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# International tempo-spatial study of antibiotic resistance genes across the Rhine river using newly developed multiplex qPCR assays

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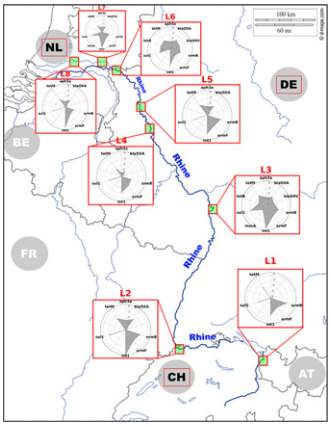
## HIGHLIGHTS

- Five multiplex qPCR assays were developed for monitoring ARGs in environmental samples.
- Rhine river locations have distinct, characteristic antibiotic resistance genes (ARG) profiles.
- ARG concentration do not continuously increase at more downstream locations.
- *Intl*1 correlates with overall ARG concentrations only due to correlations with *sul*1.
- ARG concentrations do not correlate with regional antibiotic use.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The aim of this study was to capture and explain changes in antibiotic resistance gene (ARG) presence and concentration internationally across the Rhine river. *Intl*1 concentrations and national antibiotic usage were investigated as proxies to predict anthropogenic ARG pollution. Newly-developed multiplex qPCR assays were employed to investigate ARG profiles across 8 locations (L1-L8) in three countries (Switzerland, Germany, the Netherlands) and to detect potential regional causes for variation. Two of these locations were further monitored, over the duration of one month. A total of 13 ARGs, *Intl*1 and 16S rRNA were quantified.

ARG presence and concentrations initially increased from L1(Diepoldsau) to L3(Darmstadt). A continuous increase could not be observed at subsequent locations, with the large river volume likely being a major contributing factor for stability. ARG presence and concentrations fluctuated widely across different locations. L2(Basel) and L3 were the two most polluted locations, coinciding with these locations being well-developed pharmaceutical production locations.

We draw attention to the characteristic, clearly distinct ARG profiles, with gene presence being consistent and gene concentrations varying significantly less over time than across different locations. Five genes were Rhine-typical (*erm*B, *erm*F, *Intl*1, *sul*1 and *tet*M). *Intl*1 and *sul*1 were the genes with highest and second-highest concentration, respectively. *Aph*(III)a and $bla_{OXA}$ were permanently introduced downstream of L1, indicating no source of these genes prior to L1.

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We highlight that correlations between *Intl*1 and ARG concentrations ($R^2 = 0.72$) were driven by correlations to *sul*1 and disappeared when excluding *sul*1 from the analysis ($R^2 = 0.05$). *Intl*1 therefore seems to be a good proxy for *sul*1 concentrations but not necessarily for overall (anthropogenic) ARG pollution. Aminoglycoside usage per country correlated with concentrations of *aph*(III)a and several unrelated antibiotic resistance genes ($bla_{OXA}$, *erm*B, *erm*F and *tet*M). This correlation can be explained by co-resistance caused by mobile genetic elements (MGEs), such as Tn1545.



## 1. Introduction

Water Pollution and antibiotic resistance (AR) are on the rise globally and the advancing global emergence calls for better and more extensive monitoring of environmental, urban and medical environments (UN-Water, 2009; World Health Organization, 2014). While AR has been present since the first microorganisms started producing antibiotics to protect themselves against these toxic compounds (D'Costa et al., 2011), a recent sharp increase in resistance of clinically relevant bacteria is evident (Laxminarayan et al., 2013; Mendonça et al., 2007; Ventola, 2015; Woodford and Ellington, 2007). Reasons for this increase include: overuse and inappropriate prescription of antibiotics, extensive use in agriculture and animal husbandry, severe misinformation about when antibiotics are indicated, the absence of coordinated global AR combat strategies and a low number of new antibiotics (Laxminarayan et al., 2013; World Health Organization, 2014; Ventola, 2015; Pan et al., 2016; El Khoury et al., 2017). Global and national action plans to tackle AR are developed in recent years, but they are neither fully coordinated nor incorporated into legislation (Carlet et al., 2014), partly due to gaps in knowledge (Larsson et al., 2018).

It is widely accepted that AR is largely caused by the increased use and misuse of antibiotics (Finley et al., 2013; Bell et al., 2014; Van Boeckel et al., 2014). However, the exact role of the environment is not well understood (Huijbers et al., 2015; Morris et al., 2016; Bengtsson-Palme et al., 2018). It is likely that the environment can act as a reservoir for ARGs (Sabri et al., 2018) and possible that it might facilitate transfer of AR to non-environmental microorganisms, pathogens or human microbiomes (Finley et al., 2013; Bengtsson-Palme et al., 2014). Where antibiotic pressure is high in the environment, new ARGs may emerge (Bengtsson-Palme et al., 2015).

A quantitative approach to assess the risk of ARB/ARG in the environment and possible effects on the environment, animals and humans is missing (World Health Organization, 2014). Increased exposure to AR via the environment will lead to increased risk; with a growing world population, associated water use, discharge of wastewater (Gordon et al., 2016) and the increasing reuse of wastewater (Y. Zhang and Shen, 2019; Evans et al., 2019; Koop and van Leeuwen, 2017; United Nations Environment Programme, 2017), exposure to environmental ARG/ARB will rise.

