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2021-10-15

Lai , F Y , Muziasari , W , Virta , M , Wiberg , K & Ahrens , L 2021 , ' Profiles of environmental antibiotic resistomes in the urban aquatic recipients of Sweden using high-throughput quantitative PCR analysis ' , Environmental Pollution , vol. 287 , 117651 . https://doi.org/10.1016/j.er

http://hdl.handle.net/10138/339589
https://doi.org/10.1016/j.envpol.2021.117651

Environmental Pollution 287 (2021) 117651

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Profiles of environmental antibiotic resistomes in the urban aquatic recipients of Sweden using high-throughput quantitative PCR analysis☆

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ARTICLE INFO

Keywords:
Emerging contaminants
Antibiotic resistance genes
Mobile genetic elements
Antimicrobial resistance
Surface water

ABSTRACT

Antibiotic resistance in aquatic ecosystems presents an environmental health issue worldwide. Urban recipient water quality is susceptible to effluent discharges with antibiotic resistance contaminants and needs to be protected, particularly for those as sources of drinking water production. Knowledge on aquatic resistome profiles in downstream of wastewater treatment plants allows a better understanding of the extent to which antibiotic resistance contaminants emerge and spread in recipient waters, but such information remains very limited in Sweden. The key objective of this study was to determine the resistome profiles of numerous antibiotic resistance genes (ARGs), mobile genetic elements (MGEs) and other genes in urban recipient water systems connected to Sweden's major drinking water reservoir. This was achieved through analysis of surface water samples for 296 genes using high-throughput quantitative PCR arrays. A total of 167 genes were detected in at least one of the samples, including 150 ARGs conferring resistance to 11 classes of antibiotics, 7 integrase MGEs and 9 other genes. There was a spatial difference in the resistome profiles with the greatest average relative abundance of resistance genes observed in the water body of Västerås followed by Uppsala, Stockholm and Eskilstuna, as similar to the general pattern of the antibiotic sales for these regions. ARGs against β-lactams and sulfonamides showed the highest average relative abundance in the studied water bodies, while vancomycin resistance genes were only found in the Uppsala water environment. Generally, the recipient water bodies were detected with higher numbers of genes and greater relative abundances as compared to the upstream sites. Anthropogenic pollution, i.e., wastewater discharge, in the recipient water was also reflected by the finding of *intI*, *sul1* and *crAssphage*. Overall, this study provided the first quantitative assessment of aquatic environmental resistomes in Sweden, highlighting the widespread of antibiotic resistance contaminants in urban recipient waters.

1. Introduction

Worldwide, approximately 700,000 deaths/year are caused by antimicrobial resistance, and it is estimated that this number will increase to over 10 million/year by 2050 (O'Neill, 2014). The World Health Organisation (WHO) has emphasised the current threat of antibiotic resistance to public health worldwide and the need for tackling resistance, from individuals to policymakers (World Health Organization, 2014). Recently, the United Nations Environment Programme has listed this topic as a global emerging environmental issue (UN Environment, 2017). These reveals a raising concern about the development of environmental resistance, particularly the presence and spread of antibiotic resistance genes (ARGs) in our ecosystems. Over- and miss-use of antibiotics in human disease control, as well as in the veterinary, agriculture and aquaculture sectors, are main factors contributing to the environmental evolution of ARGs (Andersson and Hughes, 2014; Berendonk et al., 2015; Larsson et al., 2018).

Wastewater treatment plants (WWTPs) are regarded as key hotspots for mixtures of antibiotics entering the environment (Karkman et al., 2018). Effluent discharges of these residues can potentially create a selective pressure on environmental bacteria. Hence, resistance can be disseminated to their new generation (vertical transfer) and/or to other

☆ This paper has been recommended for acceptance by Klaus Kümmerer.

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https://doi.org/10.1016/j.envpol.2021.117651
Received 20 January 2021; Received in revised form 13 June 2021; Accepted 21 June 2021
Available online 26 June 2021

types of bacteria (horizontal transfer), enhancing the proliferation of ARGs in the recipient water (Karkman et al., 2018). Such genetic materials, once emerged, are rather persistent and can further spread at a global level, placing ARGs as a new group of emerging contaminants in the environment (Richardson and Kimura, 2020). Their presence in effluent-receiving waters is of particular concern when it is reused for agricultural irrigation, as sources for drinking water production and leisure activities (UN Environment, 2017; Leonard et al., 2018; Richardson and Kimura, 2020). Several studies have reported high prevalence of ARGs in recipient water environments across different continents (e.g. Stoll et al., 2012; LaPara et al., 2015; Rodriguez-Mozaz et al., 2015; Xu et al., 2016; Bueno et al., 2017; Zheng et al., 2017; Freeman et al., 2018; Sabri et al., 2018; Cacace et al., 2019; Hamiwe et al., 2019). In Europe, a few studies have been performed for the occurrence of ARGs in the surface water (Rodriguez-Mozaz et al., 2015; Sabri et al., 2018; Cacace et al., 2019). A recent Europe-wide study analysed nine ARGs in recipient water across 10 countries, in which *int1* and *sul1* were the most abundant ARGs (Cacace et al., 2019). So far, in Sweden, only two studies have reported a few ARGs in the aquatic environment (Iversen et al., 2002; Khan et al., 2019). Iversen et al. (2002) isolated the vancomycin-resistant bacteria that carried *vanA* in downstream surface water from Stockholm and Uppsala, but their occurrence was sparse (detection in 1 out of 37 samples). Similarly, Khan et al. (2019) recently studied antibiotic resistance in the Svartån river in Örebro city based on cultured resistant bacteria to qualitatively monitor 84 ARGs with the majority of β-lactam resistance genes, and detected 43 ARGs in the receiving river water.

The presence of ARGs in the aquatic environment can be assessed using culture-dependent methods, which allow an enrichment of bacteria for a better gene detection (Iversen et al., 2002; Khan et al., 2019). However, culture-dependent methods have its limitation to reflect the actual, ambient diversity and abundance of resistance determinants in the aquatic ecosystem, as only a small fraction (<1%) of environmental bacteria in natural water is readily cultivated (Karkman et al., 2018). The alternative is a cultured-independent approach. For instance, the recent development of high-throughput qPCR allows to simultaneous quantification of several hundreds of ARGs, mobile genetic elements (MGEs) and other genes of interest within one single run, resulting in a high productivity and accuracy measurement (Karkman et al., 2016; Karkman et al., 2018). The SmartChip™ technology is one recent technique in the high-throughput qPCR method family (Lamas et al., 2016; Waseem et al., 2019). It has been successfully applied to reveal resistomes in various environmental matrices including recipient surface water across different locations (e.g. Karkman et al., 2016; Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017; Zhu et al., 2017; Chen et al., 2019). Besides ARGs and MGEs, it is of emerging interest that the presence of other genes, such as *crAssphage* as indicator of human faecal contamination could help understand ARG abundances in environments impacted by anthropogenic activities (Karkman et al., 2019). As recently highlighted by the NORMAN network in Europe, more studies on ARGs in natural water bodies are urgently needed for a better understanding of their presence and risks in aquatic environments and water reuse processes (Dulio et al., 2018). There remains a lack of knowledge on the occurrence and spatial evaluation of quantitative resistome profiles in the aquatic environment in Sweden.

