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Predicting selection for antimicrobial resistance in UK wastewater and aquatic environments: Ciprofloxacin poses a significant risk

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ABSTRACT

Antimicrobial resistance (AMR) is a threat to human and animal health, with the environment increasingly recognised as playing an important role in AMR evolution, dissemination, and transmission. Antibiotics can select for AMR at very low concentrations, similar to those in the environment, yet their release into the environment, e.g., from wastewater treatment plants, is not currently regulated. Understanding the selection risk antibiotics pose in wastewater and receiving waters is key to understanding if environmental regulation of antibiotics is required. We investigated the risk of selection occurring in UK wastewater and receiving waters by determining where measured environmental concentration data (n = 8187) for four antibiotics (ciprofloxacin, azithromycin, clarithromycin, and erythromycin) collected in England and Wales 2015–2018 (sites n = 67) exceeded selective concentration thresholds derived from complex microbial community evolution experiments undertaken previously. We show that selection for AMR by ciprofloxacin is likely to have occurred routinely in England and Wales wastewater during the 2015–2018 period, with some seasonal and regional trends. Wastewater treatment reduces the selection risk posed by ciprofloxacin significantly, but not completely, and predicted risk in surface waters remains high in several cases. Conversely, the potential risks posed by the macrolides (azithromycin, clarithromycin, and erythromycin) were lower than those posed by ciprofloxacin. Our data demonstrate further action is needed to prevent selection for AMR in wastewater, with environmental quality standards for some antibiotics required in the future, and that selection risk is not solely a concern in low/middle income countries.

1. Introduction

Antimicrobial resistance (AMR) is a significant threat to society. The One Health approach to combatting AMR encourages multi-sectoral and holistic efforts across clinical, animal, and natural environments (O'Neill, 2016). However, the natural environment remains one of the less well-studied aspects, despite being recognised in several national and international policy documents (EU, 2017; Government, 2019; UNEP, 2017) as a key target for future research and mitigation.

Antibiotics and antibiotic resistant pathogens are released into the environment daily from a variety of anthropogenic sources. Following incomplete metabolism within the human body, antibiotics are excreted in urine and faeces (Kummerer, 2009). These pass through the wastewater treatment plant (WWTP) system, which is not designed to remove pharmaceuticals. Therefore, active antibiotics are present in wastewater and surface waters, introduced by WWTPs. Surface waters can also be contaminated further by run off from agricultural land fertilised with human sewage sludge or animal manures containing antibiotics (Kummerer, 2009), and combined sewer overflows. Previous research indicates environmental reservoirs of AMR may pose an exposure risk in humans (Leonard et al., 2015; Leonard et al., 2018). One possible way of reducing exposure to environmental reservoirs of AMR is reducing *in situ* selection for AMR by antibiotics, thereby reducing numbers of resistant bacteria and resistance genes present in the environment (Ashbolt et al., 2013).

Though antibiotics are present at very low concentrations in the environment, a growing body of research demonstrates these can select for AMR (Gullberg et al., 2014; Gullberg et al., 2011; Kraupner et al.,

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2018; Kraupner et al., 2020; Lundstrom et al., 2016; Murray et al., 2020; Murray et al., 2018; Stanton et al., 2020). In some cases, the potency of selection can be relatively constant across a large concentration range, from environmentally relevant concentrations, up to clinically relevant concentrations (Murray et al., 2018).

However, environmental quality standards (EQS) or other recognised limits for antibiotics do not exist, meaning their release into the environment is not currently regulated. This is partly due to the lack of a standardised experimental method that can be used routinely to generate data on the lowest concentration of an antibiotic that selects for AMR in a microbial population (Ashbolt et al., 2013). To address this issue, we recently published the SELECT method (Murray et al., 2020). We showed that the growth-based SELECT method is a reliable proxy for selection for key AMR marker genes in sewage derived complex microbial communities, and it can be used to perform rapid assessment of an antibiotic's selective potential, with results similar to more complex and expensive experimental systems (see (Murray et al., 2021) for a review of different methodologies).

There is a continued debate around the most reliable method to determine selective concentrations of antibiotics (or predicted no effect concentrations for resistance, 'PNEC^{R}s') (Murray et al., 2021). Generally, PNEC^{R}s derived from exposure experiments with complex communities of bacteria are considered more environmentally representative than PNEC^{R}s based on data from individual strains, or individual species (Larsson and Flach, 2021; Murray et al., 2021).

In this study, we compared selective concentration data for four antibiotics (ciprofloxacin, azithromycin, clarithromycin, and erythromycin) with their measured concentrations in untreated and treated wastewater in England and Wales. These Measured Environmental Concentration (MEC) data were collected during the Chemicals Investigation Programme Phase 2 (CIP2), conducted by UK Water Industry Research (UKWIR), which ran from 2015 to 2018. A total of 8187 MECs across 67 sites were used to predict selection risk. These antibiotics were selected by UKWIR based on the EU Commission Water Framework Directive Watch List of potentially hazardous substances at the time (Loos et al., 2018), for which EQS may be required in the future.

2. Materials and methods

2.1. Measured environmental concentration (MEC) data

MEC data were collected by the UKWIR CIP2, which ran from 2015 to 2018. Further information detailing the sampling methods, and the different components of CIP2, can be found at the UKWIR website under supporting documents 'Technical Specification and Guidance', and 'Project Overview' (UKWIR, 2020b).

Briefly, pharmaceutical compounds were collected using decontaminated aluminium or stainless-steel buckets and cleaned glass bottles. Samples were stored at 3–5 °C for a maximum of five days before analysis. The methods of analysis used were not specifically detailed by UKWIR but were in accordance with Directive 2000/60/EC of the European Union and EN ISO/IEC-17025.