Notwithstanding the large number of recent monitoring studies, one of the main gaps in knowledge are definite numbers of ARG concentrations and their fluctuation in the environment at different locations over time and under varying conditions (Larsson et al., 2018; Zheng et al., 2018). Tempo-spatial studies of water bodies are imperative, especially for substances of emerging concern, such as ARGs (Scott et al., 2019). AR baseline levels in the environment and their increase due to varying levels of anthropogenic pollution have not been extensively studied. These numbers are needed to serve as a baseline for knowledge on environmental dissemination of ARG and to estimate the risk of observed ARG concentrations at, for example, ARG hotspots such as strongly polluted surface water bodies (Pruden et al., 2018).

ARGs and MGEs, such as *sul*1 and *Intl*,1 have been suggested as proxies to monitor ARGs (of anthropogenic origin) in the environment. *Intl*1, has been suggested as indicator for bacterial capacity for gene transfer and gene acquisition (Narciso-da-Rocha et al., 2014) as well as proxy for ARGs of anthropogenic origin (Gillings et al., 2015). *Sul*1 has been suggested as indicator for urban and agricultural pollution of ARG (Pei et al., 2006).

Closely meshed monitoring programs using regular qPCR of more than one target gene require large amounts of time, personnel and material resources. While quantitative high-throughput technologies, such as microarray technologies and HT-qPCR (high throughput qPCR) are increasingly used to determine AR in the environment (Wang et al., 2014; Karkman et al., 2016; L. Xu et al., 2016; Ahmed et al., 2018; An et al., 2018; Zheng et al., 2018; Waseem et al., 2019), there are a number of challenges and disadvantages associated with these technologies (Waseem et al., 2019). Microarray technologies suffer from batch-to-batch variability and are considered less sensitive and specific (Waseem et al., 2019). HT-qPCR is prone to instrumental sensitivity and analytical differences which can significantly impact results and individual assays tested cannot be optimized during the experimental run (Waseem et al., 2019). Our aim was to decrease the necessary resources and to maintain the accuracy of regular qPCR approaches while using a method with a higher throughput and avoiding the shortcomings of high throughput technologies. To this end five multiplex qPCR assays were developed to detect and accurately quantify three genes per qPCR assay simultaneously.

The present work is an international study monitoring a large river from the source to the ocean and is, to the best of our knowledge, the first of its kind for large western European rivers. It monitors spatio-temporal changes in ARG presence and concentration regarding the following: genes conferring resistance to aminoglycosides (*aph*(III)a), β-lactam antibiotics ($bla_{KPC}$, $bla_{SHV}$, $bla_{OXA}$, *mec*A), macrolides (*erm*B, *erm*F), quinolones (*qnr*S), sulfonamides (*sul*1), tetracyclines (*tet*B, *tet*M) and glycopeptides (*van*A, *van*B) as well as a class 1 Integrase (*Intl*1) and 16 s rRNA. The study further aims at gaining more information on ARG levels across the river Rhine at different locations as well as changes over time to identify relationships, trends and co-occurrences of environmental ARGs and their concentrations, which will provide insight needed to detect prospective causes. Potential proxies for ARG pollution of anthropogenic origin were compared.

## 2. Methods and materials

### 2.1. Multiplex development and quality control

Five Multiplex qPCR assays were developed and validated. Development steps included: optimal annealing temperature testing for primers and probes, grouping of ARGs into multiplex qPCR assays based on optimal annealing temperatures and functionality tests using SYBR Green II (for comparison of probe-based multiplex qPCR results) as well as Taqman in combination with the iQ™ Supermix (simplex) or iQ™ Multiplex Powermix (multiplex) (Bio Rad, München, DE).

Quality control steps included: computational interaction tests (for interactions between primers and probes of ARGs within the multiplex qPCR assay) using OligoAnalyzer 3.1 (Owczarzy et al., 2008), experimental interaction tests and precision tests for accurate quantification (comparing quantification results obtained by SYBR Green II assays to Taqman qPCR assays). Mock samples were prepared in 0.1xTE-buffer

or by spiking environmental samples (pond water) with gene concentrations within a specific assay varying up to 1000-fold.

Validation steps included determination of limit of quantification (LoQ) and limit of detection (LoD) (Forootan et al., 2017), calculation of intra- and interassay variation in form of the coefficient of variation (CV) (Tellinghuisen and Spiess, 2014; Svec et al., 2015) and multiplex qPCR assay efficiencies for each of the genes within a multiplex assay (Svec et al., 2015). Validation was conducted and reported according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009). More specifically: serial dilutions of the standard were prepared for the different genes and quantified multiple times to obtain at least 8 data points per dilution.

## 2.2. Sampling

### 2.2.1. Sampling sites

The Rhine is the second-largest river in Central and Western Europe. It originates at Lake Toma in Switzerland and discharges in the Netherlands into the North Sea after 1230 km and has an average flow rate of 2900 $m^3$/s. Sampling was done along the Rhine at 8 locations: L1 – L8. The samples were collected by a cooled courier car and were processed within 24 h of sampling at the processing facility.

There are six distinctive Rhine regions with different characteristics (Fig. 1).

