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Revealing antimicrobial resistance in stormwater with MinION

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Graphical abstract

Abstract

Discharge of urban stormwater containing organic matter, heavy metals and sometime human feces, to the natural aquatic reservoirs without any treatment is not only an environmental problem. It can lead to prevalence of antibiotic resistant bacteria in stormwater systems and transmission of antibiotic resistance genes to the environment. We performed antibiotic resistome identification and virus detection in stormwater samples from Stockholm, using publicly available metagenomic sequencing MinION data. A MinION platform offers low-cost, precise environmental metagenomics analysis. 37 groups of antibiotic resistant bacteria (ARB), 11 resistance types with 26 resistance mechanisms – antibiotic resistance genes (ARGs) giving tolerance to the aminoglycoside, beta-lactams, fosmidomycin, MLS, multidrug and vancomycin were identified using ARGpore pipeline. The majority of the identified bacteria species were related to the natural environment such as soil and were not dangerous to human. Alarmingly, human pathogenic bacteria carrying resistance to antibiotics currently used against them (Bordetella resistant to macrolides and multidrug resistant Propionibacterium avidum) were also found in the samples. Most abundant viruses identified belonged to Caudovirales and Herpesvirales and they were not carrying ARGs. Unlike the virome, resistome and ARB were not unique for stormwater sampling points. This results underline the need for extensive monitoring of the microbial community structure in the urban stormwater systems to assess antimicrobial resistance spread.

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1. Introduction

The urban population density and number of citizens, size of areas modified for urban needs and scale of the intrusion of human-made structures in the landscape are growing over time and that all
together accelerates the spread and creation of new antibiotic resistance mechanisms. We already know that physicochemical parameters, presence of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the runoff from the cities can have negative impact on water bodies. Despite that, the quality of water and the risk of infection are usually investigated only in municipal sewage systems and in wastewater treatment plants' (WWTPs) effluents while urban runoff water is often neglected. As Hamilton et al. (2020) reported only 15 quantitative studies focused on ARBs and ARB were published so far. Even though only few studies investigated dissemination of ARB and ARGs in urban runoffs, their results are alarming (Almakki et al., 2019).

Anthropogenic pressure together with storms changing hydro regime and overflow from WWTPs and agricultural area (waste lagoons, manure-treated fields, animal feeding lots) create a new hot-spot and at the same time vehicle for creation and dissemination of ARGs and ARB. The low concentration of antibiotics stimulate creation of ARBs and their spread via horizontal gene transfer (HGT). That is also important as potential route of ARBs transfer from environmental bacteria to pathogens (Almakki et al., 2019). Selection of resistance is highly probable in stormwater due to stimulation which can have as a piece in lead as well as wastewater other aspect is absorption of antibiotics and other medicines at organic particles (Almakki et al., 2019). Then during rain out the settled matter can get to the sewer system and to WWTPs. Such flush within stormwater can also increase amount of sediments and partially remove biofilm in the sewage network. This phenomenon is frequent in WWTPs with combined sewer systems during stormwater events, resulting in overflows and direct transfer of untreated wastewater to the receiving aquatic environment (Eggen and Vogelsang, 2015). Global climate changes and dramatic weather phenomena including severe storms have noticeable impact on water bodies. Despite that, the common strategies for ARMs stormwater investigation are bacterial culturing joined antibiotic resistance testing, screening for specific ARGs with quantitative polymerase chain reaction (qPCR), obtaining ARGs relative abundance next-generation sequencing (both environmental DNA and cultured isolates can be the input), metagenomic using short read sequencing (Hamilton et al., 2020).

We therefore analyzed ARB and ARGs in Stockholm stormwater samples and investigated whether MinION will provide new information regarding ARMs. Our analysis revealed various ARB carrying multiple antibiotic resistance mechanisms suggesting need for large-scale metagenomic studies and microbial monitoring of urban stormwater in the face of growing antibiotic resistance.