The key aim of this study was to examine the profiles of emerging microbial contaminants, including ARGs, MGEs and other genes in the urban aquatic environment connecting to Lake Mälaren, Sweden's major drinking water reservoir serving ~2 million people (about 20% of Sweden's population). Based on the SmartChip™ high-throughput qPCR, our specific objectives were to (a) determine the resistome (296 genes) in different water bodies, targeting a wide range of ARGs (n = 268), MGEs (n = 8) and other genes (n = 20), and (b) investigate changes in gene diversity and abundance measured at downstream WWTP sites as compared to its upstream water. To the best of our knowledge, this study provides the first quantitative, wide-scope screening for antibiotic resistomes in the recipient aquatic environment across four urban locations in Sweden using molecular methods. Our data help further understand potential risks of dissemination of ARGs, MGE and other genes in the aquatic environment and plan related remediation in the future.

2. Materials and methods

2.1. Water sampling

Water sampling was conducted in July 2018. Four major cities (Uppsala, Stockholm, Eskilstuna and Västerås) located around Lake Mälaren, the major drinking water reservoir in Sweden, were selected for the study. The Stockholm, Uppsala and Västerås regions are more urbanised and more densely populated than the Eskilstuna region. The hypothesis in this study is that more diverse ARGs would be observed in the aquatic environment in more urbanised, densely-populated areas, and also in their downstream water environment compared to the upstream site. Recipient surface water samples were collected at a depth of ~0.5 m at sites located about 2 km downstream of municipal WWTP effluent discharges, including the river Fyrisån in Uppsala, the archipelago in Stockholm, the river Eskilstunaån in Eskilstuna, and a bay in Västerås. All water bodies are connected to Lake Mälaren. In addition, surface water samples were collected about 2 km upstream of the WWTPs, except for Västerås as there was no upstream water source. In Eskilstuna, an extra downstream sample was collected in a wetland area, which is part of the treatment process of the WWTP. Triplicate water samples (three times at the same spot; 1 L each) were collected at each sampling site and kept on ice during the transport to the laboratory at SLU (Uppsala) for filtration. A total of 24 water samples were obtained from eight sampling sites.

2.2. DNA extraction

Water samples were concentrated using 0.22 μm Millipore Express® polyethersulfone filter membranes on the same day of sample collection. The filters were stored at −80 °C until gene extraction. Extraction of DNA in the samples was performed using DNeasy PowerWater Kit (Qiagen Sciences, Germantown, MD) according to the manufacturer's protocol. Prior to qPCR measurements, the DNA quality and concentration in the extracts (triplicates per sampling point) were determined with NanoDrop™ One Microvolume UV/Vis Spectrophotometer (Thermo Scientific™, Wilmington, DE, USA) via monitoring the absorbance ratio of 260/280 and 260/230 nm.

2.3. Gene analysis with high-throughput qPCR arrays

DNA samples, primer sets and the qPCR reagents were transferred to 100 nL reaction wells (n = 5184) of the SmartChip™ by the SmartChip™ Multisample Nanodispenser (TakaraBio, CA, USA). Briefly, the reaction mixture in each 100 nL reaction comprised 1 × SmartChip TB Green Gene Expression Master Mix (TakaraBio, CA, USA)), nuclease free PCR-grade water, 300 nM of each primer and a DNA template of 2 ng/μL.

A total of 296 primer sets was used to assay the genes of interest in this study using previously validated methods (Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017). Of all the selected primer sets, 268 primer sets were used for ARGs conferring resistance to 11 major classes of antibiotics (Table S1) that are commonly consumed by humans and animals, including aminoglycosides, amphenicol, β-lactams, sulphonamides, trimethoprim, multidrug resistances (MDR), florfenicol, macrolide-, lincosamide- and streptogramins B (MLSB), tetracycline, quinolone, and vancomycin, which cover three main mechanisms (efflux-pumps, cellular protection and deactivation) of antibiotic resistance; 8 primer sets for integrons (mobile genetic elements (MGEs) (Table S1); and 20 primer sets for other genes (Table S1) of mercury resistance, antibacterial resistance including bacitracin, colistin and antiseptic, genes associated with human faecal

contamination, and the total bacteria gene *16S rRNA*. Several primer sets were designed to target sequence diversity within a gene for a more specific assessment of the environmental resistome, and therefore, each primer set was analysed independently.

Gene detection and quantification were performed using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). qPCR cycling conditions and processing of raw data were described elsewhere (Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017). Briefly, the qPCR conditions included initial enzyme activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s and then annealing at 60 °C for 30 s for amplification. Melting curve analysis was performed for each primer set of all the samples. Amplicons with unspecific melting curves and multiple peaks based on the slope of melting profiles were considered to be false positive data and therefore discarded from the analysis. Melting curve analysis was processed using the SmartChip™ qPCR software. The threshold cycle (C_T) of 27 was set as the detection limit (Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017). Each DNA sample was analysed in three qPCR reactions (i.e. technical replicates). When a gene was detected in at least two technical replicates, mean C_T of three technical replicates in each qPCR reaction was calculated. For each replicate sample (1 L), the quantity of a detected gene was calculated and expressed as the relative gene abundance in proportion to the *16S rRNA* gene based on the equation of $2^{-\Delta C_T}$, where $\Delta C_T = \Delta C_T(detected\ gene) - \Delta C_T(16S\ rRNA)$ (Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017). Data processing and data analysis were performed using python program by Resistomap Oy (Helsinki, Finland).

2.4. Statistical analysis

Statistical analysis and visualisation in figures were performed using R Studio (Version 1.1.463). Non-metric multidimensional scaling (NMDS) analysis was performed using the Vegan package in R (Version 2.5–7) (Oksanen et al., 2020) to evaluate the degree of similarity of gene profiles across different water bodies, taking the geographical locations and sampling sites into account. Function metaMDS was used to calculate the distance matrix. Function ordispider and ordiellipse from Vegan were, respectively, used to plot the distance matrix of centroids and to add 95% confidence region of groups. Permutational multivariate analysis of variance also from Vegan was performed to determine the differences of gene profiles between sites. Genes' abundance values relative to the *16S rRNA* gene were used to calculate the distances between samples using function vegdist from Vegan based on Bray-Curtis method. Data values below detection limits were replaced with zero for the data analysis. The permutational multivariate analysis of variance was performed using function adonis2 from Vegan and default parameters with 9999 permutations to test whether the sites were significantly different. The analysis was considered to be significant at p-value<0.05. Significant correlations ($\rho > 0.8$ and p-value<0.01) between ARGs, MGEs and other genes were analysed using Spearman's correlation, and further visualised in a network analysis. Gene profiles of each site were visualised using heatmap from ggplot2.