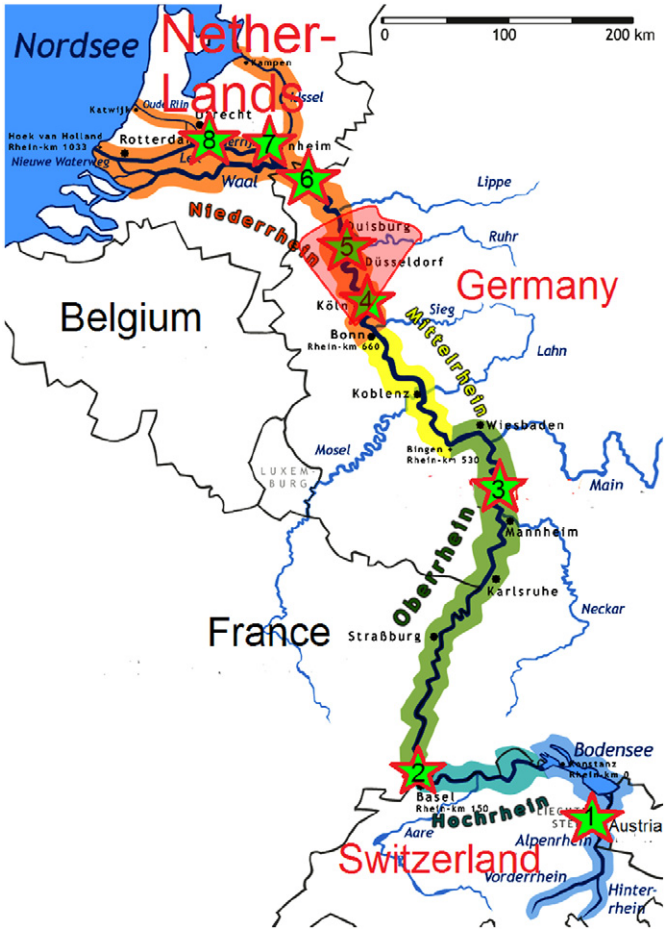


**Fig. 1.** Sections and Major Affluents of the Rhine; blues – Alpine Rhine and High Rhine, green - Upper Rhine, yellow – Middle Rhine, Orange – Lower Rhine (up to Dutch-German border) and Rhine Delta; sampling locations indicated by green stars and identified by number; approximate location of the Rhine-Ruhr-region highlighted in red.

### 2.2.2. Sampling conditions

Samples were taken at dedicated river water quality monitoring stations of the International Association of Waterworks of the river Rhine, situated in the stream-bed of the river. Locations were situated at least 3 km downstream from wastewater treatment plant discharge sites. Samples were taken at a depth of 0-30 cm. Four liters (two grab samples of 2 L) of surface water were taken. All samples were stored in identical plastic bottles at 4 °C before, during and after transport to the processing facility. Rainfall data was collected from national weather institutes (see *SI A.1*).

### 2.2.3. Temporal monitoring

Time-series sampling was performed at the locations Lobith (L6) and Utrecht (L8), approximately six months after the spatial sampling campaign (see *3.3.3*). The following samples were taken (day 1 - 24th of October 2017): day 1 (0 h, 5 h, 10 h), day 7 and day 30. Spatial samples were taken into account for temporal analysis. L6 was chosen for its high number of detected ARGs and L8 due to this being the most downstream location where potential fluctuations were hypothesized to be amplified.

### 2.2.4. Spatial monitoring

For spatial monitoring, all samples were taken on May 3, 2017. Samples were taken at the following location (increasingly downstream): Diepoldsau (L1, CH), Basel (L2, CH), close to Darmstadt (L3, DE), Cologne, Düsseldorf and Lobith (L4 – L6), Arnhem (L7, NL) and from the Lek at Utrecht/Nieuwegein (L8, NL). L2 and L3 are locations with extensive, well-developed pharmaceutical industries. L4 and L5 fall into a densely populated German region called the Rhine-Ruhr-region. Meteorological conditions were comparable across sampling locations (see *SI A.1*).

### 2.2.5. Filtration and DNA extraction

Samples were filtered (300 mL) and DNA extracted from sample duplicates. DNA extract duplicates were then pooled for further analysis and stored at −30 °C. Filtration and DNA extraction were performed as previously described (Paulus et al., 2019).

## 2.3. Antibiotic resistance gene quantification

13 ARGs, *IntI*1 and an internal control (IC) were quantified by multiplex qPCR using TaqMan. The IC is a DNA sequence added to the sample before DNA extraction to identify potential DNA loss during/ due to the DNA extraction process, to account for this loss and make data from different samples more comparable (Wullings et al., 2007). 16S rRNA gene concentrations were quantified using SYBR Green. TaqMan and SYBR Green assays were comparable as previously confirmed by comparison studies conducted using TaqMan and SYBR Green assays for all genes within the multiplex qPCR assays (see *3.1.1*). All qPCR assays were performed on a CFX96 system (Bio-Rad). Each reaction was carried out in a final volume of 50 μL containing 10 μL DNA extract. Primer and probe sequences, as well as cycling conditions, standards and controls used are documented in the supplementary material (*SI* Tables 1–3).

Each sample was analyzed by qPCR at least thrice in separate qPCR runs using duplicate wells.

Primers, standards and probes were obtained from Integrated DNA Technologies, BVBA Belgium.

## 2.4. Antibiotic consumption and environmental data

ARG concentrations were correlated to national human (European Centre for Disease Prevention and Control, 2018) and veterinary (European Medicines Agency, ESVAC, 2018) antibiotic usage data as well as to agricultural, farming and environmental data (see *SI A.3*) obtained from European and national surveillance agency reports and databases.

Numbers from human and veterinary antibiotic usage data were added to obtain the antibiotic load used for correlation analysis. Antibiotic data used was country-specific so that the same national antibiotic average was assigned to the locations within a country.

### 2.5. Data analysis

Python 3.6.0 was used to calculate descriptive statistics and correlations and to create data visualizations. R version 3.5.0 was used to perform inferential statistics, including Student's *t*-test, Welch's F-test and ANOVA.

Mean and standard deviations (std) were calculated using the results from all qPCR replicates for each sampling location for the (a) spatial samples only, and (b) all temporal samples.

