Spatiotemporal urban water profiling for the assessment of environmental and public exposure to antimicrobials (antibiotics, antifungals, and antivirals) in the Eerste River Catchment, South Africa

Elizabeth Holton [1], Edward Archer [2], James Fidal [3], Thomas Kjeldsen [3], Gideon Wolfaardt [2], Barbara Kasprzyk-Hordern [1*]

[1] University of Bath, Department of Chemistry, Bath, BA2 7AY, UK

[2] Stellenbosch University, Department of Microbiology, Stellenbosch, SA

[3] University of Bath, Department of Architecture & Civil Engineering, Bath, BA2 7AY, UK

*Corresponding author. Email address: b.kasprzyk-hordern@bath.ac.uk

---

Abstract

Antibiotic usage, excretion, and persistence are all important factors in association with the occurrence and dissemination of antimicrobial resistance. Urban water profiling was utilised in the Eerste River catchment (South Africa) to establish antibiotic usage in a catchment where comprehensive prescription records were not readily available and where portions of the community did not have sufficient access to sanitation. This technique enabled the environmental exposure to be quantified throughout the catchment area and the identification of contamination hotspots. Monitoring occurred over a 10-month period. 812 samples were processed using UPLC-MS/MS for the quantitation of 56 antimicrobials and 26 of their metabolites. Spatiotemporal trends were established, with consideration to community behaviour, seasonal changes, and physiochemical properties of the analytes. The Eerste River samples collected upstream from the town of Stellenbosch had the lowest antibiotic loads (< 4 g/day), unafflicted by industrial presence and with only small impact from farming activity. This was followed by sites downstream from a treated wastewater treatment plant (serving 178K people) discharge point (influent: 500-800 g/day and effluent 50-100 g/day), which indicates a high efficiency of wastewater treatment allowing for an effective reduction of ABs and a lower environmental burden compared to the river sites receiving untreated waste from communities in informal settlements (6-12K people) that are not connected to the sewer infrastructure (with AB levels accounting for 100-600 g/day). Temporal trends exhibited

reduced daily loads during the summer to early autumn. This is likely due to seasonal patterns in community health. However, weather patterns are also important to consider – particularly for the river sites. South Africa has notable rainfall and temperature seasonality. ARVs, emtricitabine and lamivudine, were the most prevalent drugs throughout the monitoring campaign, followed by tuberculosis drugs and sulfonamides. ARVs were, however, effectively reduced via wastewater treatment processes (>97%). This was also the case for beta-lactams, nitrofurantoin, and trimethoprim. The treatment efficacy for other drugs was more variable, that did not appear to have temporal significance.

Keywords: antibiotics antivirals and antifungals occurrence; AMR; catchment monitoring; wastewater; river

1. Introduction

Urban water profiling of pharmaceuticals, such as antibiotics, enables the evaluation of environmental contamination, as well as the health within a community (Kasprzyk-Hordern, Proctor et al. 2021). Wastewater monitoring provides temporal trends of drug class usage, typically in the form of daily mass loads (g day^{-1}). Whereas quantitation in river systems allows for the determination of spatiotemporal emergence and dissemination of contaminants and pollutants. Here, the concentration of antibiotics is of concern for several reasons. High quantities of antimicrobials have a detrimental impact on the ecosystem by disrupting the natural abundance and diversity of the microbial community. However, lower antibiotic concentrations can also be damaging, particularly in the context of antimicrobial resistance (AMR) development amongst natural microbial communities through long-term exposure. Indeed, in a study focussed on a UK catchment, several antibiotics (ciprofloxacin, clarithromycin, azithromycin, and erythromycin) were regularly found exceeding $PNEC_{enviro}$ and $PNEC_{MIC}$ in wastewater influent and effluent, and occasionally in receiving waters (Elder, Proctor et al. 2021).

Antimicrobial resistance (AMR) occurs through numerous mechanisms, e.g., where AMR genes can be acquired rapidly via lateral gene transfer. Exposure to sub-inhibitory concentrations of antibiotics in the environment is likely to impose selection, favouring the presence of these AMR genes. The presence and persistence of such pollutants can lead to the emergence and maintenance of AMR genes in the environment. Therefore, longitudinal quantification and spatial mapping of antibiotic contamination within a catchment can be used to identify pollution 'hotspots', with predicted high risk for AMR dissemination.

The inclusion of antibiotic metabolites is important for two reasons. Both in supporting the calculation of drug usage via human metabolism (versus drug disposal), as well as accounting for the antimicrobial properties of metabolites when considering AMR selective pressures. Several published papers have focused on antibiotics presence in the environment (Diaz-Cruz and Barcelo 2006, Luis Martinez 2009, Speltini, Sturini et al. 2010, Tamtam, van Oort et al. 2011, Milic, Milanovic et al. 2013, Castrignanò, Kannan et al. 2018, Kim, Ryu et al. 2018) and in wastewater treatment plants (WWTPs) (Castiglioni, Bagnati et al. 2006, Gros, Petrovic et al. 2006, Choi, Kim et al. 2008, Verlicchi, Al Aukidy et al. 2012, Michael, Rizzo et al. 2013, Polesel, Andersen et al. 2016, Yuan, Liu et al. 2019) but very few provided a comprehensive understanding of spatiotemporal distribution of antibiotics and their metabolites at a catchment level ((Castrignano, Kannan et al. 2020, Elder, Proctor et al. 2021, Proctor, Petrie et al. 2021).

This manuscript in focussed on a comprehensive monitoring of the Eerste River Catchment in South Africa for 56 antibiotics and 26 antibiotic metabolites with an aim to:

- Understand spatiotemporal antibiotics speciation in the aqueous environment, upstream and directly downstream of a section of the River Eerste with the highest level of human impact, in a one-year longitudinal study
- Identify antibiotic groups with the highest persistence and prevalence in the catchment
- Verify the fate of antibiotics and their metabolites
- Identify the main hotspots for contamination

Stellenbosch town was selected as the case study for longitudinal monitoring largely due to its diverse infrastructure. The town, situated in the Western Cape province of South Africa, is host to approximately 178k people: including about 29k university students, Cape Winelands tourism, as well as permanent residents. Kayamandi, a developing township, and Enkanini, an informal settlement, located north-west of Stellenbosch town. At the time of the study, areas within these communities did not have sufficient integrated waste removal and sewer infrastructure, therefore much of the waste was untreated and could enter the river system as surface runoff (Seeliger and Turok 2013, Ambole, Swilling et al. 2016, Meyer 2016). Sample collection of the grey water immediately downstream of these townships may be considered as pseudo-wastewater sites. Consequently, there was the opportunity to compare antibiotic usage with respect to different socioeconomic regions.

The River Eerste is the main river that traverses the town, stemming from the Jonkershoek nature reserve. Comparisons between the Eerste upstream (prior to entering the town) and downstream (as it exits the town) will indicate the impact of the community, including the effect of rapid urbanization that occurs in many developing countries. Longitudinal monitoring of the other river systems joining the Eerste enables spatiotemporal mapping of emerging contaminants, such as antibiotics.

2. Materials and methods

2.1.Materials and metadata

Analytical standards and deuterated (stable isotope-labelled) standards were purchased from Sigma-Aldrich (Gillingham, UK), TRC (Toronto, Canada), LGC (Middlesex, UK), or MCE (Cambridge, UK); the list of which is collated in SI section 3. Methanol, MeOH, was HPLC grade (Sigma-Aldrich). Water, H_2O, was of 18.2 MΩ quality (Elga, Marlow, UK). Glassware was deactivated using 5% dimethylchlorosilane (DMDCS) in toluene (Sigma–Aldrich) to mitigate the loss of basic chemicals onto −OH sites present on glass surfaces. This consisted of rinsing once with DMDCS, twice with toluene and three times with MeOH. The mobile phase buffer was formic acid (>95%, HCOOH), purchased from Sigma-Aldrich. 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).

Chemical oxygen demand (COD), conductivity, dissolved oxygen (DO), pH, and temperature measurements were collected to provide spatial-temporal indications of water quality. Methodology included COD cell tests (Merck, Germany, CAT no. 1.14541.0001); multiple DO and temperature field meter (proODO, SKU626281, YSI, USA); and Palintest Micro 800 Multiparameter field meter for pH and conductivity (Palintest Water Analysis Technologies, UK).

The quantity of suspended particulate matter (SPM) was measured in order to monitor the potential partitioning of analytes into solid phase and subsequently lost during analysis of the aqueous phase. However, this loss was also quantitively accounted for by internal standards being added, and allowed to partition for 20 min, prior to filtration. Pre-dried Whatman filters were weighed before use and the resultant solid residue was dried and re-weighed, enabling g/L to be calculated.

Wastewater influent and effluent flow rates (ML day $^{-1}$) were obtained from the municipal operators of the Stellenbosch WWTP. River flows were calculated using a hydrological model called URMOD developed by Fidal et al. (Fidal and Kjeldsen 2020). URMOD is a deterministic, lumped, conceptual rainfall-runoff model designed for simulating river runoff from catchments that include urban land cover. URMOD was calibrated on gauged sites within the Stellenbosch catchment, then estimation of ungauged river flow sites was achieved through scaling the time dependent parameters.

2.2.Study design and sampling sites

Twelve sites were trialled over the first three sampling campaigns, whereby ten sites were selected for continual monitoring for the remaining collection period. These sampling sites included the rivers that flow through the town: the Eerste river entering from the east, the Krom from the northeast, the Plankenbrug from the north, as well as the WWTP effluent via the Veldwagters tributary in the west of the town. The Krom, Plankenbrug, and WWTP effluent join the Eerste before exiting the town (Figure *1*). Sites along the Eerste, before the town, were included as environmental blanks and expected to be largely uncontaminated by human pollution. Eerste sites downstream of the town contained the combination of all water systems, and thus provides an assessment of the impact of the town on subsequent water quality. Sites along the Plankenbrug were chosen in order to account for run-off from a developing informal settlement, Kayamandi/Enkanini. At the time of the study, much of the township did not have sufficient waste removal infrastructure and consequently much of the waste entered the river. Other sites observed points where rivers systems meet, such as the confluence of the Krom and Plankenbrug, and then the Krom and the Eerste. These placements were designed to trace the origins and dissemination of pollutants via downstream accumulation.

Sampling occurred throughout 11 months, July 2018-May 2019, including six sampling campaigns. Seven samples were collected, in physical duplicate, per site, within a two-week period, every other month. Aqueous samples were collected from the WWTP via 24 h time-proportional composite samplers (50 mL homogenised aliquot, per sample), and from rivers via grab sampling (100 mL homogenised aliquot, per sample). The total number of aqueous samples, collected from all sites during six sampling campaigns, was 812.

2.3. *Sample preparation and analysis*

Samples were analysed for various physical-chemical properties, such as pH, temperature, conductivity, dissolved oxygen (DO), chemical oxygen demand (COD), and suspended particulate matter content (SPM); prior to preparation for LC-MS/MS. The method of which is described in full in an earlier publication [Holton 2021].

Samples were processed at Stellenbosch University: spiked with 50 ng internal standard (ISTD), allowed to partition for 20 min at 4 °C, and filtered, before undergoing solid-phase extraction (SPE) using Oasis HLB cartridges. Cartridges were fully dried, sealed with parafilm, and stored at -18 °C, until being shipped on ice to the University of Bath (duration of 2-3 days). Cartridges were re-dried upon arrival, eluted in methanol, dried under nitrogen, and re-suspended in mobile phase (500 µL 80:20 H2O:MeOH) into polypropylene LC vials (Waters). The analytical method for antibiotic quantification was performed using a Waters, ACQUITY UPLC^{TM} system coupled to a Xevo TQD-ESI Mass Spectrometer. The chromatography involved a 19 min gradient elution through a reverse-phase BEH C18 column (50 x 2.1 mm, 1.7 µm) with Acquity column in-line 0.2 µm pre-filter (Waters, Manchester, UK). Mass spectrometry was performed via fully targeted multiple reaction monitoring (MRM), involving two MRM transitions per analyte and one per ISTD.