MEC data for ciprofloxacin, erythromycin, azithromycin, and clarithromycin were downloaded from the UKWIR website (UKWIR, 2020a) on 8/12/2020 (Supplementary File 1). These represent all the antibiotics included in CIP2 (Supplementary Table 1), with exception of norerythromycin (which was excluded from this study, as no selective concentration data for norerythromycin exist). The WWTPs monitored

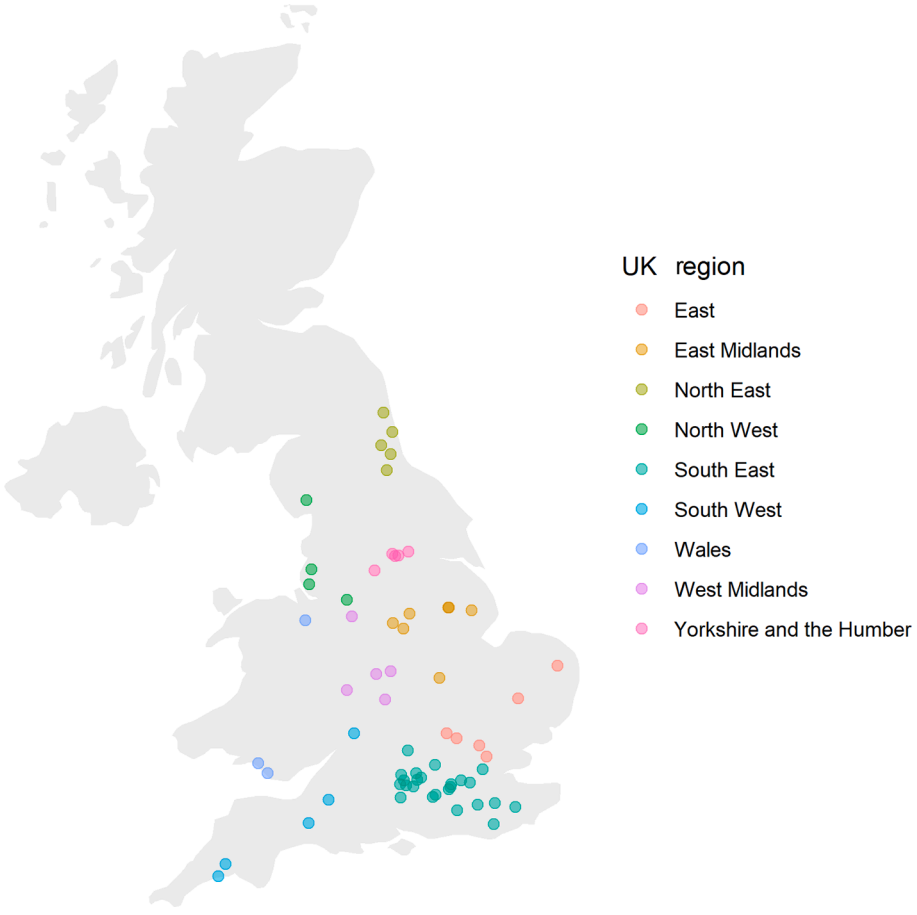

Fig. 1. Map of the UK, showing Wastewater Treatment Plants (WWTPs) used in this analysis of antibiotics in the CIP2 programme. Points are coloured corresponding to the UK region.

in CIP2 (Supplementary Table 2) were regarded as those most 'at risk', chosen to represent sites at which there was low-dilution, and other factors not under the control of the Water Industry that would likely lead to non-compliance for EQS. It is unknown if any of these treat pharmaceutical manufacturing effluent. However, these sites were considered representative of the range of sizes and types of WWTPs across England and Wales by UKWIR. We stratified the data into the nine regions of England and Wales to enable geographical analysis (Fig. 1). Fig. 1 was created in R using the maps package (v. 3.3), and ggplot2 (v 3.3.5).

2.2. Predicted no effect concentration for resistance ($PNEC^R$) data

The ciprofloxacin SELECT $PNEC^R$ was determined by culturing a complex community of wastewater influent-derived bacteria in Iso-Sensitest broth, at 37 °C, shaking, spiked with a twofold dilution series of ciprofloxacin. Note, different culturing conditions (i.e., reduced temperature and use of artificial sewage as the growth medium, as per the OECD activated sludge respiration inhibition test (OECD, 2009)) were shown to have minimal effect on the lowest observed effect concentration in the previous study, with no $PNEC^R$s differing by more than a factor of four (Murray et al., 2020). The lowest observed effect concentration was determined as the lowest antibiotic concentration where bacterial growth (measured by optical density) was significantly reduced compared to the no-antibiotic control, at the time point during exponential growth phase where the dose–response relationship between growth and antibiotic concentration was strongest. This effect concentration has been shown in good agreement with selection for key AMR genes (Murray et al., 2020). The standard SELECT $PNEC^R$ (i.e. with standard culturing conditions, Table 1, (Murray et al., 2020)) was used to calculate risk quotients (RQs) for ciprofloxacin. This $PNEC^R$ is at the lower to mid-range of available experimentally derived $PNEC^R$s currently available, and similar to $PNEC^R$s extrapolated from minimum inhibitory concentrations (MICs) of individual clinical strains (Table 1). The no observed effect concentration was taken as the concentration directly below the lowest observed effect concentration. $PNEC^R$s were determined by applying an assessment factor of 10 to the no observed effect concentration (Murray et al., 2020).

For the macrolides, we used the lowest experimental $PNEC^R$ available, also determined previously (Stanton et al., 2020), using a complex community (Table 1). Briefly, a wastewater influent community was cultured in Iso-Sensitest broth, at 37 °C, shaking, with varying concentrations of azithromycin, clarithromycin or erythromycin for 24 h, at which point 50 µl culture was transferred into fresh 5 ml media supplemented with antibiotic. After seven days serial passage, DNA was extracted, and qPCR used to determine the prevalence of macrolide specific resistance genes (normalised to 16S rRNA copy number). The $PNEC^R$s used in this study were based on the macrolide specific resistance gene *ermF* (the lowest reported (Stanton et al., 2020)) and calculated by applying an assessment factor of 10 to the no observed effect concentration.

2.3. Assessing risk of AMR selection in wastewater influent and effluent

RQs were calculated for both wastewater influent and wastewater effluent by dividing MECs by the $PNEC^R$s generated previously (Murray et al., 2020; Stanton et al., 2020). All statistical analyses were carried out in R and normality was assessed with the Shapiro-Wilk test. For comparisons of overall influent, effluent, and surface water, Kruskal-Wallis and Dunn's tests were used to calculate statistical significance between RQ values. The Wilcoxon rank sum test was used to test the effect of treatment on risk. Kruskal-Wallis and Dunn's tests were used to compare significance between RQ values between regions. Risk was assigned as 'High' (red, RQ $\geq$ 10), 'Medium' (orange, RQ $<$ 10 and $\geq$ 1), 'Low' (Yellow, RQ $<$ 1 and $\geq$ 0.1) and'Insignificant' (Green, RQ $<$ 0.1), as per Graae et al. (2016)). We used this approach to qualify particularly high risk, where the margin of safety conferred by the assessment factor is removed, i.e., a RQ of 10 indicates the antibiotic has been measured at the no observed effect concentration, not the $PNEC^R$.

