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Free-floating extracellular DNA: Systematic profiling of mobile genetic elements and antibiotic resistance from wastewater

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ABSTRACT

The free-floating extracellular DNA (exDNA) fraction of microbial ecosystems harbors antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). Natural transformation of these xenogenetic elements can generate microbial cells resistant to one or more antibiotics. Isolating and obtaining a high yield of exDNA is challenging due to its low concentration in wastewater environments. Profiling exDNA is crucial to unravel the ecology of free-floating ARGs and MGEs and their contribution to horizontal genetransfer. We developed a method using chromatography to isolate and enrich exDNA without causing cell lysis from complex wastewater matrices like influent (9 μg exDNA out of 1 L), activated sludge (5.6 μg out of 1 L), and treated effluent (4.3 μg out of 1 L). ARGs and MGEs were metagenomically profiled for both the exDNA and intracellular DNA (iDNA) of activated sludge, and quantified by qPCR in effluent water. qPCR revealed that ARGs and MGEs are more abundant in the iDNA fraction while still significant on exDNA (100–1000 gene copies mL^{-1}) in effluent water. The metagenome highlighted that exDNA is mainly composed of MGEs (65%). According to their relatively low abundance in the resistome of exDNA, ARGs uptake by natural transformation is likely not the main transfer mechanism. Although ARGs are not highly abundant in exDNA, the prevalence of MGEs in the exDNA fraction can indirectly promote antibiotic resistance development. The combination of this method with functional metagenomics can help to elucidate the transfer and development of resistances in microbial communities. A systematic profiling of the different DNA fractions will foster microbial risk assessments across water systems, supporting water authorities to delineate measures to safeguard environmental and public health.

1. Introduction

Xenogenetic pollution has become a global threat to environmental and public health (Gillings et al., 2018). Risk evaluation due to transmission and proliferation of pathogenic agents, antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), and materials from genetically-modified organisms (GMOs) via waterways and biological systems like wastewater environments are under active examination (Berendonk et al., 2015; Bürgmann et al., 2018; Pruden et al., 2013). While ARGs and MGEs are now widely targeted across investigations of sewage, their survey has primarily been made based on the molecular analysis of total DNA extracts from the microbial communities of wastewater. However, besides the intracellular DNA (iDNA), the extracellular DNA (exDNA) that is free-floating in the bulk liquid water phase may carry a sub-

List of abbreviations and acronyms: ARG, Antibiotic Resistance Gene; AICE, Actinomycete integrative conjugative element; CTAB, Cetyl trimethyl ammonium bromide; DEAE, Diethylaminoethyl cellulose; DMSO, Di-methyl sulfoxide; EPS, Extracellular polymeric substances; eDNA, Environmental DNA; exDNA, Free-floating extracellular DNA; GMO, Genetically modified organsim; HGT, Horizontal gene transfer; ICE, Integrative conjugative element; iDNA, Intracellular DNA; IS, Insertion sequence; MG-RAST, Metagenome Rapid Annotation using Subsystem Technology; MGE, Mobile genetic element; MLS, macrolide-lincosamide-streptogramin B; OTU, Operational taxonomic units; PCI, Phenol:chloroform:isoamyl alcohol; PI, Propidium iodide; qPCR, Quantitative PCR; TSS, Total suspended solids; WWTP, wastewater treatment plant.

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stantial amount of ARGs and MGEs of environmental and public health concern. The exDNA fraction has not been studied in detail, even if there are some exceptions in sludge, cattle manure and swine waste samples (Zhang et al., 2013; Zhou et al., 2019). Differential isolation and systematic profiling of iDNA and free-floating exDNA is necessary to elucidate the mechanisms of transfer of these mobile DNA contaminants that replicate. exDNA retrieved from environmental samples can be defined as the DNA fraction that is not housed within the envelope of the cell membrane (Torti et al., 2015). The acronym "eDNA" is widely intermixed to describe either environmental DNA or extracellular DNA. Environmental DNA does not necessarily discriminate between extracellular and intracellular fractions of nucleic acids. It only means that DNA extracted originates from cells that were present in the investigated ecosystem at the sampling event (Thomsen and Willerslev, 2015). We define free-floating exDNA as "all the DNA components that are neither enclosed inside cells nor adsorbed or aggregated in complex matrices and that are persistently floating in aqueous samples". This exDNA fraction can represent a genetic proxy of microbial and cellular diversity among different biogeographical areas (Corinaldesi et al., 2018; Torti et al., 2015). Isolating and analyzing exDNA from environmental samples provides insight into the dynamics, interactions, and evolutionary history of populations of microorganisms and higher organisms that are or have been present in the investigated environment. Moreover, exDNA is abundant and plays an important role as a structural component of microcolonies, stabilizing microbial bioaggregates (Dominiak et al., 2011; Rusanowska et al., 2019; Weissbrodt et al., 2013), being a key component within the matrix of extracellular polymeric substances (EPS) (Dominiak et al., 2011; Tang et al., 2013; Tetz and Tetz, 2010; Wu and Xi, 2010). However, biofilm and flocs have also been suggested as reservoirs for ARG occurrence and dissemination (Guo et al., 2018; He et al., 2019). Active or passive release out of cells are the main sources of exDNA (Nagler et al., 2018a; Torti et al., 2015). Whether exDNA is actively or passively released in these biological environments remains unsolved, although some authors provide claims about one or the other hypotheses (Flemming and Wingender, 2010; Merod and Wuertz, 2014). Until mechanistic measurements will be made available, strong debate will remain on the 'intention' of cells to release DNA to drive biofilm formation. In this study, exDNA immobilized in the EPS matrix is considered to be likely less available to gene transfer in comparison with free-floating exDNA (Karygianni et al., 2020).