2. Materials and methods

2.1. Metagenomic sequencing data

We performed ARB and ARG identification and virus detection in stormwater samples using publicly available metagenomic MinION sequencing data from Sequence Read Archive (SRA), accession number PRJEB20562 (Hu et al., 2018). Authors have taken total of 73 samples from stormwater street manholes not linked in the stormwater system in the city of Stockholm, except for the sample name ‘Bromma’ in supplementary material originating from WWTP. E. coli culturing was performed to assess potential contamination resulting from misconnections of the sanitary sewer pipes (wastewater from kitchens, toilets, and bathrooms) connected to the stormwater system. From all stormwater samples, five were randomly selected for MinION sequencing, two with high (>242,000 MPN per 100 ml, those were Sample 2 and Sample 4) and three with low (<100 MPN, Sample 1, Sample 3, Sample 5) E. coli culturing counts and sufficient DNA amount for MinION sequencing. Total of 5 samples, coming from two different sampling areas and 5 different points (Samples 1, 3, 4, 5 comes from Liljeholmen, according to Hu et al. (2018): C8, C13, C16, C21 respectively; Sample 2 is from Kungsholmen, C12 in Hu et al. (2018); geographic locations are shown in Fig. 1) The map was modified manually using images from Google Maps Map of Stockholm, 2017. Swedish urban stormwater systems were sequenced using Oxford Nanopore MinION long-read sequencing technology. FASTQ files deposited to SRA were converted to FASTA files using SRA toolkit (Leinonen et al., 2011).

The procedure of DNA extraction and sequencing was described in details in Hu et al. (2018). Briefly, after filtration through 0.22 μm pore-size polyethersulfone membranes the genomic DNA was extracted with the PowerWater® DNA Isolation kit (MO BIO Laboratories Inc.). Later the DNA was sheared and purified before the Nanopore library preparation using the MinION PCR barcoding kit (NC_48Hr_Sequencing_Run_Flo_MIN106_SQK_LSK208.py) on the MinKNOW control software (version 1.3.25) a base-calling was done on the Metrichor software (version 1.125) and 2D Base-calling...
plus barcoding program (for FLO-MIN106: “2D Base-calling plus Barcoding for FLO-MIN106 250 bp”).

2.2. Resistome investigation

ARGpore (Xia et al., 2017) was used to investigate antibiotic resistance in stormwater. The tool was designed to find AMR genes and their hosts in metagenomic data utilizing BLAST, HMMER, UBLAST using databases derived from ARDB and CARD. ARGpore uses Resistome prediction Algorithm (FASTA sequence was searched against nt-version SARG database (v1.0); valid alignment with >80% similarity over 70% alignment length were kept for further filtering of overlap regions and if two hit regions on the same read overlapped for > 50% alignment length, only the one with longest ARG hit were kept). To identify the host of ARGs ARGpore uses another algorithm - Taxa Algorithm. The Nanopore 2D reads are aligned to MetaPhlAn 2 database of taxon-defining marker genes (Segata et al., 2012). MetaPhlAn (Metagenomic Phylogenetic Analysis) maps reads against a set of clade-specific marker sequences, it compares each metagenomic read from a sample to the marker catalog to identify high-confidence matches (Segata et al., 2012). Only the best alignment (with the highest bit score) showing similarity higher than 80% over more than 70% of the marker gene length is kept for taxonomy classification. The generated output is the list of ARG-containing nanopore reads with taxa annotated (arg.w.taxa.tab). Additional classification of species was made manually based on MiDAS 2.0 (McIlroy et al., 2017).

2.3. Viruses investigation

Virus detection in sequenced samples was done using Kraken tool (Wood and Salzberg, 2014). Kraken assigns taxonomic labels to metagenomics DNA reads by examining the k-mers within a read and then querying a selected database with those k-mers. Kraken was run on the Galaxy platform using default parameters and the viruses database (Galaxy Version 1.2.4). Classification results were summarized using Kraken-report tool (usegalaxy.org).

PPR-Meta ver. 1.1 was used to identify metagenomic sequences as phages, chromosomes or plasmids (Fang et al., 2019). It uses a novel neural network architecture named as the Bi-path Convolutional Neural Network for 3-class classification of sequences based on comparison to prokaryote chromosomes, prokaryote plasmids,
and phages from NCBI genome database. The virtual machine version of PPR-Meta was run through the executable file with default parameters. FASTA files containing metagenomic sequences were split in three parts (representing phages, chromosomes and plasmid sequences) based on PPR-Meta classification using seqinr package in R and further analyzed separately using ARGpore as described above.

