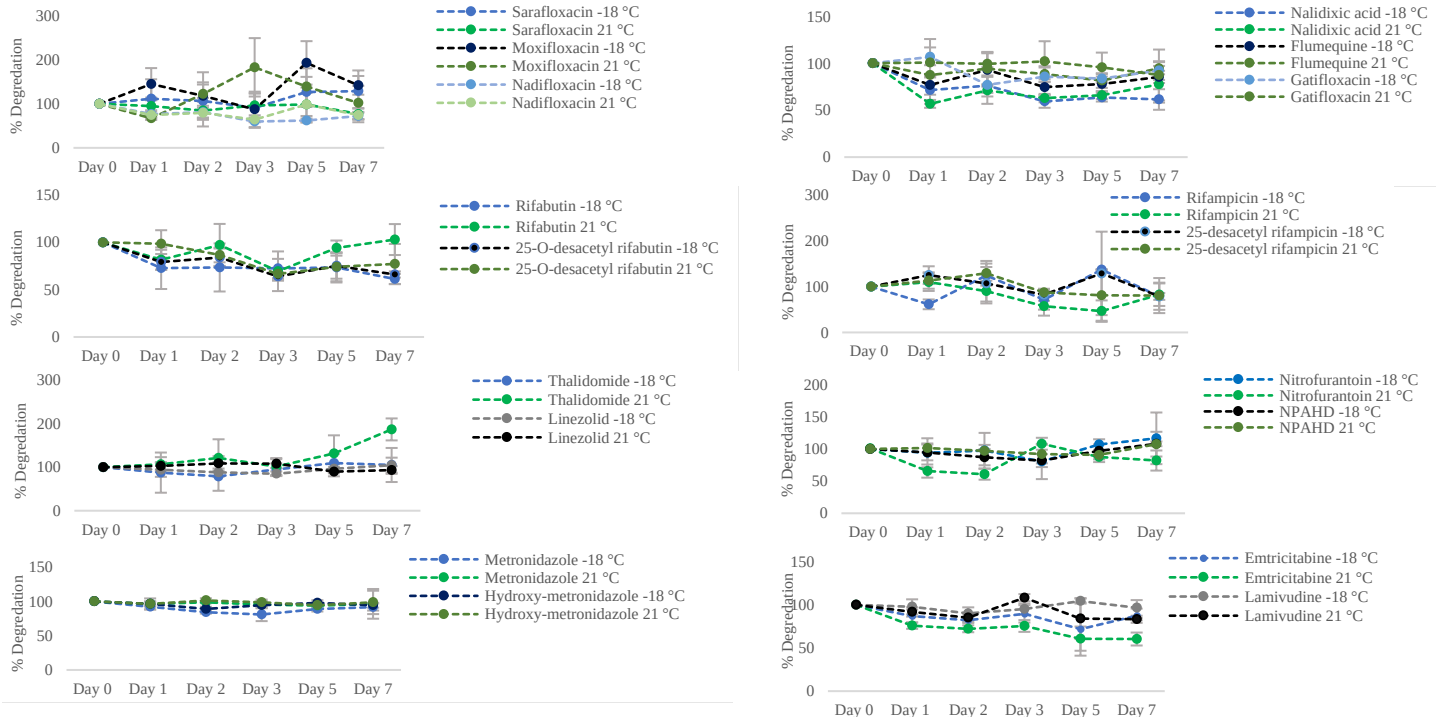


Figure 4. Week-long stability of CECs on cartridge, stored at room temperature (21 °C) and freezer temperature (-18 °C), for an initial CEC spiking concentration of 100 ng L-1. Error bars represent standard deviation, n = 2 biological replicates, injected in duplicate.

3.4 iMME application to contrasting locations in urbanized River Avon in the UK and remote Olifants River in SA (CECs in group A and B)

Samples collected from the river Avon (UK) were analyzed for group A and B CECs, and Olifants River (SA) samples were analyzed for group A CECs (table 3). River samples from the UK had a total of 45 and 13 CECs above quantifiable limits from groups A and B, respectively. Metformin was recorded in the highest concentrations (1359 ± 83 ng L-1) in the river Avon. Previous work done on this river has also reported high levels of metformin ranging from 2000-3000 ng L-1 (Petrie et al., 2015b). The reasoning for why this CEC is so high, has been attributed to its extensive usage in the treatment of type-2 diabetes, and for the lack of metabolism in the body (Gong et al., 2012). The UV filter 4-benzophenone, commonly found in sunscreens and cosmetic products, was also reported in high levels (299 ± 18 ng L-1). Its presence here is likely from a mixture of release from sewage discharge and from washed off skin from recreational activities (the river is popular among open water swimming).

Regarding group B, all AAs and metabolites from the macrolide and lincosamide class were reported in the river Avon. The macrolide AAs, clarithromycin and erythromycin, were reported at 51 ± 4 ng L-1 and 24 ± 2 ng L-1, respectively; and metabolites for both were also observed (desmethyl clarithromycin: 95 ± 32 ng L-1 and desmethyl erythromycin 0.88 ± 0.21 ng L-1). Several sulfonamides were also reported, including sulfamethoxazole at 33 ± 4 ng L-1, which is another CEC that has been widely reported in rivers across the world (Booth et al., 2020; R. Singh et al., 2019). Sulfapyridine was another of the sulfonamides detected at 75 ± 2 ng L-1. Whilst sulfapyridine is a major metabolite of sulfasalazine (prescribed as an inflammatory in human medicines), it is also utilized as an AA in veterinary medicine. It is therefore likely that sulfapyridine could be from human consumption of sulfasalazine but also some potential agricultural run-off; particularly as the river in question has a strong surrounding agricultural presence. It is worth mentioning that many AAs used in veterinary

medications do have cross-over with those in humans, so AAs could be from a combination of human and animal sources.

In comparison, the Olifants River, flowing through pristine environment of Kruger National Park, had less CECs detected with only 13 CECs from group A above quantifiable limits. Whilst very high levels of metformin were reported in the river Avon, this was not detected in the samples collected from Olifants River, as the human contribution to the river is limited. Caffeine was reported in the highest concentration for SA samples, on average 115 ± 87 ng L-1 across the sampling points. Interestingly, whilst pharmaceuticals detection was low in Olifants River, all human indicators investigated in this study were quantifiable. This is likely due to tourists' presence in Kruger Park camps. Carbamazepine has been identified as one of the most readily found CEC in the environment (aus der Beek et al., 2016), and was found in both rivers at concentrations of 51 ± 1 ng L-1 and 1.26 ± 0.65 ng L-1 for Avon and Olifants River, respectively. Interestingly, carbamazepine's metabolite, carbamazepine 10, 11-epoxide, was detected in higher concentrations on average than its parent compound in the Olifants River (2.58 ± 0.78 ng L-1); highlighting the importance of monitoring metabolites alongside parent compounds.

Table 3. Average CECs concentrations in river water from the river Avon (UK) and the Olifants River (SA)

		River Avon, UK (n=3*)		Olifants River, SA (n=4**)	
Compound	**Compound Class**	**Concentration ng L-1**	**STD**	**Concentration ng L-1**	**STD**
Methylparaben	Parabens	25.80	0.82	6.70	8.35
Propylparaben		-	-	-	-
Butylparaben		1.92	0.68	-	-
1-benzophenone	UV Filters	-	-	0.25	0.23
2-benzophenone		-	-	-	-
3-benzophenone		20.79	9.21	-	-
4-benzophenone		298.75	18.13	59.48	35.19
Bisphenol A	Plasticizer	0.00	0.00	-	-
E1	Steroid Estrogen	-	-	-	-
E2		-	-	-	-
EE2		-	-	-	-
Ketoprofen	NSAIDs	57.52	0.83	-	-
Ibuprofen		67.08	13.87	-	-
Naproxen		80.09	3.92	-	-
Valsartan	Hypertension	58.04	3.05	-	-
Irbesartan		18.21	1.64	-	-
Lisinopril		13.93	0.19	-	-
Amphetamine	Stimulants and metabolites	86.30	79.48	-	-
Methamphetamine		-	-	-	-
MDMA		1.21	0.14	-	-
MDPV		0.00	0.00	-	-
Cocaine		1.11	0.49	-	-
Anhydroecgonine methyl ester		3.12	0.01	-	-
Benzoylecgonine		42.80	2.28	-	-
Cocaethylene		-	-	-	-
Methadone	Analgesics and metabolites	0.11	0.36	-	-
Acetaminophen		108.49	40.58	6.84	3.22
EDDP		2.57	0.76	-	-

Codeine		29.51	3.04	-	-
Norcodeine		10.11	4.36	-	-
Dihydrocodeine		11.26	0.86	-	-
N-desmethyltramadol		2.10	11.80	-	-
O-desmethyltramadol		24.04	2.42	-	-
6-acetylmorphine	Opioid metabolite	-	-	-	-
Cotinine	Human Indicators	22.63	3.22	2.14	0.59
Caffeine		131.23	35.35	115.06	86.69
Nicotine		40.60	1.14	14.69	0.62
1,7 dimethylxanthine		226.93	48.89	64.89	42.02
Carbamazepine	Anti-epileptic	50.74	0.76	1.26	0.65
Carbamazepine 10, 11-epoxide		4.48	1.92	2.58	0.78
10,11-dihydro-10-hydroxycarbmazepine		5.38	1.09	-	-
Ketamine	Anaesthetic and metabolite	14.41	0.75	-	-
Norketamine		1.45	0.47	-	-
Venlafaxine	Antidepressant	22.55	0.92	-	-
Citalopram		6.39	2.58	-	-
Desmethylcitalopram		4.32	0.90	-	-
Quetiapine	Antipsychotic	0.03	0.10	-	-
Bezafibrate	Lipid regulator	38.84	1.38	-	-
Metformin	Diabetes	1358.84	82.80	-	-
Gliclazide		1.85	1.53	-	-
Atenolol	Beta blocker	17.43	0.90	-	-
Metoprolol		-	-	-	-
Cimetidine	Histamine H_2 receptor antagonist	-	-	-	-
Diltiazem	Calcium channel blocker	1.67	0.30	-	-
Azathioprine	Anticancer	21.46	0.08	-	-
Ifosfamide		-	-	-	-
Fexofenadine	Antihistamine	49.89	7.16	-	-
Sulfadiazine	Sulfonamide & trimethoprim	-	-		
Sulfapyridine		74.74	1.72		
Sulfamethoxazole		32.67	3.94		
Sulfasalazine		-	-		
Trimethoprim		10.04	1.69		
N-acetyl sulfadiazine		-	-		
N-acetyl sulfapyridine		9.81	1.93		
N-acetyl sulfamethoxazole		-	-		
4-hydroxy-trimethoprim		-	-		
Erythromycin	Macrolide & lincosamide	23.85	2.08		
Clarithromycin		51.01	4.07		
Clindamycin		15.85	0.31		
N-desmethyl erythromycin		0.88	0.21		
N-desmethyl clarithromycin		95.31	31.98		
N-desmethyl clindamycin		1.29	0.07		
Flucloxacillin	β-LACTAMS	30.97	4.53		
Gatifloxacin		-	-		

Sarafloxacin		-	-
Moxifloxacin		-	-
Flumequine		-	-
Nalidixic acid		-	-
Desethylene ciprofloxacin		-	-
Hydroxy-norfloxacin		-	-
Desmethyl-ofloxacin		-	-
Ofloxacin N-oxide		-	-
Rifampicin	TB drugs (1st line)	95.45	15.10
Rifabutin		-	-
25-desacetyl rifampicin		-	-
25-O-desacetyl rifabutin		-	-
Linezolid	TB other	-	-
		-	-
Florfenicol	Amphenicol	-	-
Nitrofurantoin	Nitrofurantoin	-	-
NPAHD		-	-
Metronidazole	Azole	-	-
Hydroxy-metronidazole		-	-
Lamivudine	Antiretroviral	-	-
Emtricitabine		-	-

*n=3 biological replicates, injected in triplicate; ** n=4, each sample had two biological replicates, injected in duplicate

3.5 Risk to the aquatic environment posed by CECs

A predicted no effect concentration (PNEC) is defined as the concentration of a substance that indicates the limit at which no pharmacological/adverse effect is expected to occur with chronic or acute exposure to a sentinel organism, mainly lower trophic-level aquatic organisms. They are widely used for environmental and risk characterisation, typically based upon single-species laboratory ecotoxicity tests. Unfortunately, for many CECs that have been detected in the environment, ecotoxicologically derived no-effect concentrations are yet to be identified, so impacts of the presence in ecosystems in often unknown (aus der Beek et al., 2016; Gunnarsson et al., 2019). Whilst environmental risk assessments are required for new pharmaceuticals entering the market in the EU (European Medicines Agency, 2006), those introduced before this regulation have this environmental aspect missing. PNECs have been compiled for a range of pharmaceuticals in the literature (Fick et al., 2010; Tell et al., 2019). Regarding AAs, PNECs are utilized to indicate areas where concentrations of AAs could be resulting in selective pressures and potential facilitation of AMR. Though, it is recognized that using PNECs for environmental risk assessments for AAs may not be fit for purpose (Murray et al., 2021; Tell et al., 2019).