Raw instrumental data was produced and integrated using MassLynx software packages (Waters Lab Informatics, UK). Data processing, validation assessment, and interpretation was performed in Excel. Further details of the sample handling, LC-MS methodology, and data processing can be found in the 'Analytical method' SI section 3.

2.4. *Quality control and data validation*

Mobile phase and matrix quality controls were used to monitor analyte calibration and physical-chemical behaviour between the varying sample compositions. All samples were spiked with internal standard in order to account for matrix effects, degradation, and any transfer loss during sample preparation. Internal standards were added prior to filtration to correct for analyte sorption to suspended particulate matter. Physical sample duplicates were prepared and analysed to improve overall confidence, particularly regarding analytes with lower inter-sample precision.

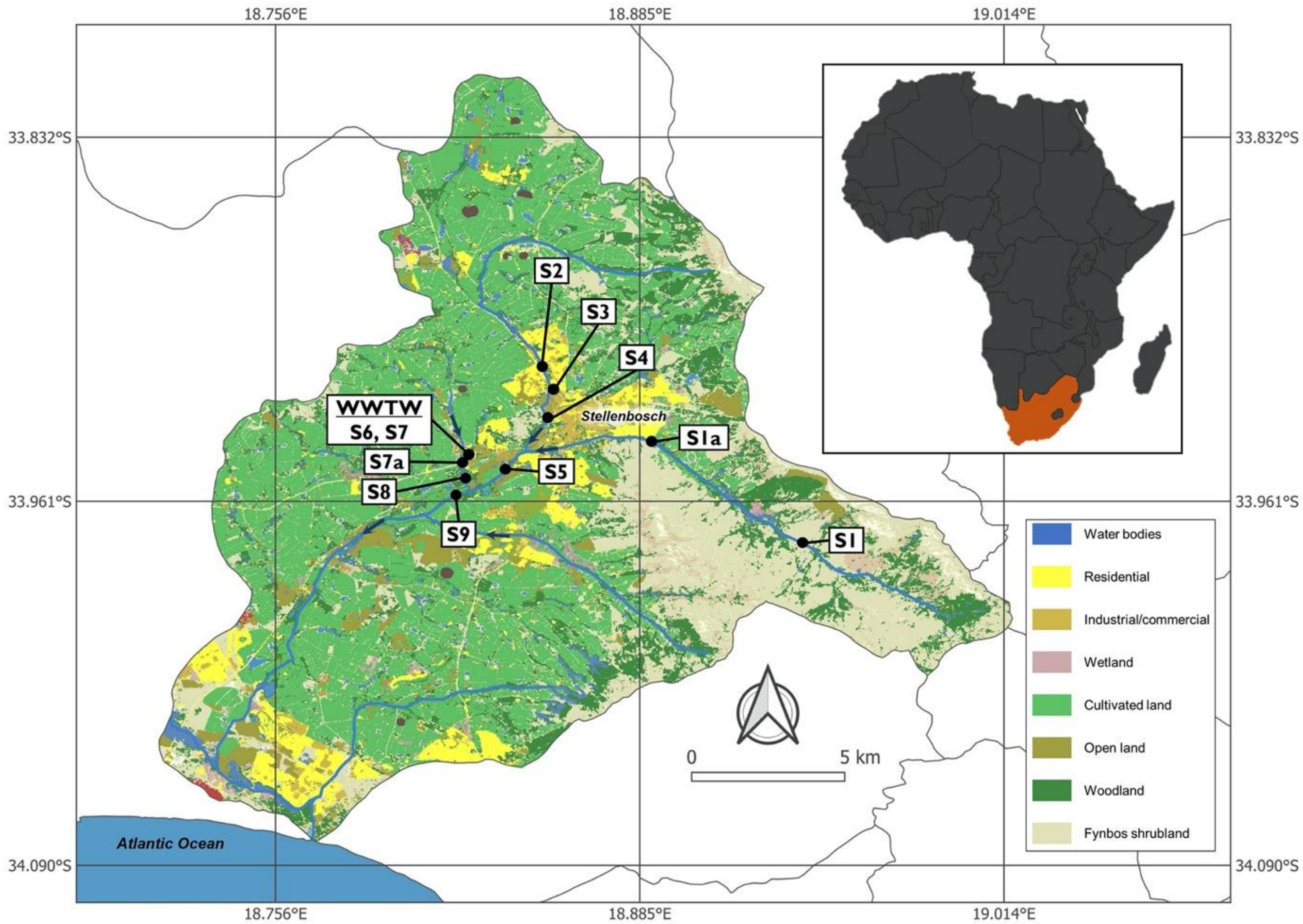

Figure 1 The Eerste catchment, including sampling sites and land use type

Upstream of the Eerste (S1,1a); Plankenbrug, downstream of informal settlements (S2,3); confluence of Plankenbrug and Krom (S4), combined waters of Plankenbrug, Krom, and Eerste (S5); WWTP influent (S6); WWTP effluent and downstream (S7, 7b); combined waters of WWTP effluent and Veldwagters (S8); downstream Eerste, combined waters exiting the town (S9). A summary of the samples collected per site and campaign is displayed in SI section 2.

2.5. Analyte stability study

The stability of analytes in influent wastewater was assessed over 24 h, at ambient room temperature (18-21 ºC) and refrigerated temperature (8-14 ºC) (SI fig.2). Seven litres of influent wastewater were collected by composite autosampler (at 15 min sampling intervals), from a WWTP in the SW of England on the morning of the study. The wastewater was homogenised, spiked with several drug standards (for a final vial concentration of 50 µg L^{-1}, SI t3), and split into bottles for analysis: two temperature variables, in physical triplicate (350 mL per replicate). Samples were taken at six time points (t= 0, 3, 6, 16, 20, and 24h), during which sample temperature and pH readings were recorded. Per time point, 50 mL aliquots were taken, spiked with 25 ng of each internal standard (25 µL of 1 µg mL^{-1} mixture), shaken, and left to partition for 30 min. Samples were then filtered using an oven dried, pre-weighed filter paper (Whatman GF/F 0.7 µm). After drying, filter papers were re-weighed, recording the mass of the suspended particulate matter removed. Samples were processed via solid-phase extraction (SPE) using Oasis HLB cartridges and analysed via LC-MS/MS as described in a previous paper (Holton and Kasprzyk-Hordern 2021).

The stability of each analyte was determined by the relative change in concentration over the 24 h time-period. The average of triplicate samples was taken for each time point, error bars representing the standard deviation. The stability/degradation of analytes were assessed in reference to drug class and the impact of sample temperature.

3. Results & Discussion

3.1. Data overview and validation

Data points were quantitatively assessed against each validation criterion: retention and relative retention time (t_R and rel. t_R), signal to noise ratio (S/N), analyte linear range, MRM ion ratio (IR), and whether the analyte was detected in duplicate. Tolerances for each criterion were established during method development (Holton and Kasprzyk-Hordern 2021) and thus, the percentages by which datapoints failed any/each criterion were documented (SI spreadsheet 1). Quantification limits are included in figures when pertinent. The results of the full dataset validation assessment are displayed in SI section 6. Each sample was analysed for the quantitation of 85 analytes, producing a total of 69,020 data points. Of these, 2,405 (8.6%) were excluded due to ISTD issues and 25,510 (37.5 %) were positive analyte identifications.

Ion ratios were used to support peak identification, however falling outside of the tolerance windows was not considered a reflection of the data's quality. Due to the complex nature of the sample matrices, the sensitivity and resolution of an analyte's MRMs were often variable. This variability was observed across the concentration range as well as being related to sample recovery and matrix interference. Thirteen analytes, which were only validated to a semi-quantitative level, were not monitored for ion ratio. Therefore, ion ratios were included in the dataset evaluation, but were not critical for target identification or quantitation.

For analytes that did not have an exact labelled analogue for an ISTD, ISTDs were allocated based on similarities in physical-chemical properties – typically from within the same drug class, such as using sulfamethoxazole-d4 for sulfadiazine. Consequently, matrix-associated chromatographic shift was generally well accounted for using relative retention time. Falling outside established tolerance windows for this relative retention time parameter was considered the most significant validation flag. These datapoints were checked manually and were predominantly observed in the semi-quantitative analytes. Other fails were attributed to non-gaussian shape caused by poorly resolved peaks, or suboptimal ISTD assignment (i.e., disparate chromatographic shift). Matrix interference is generally low in MRM acquisition mode, however, was observed in a few analytes: namely isoniazid and metabolites, pyrazinamide, sulfadiazine, and lamivudine.

3.2. Spatial-temporal observations

3.2.1. Antibiotic daily loads and concentrations

Sampling sites are grouped by their various river sources – the upper Eerste (1 & 1a), prior to the urban setting, is joined by waters from the Plankenbrug (2 & 3), Krom (4), and Veldwagters (6, 7, 7b, & 8) as it traverses the town. The confluence of the upper Eerste, Plankenbrug, and Krom is sampled via site 5 (upstream of the WWTP discharge), and the outflowing river quality is measured further downstream, as the Eerste leaves the town (site 9). The Plankenbrug sites are predicted to be contaminated by waste from the informal settlements; the Krom collects land runoff from surrounding agriculture and municipal stormwater, and the Veldwagters receives the municipal treated wastewater effluent (Meyer 2016).

Cumulative average daily loads and concentrations of ABs and AB metabolites are presented in Figure *2* and SI fig.19-24, arranged per site and sampling month. Concentrations are useful for establishing environmental impacts, in terms of relative pollution and toxicity. However, due to a vast disparity in the volumes of water flowing through the various sites at different time of year, spatiotemporal trends are difficult to observe via concentration. Consequently, river and wastewater flow rates were modelled and measured, respectively, ranging from <1 up to >1000 ML/day. By accounting for daily water volume, concentration values (g/L) were converted to daily mass loads (g/day).

Flow rates are more constant through WWTPs, varying by less than 3 ML/day throughout all sampled days. Consequently, temporal patterns are very comparable between the daily load and concentration datasets, Figure *2* and SI fig.19-22. Other sites, however, displayed inverse trends. Sites along the Plankenbrug and Krom experienced high river flow rates July-Sept 2018, resulting in low concentrations. Yet, the associated daily loads are high, suggesting contaminants may be entering the systems via land runoff (greywater).

January and March are summer months in South Africa, and dry season in the study area. It is quoted that 80% of the Eerste river's summer volume is the product of urban runoff*;* consisting of agricultural pollution, household waste water, and informal settlement grey and black water (Meyer 2016). In this case, lower river mass loads may be observed, but at high concentrations. Summer months will also result in higher thermal and photo degradation.

By observing mass loads, the general spatial trend displays an accumulation of contaminants stemming from each river system. This is somewhat counteracted by analyte degradation/biotransformation and adsorption, as well as extensive WWTP processing. An example of this spatial mass load accumulation is displayed in SI fig.25. The composition of samples collected from site 9 correlate well with the sum of analytes at site 5 and site 8. Overall, the mass loads obtained exiting the town (site 9) are approximately 200x higher than the levels entering via the Jonkershoek reserve (sites 1 & 1a) and are equivalent to about 20% (by mass) of the combined levels of municipal-wastewater from site 6 and informal settlement-wastewater from site 3.