2.4. Sankey diagram

Sankey Diagram was created using package ggvis in R (Chang and Wickham, 2018). Results of ARGpore analysis for all samples were plotted together with genus levels, resistance types and resistance genes displayed on the diagram nodes.

3. Results

3.1. Main resistance types and ARB harboring them

In total 11 resistance types with 26 resistance mechanisms were found (details are given in Table 1). Those were antibiotic inactivation of aminoglycoside, beta-lactam, chloramphenicol, rifamycin (8 such mechanisms were found); antibiotic target alteration of bacitracin, MLS, vancomycin (5); antibiotic target replacement of beta-lactam and trimethoprim (2), antibiotic efflux towards fosmidomycin, multidrug and puromycin (11).

The ARGs were present not only on chromosomes and plasmids but also on phages. Most of them were present on chromosomes. However, some of the ARGs in our study were found only on mobile genetic elements (MGEs), like aac (6’)-I, aac (3)-X, qacG and vanR. The last three were found only on plasmids.

Seven main resistance types along with species of ARB carrying them are presented in Table 2. Ten of the found ARB are usually encountered in activated sludge in WWTPs. One identified group of ARB is biofilm forming pathogen previously noted as responsible for prosthetic hip joint infections (Wildeman et al., 2016). Interestingly, identical ARB with the same resistance was found in two pairs of samples: 2 and 3; 4 and 5, independently of number of reads achieved in sequencing and the sampling point 2 (Kungsolmen) was not located next to the other sampling area (Liljestrom). Sphingomonas sp. resistant to aminoglycoside were found in samples 1, 4 and 5. Limnobotlibitans sp. were found both in sampling point 2~5, but bacteria in samples 2 and 3, presented resistance toward aminoglycoside while in 4 and 5 to beta-lactams.

All found ARB identified to genus level (with except for the unclassified representatives of Marinilabillicae and Oscillospiraceae) are presented in Fig. 2 together with carried genes and resistance type. Also Betaentomopoxviruses carrying resistance towards aminoglycoside were encountered.

3.2. Viruses

Caudovirales, Herpesvirales, Polydnaviridae were present in all tested samples. Pandoravirus dulcis, Pandoravirus salinus and

| resistance type          | resistance mechanisms                  | prevalence among the sequenced genomes and plasmids available at NCBI for 82 important pathogens | PPR-Meta prediction of resistance mechanisms presence among samples analyzed in this study (numbered from 1 to 5) on different genetic elements |
|--------------------------|----------------------------------------|-------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| aminoglycoside           | aac (3)-X                              | antibiotic inactivation                                                                       | ND                                                                                                                                      | ND                                                                                                                                      |
|                          | aac (6’)-I                             | antibiotic inactivation                                                                       | ND                                                                                                                                      | 1                                                                                                                                       |
|                          | aph (3’)I                              | antibiotic inactivation                                                                       | +                                                                                                                                        | 1, 3                                                                                                                                   |
|                          | aph (3’)IIb                            | antibiotic inactivation                                                                       | +                                                                                                                                        | 2                                                                                                                                       |
|                          | bacA                                   | antibiotic target alteration                                                                  | +                                                                                                                                        | only S. enterica (0.18%)                                                                                                                |
|                          | beta-lactam                            | class A                                                                                 | antibiotic inactivation                                                                       | ND                                                                                                                                      | ND                                                                                                                                      |
|                          | GES-23                                 | antibiotic inactivation                                                                       | ND                                                                                                                                      | 2                                                                                                                                       |
|                          | mecI                                   | antibiotic target                                                                          | +                                                                                                                                        | 3, 4                                                                                                                                   |
| chloramphenicol          | chloramphenicol                         | antibiotic inactivation                                                                       | ND                                                                                                                                      | ND                                                                                                                                      |
| fosmidomycin             | rosA                                   | antibiotic efflux                                                                          | ND                                                                                                                                      | 3, 5                                                                                                                                   |
| macrolide-lincosamide-   | ermD                                   | antibiotic target alteration                                                                 | ND                                                                                                                                      | ND                                                                                                                                      |
| streptogramin            | abfS                                   | antibiotic efflux                                                                          | +                                                                                                                                        | 5                                                                                                                                       |
| multidrug                | major facilitator superfamily transporter | antibiotic efflux                                                                           | ND                                                                                                                                      | 3                                                                                                                                       |
|                          | mexE                                   | antibiotic efflux                                                                          | +                                                                                                                                        | 3, 5                                                                                                                                   |
|                          | mexX                                   | antibiotic efflux                                                                          | +                                                                                                                                        | 4, 5                                                                                                                                   |
|                          | ompr                                  | antibiotic efflux                                                                          | ND                                                                                                                                      | 2, 3, 4                                                                                                                                  |
|                          | opcM                                   | antibiotic efflux                                                                          | ND                                                                                                                                      | 3, 4                                                                                                                                   |
|                          | oprA                                   | antibiotic efflux                                                                          | –                                                                                                                                        | 2, 4                                                                                                                                   |
|                          | oprN                                   | antibiotic efflux                                                                          | +                                                                                                                                        | 3                                                                                                                                       |
|                          | qacG                                   | antibiotic efflux                                                                          | +                                                                                                                                        | 4                                                                                                                                       |
| puromycin                | puromycin                              | antibiotic efflux                                                                          | ND                                                                                                                                      | 3                                                                                                                                       |
| rifamycin                | ADP-ribosylating                        | antibiotic inactivation                                                                       | ND                                                                                                                                      | 3                                                                                                                                       |
| trimethoprim             | dfrA12                                 | antibiotic target replacement                                                                 | +                                                                                                                                        | 2                                                                                                                                       |
| vancomycin               | vanH                                   | antibiotic target alteration                                                                 | ND                                                                                                                                      | 2                                                                                                                                       |
|                          | vanR                                   | antibiotic target alteration                                                                 | ND                                                                                                                                      | 2                                                                                                                                       |