Whilst only a few samples were investigated in this study, the concentrations of several CECs were compared with literature PNEC values (Table S7). And although the ecotoxicological effects of many CECs are still not know (aus der Beek et al., 2016), several CEC effects are well studied. For example, the steroid estrogen EE_2, causing feminization of male fish at nanograms per liter concentrations, has caught international attention (Harris et al., 2011; Kidd et al., 2007). In this study it was noted that all CECs concentrations were lower than the established PNEC values considered, in both Kruger and UK river water samples. The rivers sampled were fairly clean, located in a relatively unpopulated area in Kruger, and considered to be well-managed in the UK.

An important consideration is that PNECs are often determined on individual chemicals and rarely consider the impacts of chemical mixtures. Exposure to chemicals rarely occur in isolation and ecosystems are often exposed to complex cocktails in their environments. It is recognized that mixtures of chemicals exceeds the risk of individual chemicals (Backhaus, 2016), highlighting the importance of monitoring a range of CECs in aquatic environments. Due to the large number of CECs present in the environment it will be impossible to screen and monitor them all. It is also important to define the list of surrogate chemicals for monitoring the those that present the highest risk of eco-toxicity.

3.6 ARGs in river water samples (group C targets)

A handful of ARGs were investigated in the river Avon, UK. ARGs that were quantifiable were *16S rRNA, ermB, sul1, intI1*, and bla_{Tem} (figure 5). ARGs that were below the limits of quantification, via dPCR, were *qnrS* and bla_{CTX}. The extraction efficiency of the kit was also investigated, via spiking of TaqMan™ Universal DNA Spike in Control into the lysis step of the DNA extraction kit. Results gave a consistent extraction (58 ± 2%) across duplicate runs, over triplicate biological replicates (figure S2). The highest prevalence ARG in river water reported at 4.00E+06 ± 2.20E+06 copies/L was bla_{Tem}, which confers resistance to β-lactam antibiotics. This gene has been found in rivers worldwide (R. Singh et al., 2019) and has been identified as a potential indicator of anthropogenic AMR contamination (Narciso-Da-Rocha et al., 2014). This has been attributed to reports of increases of bla_{Tem} after wastewater treatment processes (Lachmayr et al., 2009; Rodriguez-Mozaz et al., 2015). This is in comparison to reports of decreased amounts of ARGs, including *ermB* and *tetW*, after wastewater treatment processes (Rodriguez-Mozaz et al., 2015).

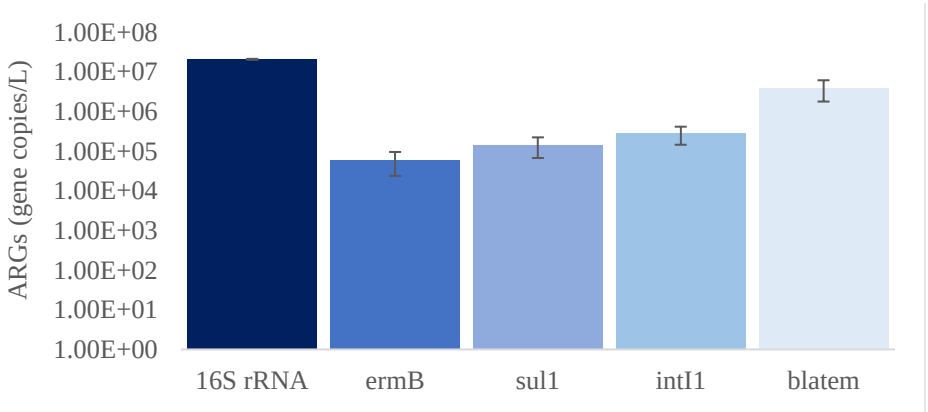


Figure 5. Absolute concentrations of quantifiable gene loads in the river Avon (UK), containing both technical and biological replicates (n=9).

Another potential indicator of anthropogenic pollution has previously been identified as *intI1*, due to its association to a number of genes that confer resistance to AAs (Gaze et al., 2011; Gillings et al., 2015). It is common for *intI1* to occur alongside sulphonamide resistance (Gillings et al., 2008), hence correlations can be observed between the two in environmental samples. In this study, *intI1* and *sul1* were found in similar levels of 2.82E+05 ± 1.36E+05 copies/L and 1.46E+05 ± 7.87E+04 copies/L, respectively.

As reported earlier, AAs from the macrolide class were well observed in the river Avon, and the ARG conferring resistance to macrolides *ermB* was also quantified in the same sample, at 6.01E+04 ± 3.62E+04 copies/L. However, it should be noted that absence of an AA in the environment could indicate a high prevalence of resistance in the bacterial communities (Murray et al., 2021). Whilst complexities lie in discerning linkages between AA presence and ARGs in environmental matrices, monitoring of both AAs and ARGs will be key for understanding the scope and scale of the problem. It is already considered that there could be a shift in surveillance in future environmental risk assessments;

for example, resistance genes monitoring in soils has been proposed by the European commission (European Commision, 2020).

3.7 Limitations of study

This study has investigated environmental water sampling using on-site SPE techniques that ensures a faster turnaround for getting samples onto a sorbent matrix and better stability at sampling areas, where processing resources are limited. However, we do acknowledge that the environmental sampling exercise was limited to very few samples in the River Avon (UK) and Olifants River (SA). Grab sampling was used in this study and is a popular choice for monitoring freshwater systems due to rapidness and ease of collecting a sample. However, care must be taken when sampling to ensure representative location and frequency. Future studies should aim to repeat and expand upon the number of samples taken in this study and investigate most appropriate times to sample; taking into account seasons and weather patterns, as well as determining the number of samples needed for statistical significance.

4. Conclusions

105 CECs and five genes were investigated in urbanized and remote locations via the iMME sampler and multi-residue trace analysis capability, utilizing liquid chromatography and triple quadrupole tandem mass spectrometry. The River Avon in the UK and the Olifants River in South Africa were used as case locations. The conclusions are as follows:

1. Several CECs showed low stability in river water, at room temperature, over a 24h sampling/transport time. It is therefore recommended that, in the absence of cooling or analyte immobilization options, environmental water samples are spiked with internal standards on site, immediately after collection. Samples should then be processed to SPE cartridge as soon as possible, to be transported to analyzing laboratory.
2. iMME has proven effective in immobilizing, concentrating, and increasing the stability of CECs at room temperature (for at least 7 days storage), allowing for sample collection at remote locations.
3. The results from the River Avon and Olifants River, indicate that the pristine environment of the Olifants catchment is largely unaffected by CECs common in the urbanized River Avon, with a few exceptions: lifestyle chemicals (e.g., caffeine, nicotine, and their metabolites); paracetamol and UV filters, due to tourism; and carbamazepine, due to its persistent nature.
4. Whilst many CECs demonstrated themselves to be stable on cartridge at room temperature (overall average of 5 ± 24% degradation after 24 hours across both groups A and B), efforts should be made to apply cooling during transport, and cartridges should be stored at freezer temperature prior to processing.
5. Using sterilized filter housing units, it is also possible to collect on-site river samples for ARG analysis, providing potential complementary data to AA concentrations. Stability of ARGs during transport and storage were not investigated during this study. Future scoping work with a few initial samples from a river in the UK and in SA have demonstrated the potential for on-site SPE sampling to quantify a range of CECs to a good level of sensitivity.
6. Further work is required to provide full integration of the device and comprehensive assessment of performance in both chemicals and biological targets.

Acknowledgements

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Credit authorship contribution statement

Natalie Sims: Conceptualisation, Methodology (sample collection from River Avon, CEC analysis with LCMS as well as dPCR analysis), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Elizabeth Holton: Writing – review editing; Edward Archer: Writing – review editing, Marelize Botes: Methodology (sample collected, Olifants river), Gideon Wolfaardt: Methodology (sample collection, Olifants river), Writing – review editing, Barbara Kasprzyk-Hordern: Conceptualisation, Methodology (experimental design, sample collection), Writing-original draft, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources.

References

aus der Beek, T., Weber, F.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment-Global occurrences and perspectives. Environ. Toxicol. Chem. https://doi.org/10.1002/etc.3339

Backhaus, T., 2016. Environmental Risk Assessment of Pharmaceutical Mixtures: Demands, Gaps, and Possible Bridges. AAPS J. https://doi.org/10.1208/s12248-016-9907-0

Bagnis, S., Boxall, A., Gachanja, A., Fitzsimons, M., Murigi, M., Snape, J., Tappin, A., Wilkinson, J., Comber, S., 2020. Characterization of the Nairobi River catchment impact zone and occurrence of pharmaceuticals: Implications for an impact zone inclusive environmental risk assessment. Sci. Total Environ. 703, 134925. https://doi.org/10.1016/j.scitotenv.2019.134925

Baker, D.R., Kasprzyk-Hordern, B., 2011. Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry. J. Chromatogr. A 1218, 8036–8059. https://doi.org/10.1016/j.chroma.2011.09.012

Batt, A.L., Kincaid, T.M., Kostich, M.S., Lazorchak, J.M., Olsen, A.R., 2016. Evaluating the extent of pharmaceuticals in surface waters of the United States using a National-scale Rivers and Streams Assessment survey. Environ. Toxicol. Chem. https://doi.org/10.1002/etc.3161

Bedner, M., MacCrehan, W.A., 2006. Transformation of Acetaminophen by Chlorination Produces the Toxicants 1,4-Benzoquinone and N -Acetyl- p -benzoquinone Imine. Environ. Sci. Technol. 40, 516–522. https://doi.org/10.1021/es0509073

Booth, A., Aga, D.S., Wester, A.L., 2020. Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. Environ. Int. https://doi.org/10.1016/j.envint.2020.105796

Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. Science (80-.). 339, 814–815. https://doi.org/10.1126/science.1226850

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2006. Identification and Measurement of Illicit Drugs and Their Metabolites in Urban Wastewater by Liquid Chromatography−Tandem Mass Spectrometry. Anal. Chem. 78, 8421–8429. https://doi.org/10.1021/ac061095b

European Chemicals Agency, 2008. European Inventory of Existing Commercial Chemical Substances (EINECS) [WWW Document]. URL https://echa.europa.eu/information-on-chemicals/ec-inventory (accessed 9.7.17).

European Commision, 2020. Update on Progress and Implementation: European Union Strategic Approach to Pharmaceuticals in the Environment.