The Eerste samples collected within, and downstream of the Jonkershoek reserve (sites 1 & 1a) had the lowest antibiotic loads (< 4 g/day), unafflicted by industrial presence and with only small impact from farming activity. This was followed by sites 7, 7b, and 8 which contained treated wastewater effluent. The latter indicates a high efficiency of wastewater treatment allowing for an effective reduction of ABs and a lower resulting environmental burden compared to the sites in the River Plankenbrug, which received untreated waste from local communities. As expected, wastewater influent (site 6), generally recorded the highest daily loads, ranging from 500-800 g/day, throughout the year. The next highest levels (100-600 g/day) were reported from the sites along the Plankenbrug that were impacted by informal settlement runoff. Waste from these latter regions, however, entered and remained in the river untreated.

Temporal trends exhibited reduced daily loads during the summer to early autumn, in South Africa (i.e., sampling campaigns in January and March 2019). This is likely due to seasonal patterns in community health. However, weather patterns are also important to consider – particularly for the river sites. South Africa has notable rainfall and temperature seasonality. Even though the relative water volumes have been accounted for via the calculation of g/day, the contribution of land run-off will vary significantly between the wet (June-Nov) and dry (Dec-May) seasons. Additionally, higher thermal and photo analyte degradation is expected in the summer months. Drug-class specific temporal trends are discussed in more detail in section 3.5. and SI section 9.

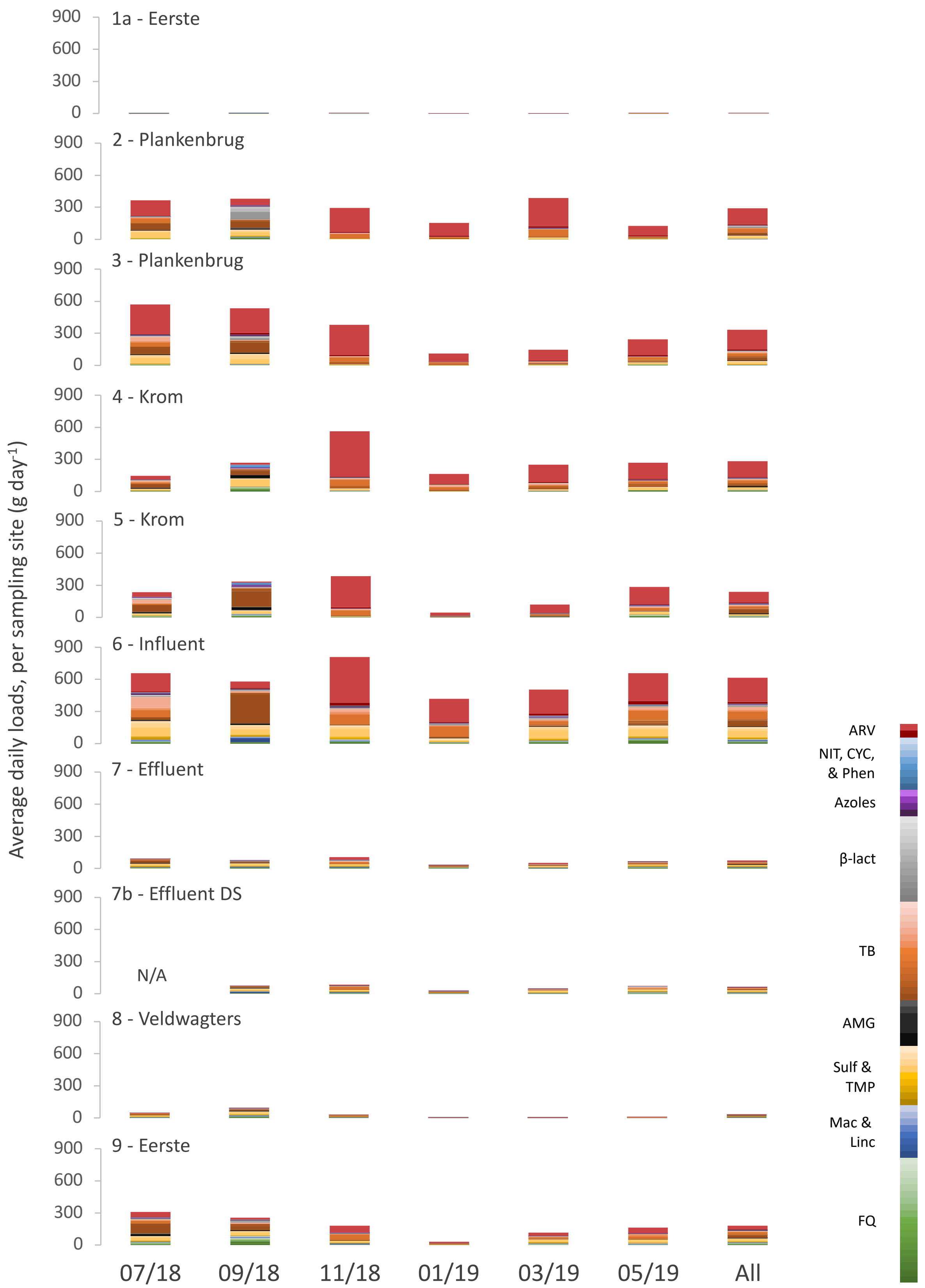

Figure 2 Spatiotemporal month-averaged antibiotics and metabolites (trends via daily mass loads)
Analyte-specific colour key, enlarged, and site-averaged figures are available in SI. The horizontal highlighted rows indicate the spatial groupings, organised by river source; and the highlighted vertical column represents the site-averaged daily loads.

3.2.2. Flows and water quality indicators

The water quality data collected also provides an overview of the spatial and temporal dynamics (SI section 5). The modelled river flows demonstrated highest values from mid-May to October; particularly in sites 4, 5, and 9, where the Krom meets the Eerste. Sites 1, 2, and 3 (upstream in the Eerste and Plankenbrug), are smaller, slower sections of the river with the lowest flow. Flow through the WWTP is more consistent, controlled by the plant.

The quantity of suspended particulate matter (SPM) was monitored in five of the six campaigns (Sept 2018-May 2019). Samples from sites 2 and 6 (wastewaters) had the highest SPM content, regularly above 0.2 g/L. Other samples were typically less than 0.05 g/L.

All measured parameters had spatial trends: sites 1 & 1a were found to be the least polluted (low conductivity and COD, and high DO levels); whereas wastewater (site 6) and the sites associated with informal settlement runoff (sites 2-3) were the most impacted by human activities (high conductivity and COD, and low DO levels). The remaining sites had intermediary measurements, following the confluences and dilutions within the river system. It is notable to mention that sites 2 and 3 were much more impacted by human-linked discharges than site 8, due to the mitigating effect of the wastewater treatment plant, treating wastewater for the whole town. The results clearly indicate that lack of waste treatment by communities upstream of sites 2 and 3 is directly linked with the most pronounced environmental burden manifested by high levels of water quality indicators tested in this study. For example, the COD in raw sewage (site 6) was measured up to 1400 mg/L, but after WW treatment was reduced substantially to 70 mg/L (site 8). In comparison, samples downstream from the informal settlements (site 3) recorded COD to almost 1000 mg/L; yet these waters are untreated, and the nutrient load persisted downstream, reading up to 600 mg/mL at site 4.

Several parameters also presented temporal trends: sample temperature was distinct between summer and winter months (ranging from 9.2 °C at site 1 in September, to 27.7 °C in the WWTP in January); and DO and pH were generally lower in the summer months (November through March). A comprehensive evaluation of spatial and temporal trends is available in SI section 5.

3.3. WWTP efficacy

Stellenbosch WWTP operated using a conventional activated sludge (CAS) setup until December 2017, after which several upgrades were performed. The conversion to ultrafiltration technology was finalised prior to our first sample campaign in 2018, by the introduction of a membrane biological reactor (MBR). The plant was estimated to serve an average of 176,523 people during 2018 and 179,463 in 2019.

The WWTP hydraulic retention time (HRT) is calculated to be approximately 18 hours. Therefore, the interpretation of daily (24-hour composite) analyte reduction efficacy is deemed acceptable for the evaluation of general trends, with WWTP conditions comparable to those outlined in other publications (Watkinson, Murby et al. 2007, Gulkowska, Leung et al. 2008, Sinthuchai, Boontanon et al. 2015, Zhu, Su et al. 2021).

South Africa is subject to load-shedding periods, whereby localised power outages are implemented to relieve the demand on the national grid. Several rounds of load shedding occurred during the March 2019 sampling campaign, causing disruption to the WWTP function as well as the composite samplers. Despite this, no significant differences were observed in the efficacy of the antibiotic reduction relative to other periods.

When influent volumes are high, untreated influent waste can enter overflow channels and potentially traverse the plant without being treated effectively (Seeliger and Turok 2013). Although no overflow events were recorded, the average analyte reduction was not significantly lower during the rainy season (July and September) compared to the dry months (Figure *3*). However, percentage reduction was observed to be lower during two of the seven sampling days from the September campaign (19th and 6th), suggesting some waste may have been released untreated, (SI fig.45). Flow data from the WWTP was higher than average throughout September, with the 19th measuring the highest of the dates sampled (25.9 ML/day). However, the 6th of Oct had typical measurements (10.6 ML/day). Consequently, one-way ANOVA tests were performed to assess whether the two dates had a statistically poorer analyte reduction. Tukey's Tests, per drug class, were conducted comparing the percentage reduction on the second and sixth dates (19th and 6th) against the other dates (17th, 20th, 25th, 28th, 7th). A significant difference was determined in the data for macrolides ($F(1,47) = [8.285]$, $p = 0.006$) and sulfonamides ($F(1,35) = [4.769]$, $p = 0.036$); but not for antiretrovirals, azoles, fluoroquinolones, trimethoprims, or TB drugs. There was not enough data to evaluate the significance in the cyclines, phenicols, nitrofurans, aminoglycosides, or beta-lactams.

