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Free-floating extracellular DNA (exDNA) in different wastewaters: Status quo on exDNA-associated antimicrobial resistance genes☆

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ABSTRACT

Wastewater treatment plants (WWTPs) have been reported as major anthropogenic reservoirs for the spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) into the environment, worldwide. While most studies mainly focus on the intracellular DNA (iDNA), extracellular DNA (exDNA) accounting for a significant proportion of the total DNA in wastewater, was usually neglected. Following the One Health approach, this study focuses on wastewaters of municipal, clinical, and livestock origins (n = 45) that undergo different treatment processes (i.e., conventional activated sludge, ultrafiltration, and ozonation). Water samples were analysed for 12 ARGs as indicators of the different compartments associated with iDNA and exDNA by quantitative real-time PCR (qPCR). Taxonomic profiling of exDNA-fractions, obtained using nucleic acid adsorption particles, was conducted by sequencing the V3–V4 hypervariable regions of the 16S rRNA gene. Notified exDNA concentrations varied between on-site WWTPs and treatment stages, and ranged from 314.0 ± 70.2 ng/mL in untreated livestock wastewater down to 0.7 ± 0.1 ng/mL in effluents after ultrafiltration. In general, influents exhibited higher concentrations compared to effluents, while wastewater treated by advanced treatment processes (i.e., ultrafiltration and ozonation) showed the lowest exDNA concentrations. Despite the lower concentrations, free-floating exDNA accounted for up to 80.0 ± 5.8% of the total DNA in effluents. Target ARGs were more common in the iDNA (100%, n = 45/45), compared to the exDNA-fractions (51.1%, n = 23/45), whereas exDNA-ARGs were mostly detected in clinical and slaughterhouse wastewaters as well as in the municipal influents. Compared to the iDNA-ARGs, the concentrations of exDNA-ARGs were in general lower. Nevertheless, significant higher concentrations for exDNA-associated genes were measured in clinical wastewaters for bla_{NDM} (4.07 ± 0.15 log gene copies (GC)/L) and bla_{VIM-2} (6.0 ± 0.2 log GC/L). Overall, our results suggest that depending on the origin of wastewater and its treatment methods, exDNA represents an important reservoir for ARGs, particularly in clinical wastewater.

1. Introduction

Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been extensively reported in the influents and effluents of wastewater treatment plants (WWTPs) worldwide (Cacace et al., 2019). The emergence and spread of antimicrobial resistances (AMR) are a natural and inevitable part of bacterial evolution influenced by vertical/horizontal gene transfer in microbial communities and applied selection pressure, as a consequence of their exposition to antimicrobial substances or residues. Antimicrobials, together with ARBs and ARGs are released to a large extent in unmetabolized form into wastewater and are discharged from WWTPs to surface water due to inadequate wastewater treatment, mainly aimed at the reduction of phosphorus, nitrogen and organic matter. Conventional WWTPs are therefore considered to be major anthropogenic reservoirs for the release of these microbial pollutants into the (aquatic) environment (Slipko et al., 2019).

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Specific ARGs such as *sul1, ermB,* bla_{TEM} and *tetM*, which encode resistances to the most commonly used classes of antimicrobial agents are frequently reported in German wastewater and surface waters (Alexander et al., 2020). Furthermore, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{CMY\text{-}2}$, $bla_{OXA\text{-}48}$, $bla_{NDM\text{-}1}$, *vanA* and *mcr-1,* which encode resistances to "Highest Priority Critically Important Antimicrobials" (HPCIA) as defined by the WHO, are regularly reported in municipal wastewater impacted by effluents from slaughterhouses and hospitals (Alexander et al., 2020; Savin et al., 2020a). Moreover, in municipal wastewater influenced by clinical effluents Voigt et al. (2020) reported high abundances for genes encoding medically important carbapenemases $bla_{NDM\text{-}1}$ and $bla_{VIM\text{-}2}$ at 2.6×10^7 gene copies (GC)/mL and 8.0×10^7 GC/mL, respectively. Similar findings were shown by Alexander et al. (2022), who consistently detected the carbapenemase genes $bla_{KPC\text{-}3}$ and $bla_{NDM\text{-}1}$ at high concentrations in hospital wastewater, indicating a continuous emergence or a steadily persistence of these resistance determinants in the prevailing clinical bacterial population.

However, one of the limitations of these studies is the fact that they mainly focus on the intracellular DNA (iDNA), whereas a significant proportion of the total DNA in wastewater samples accounts for extracellular DNA (exDNA) that derives actively from physiologically viable cells and passively from inactivated or deceased cells. It is well known that wastewater undergoes various intensive treatment steps in WWTPs resulting in 90–99% death rates of the bacterial community, which leads to high numbers of cell debris (Alexander et al., 2020). Consequently, free genetic material (exDNA) including resistance determinants (i.e., antimicrobial, disinfectants, biocides, metal etc.), is released into the environment through effluents from WWTPs. Calderón-Franco et al. (2021) reported exDNA concentrations in influent and effluent of a municipal WWTP of 12.5 μg/L and 8.6 μg/L, respectively. In another study, exDNA was detected at 0.12–2.5 μg/L in Tama River water (Liu et al., 2020). Torti et al. (2015) measured exDNA in freshwater ecosystems at concentration of up to 25.6 μg/L, whereas in monochloraminated drinking water systems, a small amount of exDNA of 33–386 ng/L accounted for a significant proportion of total DNA of up to 64% (Sakcham et al., 2019).

It is estimated that 860-14,500 tonnes of exDNA per year are discharged from diffuse sources (e.g., agricultural soils, extensive animal husbandry, urban and industrial areas) into the rivers worldwide (Overballe-Petersen et al., 2013). At the same time, WWTPs are considered the most prominent hotspots for ARGs, which receive wastewater from urban areas, industry and hospitals. Interestingly, some ARGs are present in effluents treated by advanced technologies (e.g., chlorination) in greater abundance as exDNA compared with iDNA (Liu et al., 2018). This is of particular concern, since ARGs from exDNA fraction can lead to an acquisition of AMR via transformation of naturally competent bacteria, leading to the subsequent exchange of genetic elements by horizontal gene transfer (i.e., conjugation/mobilization, transduction) among the microbial communities and a further spread of armed-up bacteria to other ecosystems by general discharge and vectors (Dodd, 2012). Transformation is an essential mechanism of the horizontal gene transfer as it is mainly responsible for bacterial diversification and/or their adaptation to natural and artificial stress responses, without necessarily resulting in a public health threat. However, especially if exDNA contains ARGs linked to mobile genetic elements (MGEs), its release into the aquatic environment can be a significant driver for the generation of opportunistic or pathogenic bacteria resistant to one or multiple antibiotics (Blokesch, 2016).

The presence and abundance of ARGs in wastewater are influenced not only by the origin of the wastewater and the treatment processes employed, but also depending on the DNA fraction being analysed. Since there is an apparent lack on the type and concentration of free-floating exDNA, especially on clinically-relevant ARGs in the exDNA fraction in wastewaters treated by different processes, we aimed to (i) determine the exDNA-concentrations; (ii) conduct taxonomic profiling of the exDNA-fractions and (iii) assess the differences in abundance of iDNA- and exDNA-associated ARGs in wastewaters of municipal, livestock and clinical origins in Germany.