The persistence of exDNA in biofilms and of free-floating exDNA (that can also sorb to surfaces) may generate hotspots for horizontal gene transfer (HGT) in microbial biocoenoses. Natural competence is a widely distributed cellular mechanism harboured by microorganisms in nature to take up molecular resources from their surroundings. Naturally competent microbes may take up free or bound exDNA (Nagler et al., 2018a). Natural transformation is a parasexual mechanism for the exchange of genetic material induced by stress conditions such as nutrient limitations or the presence of antibiotics (Claverys et al., 2009). Such conditions are found in densely populated cultures such as activated sludge samples. Natural transformation in managed environments does not implicitly result in a threat for human health since it is a mechanism generating diversity and adaptation (Jørgensen et al., 2014).

However, if ARGs, MGEs, and pathogenic islands can be found in exDNA fractions, the formation of pathogenic bacterial cells resistant to one or more antibiotics – also known as superbugs – could be induced (Domingues et al., 2012). DNA fragments that have been released by sterilization of microbial cultures using, *e.g.*, industrial and research facility methods does not lose its integrity and capacity to be re-amplified (Calderón-Franco et al., 2020a), thus making it an undesired source of xenogenetic pollution. The molecular mechanisms generating superbugs and the underlying sources of genetic materials that can prompt it need to be studied. Methods to efficiently isolate, quantify, and metagenomically characterize exDNA templates from complex biological samples are required.

In the last years, a few methods to extract exDNA have been published. Wang et al. (2016) have used an aluminum hydroxide column to adsorb aquatic exDNA. Nagler et al. (2018) have opted for sequential steps involving enzymatic treatments to extract exDNA from cattle rumen samples. Yuan et al. (2019) have integrated magnetic beads in combination with the classic DNA precipitation method involving the surfactant cetyl trimethyl ammonium bromide (CTAB), yielding a relatively good amount of exDNA of 0.2 µg out of 5 mL of wastewater. Silica solid phases similar to commercial silica resin columns have been tested to adsorb and extract exDNA from low-concentration clinical samples (Katevatis et al., 2017). Cell lysis has seldom been investigated (although often debated) across protocols for exDNA extractions. Assessing and preventing cell lysis during exDNA isolations is crucial to obtain confident analytical results from exDNA templates. Moreover, volumes previously used ($<$ 100 mL) have hindered a more detailed exDNA characterization by metagenomics due to low isolation yields. Metagenomics can be used for deciphering the distribution, mobility and microbial hosts of ARGs in both DNA fractions from environmental samples such as sludge (Zhou et al., 2019).

A mathematical model that accounts for ARGs sitting on both iDNA and exDNA fractions has been developed to predict the fate and transport of ARGs in receiving waters downstream of WWTPs (Ikuma and Rehmann, 2020). An improved quantitative understanding of these processes and of the impact of exDNA on ARG spread and occurrence requires more effective methods to extract exDNA. It will enable a more accurate risk assessment toward more targeted mitigation of antibiotic resistance.

Here, we systematically profiled xenogenetic elements from free-floating exDNA out of the complex biological environment of wastewater. We highlight for the first time the potential xenogenetic risk associated with free-floating exDNA fragments transported with sewage across urban water systems. We provide an efficient analytical method to investigate it at high resolution.

2. Material and methods

2.1. Sampling from the influent, activated sludge tank, and effluent of a wastewater treatment plant

Biological samples were collected from influent wastewater, activated sludge, and effluent water of the urban wastewater treatment plant (WWTP) Harnaschpolder (Waterboard Delfland, The Netherlands) operated for full biological nutrient removal. Grab sampling was used totest the exDNA isolation method on the different wastewater matrices, and to metagenomically characterize and molecularly quantify what ARGs and MGEs sit on which DNA fractions in these matrices, rather than performing a "mass balance" *per se*.

Influent wastewater was collected after primary treatment. Three biological replicates were collected on three different days as grab samples. A total of 1000 mL of influent wastewater was collected per replicate. All samples were processed in a timeframe of less than 2 h prior to DNA extraction.

Six biological replicates of activated sludge were collected in two different sampling campaigns as grab samples from the activated sludge tank. Each campaign consisted of three successive dry days, *i.e.,* without recent rainfall and variations in the hydraulic retention time. The first round of eDNA isolation experiments was performed with three biological replicates of activated sludge.