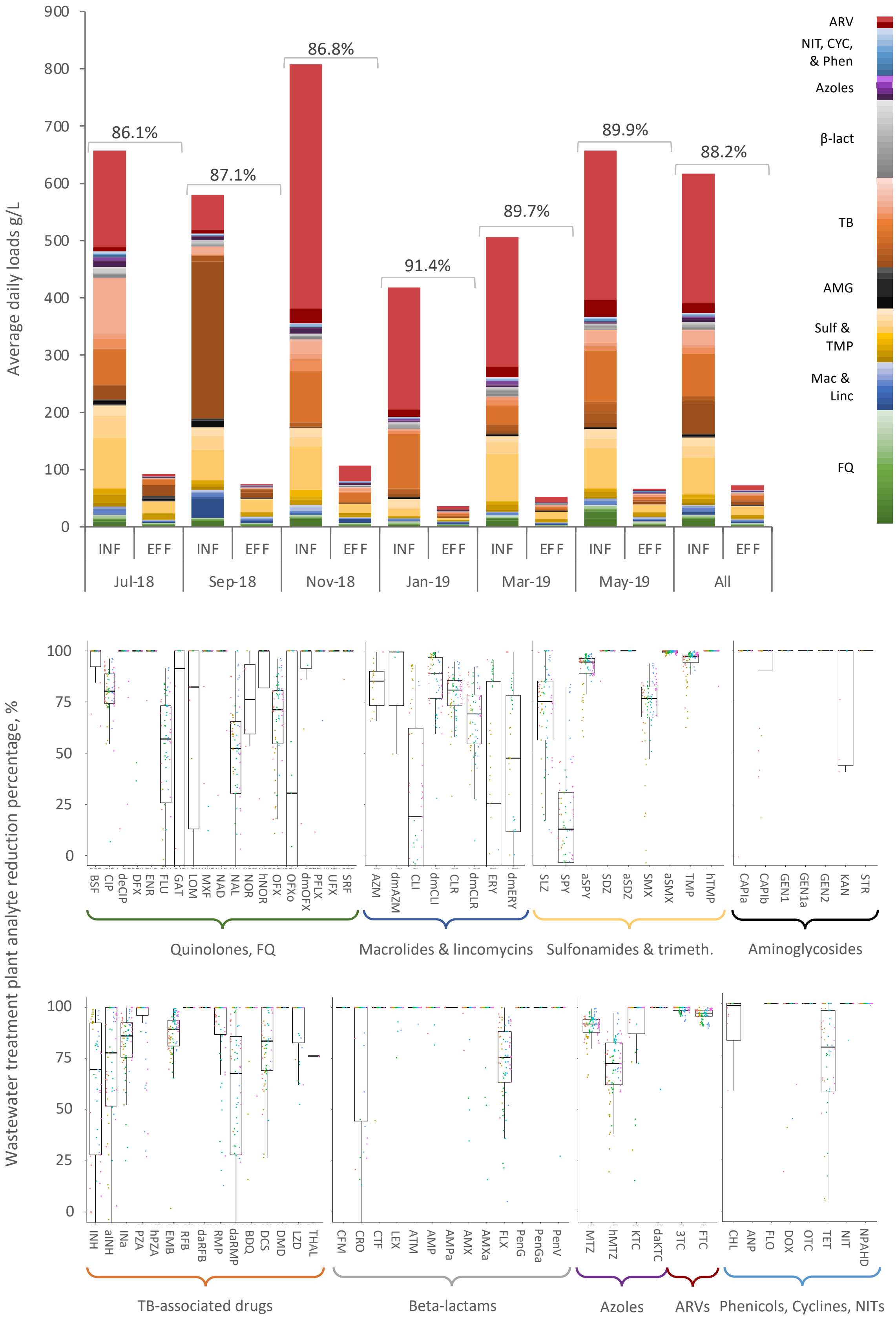

Figure 3 WWTP reduction efficacy: averaged daily loads per campaign (top) and percentage reduction (bottom). Top: wastewater influent (INF); wastewater effluent (EFF). Percentage notations indicate overall analyte reduction. Analyte-specific colour key available in SI. Bottom: data points colour coded by sampling month. Analyte abbreviations and enlarged figures available in SI sections 3 & 8.

Figure *3* bars and boxplots display the same dataset. Samples were spiked with ISTD prior to filtration, and therefore accounted for both the aqueous and SPM fractions during analysis, i.e., total antibiotic mass entering and exiting the plant. The stacked column figure demonstrates the average overall mass reduction, by comparing influent and effluent daily loads per campaign. Whereas the boxplots show percentage daily reduction of each analyte. The results presented in Figure *3* indicate a good performance from the WWTP, with an average 100% reduction for 37 of the 85 analytes. The performance of the WWTP is maintained throughout all seasons, despite any seasonal patterns in usage. The broad intra-analyte distribution observed in the boxplots may have several origins. Matrix interference in influent samples caused signal suppression in some samples, leading to an artificially lowered influent mass load and consequently higher standard deviation.

Chromatographic interference and signal suppression of some internal standards in influent wastewater artificially skewed the measured reduction of macrolides, particularly those paired to erthyromycin-$^{13}C3$. Interference was also present in the isoniazid analytes, meaning several peaks derived from influent samples were not fully resolved. This could potentially lead to underestimation of the mass loads entering the WWTP, and therefore suppress the calculated reduction efficacy. Beta-lactams, aminoglycosides, azoles, antiretrovirals, phenicols, cyclines, and nitrofurans were, on average, well removed. Whereas the sulfonamides and TB drugs had more varied reductions.

Based on data collated in reviews by Michael et al. (Michael, Rizzo et al. 2013) and Zhu et al. (Zhu, Su et al. 2021), reduction efficacies are dependent on numerous variables, including the treatment processes and operating conditions in reference to the reduction pathways of a specific drug compound. Consequently, quoted reductions vary significantly. On top of variance in the WWTP, experimental conditions may not be directly comparable – particularly the collection interval between influent and effluent, relative to treatment HRT. However, broadly, the Stellenbosch MBR-WWTP does appear to achieve comparable efficacy with other MBR plants and those operating alternative processes. Michael et al. (Michael, Rizzo et al. 2013) state a 94.4-99.9% reduction of ampicillin via MBR, concordant with our median result of 99.2%. Other comparable data was observed for amoxicillin, cefalexin, and penicillin V; obtained from CAS treatment plants. Mosekiemang et al. measured very similar reduction efficacies for antiretrovirals, emtricitabine and lamivudine, via MBR (98 and 100% (Mosekiemang, Stander et al. 2019); 96.5 and 99.5% [this study]). Compared with the literature collated by Zhu et al. (Zhu, Su et al. 2021), Stellenbosch WWTP achieved better analyte reductions for CLR, TMP, and OTC. Quinolones were, as expected, quite poorly removed during our study (72.6%), however had much higher efficacy than Hendricks et al., calculating reductions in fluoroquinolones (0-26%) from WWTPs in the Western Cape that were utilising ‘older technologies’ (CAS) (Hendricks and Pool 2012).

3.4. Municipal and settlement wastewater

Population estimation is difficult for several reasons. However, sources since the 2011 census provide a decent indication for the population growth and even settlement specific data for average household size and access to services. Very few studies address intra-annual fluctuation due to university students, holiday periods, settlement residency, or tourism. Using a range of sources, population estimates are discussed, in order to provide context to the antibiotic levels measured between municipal sewage and settlement-derived wastewaters.

The Stellenbosch region, during our sampling period of 2018 and 2019, hosted 186-187k people, in 49-53k households (Government 2018, Government 2019). Stellenbosch WWTP serves Stellenbosch town, however recent municipal reports and community surveys state that only 41-51k households had flush toilets connected to municipal sewerage/septic tanks (Statistics-SA 2016, Government 2019). Therefore, the population estimates associated with the WWTP samples were 176,523 and 179,463 for 2018 and 2019, respectively.

Households that do not have wastewater infrastructure are largely informal dwellings, located in either discrete settlements or in the backyards of formal housing. The settlements of most significance to this study were those situated along the Plankenbrug river, in and around Kayamandi: Enkanini, Azania, and Slabtown.

Enkanini, in the northwest of the town, is a relatively large informal settlement with approximately 8010 residents (Ward 121). Of these dwellings, 73.2% were considered informal (Government). As of 2013, Enkanini was predominantly populated by the overflow from the neighbouring township, Kayamandi (61%), as well as migrants from within the Western Cape (18%) and further afield (Seeliger and Turok 2013). The 2011 census indicated that 70% of the dwellings in Kayamandi were informal, housing approximately 17,252 people (Zibagwe 2016). The population estimate for Azania, 6500-8700, was calculated from estimating the number of shacks per square footage (via satellite, 2018) and an average occupancy of 3-4. Slabtown,

[1] Referencing the most recent Ward structure, since boundary amendments in 2016.

situated on the edge of Kayamandi, was host to 37 structures and 111 people in 2014 (Government 2014). There were ten working flush toilets at the time of the report, and the growth potential was quoted to be ‘very high’. Extrapolating all these figures for occupancy in 2018/19, the total number of people living in informal residence along the Plankenbrug river was approximately 30,000.

Stellenbosch municipality quoted in 2014 that 39% of informal dwellings did not have access to working sanitation (approx. 5028 people) (Stellenbosch-Municipality 2014); however data from the 2016 community survey stated that only 1096 people had no access to any type of toilet (Statistics-SA 2016). Neither report referenced the conditions that constituted reasonable ‘access’, nor the representation of the dataset acquired. Additionally, reports did not necessarily state whether a percentage represented the proportion of individuals or households. This distinction can skew the perception of the data. For instance, the average household size across Stellenbosch was quoted as 3.7 (Government 2019); however, due to the demographic of the occupants of Enkanini, the average in 2012 was 1.9 individuals per dwelling (Stellenbosch-Municipality 2012).

The accessibility of sanitation is important for numerous reasons - having significant health, social, and environmental implications. For this study, estimating the contribution of urban waste into the rivers enables better interpretation of the results. In 2012, Enkanini had a ratio of toilets/residents of 1:72. Yet the placement was not evenly distributed, with 88 households being more than a kilometre from the nearest facility (Stellenbosch-Municipality 2012). Additionally, sanitation facilities are not always considered safe, particularly at night where there may not be sufficient lighting. In fact, 92% of households in Enkanini expressed fear of using these toilets at night (Stellenbosch-Municipality 2012). Considering all the aforementioned factors, we can estimate that between 6-12k people may have been regularly contributing to nightsoil during our sampling period 2018-19, and likely entering the Plankenbrug as land runoff.

Comparisons between the municipal-wastewater (site 6) and settlement-wastewater (sites 2 & 3) may be utilised to gage socioeconomic trends. However, there are several factors to consider:

- Large difference between the size of contributing populations
    - limitations in population estimates. Especially for informal settlements, which experience rapid change

    - proportion of formal versus informal waste infrastructure difficult to quantify, including a mixture of septic tank systems and/or no sewage infrastructure
- Sample pathways and timelines
    - 'direct' entry into WWTP through sewage pipelines, versus dissemination via land runoff and leaching
    - loss/reduced rate of dissemination to rivers due to drug-specific ground sorption
    - higher weather/rainfall dependency in river samples
    - time-proportional 24-hour composite sampling (WW), versus grab sampling (river)
- Analyte stability
    - expected higher photo- and thermal-degradation in river-based runoff samples

With these considerations in mind, the clearest representation of spatiotemporal deviation between sites 3 and 6 was via heatmap, Figure 4. Municipal wastewater tends to contain a wider range of ABs at higher concentrations/daily loads; especially in macrolides, quinolones, and B-lactams. For example, CLR is found at an average of 0.16 g/day in site 3 and 5.09 g/day in site 6. This is not surprising as municipal wastewater is estimated to represent 178k inhabitants. In contrast settlement wastewater represents AB usage by approximately 6-12k inhabitants (15-30 times smaller). However, the mass loads of antiretrovirals (ARVs) were much closer between sites. The average daily load of lamivudine was 8.4 g/day at site 3, compared to 16.9 g/day in wastewater influent. Suggesting a significantly higher usage throughout the settlements.

Seasonal changes seem to be the main variable determining daily loads of ABs across sites 3 and 6, both in terms of usage and weather patterns. Samples from the enclosed sewer system will not be heavily impacted by weather changes, and so temporal trends may be more strongly attributed to AB usage. Samples from the Plankenbrug (sites 2 and 3) may be affected by both. The retention time (and consequent degradation) between the sewer system and downhill seepage/river flow will also be different between the sites, and the latter will have seasonal variance. Therefore, causality is more speculative without considering analyte photo/thermal stability.