2. Material and methods

2.1. Sampling of municipal, clinical and poultry industry wastewater

Sample acquisition was conducted in three different municipal wastewater treatment plants (WWTPs; MK1-MK3), three maximum care hospitals (UK1-UK3) and one poultry slaughterhouse PS1.

The municipal WWTPs possess different population equivalents of 285,000 (MK1), 41,000 (MK2), 80,000 (MK3) people and treat daily 47,950 m^3, 5800 m^3 and 15,200 m^3 wastewater, respectively. The WWTPs-MK1 and MK2 use conventional aerobic activated sludge process, whereas in WWTP-MK3 conventionally treated wastewater additionally undergoes ultrafiltration (pore size 0.04 μm).

Maximum care hospitals (UK1-UK3) have 950–1300 inpatient beds and treat yearly 220,000–440,000 patients (outpatient and inpatient). Daily wastewater amounts are 650 m^3 (UK1), 450 m^3 (UK2) and 400 m^3 (UK3), which are discharged without any pre-treatment into municipal WWTPs. Of note, clinical wastewater from UK1 is discharged into the municipal WWTP-MK1.

The poultry slaughterhouse exhibits a slaughtering capacity >100,000 chickens per day, and treats daily 3600 m^3 in an on-site WWTP that is based on biological and advanced oxidation (i.e., ozonation) processes. The ozone dosage used was 75 g/m^3, and the contact time varied between 15 and 30 min, depending on the water flow rate.

Biological triplicates of qualified wastewater samples (n = 45) were taken on different days in the influents (MK1: BIN, BIS; MK2: WI; MK3: GKWI; n = 12) and effluents of municipal WWTPs (MK1: BEC; MK2: WEC; MK3: GKWEU; n = 9). Influents (PIS; n = 3) and effluents after biological treatment (PEC; n = 3) and ozonation (PSEO; n = 3) were sampled in the on-site WWTP of the poultry slaughterhouse PS1. Effluents after ultrafiltration (GKWEU) were sampled from the retention tank. Clinical effluents from three maximum care hospitals (UK1-UK3; n = 9) were sampled as well. Furthermore, samples were taken in the pre-flooder of the WWTP-MK3 200 m upstream (WPU, n = 3) and downstream (WPD, n = 3) of the discharge point. The samples were collected according to the German standard methods for the examination of water, wastewater, and sludge (DIN 38402–11:2009–02) using Nalgene Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Savin et al., 2020a, 2020b). All samples were transported to the laboratory cooled in a Styrofoam box at 5 ± 2 °C and processed within 24 h after their collection.

2.2. Isolation of free-floating exDNA and iDNA

Of the samples from the influents, effluents and pre-flooders, 1L, 2L and 3L, respectively, were filtered through a 0.45 μm and subsequently through a 0.22 μm hydrophilic polyethersulfone (PES) membrane filter (GVS North America, Sanford, ME, USA). Consequently, the filtrate was collected for exDNA extraction and the filters were utilized for iDNA extraction.

The exDNA was extracted according to Wang et al. (2016) using nucleic acid adsorption particles (NAAPs), aluminium hydroxide-coated silica gel with high binding capacity. These particles were developed for an optimal adsorption-elution method to concentrate exDNA from large volumes of water. For this purpose, a glass column (1.5 × 50 cm, Bio-Rad Laboratories, Hercules, CA, USA) was sealed with 18 g of NAAPs, and then the filtrate was pumped through the column using a peristaltic pump with a flow rate of 30 mL/min. Following the passage of the sample filtrate, 100 mL of an eluent [15 g/L NaCl, 30 g/L tryptone, 15 g/L beef extract, 3.75 g/L glycine, 0.28 g/L Na(OH), pH = 9.3 ± 0.2; autoclaved at 120 °C for 20 min] was pumped through the column and collected in a centrifuge tube.

The exDNA in the eluent was then precipitated by adding an 0.6–0.7 vol of isopropanol and sodium acetate (pH 5.2; final concentration 0.3 M) and incubated at room temperature for 16 h. After precipitation, the mixture was centrifuged at 12.000 g for 10 min at room temperature, and the supernatant was decanted. The pellet was washed with 10 mL of 70% ethanol (Neofroxx GmbH, Germany) by pipetting and centrifuged once more at 12.000×*g* for 5 min at room temperature. The pellet was dried and resuspended with 10 mM Tris-HCl buffer (bioWORLD, Dublin, OH, USA).

The precipitated raw exDNA was then incubated with 100 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO, USA) at 57 °C and purified using GeneJET NGS Cleanup Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol, in order to remove possible inhibitors of DNA amplification steps. The DNA concentration was quantified fluorometrically on a Qubit fluorometer with the Qubit 1 × dsDNA HS Assay Kit (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) and analysed for its quality (absorption coefficients A260/230 and A260/280) by NanoDrop spectrophotometry (Thermo Fisher Scientific, Wilmington, DE, USA). The purified exDNA was stored at −80 °C prior to further molecular analysis.

For analytical comparison with extracellular DNA, after vacuum filtration, the 0.45 μm and 0.22 μm PES-filters were cut into fragments, and iDNA was extracted from the filters according to the NucleoMag DNA Microbiome (Macherey-Nagel GmbH, Germany) Kit manufacturer's protocol.

2.3. 16S rRNA-based metagenomic profiling of exDNA fractions from different wastewaters

16S microbial profiling of the isolated exDNA was done applying a preprocessing pipeline as previously described (Hassa et al., 2021) followed by analyses in the QIIME2 platform. Briefly, 16S rRNA gene amplicon libraries were prepared by amplification of V3–V4 hypervariable regions using the universal primer pair Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) applying the "16S Metagenomic Sequencing Library Preparation" protocol (Illumina Inc., San Diego, CA, USA) (Takahashi et al., 2014). The amplicon libraries were sequenced on the Illumina MiSeq platform applying the 2x300 bp paired-end protocol with subsequent demultiplexing. After an initial quality control of the raw sequencing reads with FastQC, forward and reverse reads were merged with FLASH (Magoč and Salzberg, 2011), primers were removed using cutadapt (Martin, 2011) and the merged reads were quality-trimmed (<Q20 discarded) with sickle (Joshi Nikhil F. J., 2011). Afterwards the QIIME2 platform (v. 2021.8) applyinq DADA2, mafft and fasttree was used (Caporaso et al., 2010; Bolyen et al., 2019), followed by the taxonomic assignment using the q2-feature-classifier against the Silva database (release 138, Quast et al., 2013). After filtering (frequencies <5) of the amplicon sequencing variants (ASVs) each sample was rarefied to a given sequencing-depth of 50,000 reads, applying the "qiime feature-table rarefy" function. Finally, biological wastewater triplicates were used to calculate median abundance values, which were afterwards normalized to 100%.