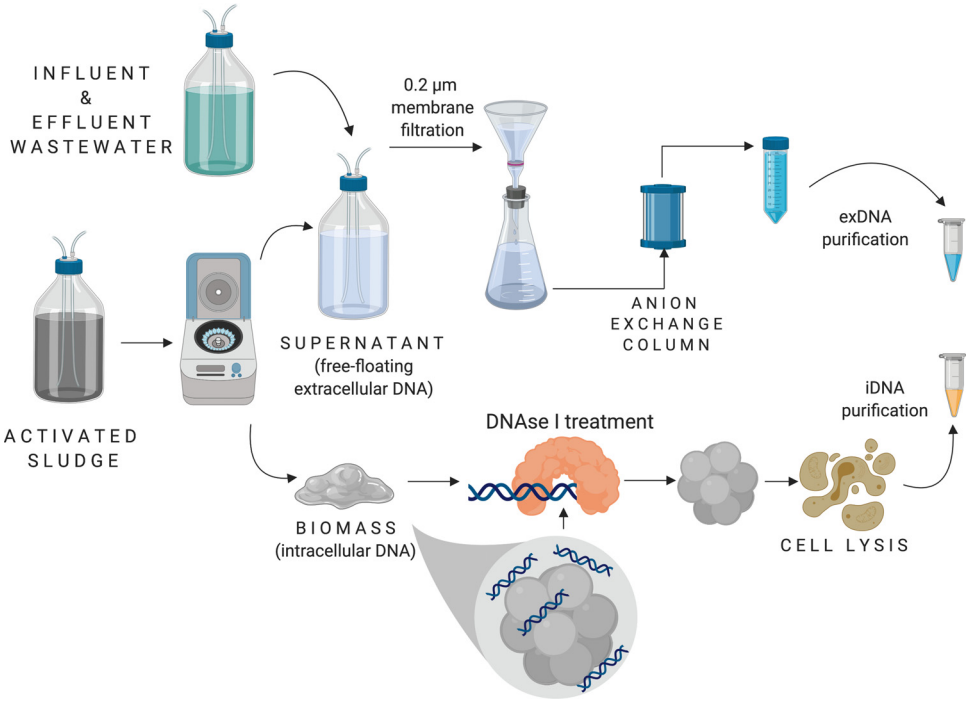

Fig. 1. Schematic representation of the free-floating exDNA and iDNA isolation method. Picture created with BioRender.

Once the method was successful, we conducteda second campaign-for which additional three samples were taken from three different days from the three different biological matrices: influent, activated sludge and effluent. All raw activated sludge samples were stored at 4 °C in a timeframe of less than 2 h prior to isolations of free-floating exDNA and extractions of iDNA. A total volume of 1000 mL of activated sludge was collected per replicate.

Effluent water was collected at the outlet of the tertiary treatment. Three biological replicates were collected in three different days as grab samples. A total of 1000 mL of treated water per replicate was collected. All samples were processed in a timeframe of less than 2 h prior to DNA extraction.

2.2. Isolation of free-floating extracellular DNA from influent, activated sludge and effluent water samples

The workflow used to isolate free-floating exDNA from influent, activated sludge, and effluent samples is sketched in Fig. 1.

Activated sludge was centrifuged at 6000 x g at 4 °C for 10 min for removing biomass in order to make the next filtration step easier. Pellet was stored at 4 °C for intracellular DNA extraction (Section 2.3.). The supernatant was used for free-floating DNA isolation.

Influent, activated sludge supernatant, and effluent samples were sequentially filtered through 0.45 and 0.2 µm 47 mm PES membrane filter (Pall Corporation, USA). The membrane filters holding the biomass were stored at 4 °C for further intracellular DNA extraction (Section 2.3.). The filtered samples (1000 mL influent, activated sludge supernatant and influent) were loaded on a positively charged 1-mL diethylaminoethyl cellulose (DEAE) chromatographic column (BIA Separations, Slovenia) using an LC-8A preparative liquid chromatography pump (Shimadzu Corporation, Japan) and 0.76 × 1.6 mm tubing (Thermo Fisher Scientific, USA). This anion-exchange column was preliminarily equilibrated at a flowrate of equilibration buffer of 0.6 mL min^{-1} while maintaining the pressure below the maximum limit of 1.8 MPa. Because of very high porosity, reusability, and flow characteristics (up to 16 mL min^{-1}), this monolithic chromatographic column is an efficient tool to separate or purify large biomolecules such as genomic and viral DNAs (Krajacic et al., 2017). Column preparation and processing were performed according to the manufacturer's instructions.

Buffers and solutions were used to equilibrate, elute, regenerate, clean, and store the column. The equilibration buffer consisted of a mixture at pH 7.2 of 50 mmol L^{-1} Tris and 10 mmol L^{-1} EDTA. The elution buffer was a mixture at pH 7.2 of 50 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA, 1.5 mol L^{-1} NaCl. The regeneration buffer was a mixture at pH 7.2 of 50 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA, 2 mol L^{-1} NaCl. The cleaning solution comprised 1 mol L^{-1} NaOH and 2 mol L^{-1} NaCl. The storage solution consisted of 20% ethanol in ultrapure water (Sigma Aldrich, USA).

The elution of exDNA was tracked over time using an HPLC photodiode array detector (Waters Corporation, USA) recording the UV–VIS absorbance at the absorbance wavelength characteristic of DNA (260 nm).