European Medicines Agency, 2006. Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use [WWW Document]. URL https://www.ema.europa.eu/en/environmental-risk-assessment-medicinal-products-human-use

Fatta, D., Achilleos, A., Nikolaou, A., Meriç, S., 2007. Analytical methods for tracing pharmaceutical residues in water and wastewater. TrAC Trends Anal. Chem. 26, 515–533. https://doi.org/10.1016/j.trac.2007.02.001

Fedorova, G., Golovko, O., Randak, T., Grabic, R., 2014. Storage effect on the analysis of pharmaceuticals and personal care products in wastewater. Chemosphere 111, 55–60. https://doi.org/10.1016/j.chemosphere.2014.02.067

Fick, J., Lindberg, R.H., Tysklind, M., Larsson, D.G.J., 2010. Predicted critical environmental concentrations for 500 pharmaceuticals. Regul. Toxicol. Pharmacol. https://doi.org/10.1016/j.yrtph.2010.08.025

Gaze, W.H., Zhang, L., Abdouslam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A.B.A., Wellington, E.M.H., 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J. 5, 1253–1261. https://doi.org/10.1038/ismej.2011.15

Gheorghe, A., Van Nuijs, A., Pecceu, B., Bervoets, L., Jorens, P.G., Blust, R., Neels, H., Covaci, A., 2008. Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry, in: Analytical and Bioanalytical Chemistry. pp. 1309–1319. https://doi.org/10.1007/s00216-007-1754-5

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., Stokes, H.W., 2008. The Evolution of Class 1 Integrons and the Rise of Antibiotic Resistance. J. Bacteriol. 190, 5095–5100. https://doi.org/10.1128/JB.00152-08

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

Gong, L., Goswami, S., Giacomini, K.M., Altman, R.B., Klein, T.E., 2012. Metformin pathways: Pharmacokinetics and pharmacodynamics. Pharmacogenet. Genomics. https://doi.org/10.1097/FPC.0b013e3283559b22

Gunnarsson, L., Snape, J.R., Verbruggen, B., Owen, S.F., Kristiansson, E., Margiotta-Casaluci, L., Österlund, T., Hutchinson, K., Leverett, D., Marks, B., Tyler, C.R., 2019. Pharmacology beyond the patient – The environmental risks of human drugs. Environ. Int. https://doi.org/10.1016/j.envint.2019.04.075

Guruge, K.S., Goswami, P., Tanoue, R., Nomiyama, K., Wijesekara, R.G.S., Dharmaratne, T.S., 2019. First nationwide investigation and environmental risk assessment of 72 pharmaceuticals and personal care products from Sri Lankan surface waterways. Sci. Total Environ. 690, 683–695. https://doi.org/10.1016/j.scitotenv.2019.07.042

Harris, C.A., Hamilton, P.B., Runnalls, T.J., Vinciotti, V., Henshaw, A., Hodgson, D., Coe, T.S., Jobling, S., Tyler, C.R., Sumpter, J.P., 2011. The consequences of feminization in Breeding groups of wild fish. Environ. Health Perspect. https://doi.org/10.1289/ehp.1002555

Holton, E., Kasprzyk-Hordern, B., 2021. Multiresidue antibiotic-metabolite quantification method

using ultra-performance liquid chromatography coupled with tandem mass spectrometry for environmental and public exposure estimation. Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-021-03573-4

Hossain, A., Nakamichi, S., Habibullah-Al-Mamun, M., Tani, K., Masunaga, S., Matsuda, H., 2018. Occurrence and ecological risk of pharmaceuticals in river surface water of Bangladesh. Environ. Res. 165, 258–266. https://doi.org/10.1016/j.envres.2018.04.030

Hughes, S.R., Kay, P., Brown, L.E., 2013. Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems. Environ. Sci. Technol. 47, 661–677. https://doi.org/10.1021/es3030148

K'oreje, K.O., Vergeynst, L., Ombaka, D., De Wispelaere, P., Okoth, M., Van Langenhove, H., Demeestere, K., 2016. Occurrence patterns of pharmaceutical residues in wastewater, surface water and groundwater of Nairobi and Kisumu city, Kenya. Chemosphere 149, 238–244. https://doi.org/10.1016/j.chemosphere.2016.01.095

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res. 43, 363–380. https://doi.org/10.1016/j.watres.2008.10.047

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Res. 42, 3498–3518. https://doi.org/10.1016/j.watres.2008.04.026

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901. https://doi.org/10.1073/pnas.0609568104

Kolpin, D., Skopec, M., Meyer, M., Furlong, E., Zaugg, S., 2004. Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. Sci. Total Environ. 328, 119–130. https://doi.org/10.1016/j.scitotenv.2004.01.015

Lachmayr, K.L., Cavanaugh, C.M., Kerkhof, L.J., DiRienzo, A.G., Ford, T.E., 2009. Quantifying nonspecific tem β-lactamase (blatem) genes in a wastewater stream. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.01254-08

Larsson, D.G.J., Andremont, A., Bengtsson-Palme, J., Brandt, K.K., de Roda Husman, A.M., Fagerstedt, P., Fick, J., Flach, C.F., Gaze, W.H., Kuroda, M., Kvint, K., Laxminarayan, R., Manaia, C.M., Nielsen, K.M., Plant, L., Ploy, M.C., Segovia, C., Simonet, P., Smalla, K., Snape, J., Topp, E., van Hengel, A.J., Verner-Jeffreys, D.W., Virta, M.P.J., Wellington, E.M., Wernersson, A.S., 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ. Int. https://doi.org/10.1016/j.envint.2018.04.041

Loos, R., Wollgast, J., Huber, T., Hanke, G., 2007. Polar herbicides, pharmaceutical products, perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern Italy, in: Analytical and Bioanalytical Chemistry. https://doi.org/10.1007/s00216-006-1036-7

Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. https://doi.org/10.1016/j.tim.2013.11.001

McLachlan, M.S., Holmström, K.E., Reth, M., Berger, U., 2007. Riverine Discharge of Perfluorinated Carboxylates from the European Continent. Environ. Sci. Technol. 41, 7260–7265. https://doi.org/10.1021/es071471p

Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010.

Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ. Toxicol. Chem. 29, 79–89. https://doi.org/10.1002/etc.27

Murray, A.K., Stanton, I., Gaze, W.H., Snape, J., 2021. Dawning of a new ERA: Environmental Risk Assessment of antibiotics and their potential to select for antimicrobial resistance. Water Res. https://doi.org/10.1016/j.watres.2021.117233

Narciso-Da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014. BlaTEM and vanA as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system. J. Glob. Antimicrob. Resist. https://doi.org/10.1016/j.jgar.2014.10.001

Neuwoehner, J., Fenner, K., Escher, B.I., 2009. Physiological Modes of Action of Fluoxetine and its Human Metabolites in Algae. Environ. Sci. Technol. 43, 6830–6837. https://doi.org/10.1021/es9005493

Peng, X., Tan, J., Tang, C., Yu, Y., Wang, Z., 2008. Multiresidue determination of fluoroquinolone, quinolone, sulfonamide, trimethoprim, and chloramphenicol antibiotics in urban waters in China. Environ. Toxicol. Chem. 27, 73. https://doi.org/10.1897/06-650.1

Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2015a. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. Water Res. 72, 3–27. https://doi.org/10.1016/j.watres.2014.08.053

Petrie, B., Youdan, J., Barden, R., Kasprzyk-Hordern, B., 2015b. Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1431, 64–78. https://doi.org/10.1016/j.chroma.2015.12.036

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Schultz, M.M., Painter, M.M., Bartell, S.E., Logue, A., Furlong, E.T., Werner, S.L., Schoenfuss, H.L., 2011. Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows. Aquat. Toxicol. 104, 38–47. https://doi.org/10.1016/j.aquatox.2011.03.011

Schwarzenbach, R.P., 2006. The Challenge of Micropollutants in Aquatic Systems. Science (80-.). 313, 1072–1077. https://doi.org/10.1126/science.1127291

Scott, P.D., Bartkow, M., Blockwell, S.J., Coleman, H.M., Khan, S.J., Lim, R., McDonald, J.A., Nice, H., Nugegoda, D., Pettigrove, V., Tremblay, L.A., Warne, M.S.J., Leusch, F.D.L., 2014. A National Survey of Trace Organic Contaminants in Australian Rivers. J. Environ. Qual. 43, 1702–1712. https://doi.org/10.2134/jeq2014.01.0012

Singh, R., Singh, A.P., Kumar, S., Giri, B.S., Kim, K.H., 2019. Antibiotic resistance in major rivers in the world: A systematic review on occurrence, emergence, and management strategies. J. Clean. Prod. https://doi.org/10.1016/j.jclepro.2019.06.243

Singh, R.R., Angeles, L.F., Butryn, D.M., Metch, J.W., Garner, E., Vikesland, P.J., Aga, D.S., 2019. Towards a harmonized method for the global reconnaissance of multi-class antimicrobials and other pharmaceuticals in wastewater and receiving surface waters. Environ. Int. https://doi.org/10.1016/j.envint.2019.01.025

Tell, J., Caldwell, D.J., Häner, A., Hellstern, J., Hoeger, B., Journel, R., Mastrocco, F., Ryan, J.J., Snape, J., Straub, J.O., Vestel, J., 2019. Science-based Targets for Antibiotics in Receiving Waters from Pharmaceutical Manufacturing Operations. Integr. Environ. Assess. Manag. https://doi.org/10.1002/ieam.4141

Vieno, N.M., Tuhkanen, T., Kronberg, L., 2005. Seasonal Variation in the Occurrence of Pharmaceuticals in Effluents from a Sewage Treatment Plant and in the Recipient Water. Environ. Sci. Technol. 39, 8220–8226. https://doi.org/10.1021/es051124k

Wilkinson, J., Boxall, A., Kolpin, D., 2019. A Novel Method to Characterise Levels of Pharmaceutical Pollution in Large-Scale Aquatic Monitoring Campaigns. Appl. Sci. 9, 1368. https://doi.org/10.3390/app9071368

Williams, M., Kookana, R.S., Mehta, A., Yadav, S.K., Tailor, B.L., Maheshwari, B., 2019. Emerging contaminants in a river receiving untreated wastewater from an Indian urban centre. Sci. Total Environ. 647, 1256–1265. https://doi.org/10.1016/j.scitotenv.2018.08.084

Wood, T.P., Du Preez, C., Steenkamp, A., Duvenage, C., Rohwer, E.R., 2017. Database-driven screening of South African surface water and the targeted detection of pharmaceuticals using liquid chromatography - High resolution mass spectrometry. Environ. Pollut. https://doi.org/10.1016/j.envpol.2017.06.043

Yao, B., Yan, S., Lian, L., Yang, X., Wan, C., Dong, H., Song, W., 2018. Occurrence and indicators of pharmaceuticals in Chinese streams: A nationwide study. Environ. Pollut. 236, 889–898. https://doi.org/10.1016/j.envpol.2017.10.032

In-situ Multi-Mode Extraction (iMME) sampler for a wide-scope multi-residue analysis of chemicals of emerging concern and antimicrobial resistance genes in the aqueous environment in urban areas and remote (off-the-grid) locations

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Table 1. International studies focused on CECs in surface waters

Compounds	Sampling/sample preparation	Transport/storage	Analysis	Stability Study done	Comments	Reference
61 pharmaceuticals including analgesics, antibiotics, antifungal, ….	1,052 sampling sites 258 rivers in 104 countries Sample preparation method described below in (Wilkinson et al., 2019)	Liquid samples frozen before transported on ice	HPLC-MS/MS Direct injection SPE (Oasis HLB)	Stability study done in prior study	-	(Wilkinson et al., 2022)
61 pharmaceuticals including analgesics, antibiotics, antifungal, ….	Grab sampling followed by filtration with 0.7 um glass microfibre filter Amber glass vials	Liquid samples frozen before transported on ice	HPLC-MS/MS Direct injection SPE (Oasis HLB)	Stability assessment at 3 different temperatures (4, 20 and 35 °C) - Spiked 1000 ng/L into LC-MS water -Samples stored for 2 or 7 days Interior temperature of polystyrene packets containing 2 x ice packs monitored over 7 days	Grab sampling Only liquid phase analyzed (fraction of analytes sorbed to SPM missing due to filtration applied before internal standard addition) Not specified when internal standard mix is added	(Wilkinson et al., 2019)
57 pharmaceuticals including antibiotics, personal care products	Grab or composite not specified in experimental Sample pretreatment specified for wastewater samples, assumed same for surface waters Acidification, followed by filtering (0.45 μm), 2 mL of Na2EDTA (5% w/v in water) was added, samples then spiked with surrogate standards (50 μL of a 1000 μg/L)	Samples transported via Oasis HLB cartridges Wrapped in foil, secured in airtight bags, shipped on ice packs to analysis laboratory Specified samples eluted upon receipt .	LC-MS/MS SPE (Oasis HLB)	Stability study done with influent wastewater 50 μL of a 1000 μg/L mixture SPE cartridges were stored at (23–27 °C) or at − 4 °C Sampling points seven, fifteen, and twenty-eight days of storage	Only liquid phase analyzed (fraction of analytes sorbed to SPM missing due to filtration applied before internal standard addition)	(R. R. Singh et al., 2019)
EU study 35 compounds, comprising pharmaceuticals, pesticides, PFOS, PFOA, benzotriazoles, hormones, and endocrine disrupters.	Grab sampling PE/PP bottles 122 rivers sampled from across 27 countries	Liquid samples collected Stored at 4 °C before sending to analysis lab (Italy) Transported with freezing elements in polystyrene boxes (arrival time generally after 2–3 days)	LC/MS/MS SPE (Oasis HLB)	Spiking river water samples 100 ng/L - 15 °C in the dark - Period of three weeks	Grab sampling Only liquid phase analyzed Samples extracted at a max of 2 weeks after sampling (not specified if frozen or kept at 4 °C during this time) -Internal standard was spiked before SPE extraction - No filtering step prior to SPE	(Loos et al., 2009)