Table 1

Resistance types, mechanisms and prevalence among the sequenced genomes/plasmids/phages in investigated stormwater samples assembled and supplemented by CARD data (Jia et al., 2017).
**Table 2**

Antibiotic resistant bacteria (ARB) identified in stormwater.

| Resistance type/ARB in sewage sample | 1 | 2 | 3 | 4 | 5 |
|-------------------------------------|---|---|---|---|---|
| aminoglycoside                       |  | Polymorphum gibum; Rhizobium etli; Sphingomonas sp.* | Azospirillum lipoferum; Acidovorax delafeldii*; Llmohabitanas sp.; Rubrivivax benzoautyliticus Bacteroides stercoris; Anaeroestes hadrus* | Sphingomonas sp.*; Leifsonia xyli |
| beta-lactam                          |  |  |  |  |  |
| fosmidomycin                         |  |  |  |  |  |
| MLS                                 |  |  |  |  |  |
| multirdrug                           |  | Pseudomonas sp.* | Acinetobacter tandoi* |  |  |
| unclassified                         |  |  | Propionibacterium avidum* | Slackia sp.*; Coprococcus sp.*; Eubacterium elieng; Prochlorococcus sp. |  |
| vancomycin                           |  |  | Eubacterium elieng; Thermoanaerobacter wiedeli |  |  |

number of reads | 56,220 | 122,506 | 44,337 | 69,316 | 31,756

Description: MLS: macrolide-lincosamide-streptogramin, species encountered in wastewater treatment plants are marked with + (based on MiDAS 2.0), opportunistic pathogen are marked with * (based on MiDAS 2.0), opportunistic pathogen are marked with ★ and pathogens are marked with ★★.

**Baculoviridae** were not found only in sample 2. **Micromonas pusilla** virus 12 T were in samples 1 and 3. **Poxviridae** in samples 2, 3, 4; uncultured phage crAssphage in sample 3. The **Coronaviridae** responsible for COVID-19 pandemics were not found in the samples. The order **Nidovirales** contains this family and was noted only in Sample 4 with the Ball python nidovirus.

**Caudovirales** and **Herpesvirales** were the most abundant orders consisting 20%–88% of all viruses in the sample. Classification sometimes even to species level and the viral abundance can be found in Krona diagram (Supplementary Figure 1) (Ondov et al., 2011).