3. Results & discussion

3.1. Gene diversity

Out of the target 296 genes, a total of 167 was detected in at least one of the replicate samples across the four studied locations. These included 150 ARGs, nine other genes, seven integron-integrase genes of class I, II and III (MGEs) and *16s rRNA* (total bacteria gene). Uppsala's downstream water (111) showed the most diverse gene detection, followed by Västerås (100) and the upstream water of Uppsala (80) (Fig. 1). A relatively lower number of genes (at least by a factor of two) were found in the Stockholm (up to 34) and Eskilstuna samples (up to 22). Higher

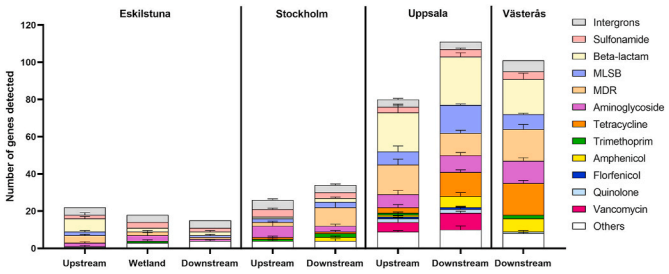

Fig. 1. Diversity of gene detected across different locations. Error bars = standard deviations of triplicate water samples. MLSB: Macrolide-, lincosamide- and streptogramins B resistance. MDR: Multidrug resistances. See detail of the genes in Table S1.

numbers of genes were generally observed at downstream than upstream sites, as showed in Uppsala (111 *vs.* 80) and Stockholm (34 *vs.* 26), indicating a potential contribution of the effluent discharges from the WWTPs to more gene varieties in the recipient. Khan et al. (2019), who investigated the Svartån river in Örebro city (Sweden), showed similar results of more ARGs detected downstream the WWTP than in the upstream water (43 *vs.* 22). However, in Eskilstuna, the number of detected genes was slightly lower in the downstream water than in the wetland and upstream samples (15 (downstream), and 18 and 22 (upstream), respectively). It has shown that wetland treatment may remove ARGs in effluent wastewater (Hsu et al., 2017) and this might be the explanation in the Eskilstuna case. Further studies in this location are needed to investigate the temporal or seasonal impact of the WWTP and also the treatment process of wetlands on removing ARGs over time.

Our results revealed, for the first time, a spatial difference in the diversity of ARGs, MGEs and other genes across different surface water bodies in Sweden, probably due to differences in the local environment, e.g. hospital settings in the community. Intergones and sulfonamide resistance genes were quite evenly present at all sites. ARGs conferring resistance to β-lactams, MLSB, MDR, aminoglycosides, tetracyclines and amphenicol were more prevalent in the Uppsala's and Västerås's waters. Trimethoprim resistance genes were mainly found in the waters of Stockholm and Västerås. Resistance genes of florfenicol and vancomycin were unique in the Uppsala waters. Similarly, quinolone resistance genes were mainly present in the waters of Uppsala followed by Västerås, but not at other sites. Besides MGEs and ARGs, other genes were also detected in the studied aquatic bodies, with Uppsala and Västerås being prevalent and revealing the occurrence of the genes (*fabK*, *nisB* and *nimE*) conferring resistance to antiseptics and antibacterial peptides as well as the genes (*crAss64* and *crAss56*) related to human faecal contamination. The mercury resistance gene (*merA*) and antiseptic-resistance genes (*qacE-delta1*) were common at all the sites, except the upstream water of Eskilstuna. The obtained data indicate that these microbial contaminants are ubiquitously distributed in the urban aquatic environment in Sweden, which agree with previous findings in other European countries (e.g. Sabri et al., 2018; Cacace et al., 2019), although it still has to be confirmed with more extensive studies covering not only the Lake Mälaren region.

3.2. Aquatic resistome profiles

Relative abundances of resistance genes and integrons to the *16S rRNA* gene were used to reflect the environmental resistome of our studied water bodies across four urbanised areas (Fig. 2). The abundance of each gene group varied with locations, and also between upstream and downstream waters (Fig. 2 and Fig. S1). On average, the total abundance of resistance genes was the highest in Västerås (1.1×10^{-1}), followed by Uppsala (1.7×10^{-2}), Stockholm (5.5×10^{-3}) and Eskilstuna (5.0×10^{-3}). This was in general similar to the pattern of the antibiotic sales for these regions (Swedres-Svarm, 2019). Similar spatial

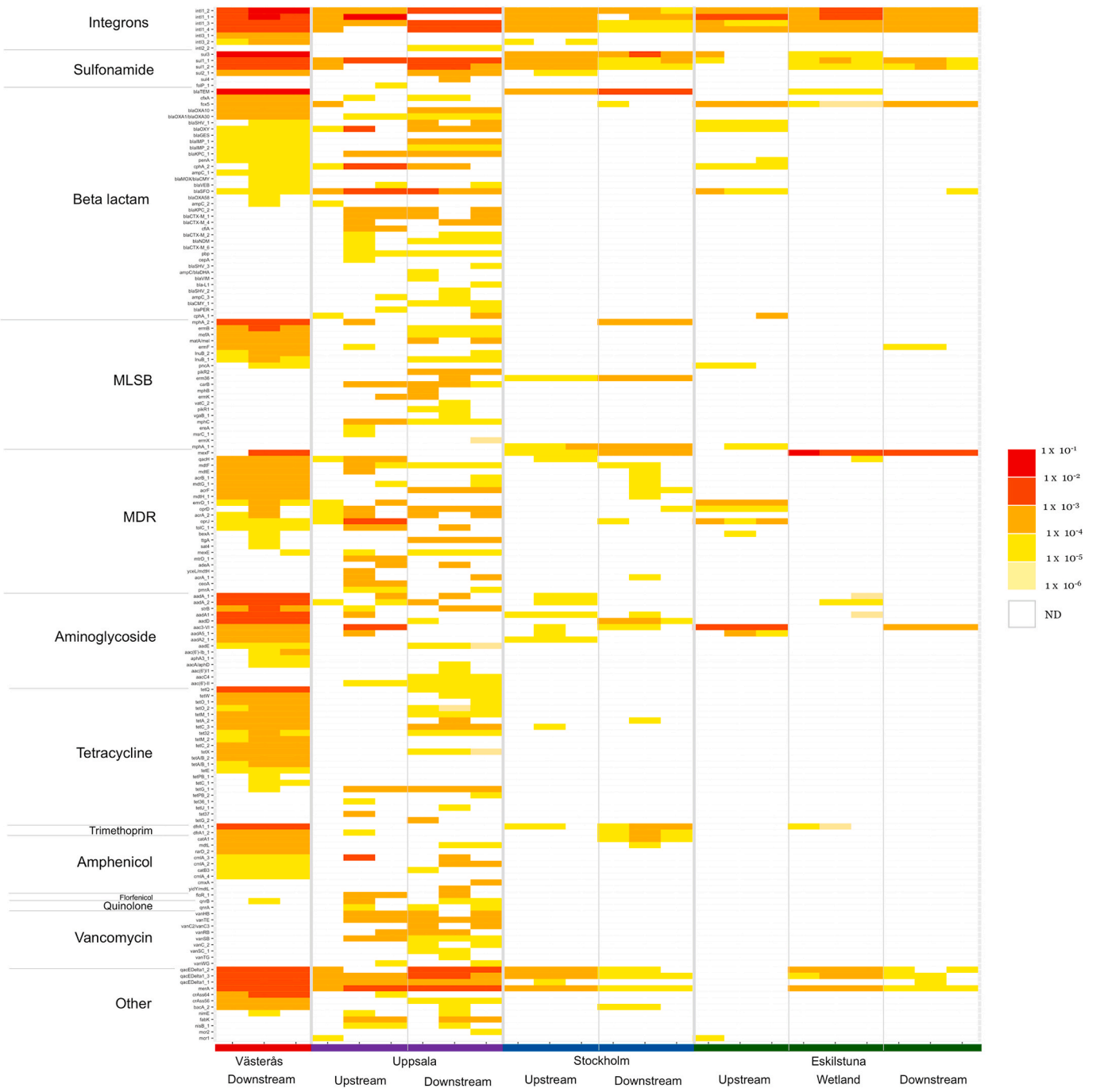

Fig. 2. Resistome profiles of upstream and effluent-receiving water bodies across the four studied locations. MLSB: Macrolide-, lincosamide- and streptogramins B resistance. MDR: Multidrug resistances. See detail of the genes in Table S1.