Perfluorinated carboxylates (PFCAs) investigated	PP/PE bottles used Grab sampling 14 major European rivers sampled	Samples collected stored at 4 °C on arrival Shipped by courier to Stockholm University with cooling elements (usually within same day)	LC-MS/MS SPE (Oasis HLB Plus)	Sample storage in PP, PE and glass investigated - 200 mL of Milli-Q-water, spiked with 1 ng of each PFCA - Stored at room temperature - Period of 36 days	Samples spiked with IS before extraction (not specified how long samples were stored at 4 °C before extraction) -Filtration not mentioned other than for particle rich waters (filtered over silane treated glass wool prior to SPE) -Seasonality of collected samples mentioned (periods of unusually high and low flows were avoided -Collected at one time point	(McLachlan et al., 2007)
Investigated in effluent of 8 WWTP in Western Europe for 36 polar compounds including household and industrial chemicals, pharmaceuticals, and personal care products	Study focuses mainly on effluent samples Single surface water grab samples from 5 rivers (Germany, Berlin, France and Spain)	Effluent samples transported in an ice chest at 3 °C within 2 days to the labs (not specified for surface waters but assumed the same) Surface water samples filtered over 0.45 µm membrane filters, and stored in a refrigerator until analyzed.	LC-MS, LC-MS/MS, GC-MS, CLSA-GC/MS	Stability not mentioned	Internal standards not mentioned Duration that surface waters were kept at 4 °C before extraction/analysis not specified Extraction protocol not mentioned (instead references to other papers for specific analytes in the supplementary)	(Reemtsma et al., 2006)
Investigation of 105 CECs in river water collected from the river Avon (UK) and Olifants river (SA)	PE/PP (check which) Grab samples Samples spiked with internal standard on site Using iSPE spiked samples were filtered and loaded on site	Cartridges collected from River Avon (UK) transported on ice to lab (<1 h). Cartridges collected from Olifants river (SA)…	HPLC-MS/MS SPE (Oasis HLB)	Stability of river water samples over 24 hours at room temperature -Analytes spiked in at 100 ng L^{-1} and 500 ng L^{-1} Stability of CECs on cartridge -Analytes spiked in at 100 ng L^{-1} -Two temperatures investigated room temp and - 18°C	No need for cold chain IS spiked on site Only liquid phase analyzed Filtered through GF/F and GF/D Samples collected in summer One time point collected	This study

PP polypropylene, PE polyethylene, SPM solid particulate matter

Figure S1. River sampling locations the River Avon, UK (top) and from the Olifants river in Kruger national park, South Africa (bottom).

Table S2. Linearity information and instrument detection limits (IDLs) and quantification limits (IQLs) for all investigated compounds

CEC Group	Compound	Compound Class	Linearity Range (ng mL-1)	IQLS/N (ng mL-1)	IDLS/N (ng mL-1)	Intra-day instrument performance Precision %	Intra-day instrument performance Accuracy %	Inter-day instrument performance Precision %	Inter-day instrument performance Accuracy %
Group A	Methylparaben	Parabens	0.06-1000	0.060	0.01	3	104	3	106
	Propylparaben		0.12-400	0.12	0.04	6	101	5	108
	Butylparaben		0.05-1000	0.050	0.015	6	100	4	107
	1-benzophenone	UV Filters	0.25-100	0.250	0.075	4	227	7	235
	2-benzophenone		0.1-1000	0.100	0.030	5	107	7	113
	3-benzophenone		0.025-300	0.025	0.008	4	98	6	96
	4-benzophenone		1.01-500	1.01	0.31	4	96	4	92
	Bisphenol A	Plasticizer	0.10-600	0.1	0.03	6	106	6	104
	E1	Steroid Estrogen	0.5-1000	0.500	0.150	5	104	5	106
	E2		1-1000	1.000	0.300	6	104	6	104
	EE2		1-1000	1.000	0.300	5	99	6	99
	Ketoprofen	NSAIDs	0.54-1000	0.54	0.11	2	107	3	108
	Ibuprofen		0.05-1000	0.05	0.01	5	132	5	136
	Naproxen		0.5-1000	0.500	0.150	7	119	5	121
	Valsartan	Hypertension	5-300	5.000	1.500	8	100	5	93
	Irbesartan		0.1-300	0.100	0.030	7	104	5	96
	Lisinopril		1-500	1.000	0.300	4	196	7	197
	Amphetamine	Stimulants and metabolites	0.25-1000	0.250	0.075	5	97	8	101
	Methamphetamine		0.05-1000	0.050	0.015	3	96	6	97
	MDMA		0.025-1000	0.050	0.015	4	108	6	112
	MDPV		0.05-500	0.050	0.015	6	98	6	100
	Cocaine		0.025-500	0.025	0.008	6	104	6	106
	Anhydroecgonine methyl ester		0.25-500	0.250	0.075	3	88	8	90
	Benzoylecgonine		0.025-1000	0.025	0.008	5	100	7	102
	Cocaethylene		0.025-1000	0.025	0.008	4	108	7	113
	Methadone	Analgesics and metabolites	0.025-400	0.025	0.008	5	109	7	110
	Acetaminophen		0.25-1000	0.250	0.075	4	100	7	104
	EDDP		0.025-1000	0.025	0.008	4	99	6	102
	Codeine		0.50-500	0.5	0.1	4	65	7	67
	Norcodeine		1-500	1	0.3	6	107	7	109
	Dihydrocodeine		0.25-1000	0.250	0.075	3	90	8	91
	N-desmethyltramadol		0.50-500	0.05	0.01	4	90	7	93
	O-desmethyltramadol		1-400	1.000	0.300	5	109	7	112
	6-acetylmorphine	Opioid metabolite	0.10-500	0.1	0.03	4	112	6	118
	Cotinine	Human Indicators	0.05-1000	0.05	0.01	5	107	5	107
	1,7 dimethylxanthine		1-500	1.000	0.300	5	98	5	99
	Caffeine		0.50-500	0.5	0.1	4	49	9	53
	Nicotine		0.5-500	0.500	0.150	5	106	6	108
	Carbamazepine	Anti-epileptic	0.05-1000	0.050	0.015	5	135	7	140
	Carbamazepine 10, 11-epoxide		0.1-1000	0.100	0.030	4	92	6	96
	10,11-dihydro-10-hydroxycarbmazepine		0.50-1000	0.5	0.05	5	107	6	110

	Ketamine	Anaesthetic and metabolite	0.05-1000	0.050	0.015	3	104	7	106
	Venlafaxine		0.04-500	0.04	0.01	4	98	7	102
	Citalopram		0.5-1000	0.5	0.05	4	103	7	107
	Norketamine		0.025-1000	0.025	0.008	4	88	6	90
	Desmethylcitalopram		0.05-500	0.050	0.015	4	110	4	113
	Quetiapine	Antipsychotic	0.025-1000	0.025	0.008	5	172	8	175
	Bezafibrate	Lipid regulator	0.1-1000	0.100	0.030	5	75	8	78
	Metformin	Diabetes	0.05-500	0.050	0.015	5	90	6	91
	Gliclazide		0.025-500	0.025	0.008	3	75	6	71
	Atenolol	Beta blocker	0.25-1000	0.250	0.075	4	102	6	105
	Metoprolol		0.5-1000	0.500	0.150	3	93	7	96
	Cimetidine	H2 receptor agonist	2.5-400	2.500	0.750	3	106	6	110
	Diltiazem	Calcium channel blocker	0.1-1000	0.100	0.030	4	107	7	110
	Azathioprine	Anticancer	0.10-500	0.1	0.03	4	96	7	99
	Ifosfamide		0.05-500	0.05	0.01	6	118	9	118
	Fexofenadine	Antihistamine	0.25-1000	0.250	0.075	4	100	6	105
Group B	Sulfadiazine	Sulphonamide & trimethoprim	0.05-1000	0.05	0.015	12	102	13	99
	Sulfapyridine		0.01-1000	0.01	0.003	6	101	8	101
	Sulfamethoxazole		0.01-200, 200-1000	0.005	0.0015	7	100	9	101
	Sulfasalazine		0.5-1000	5	1.5	10	110	14	109
	Trimethoprim		0.5-500	0.5	0.15	11	107	14	107
	N-acetyl sulfadiazine		0.05-25, 25-750	0.07	0.020979021	3	95	5	99
	N-acetyl sulfapyridine		0.05-25, 25-750	0.56	0.16	6	105	7	105
	N-acetyl sulfamethoxazole		0.06-475, 475-1500	0.06	0.019	8	101	9	101
	4-hydroxy-trimethoprim		0.01-63, 63-95	0.01	0.004	12	100	13	100
	Erythromycin	Macrolide & lincomycin	0.005-200	0.005	0.0015	8	103	11	101
	Clarithromycin		0.005-200, 200-3000	0.005	0.0015	10	105	10	106
	Clindamycin		0.5-1000	0.5	0.15	16	94	18	94
	N-desmethyl erythromycin		0.034-136	0.007352941	0.002205882	9	104	10	103
	N-desmethyl clarithromycin		0.02-1250, 1250-2000	0.016666667	0.005	6	98	9	99
	N-desmethyl clindamycin		0.005-200	0.005	0.0015	20	93	17	87
	Flucloxacillin	β-Lactams	0.5-1000	0.5	0.15	15	99	19	109
	Cefalexin	Cephalosporins	1.25-500	1.25	0.375	10	90	10	90
	Cefixime		5-750	5	1.5	22	92	20	83
	Ceftiofur		0.5-750	0.5	0.15	17	103	16	110
	Norfloxacin	Quinolone	0.005-200, 200-500, 500-1000	0.01	0.003	15	103	20	97
	Ciprofloxacin		0.5-400	0.5	0.15	10	101	20	97
	Lomefloxacin		0.1-500	0.1	0.03	21	113	21	118
	Danofloxacin		5-750	5	1.5	48	112	40	93
	Enrofloxacin		0.1-100, 100-750	0.1	0.03	13	110	15	110
	Nadifloxacin		1-400	1	0.3	19	90	24	98
	Ofloxacin (Levofloxacin)		0.1-200	0.1	0.03	8	97	11	101
	Gatifloxacin		0.01-500	0.01	0.003	7	100	14	103
	Sarafloxacin		0.05-500	0.5	0.15	11	93	14	93
	Moxifloxacin		1.25-500	1.25	0.375	16	92	17	90

Flumequine		0.01-200, 200-1000	0.01	0.003	12	103	12	103
Nalidixic acid		0.01-500	0.01	0.003	16	105	17	102
Desethylene ciprofloxacin		0.5-100, 100-500	0.5	0.15	12	92	14	89
Hydroxy-norfloxacin		1-100, 100-1000	12	3.6	16	100	17	99
Desmethyl-ofloxacin		0.5-50, 50-750	0.5	0.15	7	96	11	97
Ofloxacin N-oxide		0.5-75, 75-1000	12	3.6	9	97	14	98
Rifampicin	TB drugs (1st line)	1.25-1000	1.25	0.375	34	114	42	97
Rifabutin		0.5-400	0.5	0.15	14	100	14	105
25-desacetyl rifampicin		5-500	5	1.5	34	117	38	104
25-O-desacetyl rifabutin		0.1-100, 100-750	0.1	0.03	27	104	21	102
Linezolid	TB other	0.1-1000	0.1	0.03	12	98	19	96
Thalidomide		0.5-100, 100-750	1	0.3	16	102	17	103
Florfenicol	Amphenicol	10-200, 200-750	10	3	18	111	18	114
Nitrofurantoin	Nitrofurantoin	0.5-200, 200-1500	1	0.3	12	89	12	89
NPAHD		0.1-200, 200-1000	0.1	0.03	10	102	14	103
Metronidazole	Azole	0.1-500, 500-1000	0.1	0.03	8	103	9	104
Hydroxy-metronidazole		0.1-100, 100-750	0.1	0.03	10	99	10	94
Lamivudine	Antiretroviral	1-1000	1	0.3	12	95	12	98
Emtricitabine		0.5-200, 50-1000, 1000-3000	0.5	0.15	19	91	20	86