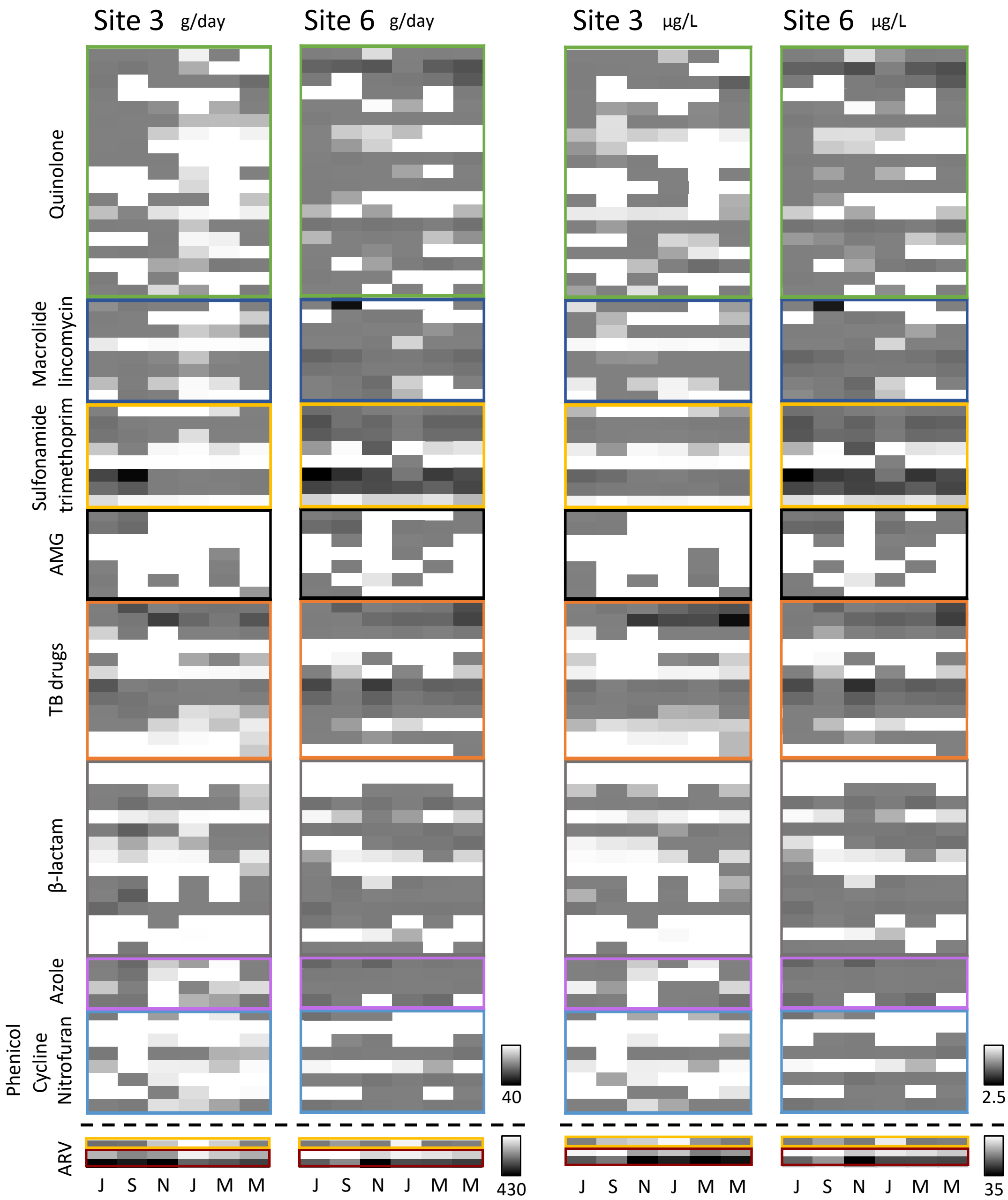

Figure 4 Heatmaps comparing spatiotemporal trends of antibiotics and their metabolites in wastewater sites 3 & 6, via daily loads and concentration. *Semi-quantitative exclusions (bedaquiline, ethambutol, isoniazid); outliers separated by dotted line (sulfamethoxazole and ARVs). X-axis: July 2018-May 2019.*

3.5. *Drug class trends*

Due to their similar chemical structure, drug classes exhibit similar physical-chemical properties. Analogues within a drug class may be utilised for related ailments and therefore may also observe comparable prescribing trends. Consequently, the spatiotemporal results have been subdivided by drug class for further interpretation. Prescription records were not accessible for the relevant catchment and time-period, and so spatiotemporal interpretations for usage versus weather patterns are currently speculative. However, relative analyte stabilities (from the 24-h study), help to establish likelihood of persistence within the river systems.

In general, TB-associated drugs and antiretrovirals were the most prevalent and their relative predominance was season independent. The TB drugs were also the most persistent, in terms of resultant mass loads exiting the town (site 9). Sulfonamides and trimethoprim had the next highest prevalence, observing much higher seasonality in the river sites.

As with the comparison of sites 3 and 6, temporal trends can be attributed to either antibiotic usage, or variables associated with seasonal change (e.g., temperature and rainfall). Antibiotic usage patterns may correspond to disease seasonality or have spatial implications within the catchment due to ease of access or commonality of different ailments. Weather-based seasonality impacts analyte movement (rainfall / land runoff) and degradation (photo / thermal). If the susceptibility of a disease is seasonal, the expected antibiotic usage would have a seasonal trend across all sites. However, if this trend is only observed at some sites, the temporal nature is less likely due to usage. During summer, if antibiotic daily loads are lower in river samples but not in WW, this may be due to greater analyte degradation; if the daily loads are higher during rainy seasons, this may be an effect of increased land runoff. Drug classes that had a seasonal trend across all sites include macrolides and TB drugs. Those that had relatively consistent levels in WW, but seasonal river patterns include quinolones, sulfonamides, B-lactams, and azoles.

3.5.1. Quinolones

The quinolone class consists of 14 parent compounds and five metabolites (SI section 3). The quinolones as a class demonstrated persistence both through wastewater treatment (72.6% average reduction) and accumulation to the most downstream site, 9. Many of the analytes studied were observed to be quite thermally stable, including the most prevalent, ciprofloxacin (SI fig.4). These properties may compliment the observation of higher prevalence in the wettest month, Sept 2018 - possible accumulation by undegraded drug traces entering the rivers via land runoff. Several quinolones (e.g., danofloxacin, besifloxacin, lomefloxacin, norfloxacin) were only sporadically detected across the catchment, both season and site dependent.

Four parent drugs and their metabolites were investigated to understand analyte ratios and spatiotemporal changes at the catchment level, OFX-dmOFX-OFXo; CIP-deCIP; NOR-hNOR, and PFLX-UFX. Norfloxacin, prulifloxacin, and their metabolites were only detected sporadically, examples of the distribution of ofloxacin and ciprofloxacin are displayed in Figure 5. Ciprofloxacin and desethylene-ciprofloxacin were detected throughout the sampling sites; however, inter-sample concordance was often low – as seen in the broad error bars. Higher relative quantities of metabolite were measured in river samples from sites 1-5, and lesser quantities in WW and downstream river sites, in comparison with the parent drug.

Ofloxacin N-oxide was validated in the analytical method to achieve quite high limits of quantification (12 µg/L), in comparison with the parent drug and desmethyl-ofloxacin (0.1 and 0.5 µg/L, respectively). Consequently, the latter two analytes were predominantly detected within quantitative linear range, but OFXo was often below the validated limits of quantification or detection, SI fig.47.

3.5.2. Macrolides and lincomycin

One lincomycin parent (clindamycin) and three macrolide parents (erythromycin, clarithromycin, and azithromycin) were monitored, alongside drug metabolites. Temporal trends were observed in both rivers and WW, suggesting variation due to usage patterns, (as well as potentially weather changes). The analytes studied had varied stability over 24 hours. Clindamycin and dmCLI, metabolite, presented temperature-dependent degradation (approx. 20% between conditions); which may have contributed to the decreased loads detected during summer. Clarithromycin and dmCLR were typically present at the highest concentrations and daily loads, averaging 1-9 g/day per campaign, at site 6. Higher levels in September in both WW (reaching 6.6 g/day) and river samples (reaching 4.2 g/day) might indicate higher usage.

Patterns for the detection of the parent drugs and metabolites are displayed in Figure 6.

Clarithromycin and dmCLR were prevalent throughout the year and amongst all sampling sites. The method detection limits for both analytes were very low, and so almost all data is within the quantitative range. Those outside this region are included, but considered semi-quantitative. The parent drug was reduced more efficiently by the WWTP plant, as observed by CLR:dmCLR ratios (influent: 1.85 ± 0.55; effluent: 1.17 ± 0.43). This could imply the occurrence of biotransformation from parent to metabolite form or be the product of matrix suppression. The parent-metabolite ratio did not show any temporal or flow rate correlations, suggesting that the physical-chemical properties were comparable, and the relationship was not impacted significantly by temperature or sorption.

Erythromycin and dmERY were detected throughout the sampling sites, particularly during September, November, and May. The parent compound was more prominent than the metabolite in WW influent, and at a ratio of approx. 1:1 in WW effluent. This, compared to upstream river sites, where the metabolite was measured at 80-90% higher loads (i.e., ERY:dmERY 0.14 ± 0.11). Clindamycin and drug metabolite are measured at much lower loads. Following WW treatment, the parent AB was measured around its respective limit of quantification.

3.5.3. Sulfonamides and trimethoprims

Sulfamethoxazole, sulfadiazine, and their metabolites were observed alongside trimethoprim and hydroxy trimethoprim, due to routine coadministration. Sulfasalazine, a human anti-inflammatory, metabolises into the antibiotic sulfapyridine. Sulfapyridine is primarily used in veterinary medicine and metabolises into acetyl sulfapyridine. All of these analogues were monitored.

In general, the mass loads in WW influent were generally consistent over the sampling period, except for a drop during January 2019. This, compared to large variation in river mass loads, suggests losses due to degradation in the summer months. This is also supported by the observation of temperature-dependent degradation. On the other hand, many of the analytes studied were observed to be quite thermally stable, including the most prevalent, SMX. This could explain the occurrence of increased mass loads during heavier rainfall periods, such as September.

Sulfadiazine was only present sporadically and its metabolite, acetyl-sulfadiazine, was detected in very few samples. Sulfamethoxazole is typically prescribed to humans with trimethoprim at a ratio of 5:1, however, may be utilised at different proportions in livestock. A literature review by Thiebault et al. presented that the final proportion measured in WW influent was 1.93:1 (median) (Thiebault 2020). The difference is a result of human pharmacokinetics, in terms of metabolism and excretion; and physical-chemical properties of the analytes, such as sorption and stability. Each catchment observed in the review had differing experimental conditions (temperature, size of catchment, matrix composition, etc.), as well as individual assay analyte recoveries, leading a broad range of SMX:TMP ratios. Our data determined a range for the drug ratio in WW influent, with a value of 3.8:1 (median). This is in line with the literature reviewed.

Data for sulfasalazine (SLZ), sulfapyridine (SPY), and acetyl-sulfapyridine (aSPY) are presented in Figure 7. All three analogues were quantitatively detected in WW influent, after which the impact of wastewater treatment was variable. The average reduction of SLZ and aSPY was 73.1 and 90.9 %, respectively, compared to only 10.1% for SPY. Comparably, SMX and TMP reduction from site 6 to 7 was fairly good, particularly in the metabolites.

3.5.4. Aminoglycosides (AMGs)

Four aminoglycosides were observed, including seven total mass analogues (capreomycin Ia and Ib, and gentamycin C1, C1a, and C2, C2a, & C2b.) During method validation, these analytes were determined to be of semi-quantitative nature. However, relative trends can be made. Daily mass loads were highest in July and Sept 2018, during the highest rainfall periods. This could suggest increased prevalence due to higher land runoff. By observing the averaged loads across all campaigns, AMGs were measured at the highest quantities at site 5 – after the confluence of upstream Eerste, Krom, and Plankenbrug.