Significant differences between experiments and/or measurements were detected by employing paired or unpaired Student's *t*-Tests and two samples/measurements were defined to be significantly different from each other when the calculated *p*-value was lower than 0.05. A test of variance between temporal and spatial data was calculated in a one-sided F-test. When necessary, data was $\log^{10}$-transformed to comply with the assumption of normal distribution of the data.

Regression plots were generated using the .regplot() function in Seaborn 0.9.0 (a Python package for statistical data visualization) (Hunter, 2007; Waskom, 2012).

Unless otherwise stated, all gene concentrations are relative concentrations normalized to 16S rRNA concentrations.

## 3. Results and discussion

### 3.1. Multiplex assays

Gene primers and probes were tested for functionality in SYBR Green II and TaqMan qPCR assays, and for potential incorporation into multiplex qPCR assays (*SI* Table 1). Genes were combined into multiplex qPCR assays depending on the optimal annealing temperatures (obtained during annealing temperature tests, *SI* Table 2), preliminary computational interaction tests (based on $\Delta G$ calculations) and, whenever possible, by amplicon length.

Out of the tested primer-probe combinations, 15 were suitable for multiplex qPCR inclusion and five multiplex assays, quantifying 3 genes each, were developed (Table 1).

Quality control tests experimentally verified that no interaction took place between primer pair and probe sequences of different genes within an assay, to ensure an independent quantification of the individual ARGs within each of the multiplex assays. All ARGs could be accurately quantified even under conditions where one of the assay genes was present in concentrations up to 1000-fold higher than the other genes. Finally, quantification results obtained by multiplex qPCR assay were compared to quantification results obtained by simplex and SYBR Green II assay. The same quantification results were obtained for multiplex assays 1, 2, 3 and 5, these assays are accurate up to one decimal number (SI Fig. 1). Multiplex assay 4 showed elevated results outside of the margin of error; gene concentrations were estimated up to 3 times higher than results obtained by SYBR Green II assays. The accuracy of this assay is therefore lower and obtained gene concentrations are order-of-magnitude rather than exact numbers (SI Fig. 1).

**Table 1**
Overview of developed multiplex qPCR assays, including ARGs in assay, optimal annealing temperature and limit of quantification (LOQ).

| Multiplex | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| ARG 1 | *sul*1 | *tet*B | *mec*A | *van*A | *erm*B |
| ARG 2 | IC | $bla_{SHV}$ | $bla_{OXA}$ | *van*B | *erm*F |
| ARG 3 | *qnr*S | *Intl*1 | *aph*(III)a | *tet*M | $bla_{KPC}$ |
| Annealing Temperature | 58 °C | 60 °C | 58 °C | 56 °C | 57 °C |
| Quantification Limit | 2.5E00 gene copies/μL DNA extract | | | | |

### 3.2. Validation of accuracy, efficiency and precision

The LoQ is 2.5E+00 gene copies/μL DNA extract for all genes, with exception of genes within multiplex assay 4 (semi-quantitative), as this was the lowest concentration at which replicates showed a CV ≤ 35%. The LoD is 5E-01 for all genes in the multiplex assays 1 and 3 as well as for *tetB* and $bla_{SHV}$. The LoD is 1.0E+00 for all genes within multiplex assay 5 as well as for *Intl1*. Standard curves for all multiplex assays including the efficiency, $R^2$, the slope and the y-intercept can be seen under SI Figs. 2–6. Concentrations to determine the LoD started from 5.0E-01 and increased by 5.0E-01 steps.

Intra – and interassay variation were determined by calculating the CV based on the $\Delta C_t$ for each gene within the five multiplex assays. Intra-assay variation was <1.5% for all genes (range: 0.26%–1.4%), while inter-assay variation was <2.5% (range: 0.49%–2.3%). Amplification efficiencies for the different genes in the multiplex assays ranged from 86% - 104%, with genes within individual multiplex assays never differing >10% in their efficiencies during one qPCR experiment (SI A.2). As efficiencies did not differ >10% between the genes within a multiplex during individual qPCR reactions, amplification of these genes is directly comparable.

### 3.3. Temporal variation of ARG concentrations

Multiplex qPCR assays were applied to river Rhine samples to monitor temporal variation of ARGs at two sites (L6 and L8).

*Intl1, sul1, ermB, ermF, TetM, blaOXA* and *apha3* were detected at both sites, while *blaSHV* and *tetB* were detected consistently at L6, and consistently not detected at L8 (Fig. 2). The temporal variance within samples at one location was largely not statistically significant ($p \geq .05$). Overall, it can be said that the observed ARG profiles are representative and consistent for both sampling locations (Fig. 2).

### 3.4. Spatial variation for ARG presence and concentrations

Out of 14 target genes, 5 were detected at the most upstream location (L1), 7 genes at L2 and 10 genes at L3. At more downstream locations, the number of detected genes was 7, with exceptions at L6 and L7 (9 and 8 genes, respectively) (Fig. 3).

The sampling location least polluted in regard to gene presence was L1 with only 5 detected genes. In regard to ARG concentrations, L6 was least polluted with total relative concentrations of 8.21E-04 ARG copies/16S rRNA gene. L3 was most polluted in terms of gene presence with 10 detected genes and L2 in regard to ARG concentrations with a total relative ARG concentration of 1.47E-02 ARG copies/16S rRNA gene (Fig. 3). Concentrations of 4 (out of 5) genes (*Intl*1, *sul*1, *tet*M and *erm*F) increased between L1 and L2.

Temporal fluctuations were significantly smaller than spatial variances and standard deviations ($p \leq .05$; see *SI Table 4* for variances and *p*-value). Exceptions to this were the *erm*B gene ($p_{L6} = 0.053$, $p_{L8} = 0.101$) at both locations and $bla_{SHV}$ at L6 ($p = .341$), where the temporal variances were not significantly smaller than spatial variances. Larger variances of the $bla_{SHV}$ gene at L6 can be explained by concentrations around the LoQ. In some samples $bla_{SHV}$ was detected but could not be quantified, which led to larger calculated variances.