4. **Discussion**

4.1. **Resistance mechanisms**

The most widespread resistance mechanism according to Zhang et al. (2009), is the target bypass, which is inaccessibility of the antibiotics to their target enzyme by mutational changes or loss on the enzyme gene (Huovinen et al., 1995; Happi et al., 2005). Surprisingly, such ARGs were not noticed in this study. We showed that in Stockholm’s stormwater most common resistance mechanisms were based on antibiotic efflux (11 out of 26), contrary to the findings of Zhang et al. (2009) for environmental ARB in the water. However, those results tie well with previous study by Nesme et al. (2014). Wherein they investigated environmental metagenomes. Mentioned study showed that bacteria with the efflux pumps were able to revoke the dosing of antibiotics used in veterinary and human healthcare namely beta-lactam, vancomycin, and tetracycline.

Others presented resistance determinants in bacterial genome have broader spectrum and are more connected to long environmental pressure than just a defense against human-produced antibiotics. Martínez (2017) remarks environmental profits like new functions obtained with ARGs as essential to keep being preserved in genome. The efflux pumps are good example of exaptation in AMR, which is organism’s procuring a new function that did not exist or differs from its original function which had been derived by evolution.

From our results on ARB having unique resistant phenotype and efflux mechanism being the most frequent one in the study, it is clear that environmental organisms may achieve resistance to antibiotics as exaptation. The efflux pumps are able to discard molecules from cytoplasm through membrane and wall of the cell. Such machinery does not target defined particles, it removes whole variety of metabolic products. What is more, in case of overexpression or mutation in pump, even antibiotics, biocides, heavy metals and pesticides can be ejected by the efflux system at the same time giving resistance to the host (Alonso et al., 1999; Baker-Austin et al., 2006; Buffet-Bataillon et al., 2016). Often it is also the reason for co-resistance to antibiotics and heavy metals in bacteria (Piddock, 2006).

Mentioned evolutionary trait was noted for the beta-lactamases. The enzymes are involved in cell wall biosynthesis as well as hydrolyzing both natural and man-made antibiotics of the beta-lactam family evolved from the same ancestor (Meroueh et al., 2003).

We found 4 other systems: (1) antibiotic inactivation through direct or indirect deactivation of antibiotic molecule (Wright, 2005); (2) modification of drug targeted structures including modification of the action sites of antibiotics (Lambert, 2005); (3) antibiotic target alteration and (4) antibiotic target replacement. It is noteworthy that the resistance to certain antibiotic may be associated with different ARGs based on more than one mechanism.

Prevalence of the identified mechanisms exclusively in chromosomes was noted in 50%, and in 17% both in chromosomes and plasmids. For most (54%) of the found ARGs such classification is still not available (Table 1).

4.2. **ARB in stormwater**

4.2.1. Environmental ARB

In line with previous studies many of the encountered ARB are associated with different environments: (1) WWTPs: half of the ARB species in this study (marked with * in Table 2); (2) freshwater:
Sandarakinorhabdus limnophila and Limnohabitans sp.; (3) lake water: Ramlibacter and Variovorax (Hahn et al., 2010); (4) soil habitat and plant growth, for example: Comamonas, Achromobacter, Azospirillum, Rhizobium, Xanthomonadaceae, Acidovorax (phytopathogen), Leifsonia xyli (sugar cane pathogen), Ralstonia (phytopathogen); (5) extreme environments: Asticcacaulis (deserts) and Caldimonas (hot springs); (6) endosymbiosis in insects: Sodalis, which is also natural hosts for Betaentomopoxvirus (present in our tested samples and carrying ARGs).

A similar pattern of results for Variovorax was obtained by Roberts (2011), where owing to efflux pumps it was resistant to tetracyclines ($tetA$ and $tetL$). Another bacteria, Asticcacaulis excen-tricus were previously recorded as resistant to beta-lactams (Kanehisa and Goto, 2000), in our study we also noted in the

\[
\text{genome the presence of genes coding protein from major facilitator superfamily able to give resistance to these and many other drugs.}
\]

Limnohabitans sp. was noted in 80% of samples, but the resistance presented by this species varied between locations (aminoglycoside in 2, beta-lactams in 3) suggesting that ARGs were obtained in effect of local environmental pressure.

4.2.2. ARB that can be transferred from/to human body environment

Most of the found taxa are not able to survive in the human body. Other like Propionibacterium are human commensals or constitute human gut microbiota like Bacteroides stercoris and Eu-bacterium eligens.