3.5.5. Tuberculosis-associated drugs and antiretrovirals (ARVs)

Parent drugs and drug metabolites associated with the treatment of tuberculosis were monitored in this study. These included first-line TB treatments (isoniazid, pyrazinamide, ethambutol, rifabutin and rifampicin), injectable second-line drugs (AMGs, previously discussed), fluoroquinolones (previously discussed), and newer/reassigned drugs (bedaquiline, D-cycloserine, delamanid, linezolid, and thalidomide). Two antiretrovirals, emtricitabine and lamivudine, were included due to the high coinfection rate of HIV with tuberculosis.

The 2020 Stellenbosch Socio-economic Profile stated the number of registered patients receiving ARVs, or treatment for TB in 2018 and 2019, respectively: 1175 and 1176 TB patients; 6064 and 6960 HIV/AIDS patients (Government 2020). It did not provide information on treatment regimens being utilised.

The spatial trends in the TB drugs and ARVs were largely comparable, with the highest prevalence in WW influent, followed by the Plankenbrug sites 2 & 3. Several analytes were persistent through the river systems, with apparent low sorption/degradation, resulting in relatively high levels measured downstream, site 9. Temporal variation was observed in both WW and river sites, suggesting seasonal differences in drug usage.

Emtricitabine was the most prevalent of all analytes monitored. This is both due to the high prevalence of HIV/AIDS in the community, but also because of the large quantity doses used during treatment. The two ARVs had a strong spatiotemporal relationship, observable when plotted on separate axes, SI fig.33.

Parent-metabolite relationships were observed for isoniazid, pyrazinamide, rifampicin, and rifabutin. Pyrazinamide, rifabutin, and their metabolites were only detected sporadically – without sufficient prevalence to assess spatiotemporal trends. Results for the isoniazids, displayed high prevalence in upstream sites as well as WW influent, yet good reduction via WW treatment. Rifampicin and daRMP were less well removed via the WWTP, Figure 8.

3.5.6. Beta-lactams

Four cephalosporins, one monobactam, five penicillins, and three penicillin metabolites were observed. These analytes were observed sporadically, and at low quantities throughout the samples; consequently, spatiotemporal trends are not observable. However, more temporal variation was noted in river samples than in WW, SI fig.30. Suggesting that weather-based fluctuation had more significance than changing community usage. Degradation was high in cephalosporins, particularly in the higher temperature condition, SI fig.9. Other beta-lactams demonstrated high intra-sample variation in both the stability and catchment study, making temporal observations harder to interpret.

3.5.7. Azoles

The antibiotic metronidazole and its metabolite were monitored, as well as the antifungal ketoconazole and metabolite. Loads were highest in September in the river samples, suggesting increased dissemination during the rainy season. No significant seasonal trend was observed in WW influent. The levels exiting the town at site 9 were quite high, relative to WW influent, suggesting accumulation through the system and little degradation/sorption. However, all analytes presented some degradation over 24 hours (approx. 20-40%), SI fig.5. Some temperature-dependent degradation was observed in MTZ; which may have contributed to the decreased loads detected during summer.
Insufficient data was obtained to assess the spatiotemporal behaviour of the antifungals, but example results are presented for metronidazole, Figure 9. The parent-metabolite ratio changed through the WWTP (site 6: 1.56 ± 0.27, versus site 7: 0.64 ± 0.21). The increased proportion of metabolite in WW effluent could indicate analyte-specific treatment efficacy, or the occurrence of biotransformation. On the 20/05/2019 sampling date, the drug and metabolite is observed to enter and persist downstream. This apparent anomaly is validated by the presence of both parent and metabolite, as well as the dissemination through downstream sites.

3.5.8. Phenicols, cyclines, nitrofurans

Analytes included chloramphenicol, its metabolite, and florfenicol (phenicols); tetracycline, oxytetracycline, and doxycycline (cyclines); nitrofurantoin and its metabolite (nitrofurans). The highest prevalence was typically doxycycline, and the lowest was the chloramphenicol metabolite - with almost no detection throughout. Higher loads were measured for July and Sept in the river samples, whereas WWTP sites observed largely constant levels, SI fig.35. Florfenicol was observed to be largely stable, whereas all other analytes degraded over the 24 hours, SI fig.5. This effect was most significant and temperature-dependant for chloramphenicol, nitrofurantoin, and their metabolites (up to 80% degradation).

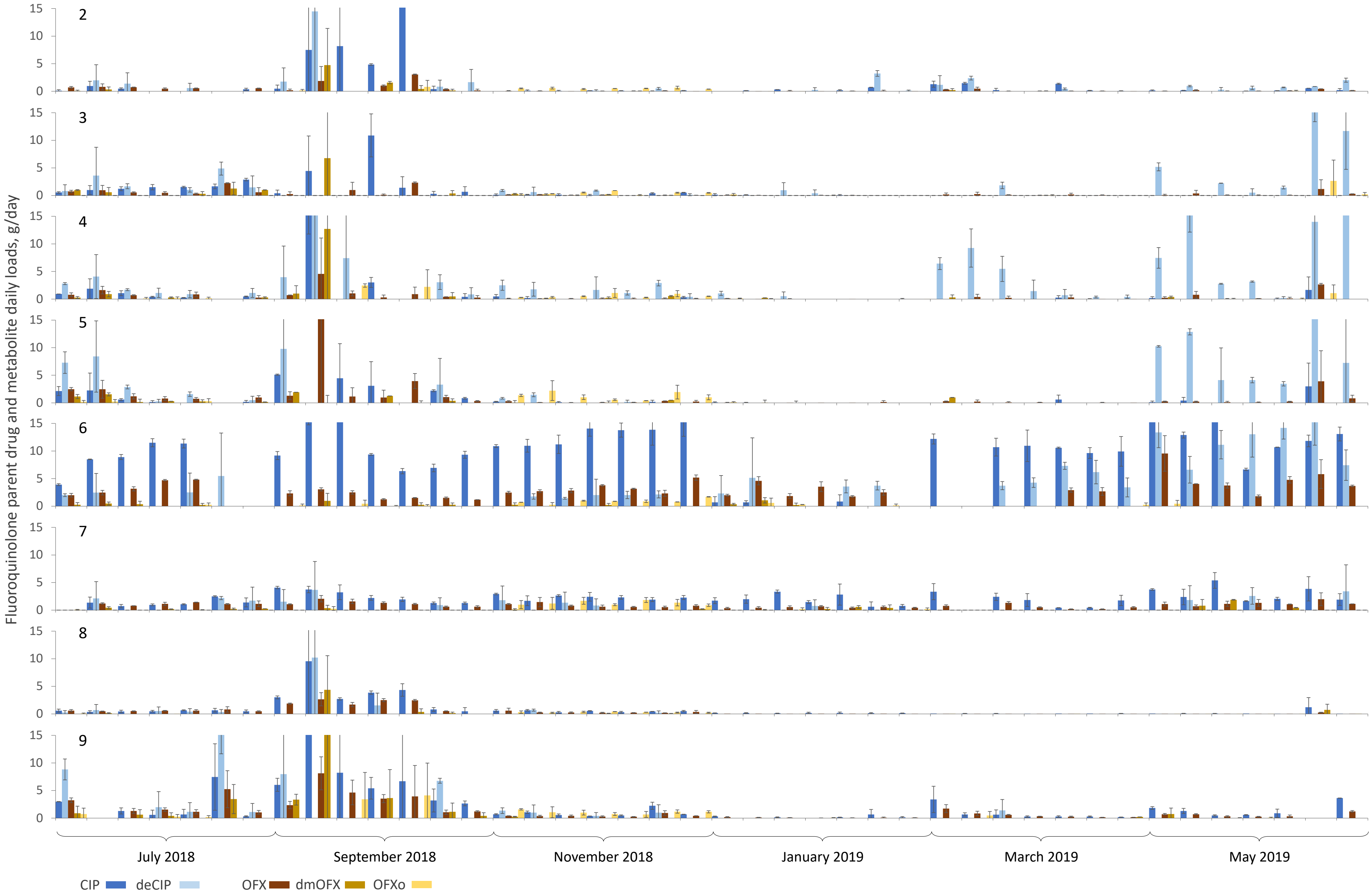

Figure 5 Fluoroquinolone parent-metabolite daily loads. The y-axes were fixed at 15 g/day (full dataset is available in SI, fig.47-49). Error bars display standard deviation, n=2.

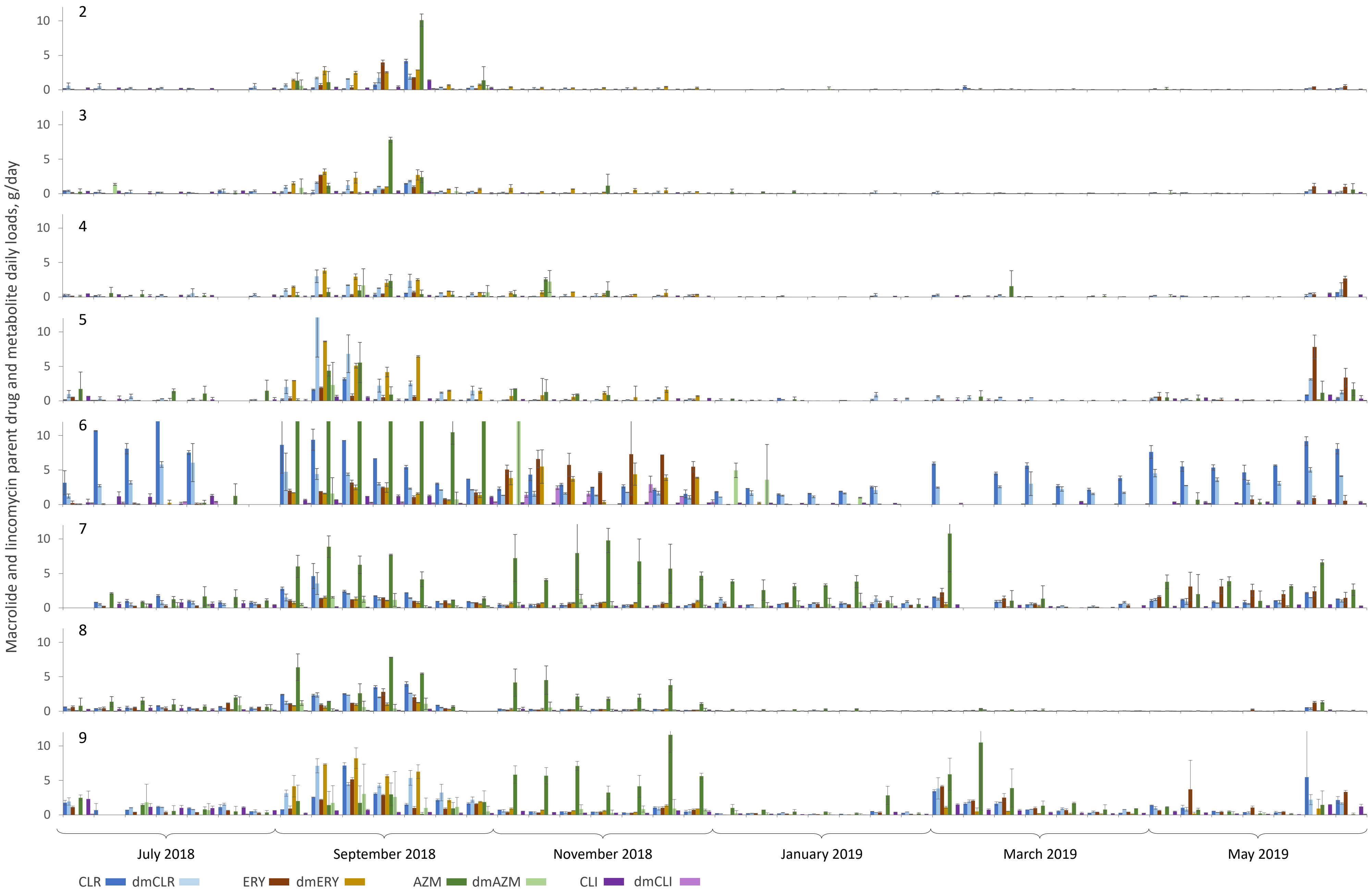

Figure 6 Macrolide and lincomycin parent-metabolite daily loads. The y-axes were fixed at 12 g/day (full dataset is available in SI, fig.50-53). Error bars display standard deviation, n=2.