Table 1
Predicted no effect concentrations for resistance ($PNEC^R$s) data, for antibiotics in this study. [1]indicates the $PNEC^R$s used in this study for generation of Risk Quotients (RQs). *indicates these are minimal selective concentrations derived using selection coefficients (i.e., not $PNEC^R$s derived using statistics to define lowest observed effect concentration with assessment factor applied to the no observed effect concentration) (Murray et al., 2021).

Antibiotic	$PNEC^R$ (µg/L)	Method	Reference
Azithromycin	50[1]	Complex community evolution experiment	(Murray et al., 2020)
Azithromycin	50[1]	Complex community evolution experiment	(Stanton et al., 2020)
Azithromycin	0.25[1]	Extrapolated from MIC data collected for individual clinical strains	(Bengtsson-Palme & Larsson, 2016)
Clarithromycin	250[1]	Complex community evolution experiment	(Murray et al., 2020)
Clarithromycin	50[1]	Complex community evolution experiment	(Stanton et al., 2020)
Clarithromycin	0.25[1]	Extrapolated from MIC data collected for individual clinical strains	(Bengtsson-Palme & Larsson, 2016)
Erythromycin	3000*	Single strain competition experiment	(Gullberg et al., 2014)
Erythromycin	1250[1]	Complex community evolution experiment	(Murray et al., 2020)
Erythromycin	50[1]	Complex community evolution experiment	(Stanton et al., 2020)
Erythromycin	1[1]	Extrapolated from MIC data collected for individual clinical strains	(Bengtsson-Palme & Larsson, 2016)
Ciprofloxacin	10.77*	Complex community evolution experiment	(Stanton et al., 2020)
Ciprofloxacin	0.78	Complex community evolution experiment	(Stanton et al., 2020)
Ciprofloxacin	0.1[1]	Complex community evolution experiment	(Murray et al., 2020)
Ciprofloxacin	0.1*	Single species competition experiment	(Gullberg et al., 2011)
Ciprofloxacin	0.004*	Single species competition experiment	(Vos et al., 2020)
Ciprofloxacin	0.064	Extrapolated from MIC data collected for individual clinical strains	(Bengtsson-Palme & Larsson, 2016)

2.4. Assessing risk of AMR selection in surface waters

Few downstream MECs (n = 60, all for one WWTP, Supplementary Table 8) were collected, preventing analysis of surface water risks across different locations and timepoints. Therefore, a dilution factor of 10 was applied to wastewater effluent MECs, to create predicted environmental concentrations (PECs) for surface water, as recommended by the European Medicines Agency (EMA, 2018). These PECs were then used to calculate RQs for surface water per region ($PEC/PNEC^R$). For comparison of risk in wastewater effluent to predicted surface water, the Wilcoxon rank sum test was used.

3. Results

3.1. Selection risk posed by antibiotics in wastewater influent and effluent

MEC data for ciprofloxacin, azithromycin, clarithromycin, and erythromycin in wastewater influent and effluent were used alongside wastewater microbial community selective concentration data generated previously (Murray et al., 2020; Stanton et al., 2020) to calculate RQs. Ciprofloxacin had RQs $\geq$ 1, indicating a high probability that selection for AMR occurs in most wastewaters sampled. MEC data for

ciprofloxacin are illustrated in Supplementary Fig. 1, and a summary is shown in Supplementary Table 3.

No RQs ≥ 1 were generated for azithromycin, clarithromycin or erythromycin using the lowest experimental $PNEC^R$s available of 50 μg/L (Stanton et al., 2020) (Supplementary Tables 4, 5 and 6). Norerythromycin was also monitored in CIP2 but RQs were not generated, as norerythromycin selective concentration data are currently unavailable. Following these findings, further analyses were conducted for ciprofloxacin only.

3.2. Wastewater treatment reduces selection risk significantly, but not completely

We compared ciprofloxacin RQs in influent and effluent. We also generated RQs for receiving surface waters by applying a dilution factor of 10 to the effluent concentration to generate a PEC. Worryingly, 30.76 % of all influents (n = 307 of 998), and 2.79 % (n = 28 of 1004) of all effluents, had RQs ≥ 10, indicating a significant risk of AMR selection, as this negates the assessment factor of 10 used to generate the $PNEC^R$. Percentages of RQ ≥ 1 for influent, effluent, and surface water were 90.08 % (n = 899 of 998), 44.82 % (n = 450 of 1004), and 2.79 % (n = 28 of 1004) respectively, indicating widespread risk of AMR selection, particularly in influent and effluent. As wastewater treatment was shown to significantly reduce risk, further analyses were conducted on influent and effluent separately.

For surface water, 42.03 % (n = 422 of 1004) of RQs were 'low risk' (Fig. 2). Risk of AMR selection significantly decreased after wastewater treatment, and decreased further in surface water, according to predicted values (both $p < 0.05$, Supplementary Table 7). To assess the reliability of the dilution factor used to calculate PECs, we compared effluent MECs with date matched downstream MECs, where available (in total, there were 60 downstream MECs, all from the same WWTP). We then compared these downstream MECs with the PECs derived in this study (calculated by dividing the effluent MEC by a dilution factor of 10, Supplementary Table 8). In general, there was good agreement between the downstream MECs and surface water PECs, with an average dilution factor of 9.68. However, some downstream MECs were far less dilute than predicted, with some having a dilution factor close to (or even less than) 1, meaning antibiotics were present in the downstream water at concentrations similar to the effluent. There were also significantly more instances where the actual dilution factor was < 10 than where the dilution factor was > 10 (Supplementary Table 9).

3.3. Risk of selection for AMR by ciprofloxacin throughout the seasons

To address the risk ciprofloxacin poses in wastewater, it is important to prioritise where mitigation strategies may have the largest impact. Therefore, we conducted further analyses to determine if risk varied significantly according to meteorological season (spring, summer, autumn, and winter) (Fig. 3). For influent, risk in autumn was significantly higher than all other seasons ($p < 0.05$, mean RQ = 14.903, median RQ = 6.3, max RQ = 99.4, see Supplementary Table 10 and Table 11). In effluent, all seasons had a medium risk of AMR selection by ciprofloxacin in most cases, yet instances of high risk still occurred. Autumnal effluent RQs were still higher on average than other seasons, but not significantly so (Supplementary Table 10). Risk in summer effluent was significantly lower than all other seasons ($p < 0.05$, mean RQ = 1.12, median RQ = 0.54, max RQ = 15.44, Supplementary Table 10 and Table 11). No other statistical differences were found between seasons and statistics were not performed on individual years due to inconsistent sample size (see Supplementary Fig. 2).