Overall, gene concentrations fluctuated with no clear trend across sampling locations (Fig. 3). Ter Laak et al. (2010) state that anthropogenic pressure increases further downstream and the cumulative upstream population impacting any given location increases with further downstream locations. Due to these reasons we assume increasing anthropogenic pollution at more downstream locations. It is to be noted that more complex dynamics are at work, as chemical and genetic anthropogenic pollutants are subjected to decay and/or transformation

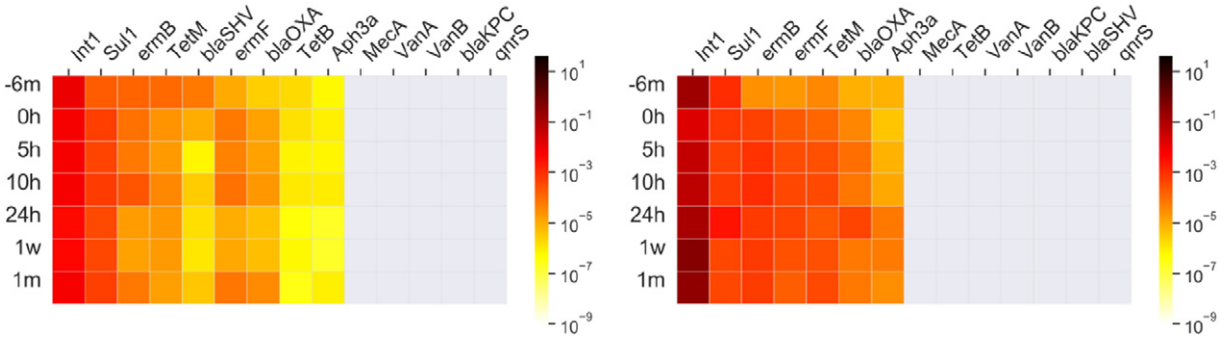


**Fig. 2.** ARG Concentration Variance; Temporal Variance at Sampling Locations L6 (left) and L8 (right), scale: gene copy number per 16S rRNA shown.

in aquatic environments and might in- or decrease the impact potential of chemical compounds (Escher et al., 2009; Fatta-Kassinos et al., 2011; Donner et al., 2013; López-Serna et al., 2013; Wallace et al., 2018). The hypothesis that ARG or *Intl*1 concentrations increase with anthropogenic pressure, could not be verified.

ARG *concentrations* do not seem to be an indicator for concentrations of these ARG downstream, as concentrations fluctuate between locations. Upstream gene *presence* seems to be an indicator of downstream gene presence for some genes, as shown for *aph*(III)a and $bla_{OXA}$, but does not necessarily determine downstream gene presence for all genes.

A lack of visible *spatial* trends has been previously observed in large rivers (Chen et al., 2015; H. Jiang et al., in press; X. Zhang et al., 2009). Previous studies that detected only partial consistency of ARG profiles were largely conducted on smaller rivers (Pei et al., 2006; Pruden et al., 2012; Y. Xu et al., 2018), but *temporal* consistency at individual locations in ARG presence and relative concentration has been shown in larger rivers (Chen et al., 2015). Similarly, ARG concentrations fluctuated in surface water samples instead of showing a steady increase with increasing downstream locations, in a spatial study of the large Pearl river (Chen et al., 2015). The large catchment area and volume of the Rhine river (flow average: 2900 $m^3/s$) and differences in local discharges (proximity of discharge to sampling location, presence of hospitals, agricultural practices) are possible explanations for the consistency of ARG profiles at individual sampling locations over time, as well as for the differences between ARG presence and concentration between locations.

Out of all monitored genes, *Intl*1 and *sul*1 were consistently detected in the highest and second-highest concentrations, with one exception at the starting location (L1) where *Intl*1 and *erm*B were detected at similar concentrations.

*Intl*1 and *sul*1 genes have previously been shown to be frequently found at high concentrations in aquatic environments (Su et al., 2012; H. Jiang et al., 2018; L. Jiang et al., 2013; Berglund et al., 2015). In our study, *Intl*1 concentrations were surprisingly high, reaching relative levels of $>10^0$ gene copies/16S rRNA copy at L2 and L7. Similarly, *sul*1 concentrations were unusually high at L2 when compared to the other locations (Fig. 3) as well as compared to previously published concentrations in river water related samples (H. Jiang et al., in press; Luo et al., 2010; Pei et al., 2006), although comparable concentrations have previously been observed in river samples (J. Xu et al., 2015).

Individual Rhine sampling locations had unique and distinctive ARG profiles which varied from each other in gene presence and concentration. Five ARGs were detected at all sampling locations, including (Fig. 3). Two additional ARGs were detected at all locations downstream from L1 (*aph*(III)a, $bla_{OXA}$). Significant sources of these genes seem to be present only downstream from L1, potential contributors include wastewater and agricultural discharges in Lake Constance or the Aare river.