The eluted exDNA fraction was further treated sequentially with absolute ethanol and a solution of 70% ethanol in ultrapure water (Sigma Aldrich, USA) (Moore and Dowhan, 2002) to precipitate the raw exDNA. The precipitated raw exDNA was incubated with proteinase K (Sigma-Aldrich, UK) at 0.85 g L^{-1} during 2 h in order to digest remaining co-extracted proteins (*e.g.*, DNA-bound proteins). The enzymatic reaction was stopped in a heat block at 50 °C for 10 min. The precipitated and protein-digested raw exDNA extract was finally purified using a GeneJET NGS Cleanup Kit (Thermo Scientific, USA). The purified exDNA isolates were stored at −20 °C pending molecular analysis.

2.3. Extraction of intracellular DNA from biomass separated from activated sludge and effluent water

iDNA was extracted from cells present in activated sludge and effluent water for analytical comparison of xenogenetic elements with exDNA. Biomass was obtained as a pellet by centrifugation (6000 x g at 4 °C for 10 min) of activated sludge and filtration

(0.45 and 0.2 μm 47 mm PES membrane filter (Pall Corporation, USA)) of effluent water.

An amount of 0.25 g wet weight of activated sludge (3.6 g TSS L^{-1}) was incubated during 1 h with 300 U mL^{-1} of DNase I in order to remove the residual exDNA from the biological sample, before extraction of the iDNA using a NucleoSpin® Soil kit (Macherey-Nagel) according to manufacturer's instructions.

The biomass from the effluent water was obtained by filtering a volume of 500 mL of effluent through a 0.22 μm PVDF membrane (Pall corporation, USA). Filters were frozen at −20 °C until extraction. The iDNA present on filters was extracted with the Power Water DNeasy kit (Qiagen, NL) following the manufacturer's instructions.

The quality and quantity of the DNA extracts were measured by NanoDrop spectrophotometry (ND-1000, USA) and Qubit® dsDNA assays (Thermo Fisher Scientific, USA), respectively.

2.4. Plasmid DNA used as chromatography selectivity control

A bacillus expression pHT01 plasmid (MoBiTec GmbH, Germany) was used as nucleic acid selectivity control for the DEAE anion-exchange column.

2.5. Biomass pre-treatment, live-dead staining, and flow cytometry analysis

Flow cytometry combined with live-dead staining was used to check for the prevention of cell lysis across the exDNA isolation workflow. Cells from the biological samples were disaggregated as a prerequisite for the migration of individual cells in the flow cytometer. Activated sludge samples were diluted at 1:500 in 1x PBS buffer. The diluted sludge samples were mild-sonicated (Branson Sonifier 250, USA) on ice in 3 cycles of 45 s at 40 W. After sonication, samples were diluted at 1:500 in 1x PBS buffer. Right after the second dilution, samples were filtered through a 10 μm syringe filter in order to remove possible residual cell debris and membranes.

The live-dead staining protocol and flow cytometry analyses were based on previous works (Boulos et al., 1999; Hammes et al., 2008; Pinel et al., 2020; Prest et al., 2013). Two staining dyes were used to track viable cells with SYBR green (Invitrogen, USA) and dead cells with red-fluorescent propidium iodide (PI) (Invitrogen, USA). Two working solutions were prepared from 10′000 × SYBR Green and 30 mmol L^{-1} PI dissolved in di-methyl sulfoxide (DMSO) stock solutions.

To obtain the total stained cell count, 10 μl of 10,000 × SYBR Green stock solution was diluted in Tris–HCl (pH 8), obtaining a final 100 × SYBR Green working solution. To obtain the viable cell count, a final working solution of 100 × SYBR Green and 6 μmol L^{-1} PI was used. Working solutions were thoroughly mixed by homogenization using a vortex and stored at −20 °C in the dark pending analysis.

Volumes of 5 μL of each of the staining working solutions were added to 495 μL of pre-treated biomass samples. After stains addition, samples were incubated at 37 °C for 10 min. After incubation, samples were kept in the dark until flow cytometry measurement.

Flow cytometry measurements were performed in a BD Accuri C6 flow cytometer (BD Biosciences, Belgium) equipped with a 50 mW laser tuned at an excitation wavelength of 488 nm. Fluorescence intensity was collected at emission wavelengths of the green (FL1 = 533 ± 30 nm) and red (FL3 > 670 nm) fluorescence detection channels. Measurements were performed at a flow-rate of 66 mL min^{-1} on 50 μL sample volume with a threshold value of 700 on FL1 to reduce the background detection noise.

2.6. Gel electrophoresis on the free-floating extracellular DNA

To analyze the different lengths of exDNA templates, the exDNA samples were analyzed by gel electrophoresis with agarose at 1% (w/v) (Sigma-Aldrich, Haverhill, United Kingdom) in 1xTAE buffer.