Table S3. Corrected SPE recoveries breakdown of all target ECs

			SPE Recoveries					
CEC Group	**Compound**	**Compound Class**	**10**	**STD**	**100**	**STD**	**500**	**STD**
Group A	Methylparaben	Parabens	104	3	101	2	95	12
	Butylparaben		119	1	128	9	118	22
	1-benzophenone	UV Filters	38	1	41	4	41	27
	2-benzophenone		52	0	45	2	45	27
	3-benzophenone		23	2	54	8	41	17
	4-benzophenone		122	1	121	9	116	45
	Bisphenol A	Plasticizer	52	4	157	16	153	73
	E1	Steroid Estrogen	110	1	105	4	93	17
	E2		137	2	100	8	93	30
	EE2		-	-	91	6	94	81
	Ketoprofen	NSAIDs	50	17	70	48	57	81
	Ibuprofen		87	1	116	3	115	12
	Naproxen		197	16	110	18	96	13
	Valsartan	Hypertension	175	3	167	23	171	71
	Lisinopril		202	1	45	2	49	14
	Amphetamine	Stimulants and metabolites	295	6	169	9	119	12
	Methamphetamine		114	1	107	2	98	9
	MDMA		147	1	133	2	122	18
	MDPV		-	-	105	4	96	15
	Cocaine		139	0	123	1	108	19
	Anhydroecgonine methyl ester		78	0	36	2	37	14
	Benzoylecgonine		165	1	149	5	132	7
	Cocaethylene		136	0	115	4	106	20
	Methadone	Analgesics and metabolites	106	1	90	4	80	16
	Acetaminophen		80	1	145	3	124	9
	EDDP		114	0	106	4	99	18
	Codeine		80	1	59	1	56	5
	Norcodeine		233	1	90	5	82	16
	Dihydrocodeine		136	0	106	4	104	11
	N-desmethyltramadol		187	2	122	8	120	25
	O-desmethyltramadol		117	1	95	3	83	20
	6-acetylmorphine	Opioid metabolite	-	-	67	1	88	37
	Cotinine	Human Indicators	15	0	135	7	121	14
	Caffeine		89	1	106	9	84	21
	Nicotine		52	2	18	2	18	5
	1,7 dimethylxanthine		-	-	50	8	61	12
	Carbamazepine	Anti-epileptic	127	2	110	5	100	16
	Carbamazepine 10, 11-epoxide		88	1	76	2	76	14
	10,11-dihydro-10-hydroxycarbmazepine		158	3	136	9	121	40
	Ketamine	Anaesthetic and metabolite	129	1	119	2	105	10
	Venlafaxine		206	3	148	13	130	37
	Citalopram		106	1	114	8	108	6

	Norketamine		125	1	110	5	100	14
	Desmethylcitalopram		104	2	81	2	96	12
	Quetiapine	Antipsychotic	118	0	112	3	98	15
	Bezafibrate	Lipid regulator	197	1	118	1	106	12
	Metformin	Diabetes	122	2	112	25	94	34
	Gliclazide		146	1	111	7	86	36
	Atenolol	Beta blocker	117	1	113	4	109	15
	Metoprolol		118	1	116	4	110	14
	Cimetidine		128	1	72	8	76	9
	Diltiazem	Calcium channel blocker	89	1	83	3	69	8
	Azathioprine	Anticancer	83	1	75	1	70	5
	Ifosfamide		-174	1	97	7	108	8
Group B	Sulfadiazine	Sulphonamide & trimethoprim	-	-	64	3	76	2
	Sulfapyridine		126	55	79	3	83	2
	Sulfamethoxazole		106	9	92	4	93	2
	Sulfasalazine		-	-	103	25	85	5
	Trimethoprim		112	20	95	11	100	3
	N-acetyl sulfadiazine		52	3	61	2	55	2
	N-acetyl sulfapyridine		81	3	76	2	66	1
	N-acetyl sulfamethoxazole		76	6	49	1	44	1
	4-hydroxy-trimethoprim		13	2	11	1	11	0
	Erythromycin	Macrolide & lincomycin	146	56	113	4	83	4
	Clarithromycin		120	60	101	14	80	4
	Clindamycin		117	3	107	1	97	4
	N-demethyl erythromycin		119	31	125	4	91	4
	N-desmethyl clarithromycin		181	161	153	28	116	2
	N-desmethyl clindamycin		62	4	55	3	59	5
	Flucloxacillin	β-Lactams	75	4	69	4	68	6
	Cefalexin	Cephalosporins	100	98	136	33	131	17
	Cefixime		-	-	77	14	62	6
	Ceftiofur		105	-	30	1	15	1
	Norfloxacin	Quinolone	-	-	134	31	187	12
	Ciprofloxacin		170	22	85	1	139	3
	Lomefloxacin		98	35	87	4	104	4
	Danofloxacin		-	-	125	5	114	7
	Enrofloxacin		114	31	123	7	97	5
	Nadifloxacin		194	6	104	4	88	2
	Ofloxacin (Levofloxacin)		88	17	83	3	85	2
	Gatifloxacin		91	29	92	6	109	3
	Sarafloxacin		-	-	46	3	53	0
	Moxifloxacin		-	-	134	12	150	21
	Flumequine		125	0	97	1	85	4
	Nalidixic acid		-	-	75	1	70	3
	Desethylene ciprofloxacin		-	-	63	11	111	15
	Hydroxy-norfloxacin		-	-	-	-	33	1
	Desmethyl-ofloxacin		95	-	46	5	78	1

Ofloxacin N-oxide		-	-	129	-	98	3
Rifampicin	TB drugs (1st line)	-	-	85	14	110	14
Rifabutin		183	19	121	14	117	11
25-desacetyl rifampicin		-	-	109	3	149	17
25-O-desacetyl rifabutin		124	32	71	3	100	17
Linezolid	TB other	83	10	72	4	66	1
Thalidomide		211	36	155	11	139	6
Florfenicol	Amphenicol	-	-	104	-	84	4
Nitrofurantoin	Nitrofurantoin	-	-	116	23	69	1
NPAHD		64	15	60	5	53	1
Metronidazole	Azole	132	3	105	2	104	1
Hydroxy-metronidazole		-	-	57	2	73	2
Lamivudine	Antiretroviral	-	-	62	3	76	3
Emtricitabine		167	29	102	4	107	2

Table S4. 24-hour stability study in river water spiked initially with 100 ng/L and 500 ng/L

			% Degradation after 24 hr			
CEC Group	**Compound Class**	**Compound**	**100 ng L-1 spiked, RW**	**STD**	**500 ng L-1 spiked, RW**	**STD**
Group A	Methylparaben	Parabens	98	1	90	6
	Propylparaben		-35	14	-16	4
	Butylparaben		12	3	1	4
	1-benzophenone	UV Filters	13	2	4	7
	2-benzophenone		9	2	-5	4
	3-benzophenone		37	1	72	22
	4-benzophenone		4	4	-39	49
	Bisphenol A	Plasticizer	46	8	-12	13
	E1	Steroid Estrogen	-13	2	-15	29
	E2		19	12	3	3
	EE2		-20	26	-2	14
	Ketoprofen	NSAIDs	15	8	-6	9
	Ibuprofen		-20	14	-10	14
	Naproxen		-2	9	3	14
	Valsartan	Hypertension	-1	15	-14	13
	Irbesartan		1	10	-4	27
	Lisinopril		-10	0	36	5
	Amphetamine	Stimulants and metabolites	31	3	8	1
	Methamphetamine		-9	0	-7	1
	MDMA		-9	0	-1	1
	MDPV		-13	0	-3	2
	Cocaine		49	0	51	1
	Anhydroecgonine methyl ester		-1	0	-12	1
	Benzoylecgonine		-63	0	-63	1
	Cocaethylene		14	0	22	1
	Methadone	Analgesics and metabolites	4	1	0	5
	Acetaminophen		-5	0	-1	1
	EDDP		-3	1	1	1
	Codeine		-19	0	2	0
	Norcodeine		-52	1	13	1
	Dihydrocodeine		-4	1	-2	1
	N-desmethyltramadol		-7	1	0	3
	O-desmethyltramadol		-3	2	-10	1
	6-acetylmorphine	Opioid metabolite	-19	1	-14	1
	Cotinine	Human Indicators	-6	0	-3	1
	Caffeine		23	3	-25	3
	Nicotine		5	1	-25	2
	1,7 dimethylxanthine		4	4	-19	1
	Carbamazepine	Anti-epileptic	-8	0	-3	1
	Carbamazepine 10, 11-epoxide		-11	0	-14	1
	10,11-dihydro-10-hydroxycarbmazepine		-6	0	-19	1
	Ketamine	Anaesthetic and metabolite	-8	0	-2	1

	Venlafaxine		-18	1	6	1
	Citalopram		-4	2	-1	1
	Norketamine		-12	0	-2	1
	Desmethylcitalopram		-13	1	5	4
	Quetiapine	Antipsychotic	-3	0	11	2
	Bezafibrate	Lipid regulator	-7	7	-2	21
	Metformin	Diabetes	11	22	0	10
	Gliclazide		29	1	8	12
	Atenolol	Beta blocker	-6	1	-1	1
	Metoprolol		-9	0	-1	2
	Cimetidine		-25	0	9	2
	Diltiazem	Calcium channel blocker	-8	1	-4	4
	Azathioprine	Anticancer	2	1	-15	2
	Ifosfamide		-7	0	16	1
	Fexofenadine	Antihistamine	10	8	10	11
Group B	Sulfadiazine	Sulphonamide & trimethoprim	32	7	32	9
	Sulfapyridine		15	15	-3	13
	Sulfamethoxazole		-2	4	5	7
	Sulfasalazine		-44	16	-57	10
	Trimethoprim		-3	18	16	9
	N-acetyl sulfadiazine		-19	5	-36	25
	N-acetyl sulfapyridine		-36	6	-85	14
	N-acetyl sulfamethoxazole		-58	45	-35	10
	4-hydroxy-trimethoprim		30	5	23	19
	Erythromycin	Macrolide & lincomycin	21	10	-28	8
	Clarithromycin		-55	14	-41	29
	Clindamycin		26	1	8	41
	N-desmethyl erythromycin		36	4	13	7
	N-desmethyl clarithromycin		19	7	8	4
	N-desmethyl clindamycin		-78	8	-76	28
	Flucloxacillin	β-Lactams	-91	16	-38	50
	Cefalexin	Cephalosporins	-	-	-	-
	Cefixime		36	27	13	24
	Ceftiofur		-42	47	-133	59
	Norfloxacin	Quinolone	-	-	-22	16
	Ciprofloxacin		-28	237	7	23
	Lomefloxacin		-13	17	15	34
	Danofloxacin		13	-	30	23
	Enrofloxacin		1	33	10	13
	Nadifloxacin		-178	62	-105	81
	Ofloxacin (Levofloxacin)		47	6	49	16
	Gatifloxacin		-26	60	8	21
	Sarafloxacin		-	-	31	19
	Moxifloxacin		-	-	30	3
	Flumequine		1	7	4	13
	Nalidixic acid		25	3	34	6

Desethylene ciprofloxacin		-	-	9	24
Hydroxy-norfloxacin		-	-	-	-
Desmethyl-ofloxacin		-90	59	-1	6
Ofloxacin N-oxide		-7	16	10	15
Rifampicin	TB drugs (1st line)	-25	-	12	11
Rifabutin		38	0	35	0
25-desacetyl rifampicin		15	11	43	9
25-O-desacetyl rifabutin		50	3	60	7
Linezolid	TB other	21	18	11	21
Thalidomide		-	-	-	-
Florfenicol	Amphenicol	-	-	48	13
Nitrofurantoin	Nitrofurantoin	25	-	-56	42
NPAHD		15	30	37	7
Metronidazole	Azole	-4	5	0	9
Hydroxy-metronidazole		37	11	55	6
Lamivudine	Antiretroviral	13	9	35	8
Emtricitabine		49	5	51	8

Tables S5. Weeklong stability study on cartridge at freezer (-18 °C) temperatures