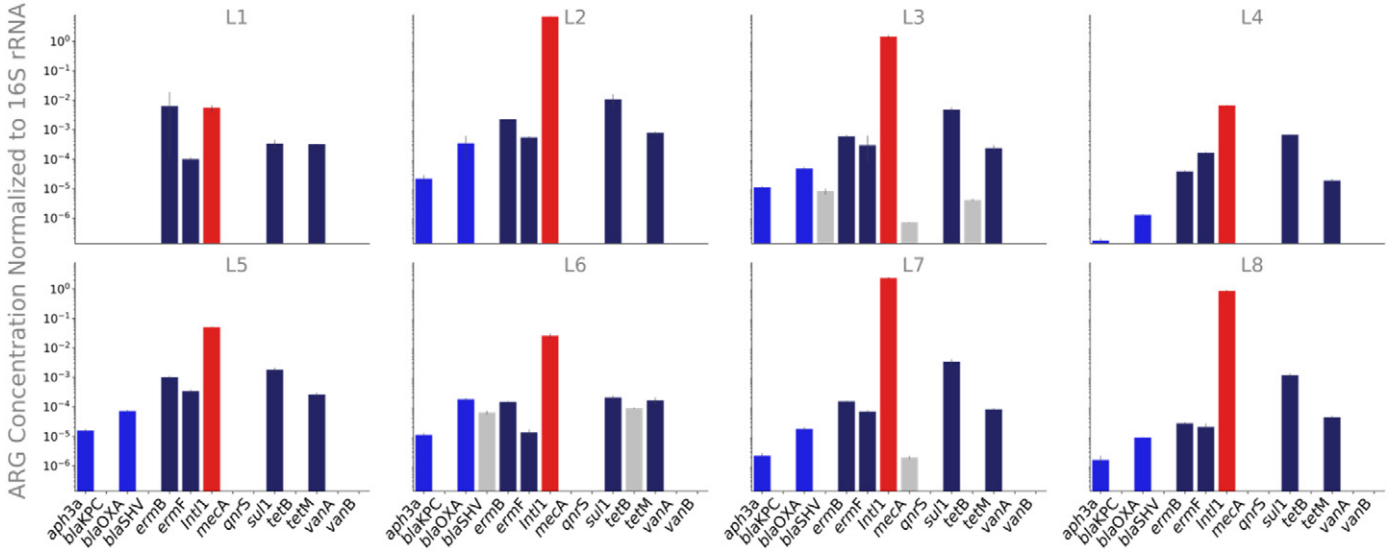


**Fig. 3.** ARG Concentrations normalized to 16S rRNA at different Rhine sampling locations (mean ± std); dark blue – common genes (all locations); blue – common genes (post-Lake Constance locations), grey – location-specific genes; red – *Intl*1;

**Table 2**
Presence and Relative Concentration of ARGs and *Intl*1; *green - least polluted sample based on parameter, red – most polluted sample based on parameter*; gene concentrations values in gene copies/16S rRNA.

| | L1 | L2 | L3 | L4 | L5 | L6 | L7 | L8 |
|---|---|---|---|---|---|---|---|---|
| Number of genes (incl. *Intl*1) | 5 | 7 | 10 | 7 | 7 | 9 | 8 | 7 |
| Gene Concentration (incl. *Intl1*) | 1.76E-02 | 6.90E+00 | 1.44E+00 | 7.61E-03 | 5.39E-02 | 2.35E+00 | 2.89E-02 | 8.59E-01 |
| ARG Concentration (excl. *Intl*1) | 6.97E-03 | 1.47E-02 | 5.89E-03 | 8.98E-04 | 3.42E-03 | 8.21E-04 | 3.71E-03 | 1.14E-03 |
| *Intl*1 concentration | 6.70E-03 | 6.90E+00 | 1.50E+00 | 6.60E-03 | 5.00E-02 | 2.60E-02 | 2.40E+00 | 8.40E-01 |
| *sul*1 concentration | 3.35E-04 | 1.07E-02 | 4.72E-03 | 6.69E-04 | 1.70E-03 | 1.99E-04 | 3.38E-03 | 1.04E-03 |

Besides the omnipresent Rhine genes, a second set of ARGs ($bla_{SHV}$, *mec*A and *tet*B) were detected sporadically at three sampling locations (L3, L6 and L7). One (L7), two (L6) or three additional genes (L3) were detected (Fig. 3). $bla_{KPC}$, *qnr*S, *van*A and *van*B were not detected in any of the samples. These genes could be of interest as potential indicator genes for specific, emerging sources of pollution within the Rhine. Examples for such specific sources of pollution are: insufficiently treated or untreated wastewater, untreated run-off from agriculture or animal husbandry or impact from hospital wastewater. $bla_{KPC}$ and *van*A have only rarely been detected in the environment but have previously been associated with hospital wastewaters (Cerdeira et al., 2017; Chagas et al., 2011; Cuzon et al., 2011; Durkin et al., 2018; Gootz et al., 2009; Hicks et al., 2015; Hu et al., 2012; Iversen et al., 2002; Paulus et al., 2019; Sellera et al., 2017), which could make them genes of priority for monitoring, to detect potential leakage from insufficiently treated hospital wastewater.

### 3.5. Estimations of ARG pollution depend on the parameter monitored

ARG pollution in river water can be expressed and compared using different parameters. The number of ARGs detected, the (total) ARG concentration (relative to the 16S rRNA gene), the number of microorganisms carrying resistance genes or *Intl*1 concentrations can all be used as indicators. In this study, the number of ARGs detected, (relative) ARG concentrations, *Intl*1 concentrations and *sul*1 concentrations were investigated (Table 2).

The lowest and highest number of ARGs could be detected in L1 and L3, respectively. The lowest and highest ARG concentrations were detected in L6 and L2, respectively. *Intl*1, which has previously been suggested as an indicator for environmental pollution with ARGs of anthropogenic origin (Gillings et al., 2015), showed high correlations with total ARG concentrations but low correlations with the number of ARGs detected per sample as well as with ARG concentrations of individual genes (Fig. 4). *Intl*1 concentrations do not continuously increase and fluctuate widely instead. In most samples (with exception of L1), *Intl*1 is present at significantly higher concentrations (usually >2 log units higher) than the other detected ARGs, which has been previously recorded in river water (Chen et al., 2015).