2.7. Quantitative polymerase chain reaction (qPCR) analysis of selected ARGs and MGEs on exDNA and iDNA from effluent water samples

A panel of genes was selected for qPCR analysis on exDNA and iDNA fractions extracted from effluent water samples. The 16S rRNA gene was selected as a proxy to quantify total bacteria. ARGs and MGEs were selected from a panel used for wastewater samples (Berendonk et al., 2015; Pallares-Vega et al., 2019). Standards for qPCR were generated from ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), a curated database of ARGs. The chosen ARGs targeted: macrolides (multidrug export protein gene *ermB*), sulfonamides (sulfonamide resistance genes *sul1* and *sul2*), fluoroquinolones (quinolone resistance gene *qnrS*) and extended-spectrum β-lactamase (cefotaxime-hydrolyzing β-lactamase bla_{CTXM}) **(Table S1)**. The class I integron-integrase gene *intI1*, known to be responsible for genes mobility (Ma et al., 2017), was included to assess the presence of MGEs. Standards, primers, and reaction conditions are listed in **tables S2** and **S3** in supplementary material.

2.8. Metagenomic profiling of exDNA and iDNA fractions from activated sludge samples

Metagenomics libraries of exDNA and iDNA samples at 50 ng μL^{-1} were sequenced using a MiSeq PE300 benchtop sequencer (Illumina, USA). Library preparation was done with a TruSeq DNA PCR-Free kit (LT Lib PREP KT-ST B PhiX control v3). Datasets of 14 million reads were obtained per sample corresponding to a sequencing depth of 6.5 GB for iDNA and 6.8 GB for exDNA fractions. Raw metagenomic data have been deposited in BioProject under accession number PRJNA632452 and ID: 632452. Activated sludge was selected for metagenomics for being a highly diverse sample, from which microbiome, resistome and mobilome differences could be assessed in order to check the applicability of the extraction method.

The quality of the acquired Illumina reads was assessed by FastQC version 0.11.9 with default parameters (Andrews, 2010). Paired-ends reads were trimmed and filtered by Trimmomatic version 0.39 with default parameters (Bolger et al., 2014). Alignments were performed by BWA-mem version 2 with default parameters (Li, 2013), generating a SAM file. To filter soft and hard clipped reads, SAM files were filtered by Samclip tool with default parameters, removing undesirable alignments that could generate downstream problems (https://github.com/tseemann/samclip). Centrifuge (Kim et al., 2016) was applied for taxonomic microbial classification (http://ccb.jhu.edu/software/centrifuge). The MEGARes 2.0 database was used to identify ARGs (http://megares.meglab.org). The metagenomes were searched for signatures of known MGEs, plasmids, prophages, and viruses through ACLAME (http://aclame.ulb.ac.be) (Leplae et al., 2009). The mobilome was searched for integrative conjugative elements through the ICEberg database (http://db-mml.sjtu.edu.cn/ICEberg/) (Liu et al., 2019), insertion sequences through ISfinder (https://isfinder.biotoul.fr) (Siguier et al., 2006), and integrons through the INTEGRALL database (https://integrall.bio.ua.pt) (Moura et al., 2009). Different databases of ARGs and MGEs of different completeness and curation levels are available in public repositories such as the aforementioned, while no consensus database is available yet. Follow-

up research will lead to a comprehensive database for wastewater environments (http://repares.vscht.cz/).

Both MEGARes 2.0 and ACLAME were used as references for the alignment of trimmed and filtered metagenomic reads. The output SAM file from the BWA-mem alignment was converted into a BAM file using SAMtools version v1.4 (Li et al., 2009) (https://github.com/samtools/samtools). Unmapped reads were removed and secondary alignments ignored in order to get a list of the best hits for further data processing. ARGs and MGEs hits were considered when 2 or more reads per variant were aligned.

2.9. Comparative subsystems analysis of the metagenomic data

Unassembled clean reads from the exDNA and iDNA fractions of activated sludge were annotated using the open-access metagenome curation and analysis platform Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) (http://metagenomics.nmpdr.org/) (Meyer et al., 2008). The subsystem classification MG-RAST ID are available for the free-floating extracellular DNA (mgm4886400.3) and the intracellular DNA (mgm4886611.3).

2.10. Statistics

Statistical analyses were performed on all molecular datasets with R 3.5.1 (R Foundation for Statistical Computing., 2018) and RStudio (https://www.rstudio.com/). For the analysis of significance on the purification effect on the yield of DNA isolation, a non-parametric Wilcoxon test was performed. For both exDNA and iDNA samples, biological replicates were used from influent water (3 replicates), activated sludge (6 replicates) and effluent water (3 replicates). A parametric two-tailed Student's *t*-test with statistical significance established at the 95% confidence level ($p<0.05$) was performed to analyze the significance of the differences in ARG and MGE compositions between exDNA and iDNA fractions. For both exDNA and iDNA samples, 3 biological replicates with 3 technical replicates were used from effluent water.

3. Results and discussion

3.1. High yields of free-floating exDNA were obtained by anion-exchange chromatography

The column performed with high nucleic acid selectivity for the retention, separation, and spectrophotometric detection ($\lambda = 260$ nm) with known concentration of pure pHT01 plasmid of *Bacillus subtilis* as control (**Figure S1a**). A sharp and well-resolved narrow peak was obtained in the chromatographic elution and detection of the plasmid control. The raw non-precipitated exDNA obtained from filtered activated sludge started to elute after 10 min for 35 min (**Figure S1b**). The chromatographic peak maximum was detected at a retention time of 18 min. The concentrated raw free-floating exDNA extract displayed a hydrogel aspect (Fig. 2). Its color related to the source of the exDNA extract, namely dark brown with influent water, yellow with activated sludge, or colorless with effluent water. The explanation could relate to either polyphenols or humic acids. Polyphenols are soluble in water and change to a red coloration when in contact with air (Hussain et al., 2015). Humic acids are abundant in wastewater and can interact with DNA (Saeki et al., 2011).