			% of original spiked concentration left (day 0 = 100%) freezer, -18 °C									
CEC Group	**Compound**	**Compound Class**	**Day 1**	**STD**	**Day 2**	**STD**	**Day 3**	**STD**	**Day 5**	**STD**	**Day 7**	**STD**
Group A	Methylparaben	Parabens	87	9	89	5	82	18	79	3	89	12
	Propylparaben		100	10	107	1	104	3	103	2	114	8
	Butylparaben		112	9	132	12	116	32	136	0	128	11
	1-benzophenone	UV Filters	79	6	87	13	71	21	101	5	73	7
	2-benzophenone		82	1	82	13	72	24	82	7	65	14
	3-benzophenone		83	14	82	4	68	12	67	10	55	9
	4-benzophenone		95	4	97	11	94	15	84	1	95	2
	Bisphenol A	Plasticizer	105	8	111	11	113	12	119	1	109	10
	E1	Steroid Estrogen	99	8	92	2	87	14	97	6	95	7
	E2		110	23	107	16	91	20	106	5	109	13
	EE2		97	14	85	9	102	16	123	13	101	24
	Ketoprofen	NSAIDs	84	10	89	9	69	9	61	6	47	13
	Ibuprofen		89	1	136	52	99	12	91	2	90	2
	Naproxen		111	2	95	3	93	10	90	2	88	7
	Valsartan	Hypertension	77	33	85	4	84	16	83	1	108	7
	Irbesartan		95	6	85	5	78	16	72	5	84	4
	Lisinopril		118	41	116	2	118	27	84	3	87	23
	Amphetamine	Stimulants and metabolites	85	6	107	20	100	20	110	7	92	6
	Methamphetamine		101	1	102	2	91	14	102	3	104	2
	MDMA		104	2	103	2	95	16	101	1	98	5
	MDPV		105	4	102	3	87	13	93	5	99	7
	Cocaine		103	5	102	6	95	19	99	1	103	1
	Anhydroecgonine methyl ester		106	94	12	87	8	68	17	92	7	109
	Benzoylecgonine		106	1	113	10	98	13	112	3	115	8
	Cocaethylene		105	2	102	5	95	18	94	2	97	2
	Methadone	Analgesics and metabolites	111	4	108	2	97	14	113	8	115	3
	Acetaminophen		95	2	147	66	133	17	101	10	122	17
	EDDP		86	36	98	36	66	2	87	5	76	34
	Codeine		101	1	100	2	94	18	104	4	102	2
	Norcodeine		105	3	107	4	94	18	123	2	102	5
	Dihydrocodeine		104	3	106	4	91	13	101	3	101	6
	N-desmethyltramadol		92	9	90	6	84	6	122	5	124	2
	O-desmethyltramadol		108	3	115	2	106	16	100	3	104	8
	6-acetylmorphine	Opioid metabolite	121	21	137	5	99	29	111	10	125	24
	Cotinine	Human Indicators	98	1	104	2	94	18	88	2	92	4
	Caffeine		82	3	95	26	88	22	75	6	75	4
	Nicotine		72	12	66	10	46	14	78	9	110	12
	1,7 dimethylxanthine		90	5	114	32	100	18	108	3	113	2
	Carbamazepine	Anti-epileptic	99	1	97	2	92	15	93	3	98	1
	Carbamazepine 10, 11-epoxide		92	108	5	87	2	89	14	85	3	99
	10,11-dihydro-10-hydroxycarbmazepine		95	105	1	104	0	98	15	95	4	99
	Ketamine	Anaesthetic and metabolite	108	2	108	4	99	17	109	6	107	4
	Venlafaxine		101	2	116	2	106	20	116	6	113	4

	Citalopram		110	14	110	6	86	16	94	5	105	13
	Norketamine		107	1	107	2	97	15	111	3	110	5
	Desmethylcitalopram		111	2	98	4	84	8	110	3	112	1
	Quetiapine	Antipsychotic	100	2	96	2	87	11	104	2	108	1
	Bezafibrate	Lipid regulator	104	7	99	6	95	16	105	3	118	11
	Metformin	Diabetes	100	4	119	23	117	3	103	1	111	2
	Gliclazide		81	5	84	7	92	2	97	2	90	7
	Atenolol	Beta blocker	100	2	108	5	99	18	104	3	105	3
	Metoprolol		104	3	107	3	96	16	98	1	97	5
	Cimetidine		92	7	81	3	75	2	101	1	84	7
	Diltiazem	Calcium channel blocker	114	6	106	12	94	16	102	4	104	16
	Azathioprine	Anticancer	108	4	110	1	103	19	96	4	104	7
	Ifosfamide		114	5	112	3	110	24	108	4	110	4
	Fexofenadine	Antihistamine	91	1	86	6	86	5	98	4	92	2
Group B	Sulfadiazine	Sulphonamide & trimethoprim	85	8	74	9	84	13	79	9	81	2
	Sulfapyridine		92	6	90	5	83	13	93	4	95	4
	Sulfamethoxazole		94	8	92	3	85	11	93	4	98	10
	Sulfasalazine		90	19	93	26	93	10	107	17	114	6
	Trimethoprim		99	8	94	4	91	10	105	4	102	7
	N-acetyl sulfadiazine		88	9	85	6	81	9	83	11	85	9
	N-acetyl sulfapyridine		88	7	88	8	80	12	82	5	86	9
	N-acetyl sulfamethoxazole		80	14	87	6	83	11	88	13	89	12
	4-hydroxy-trimethoprim		101	11	91	3	90	16	99	5	99	1
	Erythromycin	Macrolide & lincomycin	91	15	86	6	73	12	78	8	79	14
	Clarithromycin		97	5	90	10	75	2	91	4	94	8
	Clindamycin		69	10	85	26	65	17	68	16	89	16
	N-desmethyl erythromycin		95	3	86	10	80	5	84	5	86	5
	N-desmethyl clarithromycin		92	3	82	6	83	7	91	7	96	5
	N-desmethyl clindamycin		69	16	72	32	45	8	66	9	62	2
	Flucloxacillin	β-Lactams	71	8	81	29	54	13	63	4	68	11
	Cefalexin	Cephalosporins	76	20	75	9	66	14	62	19	100	25
	Cefixime		60	17	55	16	57	6	70	17	111	11
	Ceftiofur		33	20	41	11	39	1	38	5	43	10
	Norfloxacin	Quinolone	101	28	83	19	70	20	109	14	111	24
	Ciprofloxacin		97	24	82	7	74	6	100	21	101	10
	Lomefloxacin		76	21	73	3	65	20	57	9	85	20
	Danofloxacin		109	15	127	9	106	11	120	8	123	23
	Enrofloxacin		92	8	78	4	67	10	75	12	93	8
	Nadifloxacin		77	6	81	32	60	14	62	5	73	7
	Ofloxacin (Levofloxacin)		102	8	99	15	90	6	111	9	92	11
	Gatifloxacin		107	19	77	9	86	10	84	5	92	10
	Sarafloxacin		112	44	106	37	91	33	127	34	129	47
	Moxifloxacin		144	36	118	54	87	40	193	49	142	21
	Flumequine		77	11	93	18	75	8	78	8	86	7
	Nalidixic acid		71	15	76	20	59	7	64	5	61	11
	Desethylene ciprofloxacin		79	25	42	8	50	14	60	31	86	22

Hydroxy-norfloxacin		80	4	70	3	74	7	81	7	82	7
Desmethyl-ofloxacin		82	12	68	11	77	17	77	12	75	12
Ofloxacin N-oxide		96	12	81	16	78	22	73	19	95	15
Rifampicin	TB drugs (1st line)	61	11	124	19	71	22	137	1	79	30
Rifabutin		73	22	73	25	72	10	74	16	61	5
25-desacetyl rifampicin		125	19	107	43	82	13	129	91	79	8
25-O-desacetyl rifabutin		79	8	84	9	64	4	75	13	66	3
Linezolid	TB other	94	16	88	9	85	5	96	10	104	17
Thalidomide		87	46	79	33	94	14	109	21	105	39
Florfenicol	Amphenicol	102	8	93	9	77	15	95	14	109	13
Nitrofurantoin	Nitrofurantoin	94	23	97	28	81	27	107	8	117	40
NPAHD		95	12	87	13	82	10	97	10	108	4
Metronidazole	Azole	92	4	84	2	81	10	89	4	92	5
Hydroxy-metronidazole		97	8	89	3	95	2	98	4	95	4
Lamivudine	Antiretroviral	98	9	90	7	95	6	104	3	96	9
Emtricitabine		87	7	82	3	90	10	72	31	87	8

Tables S6. Weeklong stability study on cartridge at freezer (21 °C) temperatures

			% of original spiked concentration left (day 0 = 100%) room temperature, 21 °C									
CEC Group	**Compound**	**Compound Class**	**Day 1**	**STD**	**Day 2**	**STD**	**Day 3**	**STD**	**Day 5**	**STD**	**Day 7**	**STD**
Group A	Methylparaben	Parabens	87	5	91	8	73	3	86	7	94	5
	Propylparaben		106	3	122	7	98	5	119	11	95	8
	Butylparaben		127	5	150	8	126	4	135	3	133	3
	1-benzophenone	UV Filters	96	5	110	2	94	5	82	19	96	19
	2-benzophenone		89	4	95	4	75	6	75	13	69	3
	3-benzophenone		111	4	109	7	94	5	56	4	77	3
	4-benzophenone		93	5	88	8	87	4	91	12	85	6
	Bisphenol A	Plasticizer	118	8	136	13	128	16	128	10	117	8
	E1	Steroid Estrogen	99	5	103	4	95	5	98	10	102	9
	E2		120	21	115	14	107	5	116	14	108	10
	EE2		117	1	123	32	120	17	124	25	125	5
	Ketoprofen	NSAIDs	98	10	94	8	70	10	83	17	89	9
	Ibuprofen		89	1	98	2	94	2	92	2	93	2
	Naproxen		106	7	101	2	88	6	95	2	89	5
	Valsartan	Hypertension	87	1	84	6	86	4	78	2	109	6
	Irbesartan		84	3	87	3	74	5	88	13	70	7
	Lisinopril		80	6	74	3	89	6	56	3	40	11
	Amphetamine	Stimulants and metabolites	67	13	67	13	59	10	48	14	36	1
	Methamphetamine		86	14	96	5	61	3	69	9	78	1
	MDMA		94	16	101	6	68	3	72	9	76	4
	MDPV		108	16	124	6	86	8	100	16	119	6
	Cocaine		111	14	124	2	99	2	111	15	123	7
	Anhydroecgonine methyl ester		116	84	9	90	4	46	6	95	28	108
	Benzoylecgonine		112	6	118	2	109	3	114	6	114	8
	Cocaethylene		112	13	123	2	102	1	105	13	113	9

	Methadone	Analgesics and metabolites	115	19	128	3	96	1	120	16	129	8
	Acetaminophen		99	2	100	2	93	2	93	6	113	8
	EDDP		106	45	121	44	58	2	71	20	118	7
	Codeine		107	2	112	5	99	2	93	11	97	7
	Norcodeine		97	8	101	11	85	10	91	7	81	5
	Dihydrocodeine		112	10	124	6	97	2	98	10	105	6
	N-desmethyltramadol		85	9	99	14	80	3	110	5	109	2
	O-desmethyltramadol		111	8	125	4	115	1	100	7	100	6
	6-acetylmorphine	Opioid metabolite	117	24	148	10	100	10	108	16	118	12
	Cotinine	Human Indicators	106	8	113	2	103	3	96	13	100	10
	Caffeine		87	7	73	4	81	18	71	3	67	7
	Nicotine		66	3	69	1	34	4	119	43	152	12
	1,7 dimethylxanthine		92	8	82	3	86	8	101	6	101	8
	Carbamazepine	Anti-epileptic	104	6	111	1	101	2	101	5	105	7
	Carbamazepine 10, 11-epoxide		79	121	11	104	1	100	1	106	10	118
	10,11-dihydro-10-hydroxycarbmazepine		88	112	8	121	2	108	2	106	11	115
	Ketamine	Anaesthetic and metabolite	114	11	125	3	110	3	114	12	117	8
	Venlafaxine		102	8	129	7	103	4	118	8	113	4
	Citalopram		112	19	121	12	93	3	97	10	110	5
	Norketamine		116	11	122	2	100	3	113	13	116	11
	Desmethylcitalopram		101	14	95	3	73	3	83	9	108	6
	Quetiapine	Antipsychotic	104	10	108	1	97	3	114	15	130	12
	Bezafibrate	Lipid regulator	119	3	130	21	110	1	111	5	107	4
	Metformin	Diabetes	96	3	100	4	93	3	92	2	92	8
	Gliclazide		97	15	121	16	105	1	120	29	114	22
	Atenolol	Beta blocker	102	6	114	5	101	4	99	4	95	3
	Metoprolol		107	8	115	1	95	5	94	9	94	4
	Cimetidine		96	1	64	2	88	5	70	18	52	7
	Diltiazem	Calcium channel blocker	117	14	127	10	103	2	105	8	137	12
	Azathioprine	Anticancer	114	8	120	2	112	3	103	10	104	8
	Ifosfamide		140	17	141	5	125	5	131	10	129	15
	Fexofenadine	Antihistamine	92	1	92	3	97	4	88	2	99	3
Group B	Sulfadiazine	Sulphonamide & trimethoprim	82	13	85	7	77	10	71	8	73	20
	Sulfapyridine		96	6	101	5	93	4	91	1	96	15
	Sulfamethoxazole		100	6	106	6	97	5	95	4	102	17
	Sulfasalazine		115	9	118	10	99	20	95	11	95	9
	Trimethoprim		98	13	105	14	107	15	102	6	106	23
	N-acetyl sulfadiazine		90	2	93	6	85	2	83	5	85	21
	N-acetyl sulfapyridine		97	5	100	5	90	2	85	9	89	18
	N-acetyl sulfamethoxazole		90	4	98	5	89	9	83	8	91	20
	4-hydroxy-trimethoprim		99	7	94	13	97	25	83	9	93	19
	Erythromycin	Macrolide & lincomycin	98	4	101	7	89	4	86	15	77	29
	Clarithromycin		101	7	105	5	85	10	102	5	108	19
	Clindamycin		82	15	98	16	88	5	104	5	103	23
	N-desmethyl erythromycin		93	1	96	4	94	7	81	15	78	25
	N-desmethyl clarithromycin		101	5	101	8	106	10	101	4	99	23