Recent developments in next generation sequencing (NGS) and bioinformatics may be used to track the sources of environmental contamination, allowing to differentiate ARG profiles from different ecosystems. This might provide a better indication than the individual parameters discussed above. However, many ARGs appear to be present in multiple sources and it may be difficult to spatially differentiate the individual sources in a large river such as the Rhine (Li et al., 2018).

#### 3.5.1. Intl1 does not correlate with overall ARG concentrations but with sul1 concentrations

Indicators and proxies have been suggested to estimate the level of (ARG) pollution of anthropogenic origin. Two examples are *Intl* (Gillings et al., 2015) and *sul*1 (Pei et al., 2006). Missing agreements on how to classify and define ARG pollution exactly (e.g through ARG presence, the proportion of resistant bacteria in a sample, relative concentration or absolute concentration) generally reduce the usefulness of such indicators. A high correlation was observed between *Intl*1 and *sul*1 ($R^2 = 0.93$) (Fig. 4, *middle*). Strong correlations between *Intl*1 and *sul*1 have been previously described (Antunes et al., 2005; Frank et al., 2007; Shah et al., 2014; Chen et al., 2015) and are likely, at least partly, a result of the presence of *sul*1 in the conserved region of *Intl*1 (Chen et al., 2015). *Sul*1 is further often the most abundant (or among the most abundant) resistance genes in environmental samples, including samples in this study.

High correlations of *Intl*1 with ARG concentrations were observed but could be attributed largely to *sul*1 concentrations; correlations disappeared when excluding *sul*1 from the regression (Fig. 4). The results obtained during this study suggest that the strength of *Intl*1 as an indicator for ARG pollution strongly depends on the parameter used to determine ARG pollution and that it might not be the best indicator or at

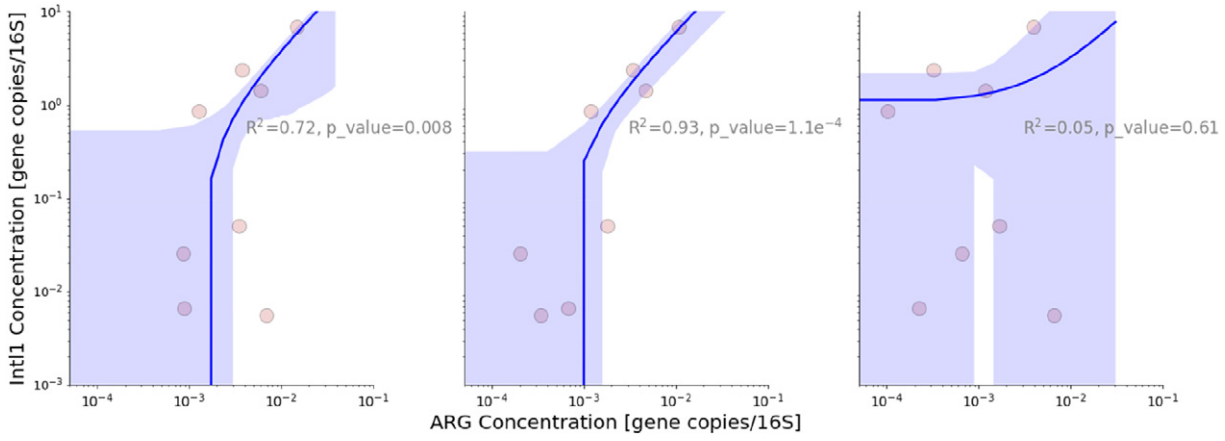


**Fig. 4.** Regression Plot Showing Correlation Between *Intl*1 and Total ARG Concentrations; shown on x-axis: left – total relative ARG concentration; middle – *sul*1 concentration; right - total relative ARG concentration excluding *sul*1; *linear least-squares regression used for calculation of $R^2$ and p-value.*

**Table 3**
Coefficient of Determination ($R^2$) for Correlations between various ARG concentrations and mean correlation per ARG; *conditions: ≥5 common quantitative datapoints.*

| | *aph*(III)a | *Intl*1 | *sul*1 | *tet*M | $bla_{OXA}$ | *erm*B | *erm*F |
|---|---|---|---|---|---|---|---|
| *aph*(III)a | 1.00 | 0.18 | 0.27 | 0.66 | 0.66 | 0.66 | 0.27 |
| *Intl*1 | – | 1.00 | 0.62 | 0.33 | 0.06 | 0.01 | 0.13 |
| *sul*1 | – | – | 1.00 | 0.41 | 0.11 | 0.02 | 0.33 |
| *tet*M | – | – | – | 1.00 | 0.38 | 0.25 | 0.25 |
| $bla_{OXA}$ | – | – | – | – | 1.00 | 0.38 | 0.11 |
| *erm*B | – | – | – | – | – | 1.00 | 0.18 |
| *erm*F | – | – | – | – | – | – | 1.00 |
| data points | 7 | 8 | 8 | 8 | 7 | 8 | 8 |
| mean | 0.53 | 0.33 | 0.4 | 0.47 | 0.39 | 0.36 | 0.33 |

the very least show that the strength of this indicator is strongly dependent on additional factors. Another such example is a study conducted by Zhang et al. (X. Zhang et al., 2009) during which *Intl*1 was found in very low abundance when compared to the *tet*A and *tet*C in samples from a highly polluted lake in China. It is to be noted, that the selection of certain genes of interest for qPCR analysis might represent a slight bias, as other ARGs are not taken into account.