2.4. Determination of abundances of exDNA- and iDNA-associated ARGs

Antimicrobial resistance genes (ARGs) that are most frequently detected in German wastewaters (*sul1, ermB,* bla_{TEM}, *tetM*), "intermediate and rare abundant" ARGs, which encode resistances to "Highest Priority Critically Important Antimicrobials" (HPCIA; $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}32}$, *mecA, mcr-1*, *vanA*) as well as genes encoding carbapenemases ($bla_{OXA\text{-}48}$, $bla_{NDM\text{-}1}$, $bla_{KPC\text{-}3}$, $bla_{VIM\text{-}2}$) were quantitatively amplified by qPCR as previously described (Hembach et al., 2017; Hembach et al., 2019). *intI1,* the class I integron-integrase gene that is known to be responsible for genes mobility, was also included for quantification (Hembach et al., 2019). SYBR Green qPCR tests were run in technical triplicates on Bio-Rad Cycler CFX96 (CFX96 Touch Deep Well Real-Time PCR Detection System, Bio-Rad, Germany). Reactions were run in volumes of 20 μL, containing 10 μL Maxima SYBR Green/ROX qPCR Master Mix (2 ×) (Thermo Scientific, Nidderau, Germany), 8.2 μL of nuclease-free water (Ambion, Life technologies, Karlsbad, Germany), 0.4 μL of the respective primers (stock concentration 10 μM, Table S1), and 1 μL of template DNA. The qPCR protocol comprised 10 min at 95 °C for activation of the DNA polymerase followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C for primer annealing and elongation. Melting curves were recorded by raising the temperature from 60 °C to 95 °C (1 °C every 10 s) to determine the specificity of the amplification. Gene copies were calculated according to Hembach et al. (2019) and normalized to 1000 mL of water sample.

2.5. Statistics

Based on the results of 16S rRNA-based metagenomic profiling, permutation multivariate analysis PERMANOVA on unweighted UniFrac data (Clarke K. R., 1993; Paliy and Shankar, 2016; Ramette, 2007), and the distance matrix was calculated using the Bray-Curtis algorithm (McQuitty, 1966). The calculated (dis)similarities were visualized as a tree diagram to define the existing clusters of the investigated wastewater samples based on their taxonomic profiles. To analyse the significance of the differences in DNA-concentrations, and abundance of ARGs between exDNA and iDNA fractions, a parametric two-tailed Student's *t*-test with 95% confidence level ($p < 0.05$) was performed using R Project for Statistical Computing (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). For both exDNA and iDNA samples, three biological replicates with three technical replicates were used.

3. Results

3.1. Concentrations of exDNA and iDNA substantially varies between WWTPs and treatment stages

The concentrations of iDNA and exDNA were confirmed to be significantly higher ($p < 0.05$) in untreated wastewater compared to those in treated wastewater. The detected exDNA concentrations varied between 0.7 ± 0.1 ng/mL and 314.0 ± 70.2 ng/mL, whereas iDNA was in the range of 1.0 ± 0.2 ng/mL to 84.3 ± 27.2 ng/mL among the different WWTPs and sampling points (Fig. 1). Effluents of a municipal WWTP-MK1 (BEC) exhibited similar or lower exDNA concentrations of 7.0 ± 2.6 ng/mL compared to the influents BIN (6.4 ± 1.4 ng/mL) and BIS (13.2 ± 1.7 ng/mL). However, its percentage in the effluents increased from 10.1 ± 1.4% (BIN) and 14.7 ± 4.0% (BIS) to 80.0 ± 5.8%. Similar results were observed in another municipal WWTP-MK2 using the conventional treatment, where the exDNA concentrations in the effluents (2.9 ± 0.9 ng/mL, WEC) were lower compared to the influents (6.1 ± 2.2 ng/mL, WI) and the exDNA percentage increased after the biological treatment from 13.9 ± 4.1% to 39.2 ± 3.7%. The waterbody receiving the treated wastewater (pre-flooder) showed an increased exDNA concentration downstream the discharge point (2.1 ± 0.4 ng/mL, WPD) compared to the sampling point upstream (0.8 ± 0.1 ng/mL, WPU). Interestingly, after ultrafiltration the exDNA concentration in the effluents of WWTP-MK3 (GKWEU) showed a notable decrease compared with the influents (GKWI) from 6.8 ± 2.5 to 0.7 ± 0.1 ng/mL. However, its percentage increased from 12.0 ± 2.3 to 41.6 ± 0.7%. This is different in the poultry slaughterhouse, where both the exDNA concentrations and its proportion were reduced after the treatment in the in-house WWTP. However, even after ozonation the exDNA percentage constituted 39.2 ± 17.7% of the total DNA. The clinical wastewaters showed comparable concentrations with the municipal influents and were in the range of 4.0 ± 1.6 ng/mL and 10.9 ± 6.3 ng/mL, whereas the exDNA percentage on the total DNA varied between 16.6 ± 5.6% and 21.3 ± 1.5%.

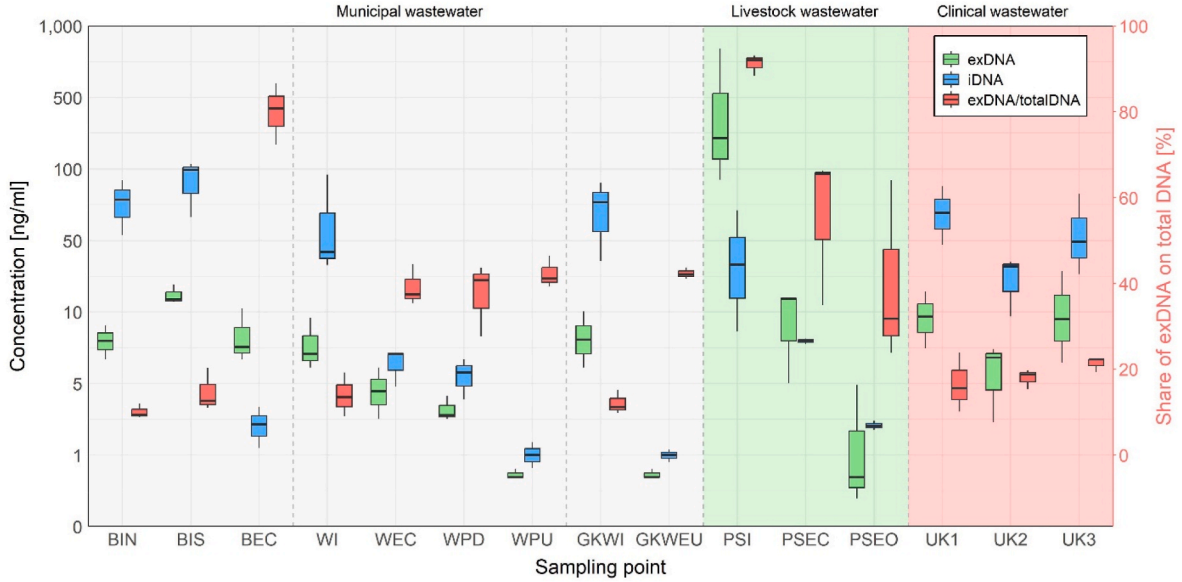

Fig. 1. Concentrations of free-floating extracellular DNA (exDNA), intracellular DNA (iDNA) and the share of exDNA on total DNA in the analysed wastewater samples (n = 45). BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

3.2. exDNA taxonomic profiling reveals substantial differences in bacterial community diversity in human and animal-associated wastewaters

Permutation multivariate analysis PERMANOVA on unweighted UniFrac data showed a significant difference ($p = 0.001$) between samples of municipal (BIN, BIS, BEC, WI, WEC, WPD, WPU, GKWI, GKWEU; n = 27) and non-municipal (PSI, PSEC, PSEO, UK1, UK2, UK3; n = 18) origin. Furthermore, samples from the slaughterhouse differ significantly from municipal and clinical wastewater samples ($p < 0.01$), while clinical wastewaters (UK1, UK3) showed a significant difference ($p < 0.01$) to municipal (BIN, BIS, BEC, WI, WEC, WPD, WPU) and slaughterhouse wastewaters.