N-desmethyl clindamycin		47	1	53	14	52	16	61	16	66	27
Flucloxacillin	β-Lactams	63	4	68	3	59	11	66	11	63	17
Cefalexin	Cephalosporins	77	12	60	17	65	16	62	12	43	10
Cefixime		72	10	73	8	112	38	93	15	91	25
Ceftiofur		33	4	36	13	31	8	41	11	39	7
Norfloxacin	Quinolone	99	22	70	18	91	34	75	12	56	26
Ciprofloxacin		97	11	74	12	103	13	85	9	75	35
Lomefloxacin		92	27	81	2	75	8	71	13	84	31
Danofloxacin		133	40	132	22	104	16	118	60	95	13
Enrofloxacin		103	17	76	12	101	12	90	8	87	24
Nadifloxacin		74	11	79	12	64	5	98	39	74	16
Ofloxacin (Levofloxacin)		112	22	113	13	98	9	105	12	107	10
Gatifloxacin		101	16	99	12	102	22	96	16	88	27
Sarafloxacin		94	20	84	20	96	28	99	26	77	12
Moxifloxacin		67	3	122	27	183	66	139	42	102	38
Flumequine		88	7	94	2	89	5	82	9	95	12
Nalidixic acid		57	4	71	7	63	4	66	4	78	20
Desethylene ciprofloxacin		85	5	48	14	77	14	71	28	44	24
Hydroxy-norfloxacin		85	11	103	7	78	5	78	16	88	8
Desmethyl-ofloxacin		93	11	62	12	86	11	71	23	39	11
Ofloxacin N-oxide		79	17	87	13	108	14	82	23	61	20
Rifampicin	TB drugs (1st line)	110	19	90	23	57	20	47	23	82	25
Rifabutin		82	10	97	22	69	21	94	8	103	16
25-desacetyl rifampicin		113	18	129	27	88	5	81	55	80	38
25-O-desacetyl rifabutin		98	14	87	8	67	7	74	15	77	21
Linezolid	TB other	103	9	108	17	108	10	90	11	93	9
Thalidomide		106	16	121	43	101	20	131	41	186	25
Florfenicol	Amphenicol	112	28	128	75	96	11	100	26	128	42
Nitrofurantoin	Nitrofurantoin	66	10	61	9	108	9	88	8	82	16
NPAHD		102	7	97	9	92	14	91	7	108	19
Metronidazole	Azole	97	3	99	2	95	5	94	3	97	22
Hydroxy-metronidazole		96	4	101	3	99	4	94	4	99	17
Lamivudine	Antiretroviral	92	5	85	5	108	4	84	24	83	1
Emtricitabine		76	4	72	4	75	7	61	14	60	7

Table S7. Quantifiable CECs in river water samples from the UK and South Africa and associated PNEC values

		UK		South Africa			
Compound	**Compound Class**	**Concentration ng/L**	**STD**	**Concentration ng/L**	**STD**	**PNEC/CEC**	**PNEC//CEC Ref**
Methylparaben	Parabens	25.80	0.82	7.33	10.11	1600	(Li et al. 2015)
Propylparaben		-	-	-	-	400	(Li et al. 2015)
Butylparaben		1.92	0.68	-	-	300	(Li et al. 2015)
1-benzophenone	UV Filters	-	-	0.15	0.14		
3-benzophenone		20.79	9.21	-	-	1320	(Kim and Choi 2014)
4-benzophenone		298.75	18.13	79.68	5.50		
Bisphenol A	Plasticizer	0.00	0.00	-	-		
Ketoprofen	NSAIDs	57.52	0.83	-	-	48978	(Fick et al. 2010)
Ibuprofen		67.08	13.87	-	-	194711	(Fick et al. 2010)
Naproxen		80.09	3.92	-	-	827999	(Fick et al. 2010)
Valsartan	Hypertension	58.04	3.05	-	-	13158	(Fick et al. 2010)
Irbesartan		18.21	1.64	-	-	50	(Fick et al. 2010)
Lisinopril		1383.42	21.45	-	-	184087	(Fick et al. 2010)
Amphetamine	Stimulants and metabolites	86.30	79.48	-	-		
MDMA		1.21	0.14	-	-		
MDPV		0.00	0.00	-	-		
Cocaine		1.11	0.49	-	-		
Anhydroecgonine methyl ester		3.12	0.01	-	-		
Benzoylecgonine		42.80	2.28	-	-		
Methadone	Analgesics and metabolites	0.11	0.36	-	-	326	(Fick et al. 2010)
Acetaminophen		108.49	40.58	6.58	3.89	24000000	(Fick et al. 2010)
EDDP		2.57	0.76	-	-		
Codeine		29.51	3.04	-	-	26620	(Fick et al. 2010)
Norcodeine		722.78	663.38	-	-		
Dihydrocodeine		11.26	0.86	-	-		
N-desmethyltramadol		2.10	11.80	-	-		
O-desmethyltramadol		24.04	2.42	-	-		
Cotinine	Human Indicators	22.63	3.22	2.06	0.70		
Caffeine		131.23	35.35	124.09	103.85		
Nicotine		40.60	1.14	15.00	0.00		
1,7 dimethylxanthine		226.93	48.89	63.42	51.34		
Carbamazepine	Anti-epileptic	50.74	0.76	1.23	0.79	346496	(Fick et al. 2010)
Carbamazepine 10, 11-epoxide		4.48	1.92	2.72	0.90		
10,11-dihydro-10-hydroxycarbmazepine		5.38	1.09	-	-		
Ketamine	Anaesthetic and metabolite	14.41	0.75	-	-		
Venlafaxine		22.55	0.92	-	-	6112	(Fick et al. 2010)
Citalopram		6.39	2.58	-	-	141	(Fick et al. 2010)
Norketamine		1.45	0.47	-	-		
Desmethylcitalopram		4.32	0.90	-	-		
Quetiapine	Antipsychotic	0.03	0.10	-	-	290938	(Fick et al. 2010)
Bezafibrate	Lipid regulator	38.84	1.38	-	-	89308	(Fick et al. 2010)

Metformin	Diabetes	1358.84	82.80	-	-	64000000	(Fick et al. 2010)
Gliclazide		100.36	174.20	-	-	2720	(Fick et al. 2010)
Atenolol	Beta blocker	17.43	0.90	-	-	792332	(Fick et al. 2010)
Diltiazem	Calcium channel blocker	1.67	0.30	-	-	27884	(Fick et al. 2010)
Azathioprine	Anticancer	21.46	0.08	-	-	18000000	(Fick et al. 2010)
Fexofenadine	Antihistamine	49.89	7.16	-	-	20222	(Fick et al. 2010)
Sulfapyridine		74.74	1.72				
Sulfamethoxazole		32.67	3.94			600	(Tell et al. 2019)
Trimethoprim		10.04	1.69			500	(Tell et al. 2019)
N-acetyl sulfapyridine		9.81	1.93				
Erythromycin	Macrolide & lincomycin	23.85	2.08			500	(Tell et al. 2019)
Clarithromycin		51.01	4.07			80	(Tell et al. 2019)
Clindamycin		15.85	0.31			100	(Tell et al. 2019)
N-desmethyl erythromycin		0.88	0.21				
N-desmethyl clarithromycin		95.31	31.98				
N-desmethyl clindamycin		1.29	0.07				
Flucloxacillin	β-Lactams	30.97	4.53			72714	(Fick et al. 2010)
Ofloxacin (Levofloxacin)		9.18	0.95			500	(Tell et al. 2019)
Rifampicin	TB drugs (1st line)	95.45	15.10			60	(Tell et al. 2019)

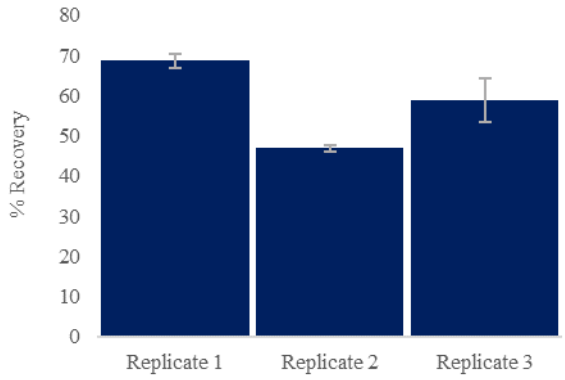

Figure S2. Percentage recovery of TaqMan™ Universal DNA Spike in Control in the three biological replicates of river water samples. Average % recovery: 58 ± 2%.

Table S8. Gene results in UK river water samples, qnrS and blaCTX were both below quantification limits

Gene	Biological replicate	Copies/L	Average Copies/L	STD
16S rRNA	1	21459536		
		19792500	20626018	1178772.46
	2	22784720		
		18524968	20654844	3012099.525
	3	25502890		
		17738140	21620515	5490507.379
ermB	1	51830.772		
		60748.968	56289.87	6306.116868
	2	24961.692		
		26910.492	25936.092	1378.009695
	3	103420.38		
		92482.74	97951.56	7734.079414
sul1	1	138537.756		
		154498.428	146518.092	11285.8994
	2	57548.064		
		77832.636	67690.35	14343.35841
	3	240099.468		
		209953.968	225026.718	21316.08747
Int1	1	287545.44		
		287277.48	287411.46	189.4763331
	2	119088.732		
		168795.312	143942.022	35147.85979
	3	434947.8		
		396848.76	415898.28	26940.08954
blatem	1	6240220		
		6345780	6293000	74642.19182
	2	1061194.68		
		2731892.8	1896543.74	1181361.97
	3	3681364.4		
		3962397.6	3821881	198720.4815
blaCTX	1	-		
		-		
	2	-		
		-		
	3	-		
		-		
qnrS	1	-		

8 Conclusions and future perspectives

8.1 Conclusions

This present work has aimed to review and expand on knowledge in the literature regarding water fingerprinting as a complimentary and innovative surveillance tool for public and environmental health.

8.1.1 Objective 1

The first objective was to give a critical perspective into how infectious disease surveillance is currently achieved and how novel WBE techniques could be utilised to provide complimentary infectious disease and antimicrobial resistance (AMR) data. The time this paper was submitted for peer review, very few cases of the novel coronavirus had been reported. It was commented in the conclusions of this publication, that WBE could be a promising tool in tracking the spread of the virus and even act as an early warning system. Since publication, along with unprecedented escalation of COVID cases, WBE has been utilised globally to gain community-wide coverage on COVID-19 circulating in a community.

8.1.2 Objective 2

Following this, a much broader perspective on WBE was presented. Here, other avenues that WBE could be utilised to support public health and its policy relevance was explored. Public health systems across the world were devasted and overwhelmed by the rapid spread of the novel coronavirus in 2020. Questions were rightly raised about countries preparedness and ability to monitor and manage such a large-scale pandemic, even with major organisations such as WHO, warning that such a pandemic was of high likelihood. WBE's popularity has largely grew during the last two years due to its low-cost nature, and its ability to give information on both asymptomatic and symptomatic patients. As a result, rapid and considerable infrastructure has been established to sample and analyse wastewater in the UK and internationally to help track and manage outbreaks. It was highlighted that WBE is a relatively young field, with only few applications of WBE ready to be implemented now. These included assessing illicit drug usage and estimation of community-wide lifestyle chemical usage, for example alcohol, nicotine, or caffeine. However, it also highlighted active and developing fields of WBE, and the promising future this area of research has in fields of disease prevalence for non-communicable and communicable diseases, and community-wide exposure to various environmental stressors.

8.1.3 Objective 3

Another objective was to explore novel endogenous biomarkers of health in wastewater. Here, a method was developed to investigate four endogenous biomarkers, specifically of oxidative stress, in wastewater. This study highlighted that oxidative stress marker, HNE-MA, was the most promising biomarker of the four. As was found to be stable in wastewater and quantifiable, averaging 48.9 ± 4.1 mg/1000/people/day. This study also highlighted challenges to analysing endogenous health markers, for example the isoprostane, 8-iso-PGF2β had poor chromatographic resolution due to matrix effects.

The biomarker 8-NO2Gua, reported low stability in wastewater (10%, t=24 hour) and 8-OHdG had poor method performance. Further research into analytical extraction techniques, with sorbents that could offer high selectivity and higher concentration factors, was recommended for 8-OHdg and 8-iso-$PGF_{2\beta}$.