3.4. Geographical differences in risk of selection by ciprofloxacin were observed

An additional factor that would be useful for mitigation would be to determine whether different geographical regions pose different risks of AMR selection. Therefore, we stratified all RQ data by UK region (Fig. 4). For wastewater influent, the East of England had the highest percentage (59.84 %) of RQs ≥ 10. For wastewater effluent, the South West had the highest percentage of RQs ≥ 10 (5.16 %), however, the East Midlands had a greater percentage of RQs ≥ 1 (91.43 %). Surface water estimations reduced the risk so that no regions had RQs ≥ 10, however, the South West had the greatest number of RQs ≥ 1 (7.77 %). All geographical regions had at least one RQ ≥ 1 for both influent and effluent. Statistically significant differences between regions for both influent and effluent are shown in Supplementary Tables 12 and 13.

For wastewater influent, four of nine UK Regions had a mean RQ ≥ 10 (East, East Midlands, South West, and West Midlands), and the remaining five regions had mean RQs ≥ 1, indicating a risk for selection for AMR. All regions also had a median RQ ≥ 1, with two regions (East, and East Midlands) having median RQs ≥ 10 (Supplementary Table 14). Means ranged from a high of 20.45 in the East of England, to a low of 3.74 in Wales. Medians ranged from 13.40 in the East of England, to 2.40 in Wales. However, several regions had significantly high RQ outliers compared to the median (Fig. 5A). For example, the South East had a median RQ of 5.31 but a maximum RQ of 93.20.

For wastewater effluent, seven regions had a mean RQ ≥ 1, indicating a medium risk of selection for AMR. All regions had a mean RQ ≥ 0.1 (Supplementary Table 15). Means ranged from a highest RQ of 3.38 in the West Midlands, to 0.48 in the North East. Medians ranged from 2.05 in the East Midlands, to 0.39 in the North East. Again, there were outliers in various regions when compared to the median (Fig. 5B), for example, the North West had a median RQ of 0.50 but a maximum RQ of 79.9. Wastewater treatment still significantly reduced the risk when accounting for UK region (Wilcoxon rank sum test, $p < 0.05$).

The risk of selection for AMR in surface water also reduced significantly compared to wastewater effluent (Wilcoxon Rank Sum Test, $p <$

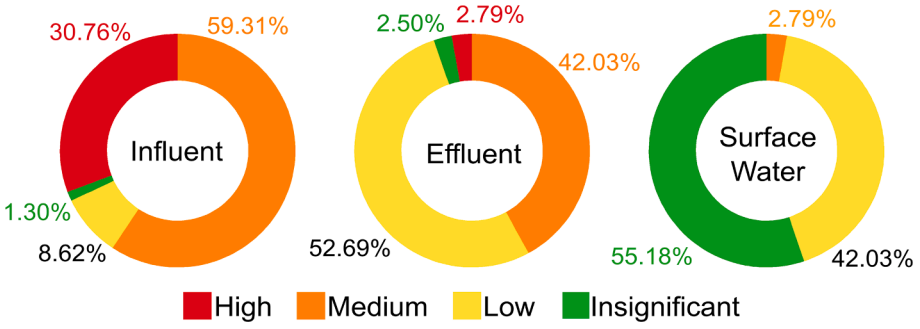

Fig. 2. Percentage of ciprofloxacin Risk Quotient (RQ) values exceeding each risk category. 'High' (Red, RQ ≥ 10), 'Medium' (Orange, RQ < 10 and ≥ 1), 'Low' (Yellow, RQ < 1 and ≥ 0.1) and 'Insignificant' (Green, RQ < 0.1) for wastewater influent, wastewater effluent (both using measured environmental concentrations) and surface water (using predicted environmental concentrations).