Taxonomic profiling of the exDNA-fractions revealed that 99.8 ± 0.2% of the detected taxa were assigned to the bacterial domain, while 0.1 ± 0.2% match to *Archaea* and 0.1 ± 0.1% to taxonomically unclassified organisms. Overall, 43 phyla were detected, whereas in the municipal and clinical wastewaters as well as in the pre-flooder, *Proteobacteria*, *Patescibacteria*, *Bacteroidota*, *Firmicutes*, *Campilobacterota* and *Fusobacteriota* were the most common phyla accounting for between 97.6 ± 0.8% and 98.7 ± 0.6% of the taxonomic profiles. In the wastewater from the poultry slaughterhouse, additionally to the previously mentioned six main phyla, *Bdellovibrionota*, *Actinobacteriota* and *Chloroflexi* were detected at relative abundances of >1%.

At the order level, among all samples, 316 orders were detected. Municipal influents (n = 12), untreated wastewater from the clinics (n = 9), and poultry slaughterhouse (n = 3) showed similar numbers of orders of 67.4 ± 31.5, 69.7 ± 20.4, and 72 ± 25.5, respectively. Noteworthy, effluents after conventional treatment (n = 6), ultrafiltration (n = 3), and ozonation (n = 3) exhibited significantly higher numbers of orders of 128.8 ± 27.6, 106.3 ± 17.2 and 155.3 ± 21.0, which were similar to those from the pre-flooder upstream (117.7 ± 19.8) and downstream (135.3 ± 22.9) the discharge point.

The most predominant taxonomic groups at the order level detected in the municipal influents were *Burkholderiales* (28.9 ± 6.9%), *Sphingomonadales* (17.9 ± 6.1%), *Flavobacteriales* (12.9 ± 2.8%), and *Chitinophagales* (10.3 ± 4.7%), whereas in the effluents after conventional treatment *Candidatus Nomurabacteria* (29.8 ± 6.7%), *Parcubacteria* (16.7 ± 5.4%), and *Candidatus Adlerbacteria* (12.4 ± 6.6%) were the most abundant orders (Fig. 2). Interestingly, in the municipal effluents after ultrafiltration *Burkholderiales* (21.9 ± 3.7%) and *Flavobacteriales* (13.5 ± 2.8%) were detected in abundances comparable to the influents. However, the share of *Enterobacterales* (11 ± 3%) was significantly higher than in the influents (0.4 ± 0.4%) and effluents after conventional treatment (1 ± 0.7%). The pre-flooder downstream the discharge point of the municipal WWTP was similar to the effluents after conventional treatment in its taxonomic composition with *Candidatus Nomurabacteria* (26.4 ± 1.5%), *Candidatus Adlerbacteria* (18.2 ± 6.4%) and *Parcubacteria* (17.3 ± 2.5%) as the most prevalent orders. However, in the pre-flooder upstream *Burkholderiales* (15.2 ± 8.6%), *Candidatus Nomurabacteria* (13.9 ± 9.2%), *Sphingomonadales* (11.2 ± 5.8%), and *Flavobacteriales* (10.5 ± 5.8%) were the most common orders. In the effluents of the poultry slaughterhouse, the shares of *Burkholderiales* and *Sphingomonadales* increased significantly after ozonation from 5.9 ± 0.4% and 1.9 ± 0.6% to 18.5 ± 6.6% and 10.7 ± 7.6%, respectively, in comparison to the effluents after conventional treatment. In clinical wastewater, *Burkholderiales* (17.7 ± 6.4%) was one of the main orders. *Pseudomonadales*, other than in municipal wastewater (1.6 ± 0.6%), accounted for 10.2 ± 8.8% of the detected orders. Furthermore, *Oscillospirales* constituted 8.3 ± 2.6%, while its share in municipal wastewater was 1.1 ± 0.3%.

Based on the results of McQuitty (dis)similarity matrix, analysed wastewater samples were divided into five clusters due to their taxonomic diversity with two samples from the poultry slaughterhouse classified as outliers (Fig. 3). Cluster I comprised clinical wastewater from UK2 and UK3 with *Burkholderiales* (15.7 ± 6.8%), *Pseudomonadales* (15.1 ± 6.8%), and *Oscillospirales* (9.8 ± 1.8%) being the most indicative taxa. Cluster II included mostly effluents after conventional treatment and pre-flooder downstream the discharge point. *Candidatus Nomurabacteria* (29.1 ± 4.2%) and *Parcubacteria* (15.5 ± 6.3%) were the most representative taxa for this cluster. Cluster III consisted of municipal and clinical wastewater from UK1, whereas *Burkholderiales* (17.9 ± 2.2%), *Campylobacterales* (14.9 ± 5.8%), and *Bacteroidales* (10.9 ± 6.8%) were identified as indicative taxa for this cluster. Samples of wastewater after advanced treatment (i.e., ultrafiltration and ozonation) were grouped into cluster IV, whereas cluster V comprised mostly municipal influents. Interestingly, orders *Burkholderiales*, *Sphingomonadales,* and *Flavobacteriales* were the most representative taxa for both

Fig. 2. Taxonomic profiles on order level of the exDNA-fractions from 45 wastewater samples. Orders with a maximal relative abundance less than 2% were summarized. BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

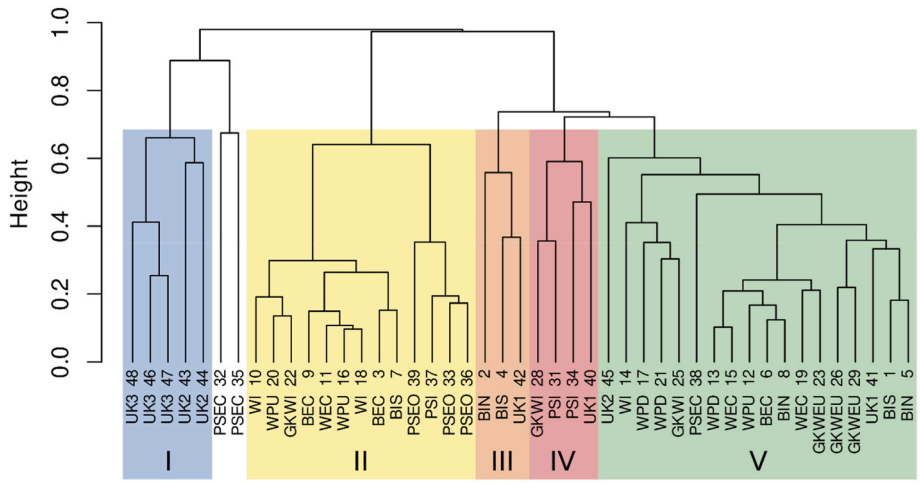

Fig. 3. Relationship based on a McQuitty (dis)similarities matrix calculated from the exDNA-associated taxonomic profiles of the analysed wastewater samples (n = 45). BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

clusters IV and V. In cluster V, the previously mentioned orders shown slightly higher percentages at 26.6 ± 6.9%, 18.2 ± 9.3%, 13.6 ± 3.8% versus 22.3 ± 3.3%, 11.2 ± 5.7% and 9.8 ± 2.6%, respectively.