patterns of the average total abundance was also noticed for integrons, with the greatest abundance in Västerås (3.3×10^{-2}) and Uppsala (1.7×10^{-2}), followed by Eskilstuna (2.8×10^{-3}) and Stockholm (1.1×10^{-3}). Average total abundances of resistance genes were about three to five times higher in downstream than upstream waters, as measured in the samples of Eskilstuna (1.3×10^{-2} *vs.* 2.4×10^{-3}) and Stockholm (8.1×10^{-3} *vs.* 2.8×10^{-3}), but showed similar abundances at both sites of Uppsala (1.9×10^{-2} *vs.* 1.6×10^{-2}). Among the different groups of genes (Fig. S2), the MGE integrons (5.4×10^{-2}) showed the greatest average total abundance, followed by ARGs against β-lactams (4.3×10^{-2}) and sulfonamides (3.2×10^{-2}). In comparison, other ARG groups were found at lower average total abundance ($\leq 1.0 \times 10^{-2}$) (Fig. S2).

3.2.1. *MGE integrons*

For the MGE group, seven *intI* genes were quantifiable in at least one of the water samples, with the abundance in a range of 1.2×10^{-5}-4.5×10^{-2} (Fig. 2). On average, the *intI1* (2.4×10^{-3}) genes were the most abundant, followed by *intI3* (1.8×10^{-5}) and *intI2* (1.9×10^{-6}). *IntI1* were present in all the water samples. The Class 2 and Class 3 *intI* genes were sporadically detected at low abundances, in which *intI2* was only measured in Uppsala downstream water and the *intI3* genes were mainly observed in Västerås water (Fig. 2). *IntI1* is widely considered as a genetic marker of anthropogenic pollution, and its occurrence has been suggested to indicate potential impact from WWTPs and human activities (Gillings et al., 2015; Cacace et al., 2019). Our results of *intI1* suggested that such impacts appeared higher in the Västerås and

Uppsala recipient waters as compared to the other two locations. The presence and enrichment of these integron genes in the water bodies poses a concern about facilitating transfer of resistance genes among the same and/or different species of bacteria in our environment (Karkman et al., 2018). While this study is the first to show various classes of integron genes in Swedish recipient waters, our data show similarity to a previous study, which found increased abundances of *intI1* in sediment downstream of the Stångån river in Linköping (Berglund et al., 2015). *IntI1* was also commonly measurable in other receiving river water across different European cities (Cacace et al., 2019).

3.2.2. Sulfonamide resistance genes

In the group of sulfonamides, six ARGs were measurable in our water samples, with an abundance ranging from 1.2×10^{-5} to 2.0×10^{-2} (Fig. 2). The receiving water bodies revealed two to four times higher average abundance than their upstream water (Uppsala: 3.9 *vs.* 1.0×10^{-3}; Stockholm: 1.7 *vs.* 1.0×10^{-3}; Eskilstuna: 2.0×10^{-4} *vs.* 8.3×10^{-5}). *Sul3* (2.4×10^{-3}) was the most dominant gene with the greatest average abundance, followed by *sul1* (9.8×10^{-4}). *Sul3* was mainly found in Västerås, Stockholm and Eskilstuna's wetland waters, but was absent in any water samples of Uppsala (Fig. 2). *Sul1* was prevalent in the studied water bodies, except for Eskilstuna upstream water. Comparatively, the other sulfonamide resistance genes, including *sul2*, *sul4* and *folP*, were less frequently detected and measured by about an order of magnitude lower in the average abundance. *Sul2* was mostly observed in Västerås and Uppsala recipient waters. *Sul4* and *folP* were rarely detected in the studied water bodies. The finding of high prevalence of *sul1* and *sul3* in the studied water bodies, especially downstream sites, was not unexpected, given the fact that *sul1* has been repeatedly measured in receiving aquatic ecosystems around the world and is regarded as a genetic marker of anthropogenic pollution (e.g. Stoll et al., 2012; Berglund et al., 2015; Sabri et al., 2018; Cacace et al., 2019). *Sul3* was originally identified in *Escherichia coli* isolates from pigs in Switzerland (Perreten and Boerlin, 2003) and was later on found in *E. coli* isolates from cattle, pigs and poultry in Germany (Guerra et al., 2003) and Denmark (Hammerum et al., 2006). While these studies appeared to suggest a widespread of *sul3* among livestock, probably due to consumption of sulfonamides in veterinary use and animal husbandry, *sul3* has also been discovered in human clinical isolates of Enterobacteriaceae (*E. coli*) from Sweden (Grape et al., 2003), as well as *Salmonella* isolates from human clinical sources in Portugal (Antunes et al., 2005). *Sul3* was also detected in river water receiving wastewater from animal farming (Al Salah et al., 2019) and in the estuary environment (Chen et al., 2020). These studies commonly reported *sul3* in lower relative abundance compared to *sul1* and *sul2*, while our study observed the opposite, especially in Västerås recipient water. There is veterinary prescription of sulfonamides in Sweden, mainly for oral use in horses, and *E. coli* isolated from dairy cows and dogs has showed resistance to sulfonamides (Svarm, 2007). Our data on *sul3* is rather a new discovery for the urban surface water environment in Sweden.