The concentration of humic acids decreases during wastewater treatment (Nemerow and Doby, 1956). For instance, humic acids adsorb onto activated sludge (Feng et al., 2008). They are less abundant in absolute concentration in effluent water (Ma et al., 2001). Therefore, we hypothesize that the loss of color in the exDNA extract through the process is due to loss of humic acids

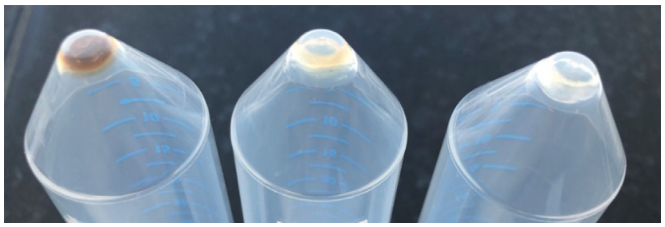

Fig. 2. Aspects of raw extracts of free-floating exDNA obtained from different wastewater environments. From left to right: (i) filtered influent wastewater (brown free-floating exDNA extract due to high presence of humic acids), (ii) centrifuged and filtered activated sludge supernatant (yellow extract), and (iii) filtered effluent water (colorless extract).

that are co-extracted with the exDNA. The diethylaminoethyl cellulose (DEAE) column efficiently isolated and concentrated the free-floating exDNA fraction from large volumes of influent wastewater, activated sludge, and effluent water of the WWTP.

The yields (*i.e.*, mass of DNA isolated in molecular biology after the isolation and purification procedures) of raw and purified (with a commercial DNA purification kit after Proteinase K treatment) free-floating exDNA isolated from influent wastewater, activated sludge, and effluent water are shown in **Figure S2**. Yields and concentrations are summarized in Table 1. The exDNA fraction corresponded to <1% of the total DNA extracted from activated sludge. Only exDNA obtained from activated sludge samples showed significant differences in the yields between raw and purified exDNA ($p<0.05$). There was no significant difference ($p>0.05$) between in yields of raw exDNA obtained from different water quality samples. However, the influent wastewater displayed a significantly ($p<0.05$) higher fraction of purified free-floating exDNA (9′000 ng out of 1000 mL initial sample volume) than activated sludge (5′631 ng out of 1000 mL initial sample volume) and effluent water (4′276 ng out of 1000 mL initial sample volume).

The raw exDNA extracted from influent wastewater and activated sludge supernatant samples yielded higher concentrations (12.5 ± 1.9 ng mL^{-1} and 12.3 ± 1 ng mL^{-1}, respectively) than after purification (9.0 ± 0.7 ng mL^{-1} and 5.6 ± 0.46 ng mL^{-1}), respectively. An average mass loss of 42% through the commercial purification kit was measured. In the method, one commercial kit was used for the purification of the exDNA extracts after digestion with proteinase K. For future works, other commercial kits or classical purification methods might also be assessed for the isolation of free-floating exDNA from low-content water samples.

It is paramount to note that the exDNA yields were substantial for the three biological matrices. The free-floating exDNA extracts fulfilled the minimal quantitative and qualitative requirements (mass $\geq$ 1 µg, concentration $\geq$ 20 ng µL^{-1}, volume $\geq$ 20 µL) required for qPCR and high-throughput metagenomics analyses. Much higher yields were obtained than with other isolation methods tested in parallel in this study or reported in literature: *ca.* 3-fold higher than with classical CTAB and PCI methods, and up to as high as *ca.* 2000-fold higher than direct precipitation with isopropanol (Table 2). Our method and the one of Yuan et al. (Yuan et al., 2019) provide the necessary yield of exDNA recovered from wastewater samples for subsequent molecular analyses.

3.2. Mild enzymatic post-treatment with proteinase K is necessary to release exDNA from bound extracellular polymeric proteins found in activated sludge

The free-floating exDNA extracted from activated sludge was characterized by an intense band at the top of the agarose gel. This suggested that exDNA was entrapped in a protein mesh that did not allow it to migrate through the gel (Fig. 3a). After mild

Table 1
exDNA yield and concentrations for different water qualities before and after commercial kit purification. The sample volume corresponds to the volume of the initial water sample from which exDNA had been extracted.

Sample	Initial sample volume(mL)	exDNA extract	exDNA yield(μg)	exDNA concentration in initial sample(ng mL^{-1})
Influent wastewater [a]	1000	Raw	12.5	12.5
	1000	Purified	9.0	9.0
Activated sludge [b]	1000	Raw	12.3	12.3
	1000	Purified	5.6	5.6
Effluent water [c]	1000	Raw	8.6	8.6
	1000	Purified	4.3	4.3

[a] Filtered influent wastewater.
[b] Centrifuged and filtered activated sludge supernatant.
[c] Filtered effluent water.