3.3. The source of wastewater and the processes used for its treatment strongly influence the proportion of the detected target ARGs in exDNA- and iDNA-fractions

exDNA-associated ARGs were detected in 51.1% (n = 23/45) of the samples, whereas all samples (n = 45/45) contained target ARGs in their iDNA-fractions (Fig. 4).

Target exDNA-associated ARGs were mostly detected in clinical and slaughterhouse wastewaters as well as in the municipal WWTP influents. No target exDNA-ARGs were detected in the effluents of two municipal WWTPs after conventional treatment (BEC), and ultrafiltration (GKWEU) as well as in the receiving waterbody upstream (WPU) and downstream the discharge point (WPD). However, effluents after conventional treatment in WEC exhibited exDNA-associated gene targets *tetM*, *ermB*, *sul1* and *intl1*. In the exDNA-fractions from the municipal influents (i.e., BIN, BIS, GKWI, WI), *tetM*, *ermB, mecA* and *sul1* were the most frequently detected ARGs.

Of note, all target genes, except for *mcr-1*, were detected in the exDNA-fraction of clinical wastewater UK3. Furthermore, all target ARGs but $bla_{\text{OXA-48}}$ (n = 1/3) and *vanA* (n = 2/3) were detected in all three biological replicates from UK3. However, exDNA-fractions from two other hospitals exhibited less variety with $bla_{\text{NDM-1}}$ (n = 3/3), *mecA* (2/3) and *ermB* (n = 2/3) in UK1, and bla_{TEM} (n = 1/3) in UK2.

In the poultry slaughterhouse, target genes, i.e., *sul1, int1*, *tetM* and $bla_{\text{CTX-M32}}$ were detected more frequently in the effluents after conventional treatment compared to the influents. However, after ozonation target genes in the exDNA-fraction were eliminated (<LOD) and only exDNA-associated *intl1* was detected. Interestingly, besides the

Fig. 4. Detection frequencies of target ARGs and *intl1* at the examined sampling sites. Colours of the sampling point IDs represent municipal (grey), livestock (green) and clinical (red) wastewater. BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

wastewater from the poultry slaughterhouse, exDNA-associated *intl1* was detected only in the effluent of a municipal WWTP after conventional treatment at a concentration of 2.79 ± 0.31 log GC/L (WEC) and in the clinical wastewater from UK3 at a higher concentration of 6.34 ± 0.34 log GC/L. In the iDNA-fractions of the analysed wastewater samples *intl1* were detected in the range of 4.2–7.8 log GC/L, whereas the highest concentrations of up to 7.29 ± 0.38 log GC/L were detected in untreated municipal and clinical wastewaters.

The abundances of target ARGs in iDNA- and exDNA fractions are shown in Figs. 5–7. As expected, the highest concentrations of iDNA-associated target ARGs were detected in untreated, and clinical wastewaters, where ARGs that are most frequently detected in German wastewaters (i.e., *sul1, ermB*, *tetM*) exhibited comparable abundances in the range of 4.5–8.5 log gene copies (GC)/L (Fig. 5). Surprisingly, bla_{TEM} was mostly detected in clinical wastewaters at high abundances of 6.2–7.5 log GC/L, whereas municipal WWTP influents and slaughterhouse wastewaters were only sporadically positive for bla_{TEM} at slightly lower concentrations of 6.0–6.8 log GC/L. Furthermore, iDNA fractions of clinical wastewaters exhibited the highest concentrations of ARGs encoding resistances to the HPCIA $bla_{CTX\text{-}M\text{-}15}$ (5.53 ± 0.10 log GC/L), *vanA* (4.28 ± 0.25 log GC/L) (Fig. 6), and carbapenemases $bla_{NDM\text{-}1}$ (5.71 ± 0.11 log GC/L), $bla_{OXA\text{-}48}$ (5.28 ± 0.06 log GC/L), $bla_{KPC\text{-}3}$ (6.77 ± 0.10 log GC/L) and $bla_{VIM\text{-}2}$ (6.0 ± 0.2 log GC/L) (Fig. 7).

Compared to the iDNA-ARGs, the concentrations of exDNA-associated ARGs were in general lower. However, in the influents and effluents of the poultry slaughterhouse *sul1*, *tetM*, *intl1* and *tetM*, *ermB*, bla_{TEM}, respectively, were detected at a higher abundance in the exDNA-fraction. Nevertheless, significant higher concentrations ($p < 0.05$) for exDNA-associated genes were measured only for bla_{NDM} and $bla_{VIM\text{-}2}$ in clinical wastewaters. Their abundances in the exDNA-fraction varied between 4.07 ± 0.15 log GC/L (bla_{NDM}) and 6.0 ± 0.2 log GC/L ($bla_{VIM\text{-}2}$). These values were either comparable to those from the iDNA-fraction (e.g., $bla_{VIM\text{-}2}$ and $bla_{OXA\text{-}48}$) or even higher. In UK3, $bla_{NDM\text{-}1}$ exhibited a 2.16 ± 0.14 log (i.e., 105–200 times) higher concentration in the exDNA-fraction compared to the iDNA-fraction.

The highest reduction efficiency based on the abundances of iDNA-associated *tetM*, *ermB* and *sul1* as the most frequently detected ARGs was measured in BEC after a conventional treatment, where the target ARGs were reduced by 4.30 ± 0.24, 4.46 ± 0.31 and 2.87 ± 0.33 log, respectively. Similar reduction rates were observed in GKWEU after ultrafiltration as well as in the conventional on-site WWTP of the poultry slaughterhouse (PSEC). Of note, after ozonation no significant reduction of target iDNA-associated ARGs was observed. However, after ozonation both exDNA concentrations and its share on total DNA were reduced, and exDNA-associated *teM*, *ermB*, *sul1* eliminated (<LOD).

4. Discussion

This study highlights that free-floating extracellular DNA accounts for a significant proportion of the total DNA in wastewaters of different origins. This applies in particular to the wastewater treated by conventional biological process, where the proportion of exDNA on the total DNA constituted up to 85.8%. Our results on exDNA in municipal wastewaters are in line with the study of Calderón-Franco et al. (2021), who reported exDNA in a Dutch WWTP at concentrations of 12.5 μg/L and 8.6 μg/L in influents and effluents, respectively. However, as this is, to the best of our knowledge, the first study in Europe to analyse the *status quo* on exDNA in wastewaters not only of municipal origin but also from hospitals and slaughterhouses, comparative data are limited.