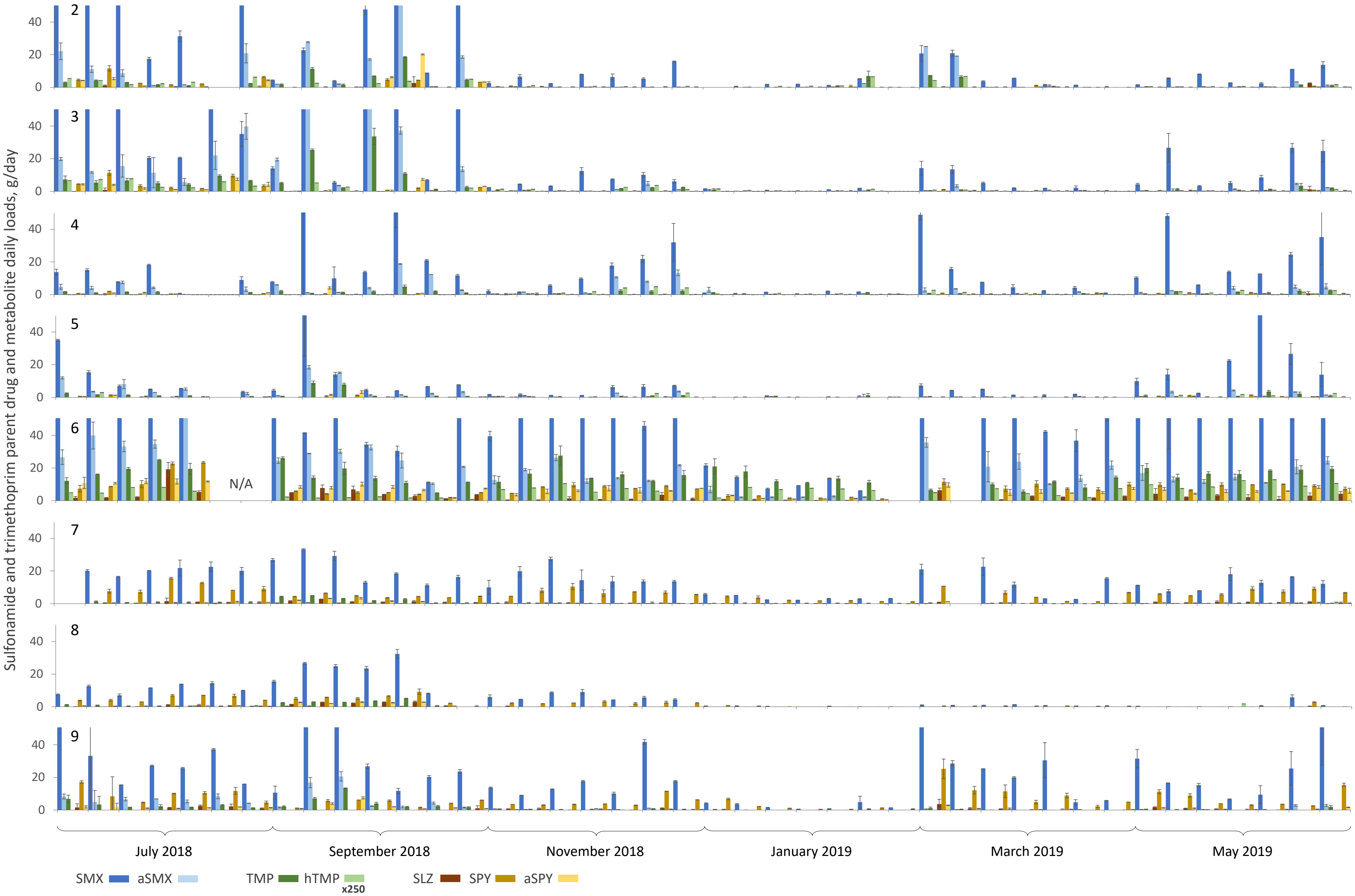

Figure 7 Sulfonamide and trimethoprim parent-metabolite daily loads. The y-axes were fixed at 50 g/day (full dataset is available in SI, fig.54-57). Error bars display standard deviation, n=2.

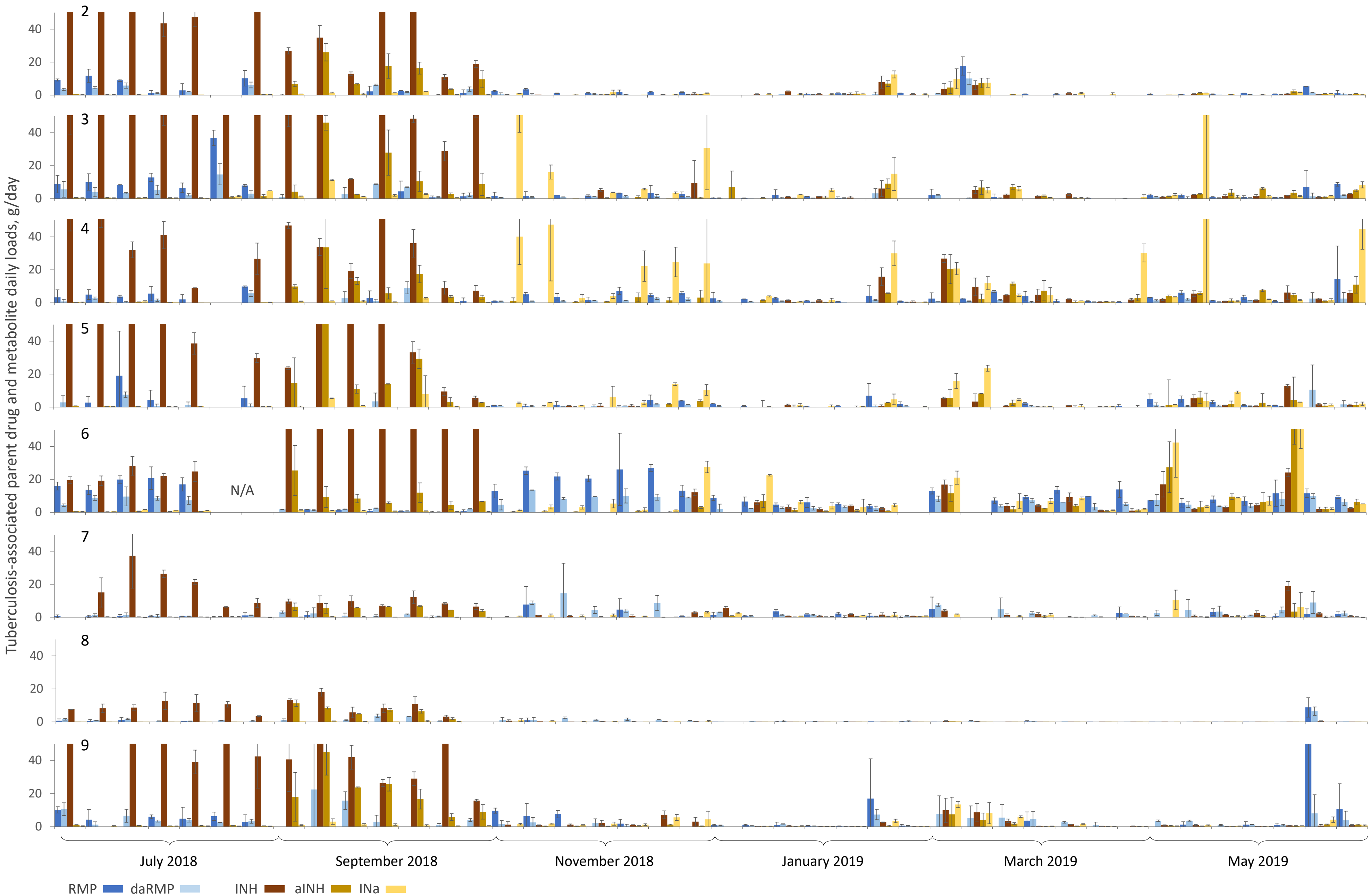

Figure 8 Tuberculosis-associated drug parent-metabolite daily loads. The y-axes were fixed at 50 g/day (full dataset is available in SI, fig.58-59). Error bars display standard deviation, n=2.

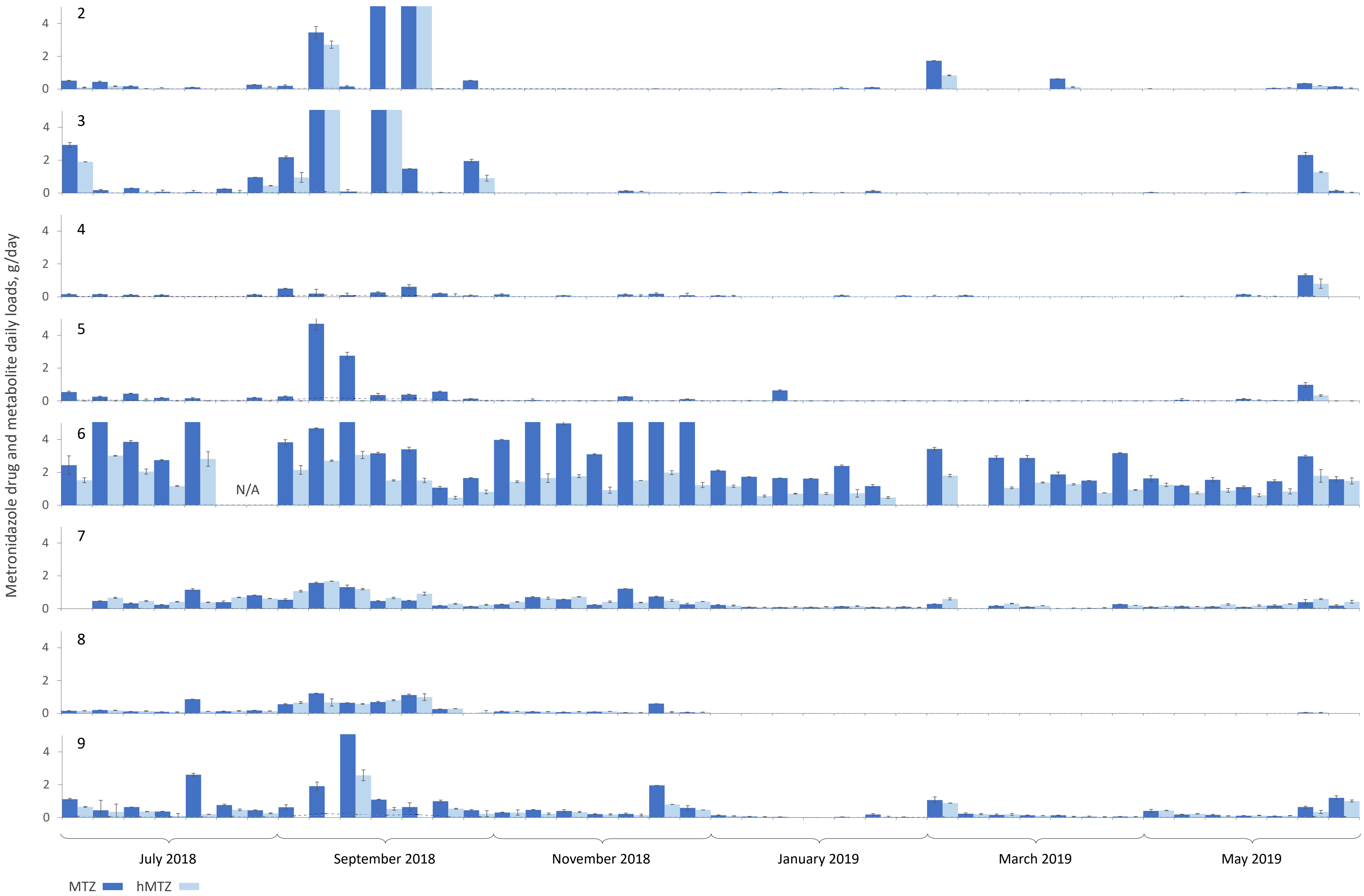

Figure 9 Metronidazole parent-metabolite daily loads. The y-axes were fixed at 5 g/day (full dataset is available in SI, fig.60). Error bars display standard deviation, n=2.