Table 2
Concentrations and yields of free-floating extracellular DNA extracts obtained during this study in comparison to other methods. Legend: cetyl trimethyl ammonium bromide (CTAB), phenol:chloroform:isoamyl alcohol and ethanol (PCI).

Method	Reference	Initial sample volume (mL)	exDNA yield (ng)	exDNA concentration in initial sample(ng mL^{-1})
AEX column	This study	1′000	12′323	12.3
CTAB, PCI	This study	800	3′630	4.53
Magnetic beads	(Yuan et al., 2019)	5	234	78.0
Vivaspin columns 100 kDa	This study	500	136	0.27
Isopropanol precipitation	This study	50	6.5	0.13
DNA extraction kit	This study	50	ND	ND [a]

[a] ND means not detected.

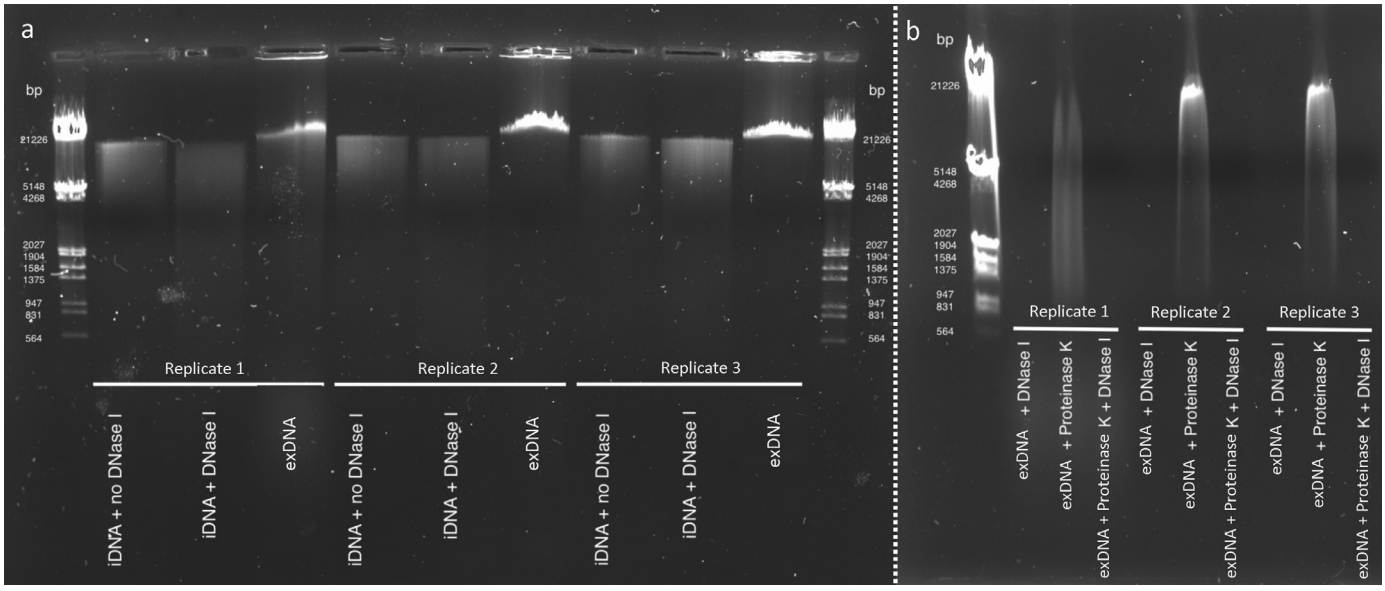

Fig. 3. The impact of enzymatic post-treatment of raw exDNA extracts with proteinase K to release the exDNA from extracellular protein mesh: (a) Agarose gel from three different biological replicates showing the fragment size distributions of intracellular DNA (iDNA) obtained without any pre-treatment, of iDNA obtained after enzymatic pre-treatment of cells with DNase I prior to cell lysis and DNA extraction, and of the raw extract of free-floating extracellular DNA (exDNA) obtained from centrifuged and filtered activated sludge supernatant samples. (b) Agarose gel of free-floating exDNA after either pre-treatment of cells with DNase I, post-treatment of exDNA extract with proteinase K, or a combination of both pre- and post-treatments. Values of DNA concentrations after DNAse I treatment are summarized in Table S4.

enzymatic treatment of these residual proteins bound to the exDNAs with proteinase K, it was observed that the purified exDNA templates were able to run through the gel (Fig. 3b). These were characterized by a distribution of fragments sizes that ranged from 0.5 kbp to >20 kbp. For comparison, the iDNA that was extracted in parallel using commercial kits exhibited DNA fragments that also ranged from less than 0.5 kbp to >20 kbp. Both DNA fractions displayed similar fragment size distribution.

Assuming that the average size of genes present in bacterial genomes is 1.5 kb long, exDNA was suggested to be large enough to contain multiple ARGs and MGEs (DeFlaun et al., 1987; Zhang et al., 2013). No significant differences in band intensity could be observed between untreated biomass and treated with DNase I prior to intracellular DNA extraction (Fig. 3b). This suggests a low exDNA content bound to biomass when DNA is extracted from 0.25 g of activated sludge. The extracted pools of free-floating exDNA fragments were suitable for further molecular analyses since purity (1.76 ± 0.02) was close to optimal the optimum absorbance ratio $A_{260\ nm}$ / $A_{280\ nm}$ of 1.8.