Wastewater treatment based on conventional activated sludge showed insignificant reduction in exDNA concentrations compared to advanced technologies of ozonation and ultrafiltration. While the biological treatment causes cell disruption and a subsequent leakage of DNA, cell wall of bacteria including intracellular substances, e.g., purines and pyrimidines of DNA, are destructed by advanced treatment such as ozonation via hydroxyl radicals and O_3. Exposure of exDNA to

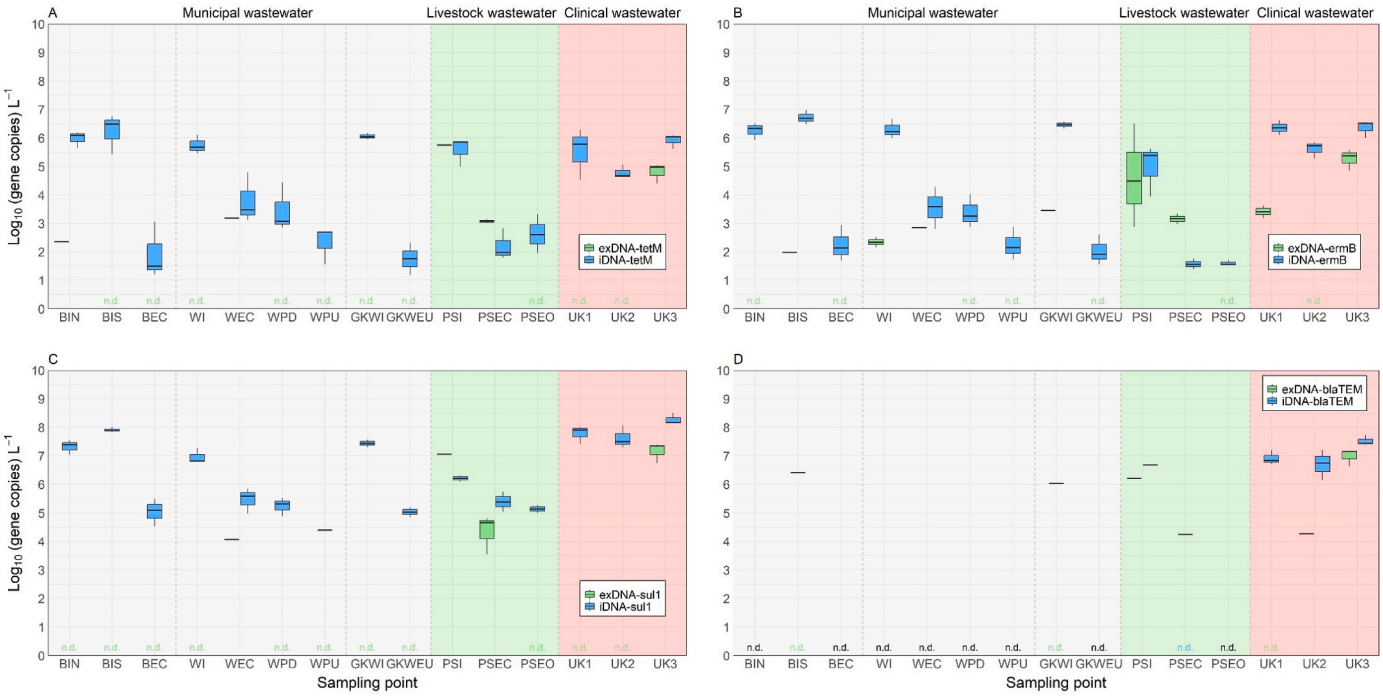

Fig. 5. Abundances of antimicrobial resistance genes *tetM* (A), *ermB* (B), *sul1* (C) and bla_{TEM} (D) in exDNA- and iDNA-fractions of analysed wastewater samples (n = 45). Measurements obtained for particular target gene from two or all three biological replicates are shown as box plots. Measurements from a single replicate are shown as lines. The n.d. labels in green, blue and black indicate that the target gene was not detected in the exDNA-, iDNA- or in both DNA-fractions, respectively. BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

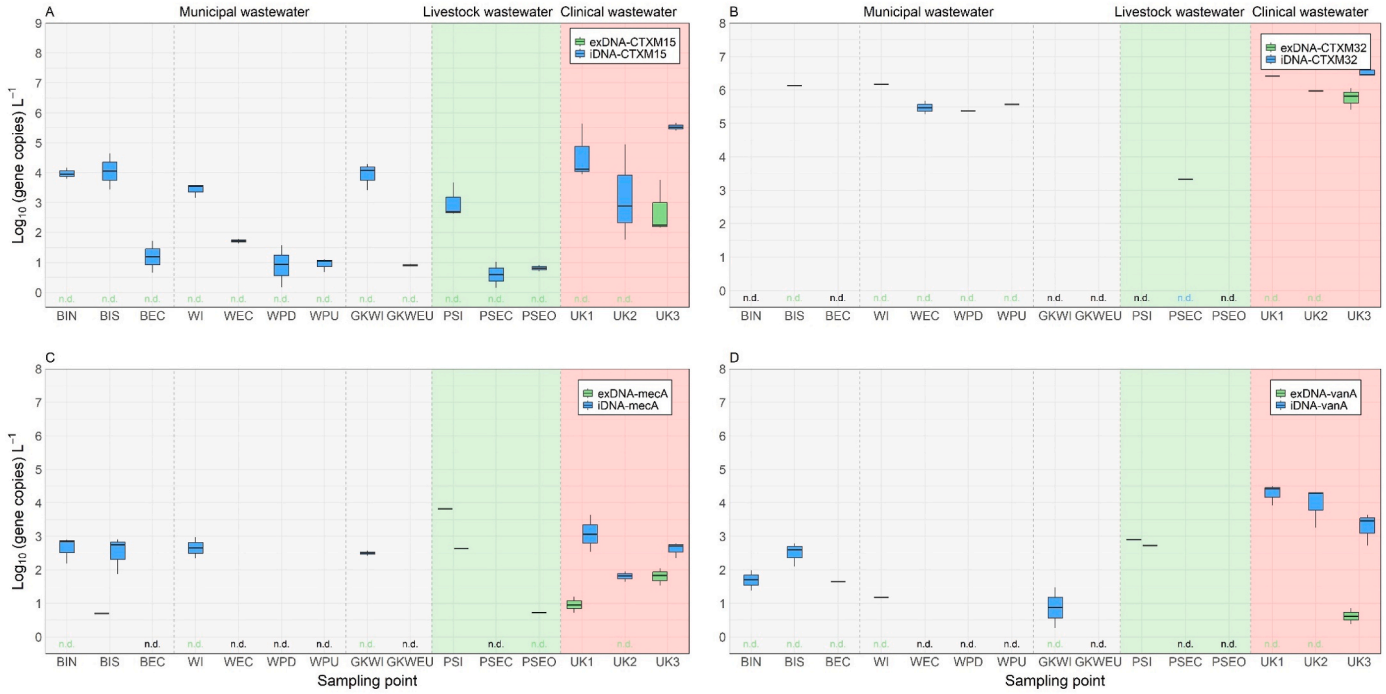

Fig. 6. Abundances of antimicrobial resistance genes *bla*$_{\text{CTX-M-15}}$ (A), *bla*$_{\text{CTX-M-32}}$ (B), *mecA* (C) and *vanA* (D) in exDNA- and iDNA-fractions of analysed wastewater samples (n = 45). Measurements obtained for particular target gene from two or all three biological replicates are shown as box plots. Measurements from a single replicate are shown as lines. The n.d. labels in green, blue and black indicate that the target gene was not detected in the exDNA-, iDNA- or in both DNA-fractions, respectively. BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