3.2.3. β-lactam resistance genes

For the gene group encoding resistance to β-lactams, 37 ARGs were quantified in our water samples, with the abundance ranging from 9.3×10^{-6} to 4.1×10^{-2} (Fig. 2). These genes were highly prevalent in Västerås and Uppsala waters. The former was on average the most abundant (3.7×10^{-2}). Both upstream (3.2×10^{-3}) and downstream (3.8×10^{-3}) sites of Uppsala showed similar average abundance. The β-lactam resistance genes were very rarely present in Stockholm waters, with mainly *blaTEM* quantified at high abundance. Particularly, this gene showed about 12 times higher abundances in the receiving water (4.1×10^{-3}) than in its upstream sample (3.2×10^{-4}) at this location. The water samples from Eskilstuna (3.9×10^{-4}) showed the lowest average abundance in the β-lactam resistance gene group. In this ARG group, *blaTEM* (4.9×10^{-4}) occurred at the greatest average abundance, with the water samples of Västerås (3.5×10^{-2}) at the highest abundance followed by Stockholm (2.2×10^{-3}) and Eskilstuna (wetland: 5.1×10^{-5}). This gene was not present in the water body of Uppsala. Four more β-lactam resistance genes, including *cfxA*, *fox5*, *blaOXY10* and *blaOXA1/blaOXA30*, were also quantified at high abundance (3.6-4.6×10^{-4}) in Västerås water. These genes were often non-detectable or at low abundance at the other sites, with an exception of *fox5* (1.8×10^{-4}) in Eskilstuna waters as well as *blaOXY10* (2.5×10^{-4}) in the Uppsala receiving water. Another 11 ARGs, including *blaSFO*, *cphA*, *blaOXY*, *blaSHV*, *blaKPC*, *blaIMP*, *penA*, *blaGES*, *ampC*, *blaVEB* and *blaMOX/blaCMY*, occurred at higher average abundance mostly in the water bodies of Uppsala (2×10^{-4}) compared to Västerås ($<5 \times 10^{-5}$) and Eskilstuna ($<4 \times 10^{-5}$). In Uppsala, three of these 11 genes were only measured in its downstream water (*blaSHV*: 3.3×10^{-4}; *blaIMP_1*: 1.1×10^{-4}; *blaIMP_2*: 1.7×10^{-5}). One gene was about two times more abundant in the downstream than upstream water of Uppsala (*blaOXY*: 7.4 *vs.* 3.5×10^{-4}), while three genes showed similar abundance between downstream and upstream waters at this location (*blaSFO*: 8.8×10^{-4}; *cphA*: 3.3 *vs.* 5.1×10^{-4}; *blaKPC*: 1.1 *vs.* 1.3×10^{-4}). There were five genes among these 11 ARGs detectable only in Västerås water at very low abundances (*penA*, *blaGES*, *ampC*, *blaVEB* and *blaMOX/blaCMY*: 2.2-4.9×10^{-5}). The rest of the quantifiable ARGs in this group were mainly observed in Uppsala's water bodies, at very low but similar abundances between its upstream and downstream sites (average abundance: 3.9×10^{-5} vs. 3.4×10^{-5}) (Fig. 2).

There are some similarities between our findings and a recent study in the river water system of Örebro city, showing a high detection frequency of β-lactam resistance genes with some of them from the group of *blaOXA*, *blaVEB*, *blaVIM* and *blaCTX-M* mainly in its downstream sites (Khan et al., 2019). Particularly, for the *blaOXA* group, a high prevalence of *blaOXA10* was observed in the receiving waters by Khan et al. (2019) and in this study. *blaOXA58*, *blaVEB*, *blaVIM* were rarely detected in our studied water bodies, so were Örebro (Khan et al., 2019), whereas *blaCTX-M_1* appeared to be more consistently present in the Örebro's river system than in this study. *BlaCTX-M* has been observed to be increasingly enriched along the conventional treatment process, with an average relative abundance of about 1.2×10^{-3} measured in effluent wastewater from three WWTPs in Sweden (Bengtsson-Palme et al., 2016), which is at least an order of magnitude higher than the relative abundance of *blaCTX-M* found in the water bodies of our study. While *blaOXA58* was suggested to be another important marker of anthropogenic pollution by a recent Europe-wide study (Cacace et al., 2019), the occurrence of this gene in the recipient water environment was very scarce in both this study and Khan et al. (2019)'s study. However, our result of the most dominant β-lactam resistance gene, *blaTEM*, was similar to other European receiving water environments with high prevalence and abundance of this gene (Cacace et al., 2019). On the other hand, a low occurrence and abundance was observed for *blaKPC* group, such as *blaKPC_3*, in this study as well as in the previous study (Cacace et al., 2019).

3.2.4. MLSB resistance genes

For the MLSB resistance gene group, 21 ARGs were quantified in this study, with an abundance ranging from 9.6×10^{-6} to 3.3×10^{-3} (Fig. 2). Most of these ARGs were consistently detected in the water bodies of Västerås (average abundance 5.2×10^{-3}) and Uppsala (upstream: 5.0×10^{-4}; downstream: 1.2×10^{-3}), but were very rare in those of Stockholm and Eskilstuna. Eight ARGs showed higher abundance, especially in Västerås, in which *mphA_2* (2.8×10^{-3}) was the most abundant followed by *ermB* (6.4×10^{-4}), *matA/mel* (5.2×10^{-4}), *ermF* (5.0×10^{-4}), *mefA* (4.8×10^{-4}), *InuB_2* (1.3×10^{-4}), *InuB_1* (1.1×10^{-4}) and *pncA* (3.1×10^{-5}). These genes were also observed in the Uppsala water, with a higher detection and average abundance at the downstream site (4.6×10^{-5}) in comparison to the upstream site (1.6×10^{-5}), and five (*ermB*, *matA/mel*, *mefA*, *InuB_1*, *InuB_2*) of these ARGs were detected only in the receiving water body of Uppsala. In the Stockholm water bodies, *mphA_2* (3.7×10^{-4}) was also present but only

in its downstream water, together with the other two genes *erm36* (5.1×10^{-4} *vs.* 5.8×10^{-5}) and *mphA_1* (4.4×10^{-4} *vs.* 8.6×10^{-5}) which showed about five to eight times more abundant in the receiving water than its upstream site. The rest of the quantifiable MLSB resistance genes was mainly found in the Uppsala water bodies, with a higher occurrence and abundance in its downstream than upstream water (6.8 *vs.* 3.4×10^{-5}). Compared to the Örebro's recipient with only *ermB* detected out of five targeted genes (Khan et al., 2019), our finding demonstrated that more macrolide and MLSB resistance genes actually existed in the aquatic environment.

3.2.5. MDR resistance genes

For the resistance gene group of MDR, 23 related ARGs were found at the studied sites with the abundance ranging from 1.3×10^{-5} to 2.2×10^{-2} (Fig. 2). These genes had the highest average abundance in the samples from Västerås and Uppsala in comparison to Stockholm and Eskilstuna. However, the most abundant gene, *mexF*, was observed in the Eskilstuna water at the two downstream sites (wetland: 9.6×10^{-3}; downstream: 1.5×10^{-3}). The water body of Västerås showed also a high abundance for this gene (1.1×10^{-3}), as well as that of Stockholm with two times higher abundance in the receiving water (1.4×10^{-4}) than at its upstream (6.8×10^{-5}) site. In contrast, *mexF* was absent in the Uppsala waters. Seven MDR resistance genes, including *qaH*, *mdtE*, *mdtF*, *acrF*, *acrB_1*, *mdtG_1*, *mdtH_1*, were more consistently measured at a high abundance (2.3-4.4 $\times 10^{-4}$) in Västerås as compared to the other sites. These ARGs were also sporadically detected at low abundances in the water bodies of Uppsala ($<8.0 \times 10^{-5}$) and Stockholm ($<1.6 \times 10^{-5}$, mainly downstream). Another five ARGs (*oprJ*, *oprD*, *acrA_2*, *emrD_1*, *tolC_1*) were also frequently observed in Västerås (average 6.1×10^{-5}) and Uppsala (upstream: 4.5×10^{-4}; downstream: 2.2×10^{-4}) waters. Three of them were occasionally found in the upstream water of Eskilstuna (*emrD_1*: 1.5×10^{-4}; *oprJ*: 1.4×10^{-4}; *oprD*: 7.7×10^{-5}). The remaining ARGs were mainly found in the water bodies of Uppsala at very low abundances ($<6.0 \times 10^{-5}$). Two MDR genes (*oprJ*, *oprM*) were detected in the Örebro's downstream water (Khan et al., 2019) similar to our findings.