*3.5.2. Antibiotic resistance gene correlations with other factors*

Detected ARG classes coincided with the most used antibiotics in the region [penicillins, tetracyclines, sulfonamides and macrolides] (European Medicines Agency, ESVAC, 2018; European Centre for Disease Prevention and Control, 2018). Antibiotic sales and usage within a region has previously been shown to influence antibiotic concentrations in aquatic environments (ter Laak et al., 2010), so that this data can potentially be used as an estimate for expected variation of antibiotic concentrations between countries.

Antibiotic usage and the preferred class of antibiotic used varied widely between the three countries (European Medicines Agency, ESVAC, 2018; European Centre for Disease Prevention and Control, 2018). Nevertheless, ARG concentrations in the different countries showed little to no correlation with antibiotic use (*SI Table 5*), as has previously been observed (Brown et al., 2019) There were two exceptions (*SI Table 5)*: sulfonamide concentrations correlated weakly to moderately with *Intl*1 and *sul*1 ($R^2 = 0.25$ and $R^2 = 0.17$, respectively); and aminoglycoside concentrations did interestingly not only correlate with *aph*(III)a ($R^2 = 0.58$), but also with $bla_{OXA}$ ($R^2 = 0.58$), *erm*B ($R^2 = 0.68$), *erm*F ($R^2 = 0.42$) and *tet*M ($R^2 = 0.68$). $bla_{OXA}$, *erm*B, *erm*F and *tet*M resistance should not be impacted by aminoglycoside activity as their targets in the cells differ, so we don't see direct causality behind the observed correlation. Further, individual ARG concentrations correlated most with *aph*(III)a gene concentrations. The highest correlations could be observed between *aph*(III)a and, *tet*M, $bla_{OXA}$ and *erm*B ($R^2 = 0.66$) (Table 3). A possible explanation is co-resistance mediated by transfer on MGEs also carrying *aph*(III)a, such as the conjugative transposon Tn1545 (Seral et al., 2001). This would account for correlation with aminoglycoside usage and *aph*(III)a concentrations, both. Co-resistance due to MGEs is well-documented (Pal et al., 2015; Imran et al., 2019; Baker-Austin et al., 2006; Di Cesare et al., 2016; Seiler and Berendonk, 2012) and other Tn1545-like transposons (Tn6263 and Tn6331) have previously been found to confer aminoglycoside/macrolide co-resistance (Kambarev et al., 2018).

For a more in-depth picture, correlation analyses on regional parameters (provincial or municipal level), including non-class-segregated antibiotic sales, farming intensity, nitrate pollution, population density and untreated wastewater discharge, were conducted (see *SI A.3*). Only farming intensity (represented by farm animals per $km^2$) positively correlated with all discussed AR parameters ($R^2 = 0.11$–$0.52$; see *SI A.4*).

While ARG concentrations do not necessarily correlate with antibiotic concentrations (Takasu et al., 2011), pharmaceutical pollution, including disinfectant and heavy metal pollution can be an influencing factor, especially in *Intl*1 selection (Hegstad et al., 2010; Khan et al., 2013; Koenig et al., 2009; Rosewarne et al., 2010; Stepanauskas et al., 2006; Q. Wang et al., 2015). L2 and L3 are locations with an extensive pharmaceutical industry (incl. L2 – Novartis, Basilea etc., L3 – Merck, Bayer, Steigerwald etc.).

Discharges from antibiotics manufacturing have previously been shown to be able to increase ARG concentrations locally (Khan et al., 2013) and have been mentioned as being higher-risk hotspots (Bengtsson-Palme and Larsson, 2015). It is not known whether pollution from the pharmaceutical industry (Ruff et al., 2015) is responsible for the observed increases in ARG and Intl1 presence (L3) and abundance (L2) at these locations. Other influencing factors are antibiotic use/sales and farming intensity. While L2 is in a region with intense farming, L3 is characterized by low antibiotic sales and little farming (see SI A.3).

## 4. Conclusion

Multiplex qPCR assays are an efficient method to monitor antibiotic resistance genes in the environment. While second- and third generation sequencing are the high-throughput methods of choice for gene detection today, they are not considered quantitative. Other quantitative high-throughput methods like microassays and high-throughput qPCR are prone to issues, including sensitivity issues. Multiplex qPCR assays increase the throughput while maintaining the accuracy and quantitative nature of classic qPCR assays. Higher throughput methods are of special interest in environmental antibiotic resistance research as there is a continuously increasing number of genes of interest.

Antibiotic resistance in the Rhine river may be influenced by anthropogenic pollution to a certain extent as the AR rise between L1 and L3 suggests, but does not continuously increase over the course of the Rhine. Factors influencing local ARG concentrations can be river size, as data suggests that smaller rivers are more directly impacted by anthropogenic pollution, but also proximity and type of discharges of wastewater treatment plants, presence of hospitals, agricultural/farming discharges and run-off. A general link between the most used antibiotics in the Rhine catchment (penicillins, tetracyclines, sulfonamides and macrolides) and presence of ARG classes was observed.

*Intl*1 concentrations are a good indicator for *sul*1 concentrations but not for overall anthropogenic ARG pollution. It is necessary to determine which factors are most relevant for the quantification of ARG pollution. Investigating correlations between *Intl*1 concentrations and data of NGS of ARG presence might be of further interest as the selection bias caused by gene selection for qPCR analysis would be minimized. The combined presence of certain ARGs on MGE could explain the observed correlation between those specific ARGs and are also relevant in health risk assessment (Oh et al., 2018).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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