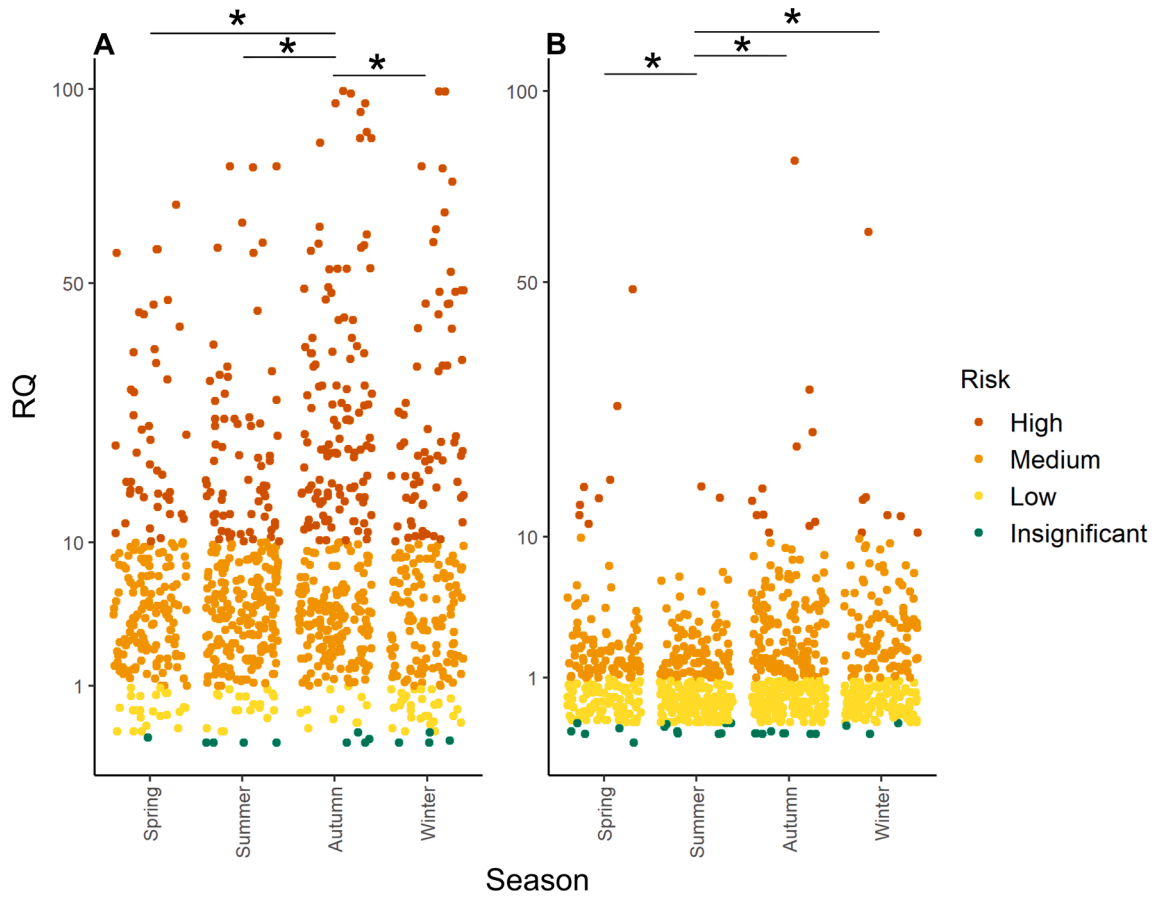

Fig. 3. Ciprofloxacin Risk Quotient (RQ) values for each season for wastewater influent (A) and wastewater effluent (B) with square root transformed y axis. RQ has been categorised into 'High' (Red, RQ $\geq$ 10), 'Medium' (Orange, RQ $<$ 10 and $\geq$ 1), 'Low' (Yellow, RQ $<$ 1 and $\geq$ 0.1) and 'Insignificant' (Green, RQ $<$ 0.1). *Indicates significant differences between seasons, $p < 0.05$, Dunn's test (Supplementary Table 5).

0.05) when assuming a 10-fold dilution. However, there are varying levels of dilution in wastewater receiving rivers, so in some cases, risk may be higher or lower than reported here. Nevertheless, RQs $\geq$ 1 were still found in six regions (Fig. 4C), indicating a risk of AMR selection in these wastewater receiving environments. The North East, Yorkshire and the Humber, and Wales did not have any estimated RQs $\geq$ 1 in surface water.

4. Discussion

Using previously published selective endpoint data (Murray et al., 2020; Stanton et al., 2020) and recent MEC data collected by the UKWIR CIP2 programme, we found that a high selection risk is posed by ciprofloxacin in wastewater influent and wastewater effluent. Using the classification of RQ $\geq$ 10 indicating high risk (Graae et al., 2016), there were many instances where MECs of ciprofloxacin were present at or greater than the no observed effect concentration (i.e., negating the assessment factor applied to generate the $PNEC^R$). The overall reduction in the risk in effluent compared to influent demonstrated that wastewater treatment significantly reduces the risk of selection in effluents and receiving waters for AMR by ciprofloxacin, but not entirely. When wastewater effluent MECs were diluted into surface water PECs, risk was further reduced, but again not entirely, with surface water RQs $\geq$ 1 in some cases. The comparatively higher RQs for ciprofloxacin in this study are likely due to its very low $PNEC^R$ (Murray et al., 2020). However, many other studies have suggested similarly low selective concentrations for ciprofloxacin using different methodologies (Table 1). (Bengtsson-Palme and Larsson, 2016; Kraupner et al., 2018; Stanton et al., 2020; Vestel et al., 2021).

Risk of ciprofloxacin selection is also driven by high ciprofloxacin MECs. The Umweltbundesamt Pharmaceuticals in the Environment database brings together MECs for pharmaceuticals from studies conducted across the world (aus der Beek et al., 2016; UmweltBundesamt, 2019). The maximum and median MECs for ciprofloxacin in this database were as follows: the maximum MEC was 246.1 μg/L (in Indian wastewater influent ('WWTP inflow (untreated)'), measured using resolution liquid chromatography-tandem mass spectrometry (Mohapatra et al., 2016)). The median MEC across the whole database was 0.458 μg/L (UmweltBundesamt, 2019). In effluent, the maximum MEC recorded was 14.11 μg/L (measured in India using solid phase extraction and high-performance liquid chromatography (Archana et al., 2016)), and the median ciprofloxacin MEC across the database was 0.112 μg/L (UmweltBundesamt, 2019). In this study, the maximum and median MECs for ciprofloxacin in wastewater influent were 9.94 μg/L and 0.56 μg/L, and in effluent were 7.97 μg/L and 0.08 μg/L. Though we note it can be difficult to draw comparison across studies as the same extraction methods may not have been used, the fact that the median MEC in influent was higher in this study than globally demonstrates the importance of localised monitoring of antibiotic concentrations to fully appreciate environmental risk. It also illustrates that concentrations found in high-income countries such as the UK can be comparable to those reported globally. However, low/middle income countries (LMICs) have increasing antibiotic consumption rates (114 %, year 2000–2015) (Klein et al., 2018), inadequate sanitation infrastructure (Nadimpalli et al., 2020) and lack of MEC data. Generally, LMICs also have greater numbers of pharmaceutical manufacturing plants, particularly in India, which would contribute to locally higher MECs (Larsson et al., 2007). This could result in higher concentrations of antibiotics in