3.2.6. Aminoglycoside resistance genes

For the aminoglycoside resistance genes, 15 related ARGs were measured in the water samples with an abundance ranging from 7.3×10^{-6} to 4.7×10^{-3} (Fig. 2). These genes were mostly prevalent in the Västerås water at high abundances (average 8.0×10^{-3}), especially *aadA_1* (1.9×10^{-3}), *aadA_2* (1.6×10^{-3}), *aadD* (1.2×10^{-3}), *aadA1* (1.1×10^{-3}) and *strB* (9.5×10^{-4}). These five ARGs were also found, albeit less often and with lower abundance, in the water bodies of Uppsala (upstream: 3.2×10^{-5}; downstream: 5.9×10^{-5}) and Stockholm (upstream: 2.3×10^{-5}; downstream: 4.0×10^{-5}). Another three genes, *aac3-VI*, *aadA5_1* and *aadA2_1*, were also commonly detected in the different water bodies. Particularly, a high abundance of *aac3-VI* was measured in Eskilstuna (upstream: 1.1×10^{-3}; downstream: 1.7×10^{-4}), Uppsala (upstream: 2.9×10^{-3}) and Västerås (4.9×10^{-4}). The remaining seven measurable aminoglycoside resistance genes were occasionally observed in Västerås and Uppsala receiving water bodies at low abundances ($<1.0 \times 10^{-4}$). While *aacC2* was detected in Örebro's downstream water (Khan et al., 2019), this gene was not quantifiable ($<1.0 \times 10^{-6}$) in any of our samples. On the other hand, *aadA1* was more frequently detected than *aacC4* in the water samples from Örebro (Khan et al., 2019), similar to our results.

3.2.7. Tetracycline resistance genes

For the resistance genes against tetracycline, 22 ARGs were quantified in this study, with an abundance ranging from 9.5×10^{-6} to 1.9×10^{-3} (Fig. 2). These genes were mainly found in the Västerås and Uppsala downstream sites, with *tetQ* at the greatest abundance (1.5×10^{-3} and 6.5×10^{-5}, respectively). Twelve more genes, including *tetW*, *tetA_2*, *tetC_3*, *tetO_1*, *tetM_1*, *tetO_2*, *tetC_2*, *tetA/B_2*, *tetX*, *tetM_2*, *tet 32*, *tetA/B_1*, were also present at a high abundance in the water body of Västerås ($1.2\text{-}5.1 \times 10^{-4}$). Four of them (*tetC_2*, *tetA/B_2*, *tetM_2*, *tetA/B_1*: $1.2\text{–}1.7 \times 10^{-4}$), together with another three genes (*tetE*, *tetPB_1*, *tetC*: $1.7\text{-}6.9 \times 10^{-5}$), were only found in the Västerås water. Eight of those 12 ARGs were also observed at the Uppsala site, but only in its receiving water (7.3×10^{-6}-4.5×10^{-5}). There were a few genes (*tetG_1*, *tet37*, *tetU_1*, *tet36_1*, *tetPB_2*) mainly found in Uppsala waters. However, their occurrence was rather sporadic, except for tetG_1 (1.6×10^{-4}), which was consistently present at the upstream and downstream site in Uppsala. These ARGs were very rare or even absent in Stockholm and Eskilstuna waters. The occurrence of *tetA* in this study was also observed in Örebro's river system (Khan et al., 2019). While *tetM* was highly prevalent in other European receiving water environments (Cacace et al., 2019), this gene was only detected in two of our studied locations. This may suggest that the environmental resistance of tetracycline can be varied across different European countries.

3.2.8. Trimethoprim resistance genes

Only two genes (*dfrA1_1*, *dfrA1_2*) conferring resistance to trimethoprim were quantifiable in the studied sites, and constantly appeared in Västerås and Stockholm downstream waters. The Västerås water showed the highest abundance for both genes (*dfrA1_1*: 1.6×10^{-3}; *dfrA1_2*: 7.6×10^{-4}). Relatively lower abundance of these two genes (*dfrA1_1*: 1.2×10^{-4}; *dfrA1_2*: 6.2×10^{-6}) was observed in the downstream water of Stockholm. Both genes were very rarely detected in the other water bodies. *dfr1* was reported in downstream sediment of the Stångån river in Sweden (Berglund et al., 2015). In general, data on the aquatic occurrence of trimethoprim resistance genes in the recipient water remain very limited in Sweden. Therefore, our results showed an initial awareness of these ARGs present in such aquatic environment.

3.2.9. Amphenicol resistance genes

For the resistance gene group of amphenicols, nine genes were quantifiable in this study, with the abundance ranging from 2.6×10^{-5} to 2.4×10^{-3} (Fig. 2). Seven of them were frequently present in Västerås water, in which three (*catA1*, *mdtL*, *rarD_2*: $1.1\text{-}7.0 \times 10^{-4}$) were by about an order of magnitude higher in abundance than the others (*cmlA_3*, *cmlA_2*, *catB3*, *cmlA_4*: $3.0\text{-}5.9 \times 10^{-5}$). The most abundant gene, *catA1*, was also consistently quantified in Stockholm, but only in the downstream water (9.1×10^{-5}). These ARGs (i.e., those seven ones, *yidY/mdtL* and *cmxA*) were only occasionally measurable in the Uppsala water, mostly at its downstream site, whereas none of them occurred in the water environment of Eskilstuna. To the best of our knowledge, this is the first study on amphenicol resistance genes in the Swedish water environment.

3.2.10. Florfenicol and quinolone resistance genes

For the florfenicol resistance gene, only *floR_1* was targeted and present sporadically in Uppsala with comparable abundances between its upstream and downstream waters ($\sim 1.0 \times 10^{-4}$). Similarly, the two targeted resistance genes (*qnrA*, *qnrB*) against quinolones were mainly observed in the Uppsala water, at low abundances ($<5 \times 10^{-5}$) in both downstream and upstream sites. These three ARGs were extremely rare (*qnrB*) or even absent (*floR_1*, *qnr A*) at the other sites in this study. *qnrA* was rarely detected in the Örebro's river system (Khan et al., 2019). However, *qnrB* genes were more frequently detected in Örebro's river system as compared to this study.