Another species, Mycobacterium parascrofulaceum is an

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**Fig. 2.** Sankey Diagram showing results of ARGpore classification analysis. The nodes represent genus levels of bacteria with antibiotic resistance mechanisms and genes found in the metagenomic sequencing data from Samples 1–5.
opportunistic pathogen, like many other nontuberculous mycobacterial species (Tortoli et al., 2005). Even though *M. parascrofulaceum* have rosA gene, cationic antimicrobial peptides (CAMPs) efflux/K+ antiporter, it is not additional danger when it comes to spread. Described subunit typical for Mycobacteria. *M. tuberculosis* is not affected by fosmidomycin due to a lack of its uptake, not thanks to rosA. As it was described by Brow and Parish (Brown and Parish, 2008) mycobacteria are naturally resistant at the cell level, not connected to efflux pump. Usually Mycobacteria are resistant to antibiotic owing to cell wall rich in long-chain fatty acids covalently linked to the arabinogalactan-pectidoglycan layer. Moreover, the porins, which are specific protein channels allowing hydrophilic molecules to enter the cell via diffusion, are rare in portrayed taxa (Trias et al., 1992).

Our analysis found evidence for the presence of pathogenic ARB in stormwater. It is *Bordetella* causing ‘whooping cough’ respiratory diseases, which can develop to vomiting, convulsions, coma and death (Matto and Cherry, 2005). Roberts (2011) reported resistant *Bordetella* with tetA and tetC genes. Macrolides are recommended for ‘whooping cough’ treatment (erythromycin, clarithromycin, or azithromycin (Altuñaji et al., 2007)), therefore it is disturbing that in investigated samples *Bordetella* were carrying mexX giving resistance also to this group of drugs.

*Propionibacteria* are members of the human skin microbiota, but are also opportunistic pathogens responsible for prosthetic hip joint infections (Achermann et al., 2014, 2018; Wildeman et al., 2016). Lately, Achermann et al. (2018) reported large series of periarticular joint infections in Sweden caused by *P. avidum*. 11 out of 12 tested strains were susceptible to clindamycin, levofloxacin, and rifampin. The authors also used whole genome sequencing and found EPS-encoding island, probably obtained by HGT (flanked by transfer RNA genes). They connected the capacity to initiate biofilms on medical implants with potentially key virulence trait of *P. avidum*. However, the authors did not manage to point out a specific risk factor for the increasing number of *P. avidum* periarticular joint infections in recent years. Interestingly, we found resistant representatives of *P. avidum* in stormwater in the samples from year 2013, which was within the period (1997–2015) of Achermann et al. (2018) investigation. What is more, the mexX mechanisms characterizing *P. avidum* in our study gives resistance towards drugs tested in Achermann et al. (2018) study: penman (penicillin), lincomamide (clindamycin), fluoroquinolones (ciprofloxacin and levofloxacin) and cephalosporin (cefoxirine), but not to lincomamide (clindamycin) and rapamycin (rifampin) for which the lowest minimal inhibitory concentrations were noted in the mentioned study (Achermann et al., 2018 – Table 3 in original work).

Risk assessment is essential to unravel the relation between human exposure to ARB potentially infecting humans and environmental ARGs (Pruden et al., 2018). We identified the pathogens carrying ARGs, the question of human exposition to bacteria from urban stormwater stays open.

### 4.3. Comparison of ARGs in stormwater and wastewater

These basic findings are consistent with research showing ARGs occurrence in stormwater. We found genes giving tolerance to the aminoglycosides, beta-lactams, fosfomycin, MLS, multidrug and vancomycin. Some of them may be the source of spread of AMR to those drugs to WWTPs with stormwater influent. The result of our analysis was compared with the first broad-spectrum metagenomic investigation of WWTP’s resistome Yang et al. (2014). He found the same ARGs types giving resistance toward drugs listed above and 12 more. Those additional groups were acridine, acriflavine, bacitracin, bicyclomycin, chloramphenicol, fosfomycin, polymyxin, quinolone, sulfonamide, tetracycline and trimethoprim.