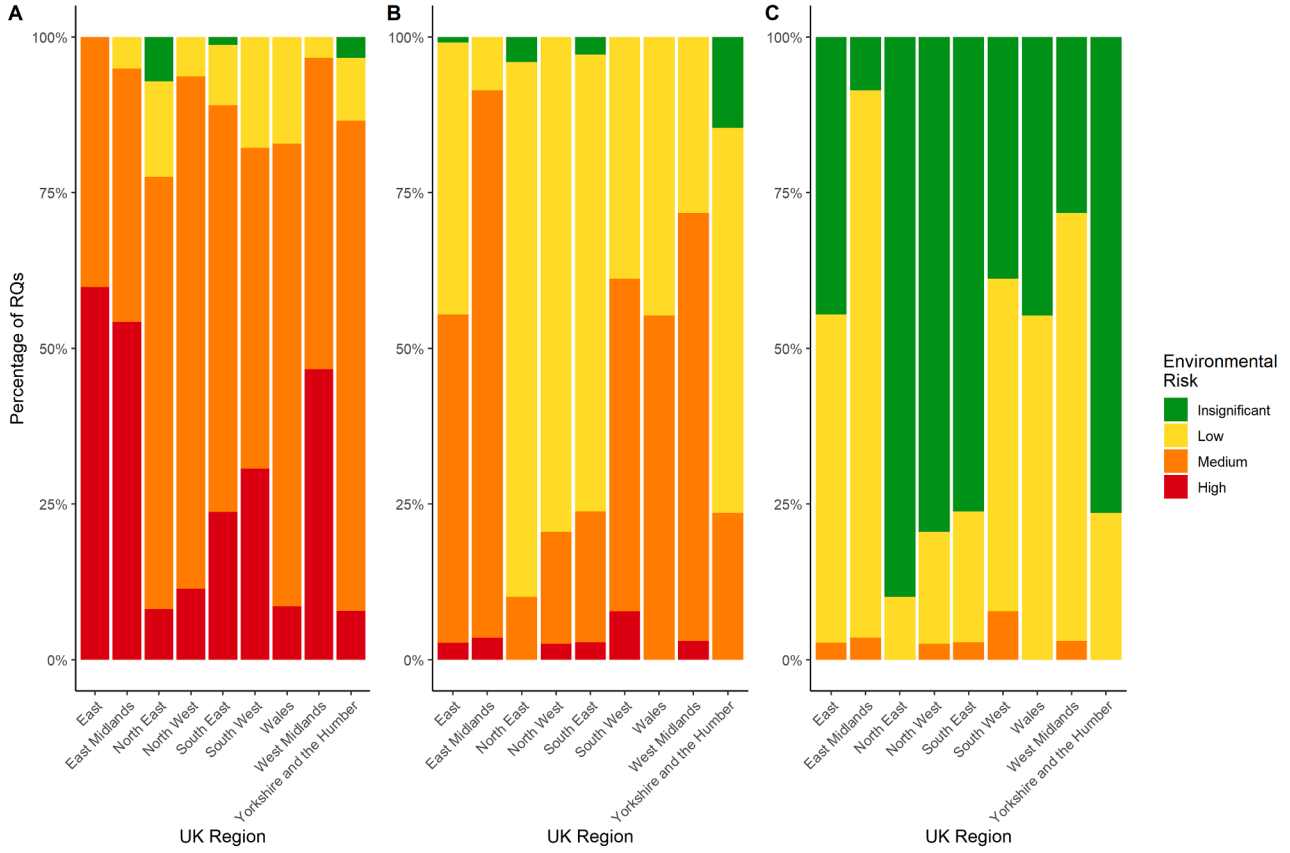

Fig. 4. For ciprofloxacin, A – Risk associated with wastewater influent, B - Wastewater effluent (both using measured environmental concentrations) C –Surface water (using predicted environmental concentrations). Ciprofloxacin Risk Quotient (RQ) has been categorised into 'High' (Red, RQ $\geq$ 10), 'Medium' (Orange, RQ $<$ 10 and $\geq$ 1), 'Low' (Yellow, RQ $<$ 1 and $\geq$ 0.1) and 'Insignificant' (Green, RQ $<$ 0.1).

wastewater receiving environments than indicated from the database used above. For example, a recent global survey reported a maximum concentration of ciprofloxacin of 1.57 μg/L in a river in Angola (Wilkinson et al., 2022). Therefore, the risk of AMR selection due to exceedance of $PNEC^{R}$s is likely to still be higher in LMICs.

Previous studies from other countries have identified relationships between season and antibiotic concentration in different environmental compartments. For example, concentrations of antibiotics, including quinolones, were found to be highest in the autumn in surface water and sediment samples in North Carolina (Gray et al., 2020). Of the WWTPs studied in seven countries by Rodriguez-Mozaz et al. (2020)), Cyprus WWTPs also had the highest concentrations of antibiotics in autumnal effluent compared to spring. Conversely in the same study, higher levels of antibiotics were found in spring effluents, and lower levels in autumn effluents for Spain, Ireland, and Finland. These differences may be explained by the sampling times of the study which were early autumn (the end of summer) and early spring (the end of winter). These findings agree with our data, where we find risk for AMR selection by ciprofloxacin is greatest in autumnal influent. We also found summer effluents had the lowest risk across seasons, possibly due to antibiotic degradation due to higher temperatures (Liao et al., 2016) and/or lower antibiotic use. Based on these findings, to reduce the risk of AMR selection in influent and WWTPs, reduced prescribing, appropriate disposal, and other pre-wastewater treatment mitigation strategies may be best prioritised for the autumn and winter months. This study also suggests end-of-pipe mitigation strategies would be beneficial across most seasons.

Antibiotics are often only partially metabolised in the human body, and can enter WWTPs as a high percentage parent compound (Danner et al., 2019). WWTPs have not been historically designed to remove antibiotics and other compounds from the water system. The ability of different wastewater treatments to reduce antibiotic load has been thoroughly reviewed, e.g., (Le-Minh et al., 2010; Michael et al., 2013). Persistence of antibiotics in the water system will depend upon various factors, such as their chemical properties, but also the pH, light, temperature, and any degradation activity of microorganisms within the WWTP. Sludge retention time is important in the removal of antibiotics, and removal efficiency is dependent upon the chemical properties of the pharmaceutical (Patel et al., 2019). For example, antibiotics that can sorb to solid particles, such as ciprofloxacin (Córdova-Kreylos and Scow, 2007), may be more easily removed from the liquid phase of treatment. However, throughout the literature, there are variations in removal efficiencies of antibiotics. For example, it was found that the variation in the removal of ciprofloxacin can be large, with a removal efficiency of 90 % in one study during activated sludge treatment followed by chemical coagulation/flocculation, to a removal efficiency of 51 % in a treatment plant processing hospital wastewater (Michael et al., 2013). This review also highlighted the challenges in removing different compounds using the same treatment, with cited studies demonstrating an almost 100 % removal of ciprofloxacin and tetracycline from wastewater treated with high doses of UV, but a removal of only 24–34 % for the macrolides clarithromycin, erythromycin, and azithromycin (Michael et al., 2013).

We observed significant differences in the risk for ciprofloxacin between regions in this study (Supplementary Tables 12 and 13). Differences in influent RQs observed in this study could be due to differential antibiotic prescribing for the population in that region, differences in population sizes served by the WWTP, weather differences between regions, and/or water flow (amongst other factors). As well as being affected by influent, differences in RQ for effluent may be due to