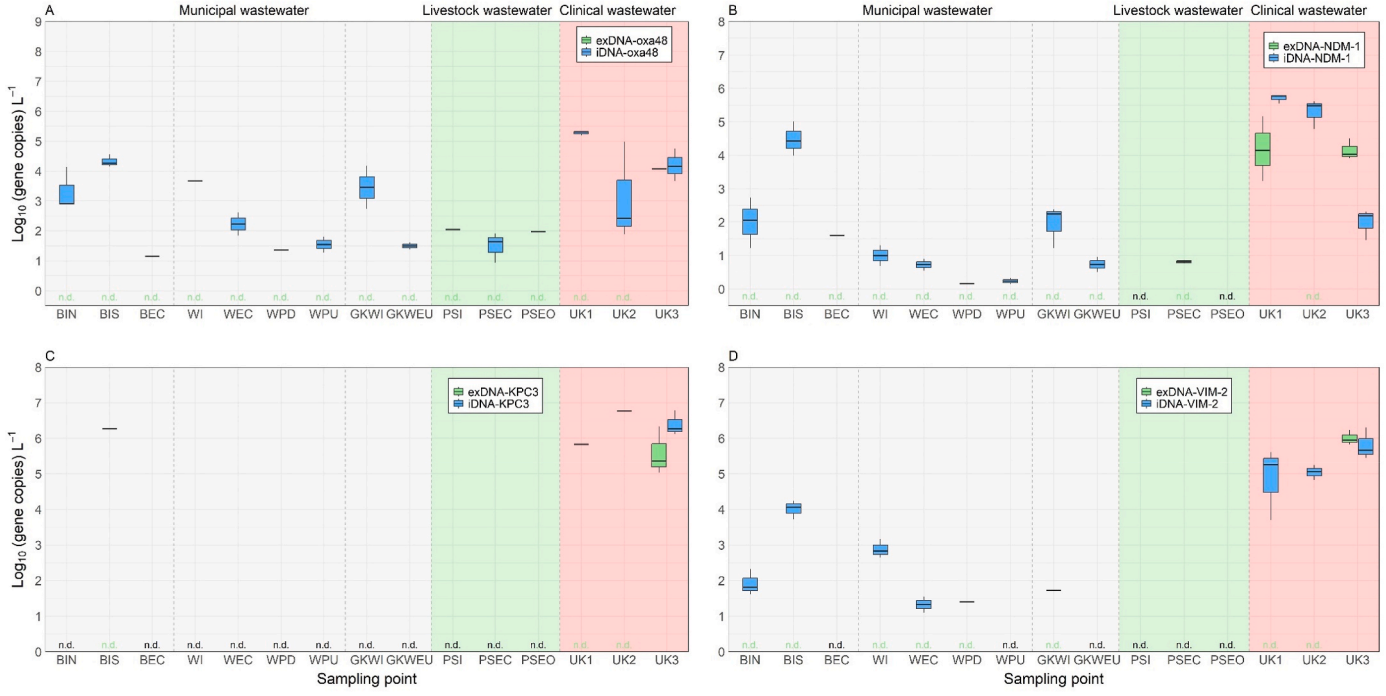

Fig. 7. Abundances of antimicrobial resistance genes *bla*$_{\text{OXA-48}}$ (A), *bla*$_{\text{NDM-1}}$ (B), *bla*$_{\text{KPC-3}}$ (C) and *bla*$_{\text{VIM-2}}$ (D) in exDNA- and iDNA-fractions of analysed wastewater samples (n = 45). Measurements obtained for particular target gene from two or all three biological replicates are shown as box plots. Measurements from a single replicate are shown as lines. The n.d. labels in green, blue and black indicate that the target gene was not detected in the exDNA-, iDNA- or in both DNA-fractions, respectively. BIN, BIS, WI, GKWI: municipal influents; BEC; WEC: municipal effluents after conventional treatment; GKWEU: municipal effluents after ultrafiltration; PIS, PEC, PSEO: influents, effluents after biological treatment, and ozonation, respectively, from the on-site WWTP of the poultry slaughterhouse; UK1, UK2, UK3: clinical wastewater; WPU, WPD: pre-flooder of a municipal WWTP upstream and downstream of the discharge point, respectively.

degradation agents results in higher rates of reduction compared to iDNA (Gajdoš et al., 2023).This explains the reduction of the exDNA concentration by 73.3 ± 15.0% in wastewater from the poultry slaughterhouse after treatment with ozone and the removal of exDNA-associated ARGs *tetM*, *ermB*, *sul1,* bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$. Furthermore, as long as there are bacteria in wastewater, exDNA will inevitable occur after their death and subsequent cell lysis. Thus, removal of bacteria by ultrafiltration would greatly contribute to the reduction of the initial bacterial load that possibly serves as an exDNA precursor. This is consistent with our results, where the exDNA in effluents after ultrafiltration was reduced to 0.63–0.77 μg L^{-1} to the level of the receiving water body upstream the discharge point of WWTP which was unaffected by treated wastewaters.

Besides the direct input of exDNA from WWTPs and other diffuse sources of anthropogenic activities (e.g., run-offs from fields fertilized with manure, stormwater overflow basins), free-floating exDNA in aquatic environments can also arise during cell lysis of bacteria that are less adapted to stressful environmental conditions (Johnston et al., 2014). This includes enteric microorganisms, which are present in wastewater in high numbers and partially reflects the human microbiome. ESKAPE bacteria (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) and extended spectrum β-lactamase (ESBL)-producing *E. coli*, which account for ca. 60% of healthcare-associated infections in Europe and the USA, are commonly reported in Germany not only in untreated wastewaters, but also in effluents and receiving water bodies (Müller et al., 2018).

Since enteric bacteria often carry ARGs on mobile genetic elements (MGEs) such as plasmids, integrons, and transposons, their leaked DNA could possibly serve as a potential transformation template. It has been reported that the efficiency of bacterial uptake of free-floating exDNA is slightly lower compared to adsorbed exDNA, which is also more likely to be prevented from degradation by extracellular nucleases though adsorption onto colloids, humic substances, clay minerals and sediments (Torti et al., 2015). Deng et al., 2023 reported higher values for exARGs in river sediments compared to municipal wastewater with aminoglycoside resistance genes (*aadA*) being the most abundant ARG (1.27×10^6 to 7.23×10^8 GC/L). Nevertheless, study of Zhang et al. (2018) reported a high persistence and a low decay rate of exDNA-associated ARGs in wastewater treated by different processes (i.e., conventional biological treatment, ultrafiltration, ozonation, and chlorination) suggesting an important role of exDNA-based transformation in aquatic environments.