8.1.4 Objective 4

The potential of WBE for AMR surveillance was also explored by investigating AAs, metabolites and ARGs in the wastewater of two different sized communities. Winter months observed in general higher loads of AA and respective metabolites, with some AAs showing significantly increased loads in winter when compared to summer, for example, clarithromycin and its major metabolite desmethyl clarithromycin. This is in line with known increases in prescribing for AAs because of increased winter respiratory infections in the UK. Several ARGs were also investigated in the city studied, however strong correlations were not observed between AAs and ARGs. This indicated that selective pressures of AAs on bacterial communities likely occurred over periods of years rather than on shorter time periods. Whilst both hospital and community wastewater had AA concentrations that exceeded PNEC values, hospital effluent had a larger number of AAs that exceeded PNECs. This was theorised to be due to the unique environment hospital environments pose, with short sewage resident times, lower volumes of flow and higher concentration of individuals at the source using AAs. Hospitals therefore are an important consideration for AMR surveillance.

8.1.5 Objective 5

This study was then built upon further, by using data triangulation to combine wastewater data with catchment specific prescription data to further explore how AAs are used within communities. Several key conclusions were identified, all studied parent AAs and metabolites observed positive correlations in both studied sites. Normalising AA loads to population size demonstrated that even with the close proximity of the two studied sites, there were spatial variation with AA usage. AA usage was in general found to be higher in Bath than in Keynsham, again this was theorised to human population being a key driver of AAs. Correction factors were applied to several AAs and metabolites in order to back-calculate and estimate consumption at the community level (population normalised daily intakes, PNDIs), these were then compared to intake estimates generated from the catchment prescription data (population normalised daily prescription, PNDPs). Several AAs reported good agreement with the prescription data, including clarithromycin, oxytetracycline and hydroxy-metronidazole. Differences between prescribing patterns and wastewater data were attributed to variable prescribing patterns and/or lack of compliance. It was highlighted that using WBE for AMR surveillance combined with prescription data provided a comprehensive approach for understanding AA usage in a community.

8.1.6 Objective 6

Whilst primarily this thesis had a focus on WBE and AMR, the last chapter explored the environmental perspective of water fingerprinting. It has been previously highlighted that there is a significant knowledge gap on chemical pollution in surface waters in low to middle income countries. With many current studies focused on the US, UK and European countries. This has been attributed to lack of specialised infrastructure and lack of appropriate standardised sampling and analytical methodologies for sampling in remote locations. This study explored using an integrated and powerless, in-situ multi-mode extraction (iMME) sampler for the analysis of >100 chemicals of emerging concern (CECs) and five ARGs. The stability of chemicals immobilised on cartridge and in river water was investigated, a significant consideration when sampling in remote areas with a potential lack of cold chain for transportation. The iMME sampler proved itself to be effective in immobilization, concentration and increased stability of many of the CECs at room temperature (with up to a least seven days storage). Several on-site river samples were collected using the iMME sampler to investigate it in action. River samples were collected from the river Avon (UK) and Olifants river (South Africa). The urbanised river Avon reported many quantifiable CECs in comparison to the largely pristine environment of the Olifants river. Whilst the Olifants river reported a low number of CECs (13 in total), lifestyle chemicals were observed (including caffeine and nicotine) and UV filters. The iMME was also investigated to sample ARGs from river water, with the river Avon reporting concentrations for four of the ARGs studied (*ermB, sul1*, bla_{TEM} and *intI1*). This work provides exciting new opportunities to investigate chemicals and biological targets in surface waters of remote areas.

8.2 Future work

There are many mounting pressures emerging in today's world, including unprecedented rises in anthropogenic pollution, emerging and re-emerging pathogens, and increase in prevalence of AMR. Water fingerprinting is a field that is continually developing and maturing as a public and environmental health surveillance tool. With regards to WBE, in the wake of the COVID pandemic the analysis of viral genetic material in wastewater allowed hotspots to be identified and the idea of a potential early warning system to become a reality. WBE therefore provides an innovative surveillance technology with increasing promise to monitor disease spread and act as an early warning system for future threats. Having this data available and easily accessible for use by decision-makers can help reduce costs and benefit society through improved health management. However, further research is essential to address key challenges for water fingerprinting.

Regarding environmental health and analysis of chemical and biological makers in aquatic systems, a key focus for future work is building and working towards standardised methods and procedures, as well as novel approaches to sampling. It is well understood in the literature that there is a lack of environmental studies in developing areas of the world in comparison to developed countries. In the

case of AMR, infectious diseases, and chemical pollution, this lack of geographic knowledge could be problematic for surveillance and result in significant public health threats. This is because resistance genes, pathogens and chemicals are all transboundary pollutants, which do not recognise boarders. Effort to increase both collaboration and environmental data across the world is critical. In the case of the iMME sampler, future work should be focused on utilising the sampler out in the field in larger sampling campaigns. By expanding on the number of samples and sites, with care being taken to ensure that samples collected are representative of that environment. This will allow further assessment of the iMME in larger, international sampling campaigns to be evaluated, and its ability to provide comprehensive environmental data.

Regarding WBE, accurately estimating population size is a challenge, and has led to an active and thriving field. Future work is focused on identifying novel population biomarkers to account for temporal population fluctuations, allowing more accurate assessment for WBE. Other key gaps include a lack of understanding of stability of pharmaceuticals and their metabolites in wastewater, and a limited understanding of metabolism to undertake back-calculation of exposure. Consequently, it is difficult calculating metabolism correction factors to quantitatively analyse a community-wide exposure or the public health status. Another key gap is the lack of pipeline for both public health relevant chemical and biological markers. For example, endogenous biomarkers are an understudied area, yet general markers on inflammation combined with suite of specific disease markers could more accurately inform on prevalence or spread of certain diseases. Further research in these areas are needed to tackle these knowledge gaps.

Other areas of future work that should be explored are new statistical approaches for data triangulation between public health data and wastewater results. As evidenced by chapter 6 in this thesis, prescription and wastewater data can provide complementary evidence to each other. By combining multiple data sources together allows a broader picture to be built up and can better inform policy-makers. An example of a statistical approach that could be applied with multiple sets of data is multivariate analysis. Data is often multifaceted with many different factors at play, statistical techniques such as this can be used to study complex sets of data with multiple variables and allow correlations to be explored.

One Health is a collaborative, multisectoral and integrated approach that has been hailed as critical for tackling threats to both environmental and health. Human health is inherently linked with the environment and the health of animals, therefore, to effectively manage public health it is essential that a broader understanding of the wider environment is met. Water fingerprinting can provide key data to research in the One Health domain. It is important that collaboration across multiple disciplines is continued to be facilitated and supported, on a local, regional, national and international level, and that

data collected from water fingerprinting can be easily shared to decision making bodies. This will allow policy decisions, regarding public and environmental health interventions, to be informed by science.

Finally, further development of analytical tools is needed for multi-residue analysis of a diverse range of chemical and biological markers. It is crucial too that these tools are cost-effective and highly sensitive. High resolution mass spectrometry could provide an untargeted approach for identifying novel biomarkers for use in water fingerprinting. Effective frameworks for identifying priority biomarkers will also be vital as the number of chemicals used in everyday life continues to grow, and many of these can end up in the natural environment. Given this diverse array of chemicals, it is challenging knowing which should be monitored. This is made more complex by the fact that chemical risks aren't equal, as the potential exposure risk is not equal. Toxicity values will vary for different chemicals of concerns, and these will exist in complex mixtures in the environment, potentially causing synergistic effects. These concerns around chemical pollution are receiving global attention, as evidenced by the new United Nations resolution and agreement for a science-policy panel focused on chemicals pollution and waste. To measure is to manage, and understanding the scale of a problem is one of the first steps to providing effective solutions.

8.3 Publication and PhD activities

8.3.1 Publications

Journal Articles

Holton, E., Sims, N., Jagadeesan, K., Standerwick, R. and Kasprzyk-Hordern, B., 2022. Quantifying community-wide antimicrobials usage via wastewater-based epidemiology.

Chau, K., Barker, L., Budgell, E., Vihta, K.D., Sims, N., Kasprzyk-Hordern, B., Harriss, E., Crook, D., Read, D., Walker, S. and Stoesser, N., 2021. Systematic Review of Wastewater Surveillance of Antimicrobial Resistance in Human Populations.

Sims, N. and Kasprzyk-Hordern, B., 2020. Future perspectives of wastewater-based epidemiology: monitoring infectious disease spread and resistance to the community level. Environment international, 139, p.105689.

Fabunmi, I., Sims, N., Proctor, K., Oyeyiola, A., Oluseyi, T., Olayinka, K. and Kasprzyk-Hordern, B., 2020. Multi-residue determination of micropollutants in Nigerian fish from Lagos lagoon using ultrasound assisted extraction, solid phase extraction and ultra-high-performance liquid chromatography tandem mass spectrometry. Analytical Methods, 12(16), pp.2114-2122.

Sims, N., Rice, J. and Kasprzyk-Hordern, B., 2019. An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater. Analytical and bioanalytical chemistry, 411(11), pp.2261-2271.

Policy report

Natalie Sims, Lisa Avery, Barbara Kasprzyk-Hordern (2021), Review of wastewater monitoring applications for public health and novel aspects of environmental quality (CD2020_07). Scotland's Centre of Expertise for Waters (CREW). Available online at: crew.ac.uk/publications

Journal articles prepared for submission

Sims, N., Kannan, A,. Holton, E,. Jagadeesan, K., Mageiros, L., Standerwick, R., Craft, T., Barden, R. Feil, J. E., Kasprzyk-Hordern, B., Wastewater-Based Epidemiology for Monitoring Community derived Antimicrobials and Resistance genes: a one-year longitudinal study

Sims, N., Holton, E,. Jagadeesan, K., Standerwick, R., Barden, R. Kasprzyk-Hordern, B., City's infectious disease treatment with antimicrobial agents – a longitudinal one year study of antimicrobials in two cities via wastewater-based epidemiology

Sims, N., Holton, E,. Archer, E., Botes, M., Wolfaardt, G,. Kasprzyk-Hordern, B., In-situ Multi-Mode Extraction (iMME) sampler for a wide-scope multi-residue analysis of > 100 chemicals of emerging

concern (CEC) and antimicrobial resistance genes in the aqueous environment in urban areas and remote (off-the-grid) locations

8.3.2 Conferences

Sims, N., Kannan, A., Holton., Jagadeesan, K., Standerwick, R., Barden, R., and Kasprzyk-Hordern, B., Wastewater-Based Epidemiology for Monitoring Community derived Antibiotic and Resistant Genes, Testing the Waters 5 2021, Brisbane, Australia.

Sims, N., Kannan, A., Holton, E., Jagadeesan, K. K., Standerwick, R., Barden, R. & Kasprzyk-Hordern, B., Wastewater-Based Epidemiology for Monitoring Community derived Antibiotics and Resistant Genes, 2020, SETAC SciCon "Open Science for Enhanced Global Environmental Protection", virtual

Sims, N., Kannan, A., Holton, E., Jagadeesan, K. K., Standerwick, R., Barden, R. & Kasprzyk-Hordern, B., Wastewater-Based Epidemiology for Monitoring Community derived Antibiotics and Resistant Genes, 2020, RSCs "Antibiotics in the Water Environment, Occurrence, Detection and Fate", London

Sims, N. Arnot, Tom. Kasprzyk-Hordern, B., Fate of Pharmacologically Active Compounds in Pilot Scale High Rate Algae Ponds. 2018, EU Water JPI Conference "Emerging pollutants in freshwater systems", Helsinki

Sims, N., Rice, J. & Kasprzyk-Hordern, B.,. A Novel Route for Determining Public Health: Analysis of Oxidative Stress Biomarkers in Wastewater, 2018. Emerging Analytical Professional Conference, Knutsford

Sims, N., Rice, J. & Kasprzyk-Hordern, B.,. A Novel Route for Determining Public Health: Analysis of Oxidative Stress Biomarkers in Wastewater, 2017,. 18th UK-IWA Young Water Professional Conference "A Water World Without Boundaries"

Sims, N., Rice, J. & Kasprzyk-Hordern, B.,. A Novel Route for Determining Public Health: Analysis of Oxidative Stress Biomarkers in Wastewater. Testing the Waters 3, 2017, Lisbon, Portugal

8.3.3 Secondments

INTERWASTE Secondment to Queensland Alliance for Environmental Health Sciences – QAEHS, Brisbane (Australia), January 2020-March 2021.