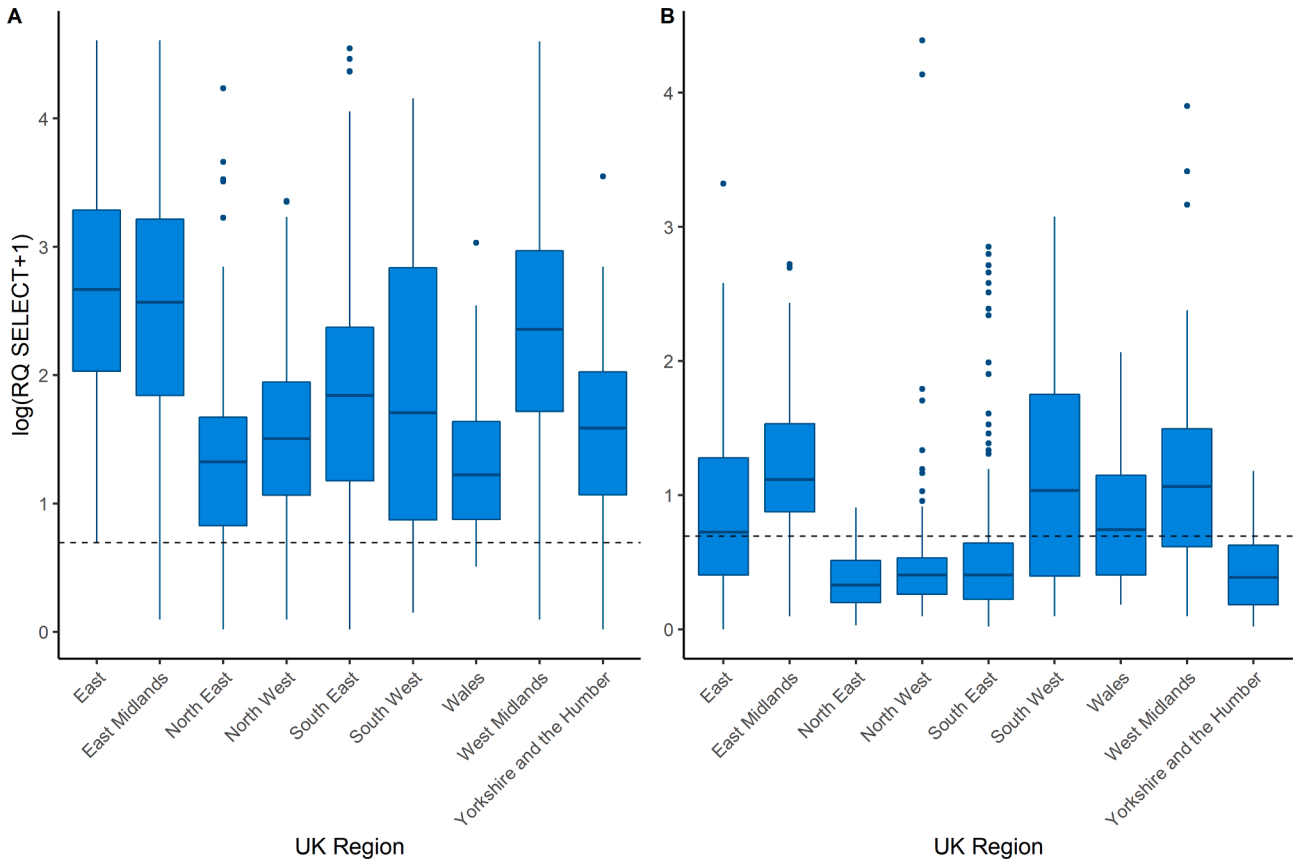

Fig. 5. Ciprofloxacin log + 1 Risk Quotients (RQs) for each UK region. A – Wastewater influent, B – Wastewater effluent. Dotted line represents RQ = 1.

differences in the water treatment in the WWTPs for these regions, since not all WWTPs use the same technologies. As specific details of treatments are often not readily available for individual WWTPs (including for the sites included in CIP2), it is difficult to fully understand the relative importance of these different factors. If treatment information was made available for individual plants, it would be beneficial to see if the risk of AMR selection was significantly reduced by some treatment processes and not others. The capacity of the WWTP (in terms of influent characteristics, physical characteristics, such as reactor volumes, and operational factors, such as sludge retention time) can also greatly affect the microbial communities within activated sludge, thereby varying the effective removal of contaminants (Kim et al., 2019), so this may have also contributed to the differences in effluent RQs across regions. It may be that upgrade of only a few WWTPs could have a significant impact on the probability of AMR selection.

We found erythromycin, azithromycin, and clarithromycin were unlikely to select for AMR in England and Wales in wastewater influent or effluent, according to this study. This supports the recent removal of these antibiotics from the EU WFD Watch List (Gomez Cortes et al., 2020), from an AMR standpoint (though their initial addition and subsequent removal was based on ecotoxicological data). It also supports recent findings from a study in France, which found that ciprofloxacin posed high selection risk, compared to the macrolides, which posed low risk (Haenni et al., 2022). However, lower $PNEC^R$s for macrolides than those used in this study have been published elsewhere and adopted as voluntary targets for manufacturing waste by the AMR Industry Alliance (Tell et al., 2019; Vestel et al., 2021). These $PNEC^R$s are modelled from the MICs of susceptible, clinical pathogens (hereon, called $PNEC^R$-MICs) (Bengtsson-Palme and Larsson, 2016). In some cases, there is good agreement between experimental $PNEC^R$s and $PNEC^R$-MICs (for example, for ciprofloxacin, the $PNEC^R$ is 0.1 μg/L (Murray et al., 2021; Murray et al., 2020) and the $PNEC^R$-MIC is 0.064 μg/L). However, for other antibiotics, such as the macrolides, there are larger discrepancies (the $PNEC^R$s used in this study were 50 μg/L, compared to $PNEC^R$-MICs of 0.25–1 μg/L).

Though complex bacterial community experiments are expected to be more environmentally representative (Larsson and Flach, 2021; Murray et al., 2021), we also repeated the initial assessment using the $PNEC^R$-MICs (Bengtsson-Palme and Larsson, 2016). In this assessment, macrolides did pose a risk (Table 2). Clarithromycin posed a generally higher risk when comparing across maximum, median and mean RQs but azithromycin was the only macrolide with a RQ ≥1 in surface water. However, the maximum, median and average RQ values for the macrolides were still much lower than those for ciprofloxacin, even when using the lower $PNEC^R$-MICs, indicating that risk of selection for AMR posed by the macrolides may be less of a concern than that posed by fluoroquinolones. Interestingly, the effect of wastewater treatment on risk was less pronounced for the macrolides than ciprofloxacin, which agrees with previously recorded poorer removal rates for macrolides during wastewater treatment (Michael et al., 2013).