In our study, abundances of genes encoding carbapenemases in the exDNA-fraction of clinical wastewaters were similar or even higher compared to the iDNA-fraction. Oliveira et al. (2020), who conducted one of the few studies in the field of exDNA-associated carbapenemases in Europe, reported $bla_{OXA\text{-}48}$, $bla_{VIM,}$ and bla_{KPC} in municipal wastewater at concentrations ranging from 2.6 x 10^2 to 1.2 x 10^5 GC/mL bla_{VIM} was detected in the discharged and reused effluents, at concentrations of 5.3 x 10^3 GC/mL, and 3.2 x 10^3 GC/mL, respectively. Zhang et al. (2023) reported high values for $bla_{GES\text{-}5}$ of 1.05 x 10^7 GC/L in exDNA-fractions from hospital wastewater. This is of particular concern, since ARGs from exDNA fraction can lead to the dissemination of AMR in the environment through natural transformation (Dodd, 2012), which is one of the functionally important mechanisms in (aquatic) environment due to the high phylogenetic divergence of bacteria. Furthermore, during transformation no cell-to-cell contact is needed, which can be crucial under turbulent flow conditions in wastewater. Such an essential mechanism of horizontal gene transfer as transformation is characterized by the uptake of free DNA by a recipient bacterium, its possible integration into chromosome, and expression that potentially leads to a new phenotype. It does not necessarily result in a public health threat, since this mechanism is primarily responsible for bacterial adaptation and diversity generation. However, if exDNA contains ARGs and MGEs, its release into the aquatic environment can be one of the significant drivers of formation of pathogenic bacteria resistant to one or multiple antibiotics contributing to the sources of antibiotic resistance both in environmental and pathogenic bacteria (Blokesch, 2016).

Phyla detected in this study by 16S rRNA gene profiling of the exDNA-fractions, have already been reported worldwide in different types of water such as surface water, drinking water, urban domestic wastewater, industrial and animal wastewaters (Vaz-Moreira et al., 2014). The major orders identified could be divided into enteric bacteria (e.g., *Campylobacter*, *Clostridium*, *Escherichia-Shigella*, *Yersinia*), waterborne and water-transmitted bacteria (e.g., *Legionella*, *Leptospira*, *Mycobacterium*), and environmental bacteria (e.g., *Acinetobacter*, *Aeromonas*, *Pseudomonas*) which include important nosocomial pathogens able to acquire multi-drug resistance. Species previously identified as common members in activated sludge and suggested of being involved in nutrient removal (e.g., nitrite oxidation by *Nitrospira*) have also been detected (Numberger et al., 2019).

Interestingly, the highest proportions of *Acinetobacter*, *Aeromonas* and *Pseudomonas* were detected in clinical wastewaters, where the abundance of exDNA-associated genes encoding carbapenemases (i.e., $bla_{VIM\text{-}2}$, $bla_{NDM\text{-}1}$, $bla_{KPC\text{-}3}$ and $bla_{OXA\text{-}48}$) was highest. This is consistent with ECDC data on carbapenem resistance in invasive isolates. In 2020 in EU/EEA, the highest population-weighted mean percentages of resistance to carbapenems (IMP/MEM) were observed among isolates of *Acinetobacter* spp. (38.0%) and *P. aeruginosa* (17.8%), whereas in Germany *P. aeruginosa* exhibited the highest resistance rate (13.8%), followed by *Acinetobacter* spp. (3.5%) (European Centre for Disease Prevention and Control, 2019). This is of particular importance, since carbapenem-resistant *P. aeruginosa* and *A. baumannii* are prone to cause outbreaks in healthcare facilities, compromising the effectiveness of life saving medical interventions such as intensive care, cancer treatment and organ transplantation. *Acinetobacter* is likely common member of WWTPs worldwide and is well adapted to environmental stressors (Numberger et al., 2019). Considering this and the fact that bacteria from the genus *Acinetobacter* are naturally competent, they could serve as "environmental backups" for clinically relevant ARGs resulting in active dissemination of resistances and thereby aggravate the environmental resistome.

Based on our results, it can be assumed that especially in clinical wastewater or municipal wastewater influenced by hospitals transformation of exDNA-associated ARGs could play an important role in dissemination of genes encoding carbapenemases. To date, about 90 bacterial species are known to be naturally competent including waterborne, and human pathogens (e.g., *Acinetobacter* spp., *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Vibrio cholera, Haemophilus influenza*, *Helicobacter pylori*) (Blokesch, 2016; Johnston et al., 2014). *In vitro* experiments have already demonstrated the ability of these species to transform ARGs conferring resistances to different antimicrobial classes including macrolides, ansamycins and quinolones (Johnston et al., 2014), nevertheless data on species being permissive for exDNA-associated plasmids encoding carbapenemases under conditions prevailing in different (waste)waters lack, once again reinforcing the need for more research.

However, not only the environmental conditions (e.g., nutrient restrictions, presence of antimicrobials, temperature fluctuations) but also the genetic traits of exDNA (plasmid or linear DNA, fragmentation and damage grade, methylation profile) can influence microbial competence and uptake of exDNA (Dong et al., 2019; Johnston et al., 2014; Torti et al., 2015). Stress conditions such as nutrient limitations or presence of antimicrobials, which are commonly found in surface waters, are known to induce natural transformation (Ikuma and Rehmann, 2020). Voigt et al. (2020) reported the presence of antimicrobials, which are moderately persistent in water (i.e., ciprofloxacin, sulfamethoxazole and clarithromycin), in German surface waters at concentrations exceeding their environmental predicted no-effect concentrations (PNECs). They can potentially contribute to the formation and persistence of carbapenemase-producing bacteria in aquatic environment, since (fluoro)

quinolone resistance genes of type *qnr*A, *qnr*B, *qnr*S as well as *sul1*, *ermB* are frequent co-localized on plasmids. Thus, exposure to only one of these antimicrobials could potentially lead to their simultaneous uptake.

5. Conclusions

In this study, it was clearly demonstrated that wastewater, especially of clinical origin, is a relevant source of ARGs associated with free-floating exDNA. Conventional treatment of wastewater was found to be inefficient in reduction of exDNA-concentrations and exDNA-associated ARGs compared to ozonation and ultrafiltration. In order to reduce the input of free-floating exDNA and exDNA-associated ARGs into the municipal wastewater system and subsequently into the aquatic environment, multiple-barrier approaches including on-site pre-treatment of clinical wastewaters and advanced treatment technologies in municipal WWTPs should be implemented. This would lower the risk of dissemination of clinically-relevant ARGs (e.g., genes encoding carbapenemases) through natural transformation, especially in wastewater where the prevailing conditions could promote the uptake of exDNA-associated resistance determinants by environmentally-adapted microbial communities. However, further studies on the conditions promoting natural transformation in wastewater and elucidating the actual transformation rate for exDNA-associated ARGs in complex biological systems are needed. Systematic isolation and molecular profiling of free-floating exDNA should complement the One Health surveillance approach in order to ensure the health of urban and natural water systems and to prevent the possible dissemination of antibiotic resistant bacteria and genes back to the human populations.

Authors' individual contributions

Mykhailo Savin: Conceptualization, Methodology, Investigation, Writing – Original Draft. Jens Andre Hammerl: Investigation, Writing – Review&Editing. Julia Hassa: Investigation; Norman Hembach: Investigation; Jörn Kalinowski: Investigation; Thomas Schwartz: Investigation, Writing – Review&Editing; Felix Droop: Visualization, Writing – Review&Editing; Nico T. Mutters: Conceptualization, Writing – Review&Editing. All authors have approved the final article.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mykhailo Savin reports financial support and article publishing charges were provided by University Hospital Bonn Institute of Hygiene and Public Health.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2023.122560.

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