While only ARGs giving resistance towards sulfonamides (sul 1), tetracyclines (tetM), beta-lactamases (*bla*<sub>NASS</sub>, *bla*<sub>TEM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>CTX-M-32</sub>, *bla*<sub>KPC-3</sub>) and colistins (*mcr-1*) were monitored and reported by Cacace et al. (2019) with qPCR in the European WWTPs. Unfortunately, Sweden being in focus in our article was not included in the survey. The resistance to aminoglycoside (*aacA*, *aadA*, *strA*, *strB*, apha) (Ferreira Da Silva et al., 2007; Moura et al., 2012); macrolides (*ermA*, B, F) (Agga et al., 2015; Marti et al., 2013; Szczepanowski et al., 2009); glycopeptidases (vancomycin, vanA) (Araujo et al., 2010; Morris et al., 2012; Rosenberg Goldstein et al., 2014); trimethoprim (dfr) (Ferreira Da Silva et al., 2007; Moura et al., 2012; Schwartz et al., 2006) and quinolones (aacA6-ib-cr, qrrA, qrnB, qrnS) (Agga et al., 2015; Figueira et al., 2011a; Szczepanowski et al., 2009) were also noted in different studies.

Our study suffers from the limitations associated with a lack of data regarding HGT and sources of ARGs in various environmental compartments.

The ARGs found in stormwater may be considered worth investigating in WWTP. Additional analysis of ARB carrying the genes may show if there was a potential transfer of resistance from one bacterial group to another. Also similarities in the sequences responsible for resistance can indicate the route of ARGs spread.

### 4.4. Viruses and potential HGT in stormwater

Others confirmed that CrAssphage can be a marker of human fecal contamination (Karkman et al., 2019), which implicates the sampling point 3 to be contaminated with human feces through sewer pipe damage. However, we do not see the typical fecal ARB composition which is also identical in the sample 2, where we did not find crAssphage.

*Caudovirales* order constitute a majority in phages. The *Siphoviridae* phage is noted in highest abundance in many habitats, also WWTPs (Nigro et al., 2017; Parmar et al., 2018; Wang et al., 2018). *Siphoviridae* constituted 3–25% of all viruses and 30–57% of *Caudovirales* in tested samples (Supplementary material).

Order *Caudovirales* was the most abundant in the hospital wastewater investigated by Subirats et al. (2016) and in aquaculture wastewater by Colombo et al. (2016), both studies focused on AMR.

The relative abundance of DNA from chromosome, plasmid and phage DNA was determined for each sample (Fig. 3) using prediction algorithm, indicating highest contribution of plasmid in samples 1, 4, 5, with 40%, 44%, 42% respectively. Phages were most abundant in samples 2, 3 and 4 (with 28%, 28%, 29% respectively).

High percentage of MGEs and phages may be associated with frequent HGT. Especially that 3 ARGs were found exclusively on plasmids. The resistance mechanisms aac (6')-I was present both on plasmids and phages sequences. Six other ARGs were also identified on phages contigs without confirmation of their presence in other contigs on chromosomes (Table 1). One can assume that they could have been transferred by those phages.

In the Subirats et al. (2016) hospital wastewater study the relative abundance of ARGs in the phages was increased by half in comparison to the bacterial DNA fraction. As the phages are the most abundant biological entities around the world and they are able to transfer genes among bacterial hosts, the transduction should be carefully investigated in AMR studies. The transposons, integrative conjugative elements, plasmids and bacteriophages and other MGEs may be involved in HGT. Especially phages are directly involved in pickup and recombination of foreign DNA by bacteria (Marti et al., 2013). To verify the frequency of the HGT and confirm which ARGs were obtained this way, additional experiments performed on live microbes are required.
carbapenems and trimethoprim and sulphamethoxazole (Krishna and susceptible to tetracycline, chloramphenicol, aminoglycosides, cephalosporin, third generation cephalosporin and previously reported resistant toward penicillin and by Vaz-Moreira et al. (2011) as isolates from a drinking water, treatment plant, tap water, cup demineralization out of 3 sampling point - 1 and 3.