There are clear advantages and disadvantages to both the $PNEC^R$-MIC method, and complex community experiments, recently reviewed in part elsewhere (Murray et al., 2021). Some of the disadvantages of $PNEC^R$-MICs are that they assume a constant relationship between minimal selective concentration (MSC) and minimum inhibitory concentration (MIC). However, in single species competition experiments, the MIC can range from 4x to 230x the MSC (Gullberg et al., 2011). Furthermore, the MIC data, though collected under standardised experimental conditions, reflect MICs of individual, clinical pathogens growing in single species cultures. Previous research has demonstrated that a complex community context may offer a 'protective effect' (Murray et al., 2018). One study investigated this directly, by comparing selection for isogenic strains in the presence and absence of a complex community, and found that the selective concentration increased in the

Table 2
Maximum, median, and mean risk quotients (RQs) for macrolides in all environmental matrices, calculated using the PNEC^R-MICs (Bengtsson-Palme & Larsson, 2016) and measured environmental concentrations for influent and effluent and predicted environmental concentrations for surface water.

Antibiotic	Matrix	RQ Max	RQ Median	RQ Mean
Azithromycin	Influent	39.68	0.94	1.71
	Effluent	13.40	0.80	1.17
	Surface water	1.34	0.08	0.12
Clarithromycin	Influent	52.40	3.60	4.89
	Effluent	12.72	1.44	1.89
	Surface water	0.13	0.14	0.19
Erythromycin	Influent	14.78	0.58	0.89
	Effluent	4.13	0.33	0.39
	Surface water	0.41	0.03	0.04

presence of the community (Klümper et al., 2019). Further, not all clinical pathogens will be able to survive and/or grow in the environment (Tello et al., 2012). Therefore, PNEC^{R}s based on single species may overestimate environmental risk. Disadvantages of the complex community experiments used to derive the PNEC^{R}s used in this study (Murray et al., 2020; Stanton et al., 2020) include the potential underestimation of risk, due to composition of the community used. For example, the sewage communities used in these experiments are comprised of predominately Gram negative organisms, though Gram positive organisms are also present (Stanton et al., 2020). Therefore, for certain antibiotics (such as the macrolides which are less effective against Gram negative bacteria), there may insufficient diversity or prevalence of susceptible organisms present in the community to be negatively selected. However, environmental relevance is important – the use of sewage derived bacteria communities in risk assessment for wastewater and wastewater receiving environments is logical, compared to clinical pathogens, which may be unable to grow or survive in the environment. Another disadvantage of complex community experiments is that there is higher variation than in single species assays. Though expected due to the complexity of the community, there is the possibility that selection could be occurring at lower concentrations than where this can be detected experimentally. Increasing the assessment factor from 10 to 100 could account for more of this uncertainty.

To summarise, the risks posed by ciprofloxacin as reported in this study should be uncontroversial, due to good agreement between experimental PNEC^R and modelled PNEC^R-MIC data. However, for the macrolides, PNEC^R data vary significantly according to the different methods used to define the selective thresholds, so the estimated risks calculated in this study are less certain. Therefore, potential AMR selection risk posed by macrolides should not be discounted based on this study alone. As more PNEC^R data emerge, this risk assessment should be updated. Risk assessments should also be updated once PNEC^{R}s considering mixture effects are available. Additive or synergistic effects within antibiotic class, or due to presence of co-selective compounds (such as metals or biocides) could increase the selective effect, reducing PNEC^{R}s of antibiotics in mixtures, compared to individual antibiotics. This study suggests this may be of particular concern for the quinolone antibiotics, where additive effects between ciprofloxacin and other quinolones could increase selective pressure.

It is important to note that regardless of PNEC^R used, risks reported here may still be underestimated. Particularly, for surface water, we generated PECs by dividing the MECs by a dilution factor of 10. Though recommended by the EMA (EMA, 2018), this is a clear oversimplification given this can be impacted heavily by water use, rainfall and other factors. Our brief comparison of available downstream MECs and the PECs derived in this study (Supplementary Tables 8 and 9) showed that on average, a dilution factor of 10 may be acceptable for the one WWTP where the data were available. However, effluent dilution downstream of this WWTP still varied significantly, with many more instances where the dilution factor was < 10 (Supplementary Table 9), indicating risk of AMR selection in surface water in this study could have been underestimated more often that overestimated. Future studies could employ spatially resolved dilutions factors, as described previously (Zhu et al., 2019). Comprehensive MEC data for surface waters would be preferable, but these are not currently available. This, combined with lack of data on contribution of other sources of antibiotics (e.g., diffuse pollution from agriculture or aquaculture), means that risks in surface water may be higher than reported here. The underestimation of risk may also be exacerbated by the fact many receiving rivers will also contain combined sewer overflows, resulting in untreated wastewater being released directly into rivers. The extent to which untreated wastewater will be diluted with stormwater is likely to vary significantly according to site and could be negated by increased river flow during heavy rainfall, but little research has been done in this area.

5. Conclusions

Our data support the inclusion of ciprofloxacin on the current version of the EU Commission Water Framework Directive Watch List (Gomez Cortes et al., 2020), from an AMR selection standpoint. This study demonstrates the value of widespread and routine antibiotic monitoring data, not just for AMR risk assessment, but for environmental risk assessment in general. We show there is a risk of AMR selection by ciprofloxacin in wastewaters and in surface waters in England and Wales, so this is not an issue unique to LMICs. This was further supported by the observation that the median MECs of ciprofloxacin in influent and effluent in the UKWIR monitoring were remarkably similar to global median MEC values. The approach used in this study could be combined with data on WWTP type, if available, to target end-of-pipe solutions. Reduction of antibiotic use pre-wastewater treatment would also significantly reduce AMR selection risk and further research is needed to understand the relative contribution of different pollution sources (wastewater, agriculture, aquaculture) to MECs in surface waters. Currently, lack of agreement on selective thresholds for the macrolides introduces uncertainty regarding selection risk assessment for macrolides in wastewater. Improved and continual monitoring of antibiotic concentrations in aquatic environments and PNEC^R data for more antibiotics are required to fully appreciate the AMR selection risks posed by individual compounds. Future research should aim to understand the impacts of complex mixtures of antibiotics and other antimicrobials on AMR selection in the environment.

CRediT authorship contribution statement

April Hayes: Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing, Visualization. **Laura May Murray:** Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing, Visualization. **Isobel Catherine Stanton:** Validation, Writing – review & editing. **Lihong Zhang:** Writing – review & editing, Supervision. **Jason Snape:** Writing – review

& editing, Supervision, Project administration, Funding acquisition. **William Hugo Gaze:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Aimee Kaye Murray:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jason Snape is an employee and shareholder of AstraZeneca PLC. All remaining authors declare no competing interests.

Data availability

Data will be made available on request.

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Data access statement

The research data supporting this publication are provided within this paper and the supplementary information accompanying this publication.

Appendix A. Supplementary data

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