3.2.11. Vancomycin resistance genes

The genes encoding resistance to vancomycin were only quantifiable in Uppsala with an abundance ranging from 1.0×10^{-5} to 3.4×10^{-4} for nine gens (Fig. 2). Four (*vanTE*, *vanHB*, *vanC2/vanC3*, *vanRB*) of them showed relatively high and comparable abundances ($\sim 1.0 \times 10^{-4}$) at the two sites in Uppsala, in which *vanC2/vanC3* only occurred in the

downstream water. The other five genes had a rather low abundance ($<5.0 \times 10^{-5}$), with three genes (*vanC_2*, *vanSC_1*, *vanTG*) existed only in the downstream water. Vancomycin resistance genes were not observed in the Örebro's river system (Khan et al., 2019). In Sweden, the usage of vancomycin as growth promoters (e.g., avoparcin) have been banned since 1986 (Iversen et al., 2002), but clinical treatments. Previously, only in 1 of the 37 recipient surface water samples from Stockholm and Uppsala, a vancomycin resistance gene (*vanA*) was detected in 1999 (Iversen et al., 2002). Our finding of vancomycin resistance genes in Uppsala was perceived as high concern, as vancomycin is of clinical relevance in usage. The detection of these genes in the recipient water could be explained by the poor removal of vancomycin antibiotics and its resistant bacteria in conventional WWTPs (Iversen et al., 2002; Khan et al., 2019), and therefore proliferation of such resistance.

3.2.12. Other genes

In the group of other genes, 12 genes were detected at the studied sites, with an abundance ranging from 1.1×10^{-5} to 9.0×10^{-3} (Fig. 2). The three antiseptic-resistance genes (*qacE-delta1* group) and mercury resistance gene (*merA*) were frequently present in almost all the locations. The greatest average abundance was observed in the Västerås water (*qacE-delta1*: 6.1×10^{-3}; *merA*: 3.2×10^{-3}). High abundances of these genes were also found at the Uppsala site, where the *qacE-delta1* genes (1.4×10^{-3} *vs.* 1.9×10^{-4}) were by an order of magnitude higher abundant in the downstream water compared to the upstream water, while the mercury resistance gene (1.5×10^{-3} *vs.* 1.2×10^{-3}) was similar at both sites in Uppsala. In Stockholm, the downstream water generally showed a lower abundance of these genes as compared to its upstream water (*qacE-delta1*: 2.0×10^{-5} *vs.* 2.5×10^{-4}; *merA*: 4.0×10^{-5} *vs.* 2.0×10^{-4}). Not any of these genes were detected in the upstream water of Eskilstuna, but they frequently occurred in the wetland (*qacE-delta1*: 8.5×10^{-5}; *merA*: 2.9×10^{-4}) and downstream (*qacE-delta1*: 3.2×10^{-5}; *merA*: 7.7×10^{-5}) sites. Two *crAssphage* genes as the marker of human faecal contamination and a bacitracin resistance gene were consistently detected at high abundances in Västerås water (*crAss64*: 1.0×10^{-3}; *crAss56*: 3.2×10^{-4}; *bacA_2*: 2.2×10^{-4}). Also, *crAss56* (2.0×10^{-5}) was only present in the receiving water of Uppsala, but *bacA_2* was rarely detected in the Uppsala water. *bacA_2* (1.8×10^{-5}) was occasionally detected in the Stockholm downstream water at a low abundance but not the two *crAssphage* genes. The remaining five genes, including *fabk*, *nisB_1*, *nimE*, *mcr1* and *mcr2*, sporadically occurred in Uppsala and very rarely in the other water bodies. Very low to non-detectable abundances of *mcr1* were also found in other European receiving water bodies (Cacace et al., 2019). Recently, faecal pollution has been confirmed to be associated with enhanced abundances of ARGs in the downstream environments (Karkman et al., 2019). Hence, the presence of *crAssphage* in this study indicates faecal pollution in the studied recipient water, and possibly may play an important role in the fate of ARGs in the aquatic environment.

3.3. Similarity of gene profiles

The ARG profiles across different water bodies were assessed for their relationship through the NMDS analysis (Fig. 3). The obtained stress value for NMDS was 0.056, indicating a well-fitting ordination based on the ARG profiles. There were four distinct clusters of samples in the NMDS plot, separating clearly the four different studied locations with the geographical variation in their resistome, as potentially influenced by different sources. Within a NMDS cluster, the water samples from both sites in one area were likely subjected to same or very similar sources. This implies that the upstream and receiving water bodies in Uppsala, Stockholm and Eskilstuna appeared to be impacted by its own local source of pollution. Often, the upstream and downstream sites showed an opposite shift within the NMDS cluster, which can be explained by the difference in ARG abundance between the two sites in each area.

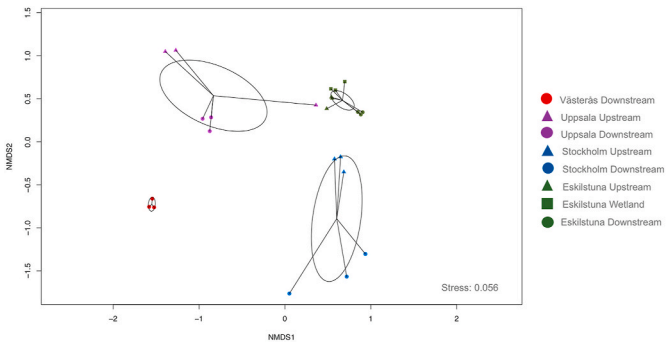

Fig. 3. Cluster analysis of the similarity of resistome profiles among different locations using non-metric multi-dimensional scaling (NMDS). Lines show the labelled group centroids of the samples. Ellipses surrounded the centroids show the 95% confidence area for the standard error of the centroids. Stress NMDS value was 0.056. The distribution of the genes' relative abundance detected in the surface water among different locations is significantly different (permutational multivariate analysis of variance $R^2 = 0.51$; p-value<0.01).

3.4. Co-occurrences of resistance genes and MGEs

Relationships among the targeted genes were evaluated by Spearman's correction and presented using network analysis for those with high significance ($\rho > 0.8$ and p-value<0.01) to determine co-occurrences of ARGs with MGEs, and other genes with MGEs across all the water bodies (Fig. 4). The network analysis showed four sub-networks, with connections between various ARGs and integrons of each class. Three of the Class I integron genes (*intI1_2*, *intI1_3*, *intI1_4*) were in a significant joint with 22 genes, including ARGs against sulfonamides, β-lactams, aminoglycosides, MLSB and tetracyclines, as well as other genes. The sulfonamide resistance genes were co-occurred only with Class I integrons in this sub-network. This observation is common, as *sul1* and *sul2* in sulfonamide resistant bacteria are usually attached to MGEs (Stoll et al., 2012). Another Class I integron gene (*intI1_1*) was also connected with two other ARGs against MDR and aminoglycosides, forming the smallest sub-network. The largest sub-network presented the linkage of Class III integron genes (*intI3_1*, *intI3_2*) with 25 ARGs referring to β-lactams, MDR, aminoglycosides, amphenicols, MLSB and tetracyclines. Only this sub-network revealed the co-occurrence of amphenicol resistance genes with integrons. The two Class III integron genes were also observed to strongly correlate with *crAssphage*. Another small sub-network illustrated a significant connection of the Class II integron gene (*intI2_2*) with six ARGs. Particularly, the vancomycin resistance genes were found to only co-occur with this Class II integron gene. ARGs against florfenicols and quinolones were not significantly correlated with any of the integron genes. Overall, the observed co-occurrences suggest that the dissemination of antibiotic resistance is facilitated by the integron mobile elements in the studied water environment. Particularly, our data on the specific linkage of some ARGs with different classes of integron genes could be meaningful for future studies that extent to other integrons' classes, as mainly the Class I integron genes have been commonly focused (e.g. Sabri et al., 2018; Cacace et al., 2019). For instance, it appears important to better understand the dissemination of vancomycin resistance with Class II integron, and aminoglycoside resistance with Class III integron in the water bodies in the future. Based on the obtained network results, the studied water bodies may be an important reservoir of ARG dissemination. Those co-occurring resistance genes with integron mobile elements should be prioritised in future assessment to further evaluate the degree of dissemination over time. Our data may also assist in planning strategies to minimise the presence of antimicrobial contaminants in the