The sequences of phages, chromosomes, and plasmids in the metagenomic data obtained by long-read sequencing were predicted using PPR-Meta and the sequence percentages of phages, chromosomes, and plasmids were calculated. The results demonstrated in this chapter match state of the art methods. ARGs detection based on sequencing can help to avoid ARG primers limitation or to design primers adjusted to environmental variety of ARGs. AMR studies based on high-throughput sequencing is already popular in environmental studies on the distribution of ARGs in various ecosystems (Freilich et al., 2010; Li et al., 2015). One limitation is found in this case, most of the sequencing techniques (e.g., Illumina, 454) could hardly achieve the real-time resistome profiling which is required to guide the resistance control measures. The genes acquired by HGT including bacterial virulence and antibiotic resistance genes, have an inherent repetitive nature resulting in numerous gaps in the short-read assemblies. Due to those obstacles, the assemblies of the short reads are also fragmented due to their nature. It hinders the identification of the ARB carrying found ARGs.

The long-reads provided by third generation sequencing techniques like nanopore i.e. MinION contribute in high quality assemblies of viral and bacterial genomes. The longer the read, the bigger chance of finding a matching sequence to the reference database (Huson et al., 2018). MinION sequencing was already used by Xia et al. (2017) for multiple antibiotic resistant coliform bacteria detection in wastewater. It was a sufficient tool for quick monitoring and parallel phylogenetic identification. It is also helpful in monitoring genomic divergence obtained by HGT. Due to the inherent repetitiveness or flanking of genome parts acquired by HGT the short read sequencing (like MiSeq, Illumina), would leave numerous gaps in the in assemblies of resistance and pathogenicity islands (Ashton et al., 2015).

The ultimate goal of this study was to verify if the genomic DNA MinION sequencing can be used in metagenomic identification of bacterial and viral AMR in stormwater using publicly available data and open source bioinformatics tools. The data from the repository were used in the study of E. coli in stormwater, thus many of the metadata regarding many aspects that would be useful in our study were not collected. Issues connected to the lack of metadata in stormwater studies have been lately described in review by Hamilton et al. (2020), some of them were mentioned in chapter 4.5. The authors advocates for filing the well-defined knowledge gaps regarding (1) characterization of background conditions; (2) survey of AMR stressors in stormwater environments; (3) survey of hydrological conditions; (4) global agreement on needed defined sampling targets. Our study has contribution in the latest gap. We presented spectrum of ARB and ARGs occurring in stormwater in European capital, the region neglected in studies listed in Hamilton et al. (2020). In their review only European representative was Italy, other studies were performed in USA and China. Other limitations are connected to the MinION sequencing technique. It is still not perfect and often characterized by high error rate. However, it according to Loman et al. (2015) it has no significant impact the ARC/ARB prediction. There are plenty of bioinformatic algorithms addressing issues with error correction and de novo assembly of nanopore long reads (Loman et al., 2015). First of all MinION sequencing can have a low accuracy (typically...
Acknowledgements

We used ARGgore, as the problem of high error rate for ARGs was already explained by authors of the tool in their paper (Xia et al., 2017). Despite the quite high error rate (assess for their study as 9% by Phred Score and 15.4% by mapping to Illumina assembly), reliable ARG survey was possible using MinION sequences as their length (on average > 800 bp) was almost equal to the whole length of reference sequences in SARG database (1131 bp on average).

5. Conclusions

The study concludes how to process long read metagenomes in search of ARB connected with defined resistance type. Coexistence of environmental and pathogenic bacteria in the same niches in stormwater and detailed investigation regarding horizontal gene transfer of extracellular ARGs between those groups should be continued. At this point the possibility of identifying not only pathogens but also resistance encoding genetic material that may be integrated by pathogens is crucial. MinION sequencing and work frame presented in the study allowed not only identification of ARGs (resistance mechanism), but also ARB carrying them. This assumption might be addressed in future studies on more samples and comparison between short and long read sequencing.

Overall, our findings indicate that ARB in Swedish stormwater are organisms typical for WWTPs, associated with soil, lake and freshwater, plant growth as well as insects’ endosymbionts. Alarmingly Bordetella causing ‘whooping cough’ resistant to macrolides was identified in two samples. To our knowledge, this is the first report of source of Propionibacterium avium carrying mexX that may be connected to large series of peri-prosthetic joint infection in Sweden.

Resistome and ARB were not unique for stormwater sampling points, but the virome was. Caulovirales, Herpesvirales, Polydnaviridae were present in stormwater, in most samples Betaproteobacteria (family Poxviridae) carrying resistance towards aminoglycoside was noted.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127392.

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