4. Conclusion

The spatiotemporal patterns for several antimicrobial groups (quinolones, macrolides, sulfonamides, beta-lactams, aminoglycosides, azoles, antiretrovirals) have been mapped for the Eerste river, considering both community behaviour as well as the antibiotic physical-chemical properties. The monitoring methodology, in terms of sampling locations and frequency, resulted in a comprehensive overview the catchment. Although the population estimates for those contributing to municipal sewage wastewater (178k) versus settlement-derived waste (6-12k) were approximated, they enabled better context for the catchment dynamics. The significance being that despite the large disparity in number of individuals with and without access to sanitation, the waste entering the Plankenbrug river remained untreated as it disseminated through the town.

ARVs, emtricitabine and lamivudine, were the most prevalent drugs throughout the monitoring campaign, followed by tuberculosis drugs and sulfonamides. ARVs were, however, very well reduced via WW treatment processes (>97%). This was also the case for beta-lactams, nitrofurantoin, and trimethoprim. The WWTP efficacy for other drugs was more variable, that did not appear to have temporal significance.

Spatial trends were well mapped, via sampling locations that were designed to account for each river confluence and possible hotspots for contamination. Analyte dissemination was traceable throughout the system. Temporal variability was also demonstrated, however there were many associated factors to consider, including drug intake due to seasonal ailments; drug intake due to population fluctuation; weather patterns impacting dissemination; and weather patterns impacting analyte stability. Sewage is less likely to experience the effect of weather changes. Therefore, temporal comparisons for river and WWTP influent suggested whether a pattern was weather dependent or independent. An example of this was that azoles and cephalosporins had largely consistent levels in WWTP influent, but lower than average levels in river samples during summer months/higher than average during rainy season.

Acknowledgements

Elizabeth Holton would like to acknowledge University of Bath for funding her PhD. The support of Engineering and Physical Sciences Research Council (EP/P028403/1) is greatly appreciated.

Conflicts of interests/Competing interests

Authors declare no conflict of interests

Supplementary material:

Content:

Author statement:

Elisabeth Holton: Conceptualisation, Methodology (experimental design, liquid chromatography and mass spectrometry), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Edward Archer (sampling strategy), Conceptualisation, Methodology (experimental design, sampling), Writing - review editing James Fidal: Conceptualisation, Methodology (flow modelling), Writing - review editing, Thomas Kjeldsen: Conceptualisation, Writing – review editing; Gideon Wolfaardt: Conceptualisation, Writing - review editing, Project administration, Resources; Barbara Kasprzyk-Hordern: Conceptualisation, Writing-original draft, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources.

References

Ambole, A., M. Swilling and M. M'Rithaa (2016). "Designing for informal contexts: A case study of Enkanini sanitation intervention." **10**: 75-84.

Castiglioni, S., R. Bagnati, R. Fanelli, F. Pomati, D. Calamari and E. Zuccato (2006). "Removal of pharmaceuticals in sewage treatment plants in Italy." Environ Sci Technol **40**(1): 357-363.

Castrignanò, E., A. M. Kannan, E. J. Feil and B. Kasprzyk-Hordern (2018). "Enantioselective fractionation of fluoroquinolones in the aqueous environment using chiral liquid chromatography coupled with tandem mass spectrometry." Chemosphere **206**: 376-386.

Castrignano, E., A. M. Kannan, K. Proctor, B. Petrie, S. Hodgen, E. J. Feil, S. E. Lewis, L. Lopardo, D. Camacho-Munoz, J. Rice, N. Cartwright, R. Barden and B. Kasprzyk-Hordern (2020). "(Fluoro)quinolones and quinolone resistance genes in the aquatic environment: A river catchment perspective." Water Research **182**.

Choi, K., Y. Kim, J. Park, C. K. Park, M. Kim, H. S. Kim and P. Kim (2008). "Seasonal variations of several pharmaceutical residues in surface water and sewage treatment plants of Han River, Korea." Sci Total Environ **405**(1-3): 120-128.

Diaz-Cruz, M. S. and D. Barcelo (2006). "Determination of antimicrobial residues and metabolites in the aquatic environment by liquid chromatography tandem mass spectrometry." Analytical and Bioanalytical Chemistry **386**(4): 973-985.

Elder, F. C. T., K. Proctor, R. Barden, W. H. Gaze, J. Snape, E. J. Feil and B. Kasprzyk-Hordern (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 Research **203**: 117533.

Fidal, J. and T. Kjeldsen (2020). "Operational Comparison of Rainfall-Runoff Models through Hypothesis Testing." Journal of Hydrologic Engineering **25**: 04020005.

Government, S. "Ward Boundaries of Stellenbosch Municipality." 2021, from https://stellenbosch.gov.za/wards/.

Government, W. C. (2014). Settlement Profile: Stellenbosch Municipality.

Government, W. C. (2018). Socio-economic Profile: Stellenbosch Municipality. Stellenbosch.gov.za.

Government, W. C. (2019). Socio-economic Profile: Stellenbosch Municipality. Stellenbosch.gov.za.

Government, W. C. (2020). Socio-economic Profile: Stellenbosch Municipality. Stellenbosch.gov.za.

Gros, M., M. Petrovic and D. Barcelo (2006). "Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters." Talanta **70**(4): 678-690.

Gulkowska, A., H. W. Leung, M. K. So, S. Taniyasu, N. Yamashita, L. W. Yeung, B. J. Richardson, A. P. Lei, J. P. Giesy and P. K. Lam (2008). "Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China." Water Res **42**(1-2): 395-403.

Hendricks, R. and E. J. Pool (2012). "The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues." Journal of Environmental Science and Health, Part A **47**(2): 289-297.

Holton, E. and B. Kasprzyk-Hordern (2021). "Multiresidue antibiotic-metabolite quantification method using ultra-performance liquid chromatography coupled with tandem mass spectrometry for environmental and public exposure estimation." Analytical and Bioanalytical Chemistry.

Kasprzyk-Hordern, B., K. Proctor, K. Jagadeesan, L. Lopardo, K. J. O'Daly, R. Standerwick and R. Barden (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**: 106331.

Kim, C., H. D. Ryu, E. G. Chung and Y. Kim (2018). "Determination of 18 veterinary antibiotics in environmental water using high-performance liquid chromatography-q-orbitrap combined with on-line solid-phase extraction." Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **1084**: 158-165.

Luis Martinez, J. (2009). "Environmental pollution by antibiotics and by antibiotic resistance determinants." Environmental Pollution **157**(11): 2893-2902.

Meyer, G. M. (2016). Methods of engagement;

a transdisciplinary learning approach to the problem of the Eerste River. Master of Philosophy Masters, Stellenbosch University.

Michael, I., L. Rizzo, C. S. McArdell, C. M. Manaia, C. Merlin, T. Schwartz, C. Dagot and D. Fatta-Kassinos (2013). "Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review." Water Res **47**(3): 957-995.

Milic, N., M. Milanovic, N. G. Letic, M. T. Sekulic, J. Radonic, I. Mihajlovic and M. V. Miloradov (2013). "Occurrence of antibiotics as emerging contaminant substances in aquatic environment." International Journal of Environmental Health Research **23**(4): 296-310.

Mosekiemang, T. T., M. A. Stander and A. de Villiers (2019). "Simultaneous quantification of commonly prescribed antiretroviral drugs and their selected metabolites in aqueous environmental samples by direct injection and solid phase extraction liquid chromatography - tandem mass spectrometry." Chemosphere **220**: 983-992.

Polesel, F., H. R. Andersen, S. Trapp and B. G. Plósz (2016). "Removal of Antibiotics in Biological Wastewater Treatment Systems—A Critical Assessment Using the Activated Sludge Modeling Framework for Xenobiotics (ASM-X)." Environmental Science & Technology **50**(19): 10316-10334.

Proctor, K., B. Petrie, L. Lopardo, D. C. Muñoz, J. Rice, R. Barden, T. Arnot and B. Kasprzyk-Hordern (2021). "Micropollutant fluxes in urban environment - A catchment perspective." J Hazard Mater **401**: 123745.

Seeliger, L. and I. Turok (2013). "Averting a downward spiral: building resilience in informal urban settlements through adaptive governance." Environment and Urbanization **26**(1): 184-199.

Sinthuchai, D., S. K. Boontanon, N. Boontanon and C. Polprasert (2015). "Evaluation of removal efficiency of human antibiotics in wastewater treatment plants in Bangkok, Thailand." Water Science and Technology **73**(1): 182-191.

Speltini, A., M. Sturini, F. Maraschi and A. Profumo (2010). "Fluoroquinolone antibiotics in environmental waters: Sample preparation and determination." Journal of Separation Science **33**(8): 1115-1131.

Statistics-SA (2016). Provincial Profile: Western Cape Community Survey 2016**:** 99.

Stellenbosch-Municipality (2012). ENKANINI (KAYAMANDI) HOUSEHOLD ENUMERATION REPORT, Community Organisation Reseource Centre & ISN**:** 19.

Stellenbosch-Municipality (2014). Municipal Profile**:** 1-8.

Tamtam, F., F. van Oort, B. Le Bot, T. Dinh, S. Mompelat, M. Chevreuil, I. Lamy and M. Thiry (2011). "Assessing the fate of antibiotic contaminants in metal contaminated soils four years after cessation of long-term waste water irrigation." Sci Total Environ **409**(3): 540-547.

Thiebault, T. (2020). "Sulfamethoxazole/Trimethoprim ratio as a new marker in raw wastewaters: A critical review." Science of The Total Environment **715**: 136916.

Verlicchi, P., M. Al Aukidy and E. Zambello (2012). "Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment—A review." Science of The Total Environment **429**: 123-155.

Watkinson, A. J., E. J. Murby and S. D. Costanzo (2007). "Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling." Water Research **41**(18): 4164-4176.

Yuan, S. F., Z. H. Liu, H. Yin, Z. Dang, P. X. Wu, N. W. Zhu and Z. Lin (2019). "Trace determination of sulfonamide antibiotics and their acetylated metabolites via SPE-LC-MS/MS in wastewater and insights from their occurrence in a municipal wastewater treatment plant." Sci Total Environ **653**: 815-821.

Zhu, T.-t., Z.-x. Su, W.-x. Lai, Y.-b. Zhang and Y.-w. Liu (2021). "Insights into the fate and removal of antibiotics and antibiotic resistance genes using biological wastewater treatment technology." Science of The Total Environment **776**: 145906.

Zibagwe, S. (2016). Struggle for urban citizenship in South Africa: Agency and politics in the Enkanini upgrading project, Stellenbosch. Doctor of Social Anthropology Dissertation, Stellenbosch University.