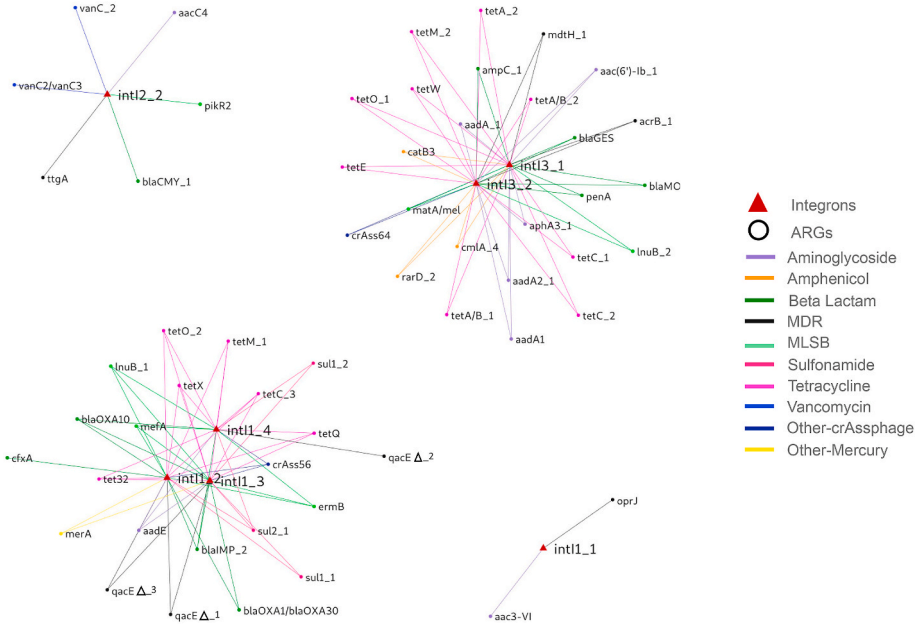

Fig. 4. Network analysis presenting co-occurrence patterns between ARGs and the class 1 integron-integrase genes in all locations. A joint means significant correlation (Spearman's coefficient $\rho > 0.8$ and p-value<0.01). Coloured labels and lines indicate the genes from different classes of antibiotics.

aquatic environments.

3.5. Potential limitations

Several caveats may apply to the adequate interpretation of our findings. The hydrologic variation in the studied water environments can be dynamic and complex to fully elucidate the gene occurrence and abundance. Dilution effects in the receiving water bodies cannot be excluded to explain our findings of the genes that were no longer present or were measured at lower abundances in the downstream water compared to its upstream site. Effluent discharges lead to higher water volumes in the receiving water body, and therefore a dilution with increased water flow. Moreover, there are possible effects of in-water processes, such as (re-)partitioning of bacteria into sediment along the upstream to downstream section, resulting in potential sinks of genes for their disappearing and reduced abundance in the receiving water bodies. This may be even facilitated over the mixing zone of both effluent and downstream water with faster current. Despite those mentioned environmental scenarios, the positive detection of aquatic resistomes in upstream water indicates the existence of potential sources of anthropogenic pollution in the upstream catchment. This may particularly be important at Uppsala city as its upstream and downstream sites showed similar resistome profiles. Such anthropogenic activities upstream of the WWTP could be attributed to effluent releases from medium-scale WWTPs and effluents and infiltration from small-scale on-site sewage facilities (e.g. septic tanks), which is known to occur in the Fyrisån river catchment upstream the Uppsala WWTP (Gago-Ferrero et al., 2017). This was also observed for chemical pollution in our previous studies (Gago-Ferrero et al., 2017; Blum et al., 2018; Rosenmai et al., 2018; Sörengård et al., 2019). Nevertheless, the impact of WWTPs on the aquatic occurrence of such microbial contaminants cannot be ruled out since our results showed consistently high detection frequencies and relative abundances of some genes in (only) the receiving water bodies. Our study reflects a snapshot of the resistome profile in the studied environment. Further investigation of any temporal or seasonal trends in the resistome and their relation to other microbial and environmental factors, as well as potential impacts of effluent discharges from WWTPs, will be needed in future studies. Furthermore, comparison of our findings with other studies (e.g. Cacace et al., 2019; Khan et al., 2019) should be interpreted with the potential difference in the measurement sensitivity and the local environment.

4. Conclusions

This study provided new data on the environmental prevalence and variety of different ARGs, MGEs and other genes, addressing the limited availability of such knowledge in the urban aquatic environment in Sweden. Our hypothesis was confirmed by the finding that ARGs were more diverse in more urbanised, densely-populated regions and in their downstream water environment. The occurrence of some ARGs was revealed for the first time in our freshwater environments, including those with sulfonamide (*sul3*), trimethoprim and amphenicol resistances. Also, this study showed a spatial difference in the aquatic resistome, with distinctive findings observed only in the Uppsala waters for the resistance genes against florfenicol, quinolone and vancomycin antibiotics. As the studied recipient water is part of Lake Mälaren, a drinking water source and also used for leisure activities (e.g. swimming and fishing), the presence of certain genes that are considered as indicators of anthropogenic pollution (e.g. *intI*, *sul1*, *blaOXA*) and human faecal contamination (*crAssphage*) and as clinically relevant (i.e., β-lactam, quinolone and vancomycin resistance genes) pose a concern to the water quality and human health in exposure to bacteria carrying these resistance genes. As a new class of emerging contaminants (Richardson and Kimura, 2020), more attention to the source tracing and reducing the aquatic occurrence of ARGs is needed in future studies. Such attention is probably high to the water bodies in Uppsala and Västerås. Continuous monitoring of these two hot-spots in the Lake Mälaren region will be worthy for further studies to better characterise the aquatic fate of these emerging contaminants in the future.

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.

Acknowledgements

We would like to thank Johannes Kikuchi and Daniel Carlson for their assistance in field sampling, as well as Sebastian Lungu-Mitea and Johan Lundqvist for the access of −80 °C storage. Also, we acknowledge the financial support from FORMAS (project no: 942-2015-1586) and the Swedish Environmental Protection Agency (grant no: 219-18-007).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117651.

Author contributions

Foon Yin Lai: Conceptualisation, Formal analysis, Investigation, Writing – original draft, Writing - Critical Writing – review & editing, Visualisation, Project administration, Windi Muziasari: Methodology, Validation, Formal analysis, Writing - Critical Writing – review & editing, Visualisation, Marko Virta: Methodology, Writing - Critical Writing – review & editing, Karin Wiberg: Investigation, Writing - Critical Writing – review & editing, Lutz Ahrens: Conceptualisation, Resources, Writing - Critical Writing – review & editing

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