3.3. Cell lysis is not induced during the isolation of free-floating exDNA

Cell lysis measurements and control were conducted on the processing of activated sludge since exhibiting the highest microbial density among the wastewater environment samples. Thus, activated sludge was considered as more prone to potential cell lysis and cross-contamination of the free-floating exDNA extract with

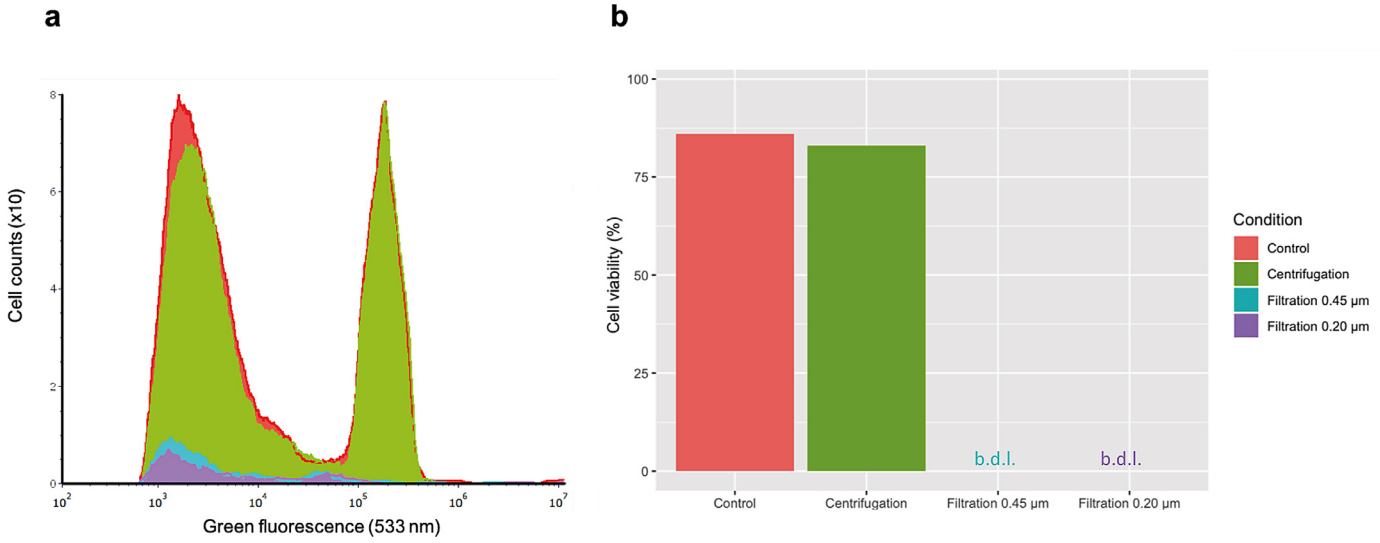

Fig. 4. **(a)** Direct comparison of the green fluorescence histograms of activated sludge supernatant water samples with the different protocol extraction steps. Cell counts correspond to the number of events in 50 µL. **(b)** Live/cell staining showing cell viability of samples after the different protocol extraction steps. Legend: **bdl:** below the detection limit (cell lower limit was achieved when both filtrations were applied). An electronic gate on the green (533 nm)/red (670 nm) fluorescence density plot was used to select the signals of cells. Dot-plots can be found in **Figure S3** in supplementary material.

iDNA residues. Flow cytometry was used to measure the total cells counts after each biomass processing step in the developed protocol (Fig. 4a) together with the relative abundances of live/dead cells measured after stainings with SYBR Green and propidium iodide fluorophores, respectively (Fig. 4b). After filtration of centrifuged activated sludge supernatant through the 0.45 µm membrane filter, the number of flow cytometry events mL^{-1} was below 10; after second filtration on 0.2 µm, below 1 event. The diluted activated sludge control that consisted of a non-centrifuged and non-filtered activated sludge sample had an average of 1606 events mL^{-1}. Cells retained in the membrane filters were assumed to maintain their robustness and viability (Tanny et al., 1980; Wu and Xi, 2010).

Initial cell viability was preserved and bacterial cells were removed upfront by centrifugation and filtration. Depending on the type of biomasses targeted, even smaller filter pore sizes than 0.2 μm could be used.

Besides high yield, the free-floating exDNA template was therefore of high quality, being deprived of iDNA contamination. The isolation method did not induce cell lysis during the extraction: a cell-free and iDNA-free exDNA fraction was isolated. This quality control ensured that in the exDNA fraction there was no genomic DNA contamination caused by the extraction. Hence, we demonstrated the isolation of free-floating exDNA at high yield and high quality from complex microbial community matrices of activated sludge, across a workflow exempt of cell lysis.

3.4. Free-floating exDNA displays lesser ARG copies than iDNA

qPCR results highlighted that molecular analysis of five selected ARGs (*sul1, sul2, ermB, qnrS,* bla_{CTXM}) and one MGE (*intI1*) could be performed from both the iDNA and the free-floating exDNA fractions of wastewater environments, being effluent wastewater in this case (Fig. 5). All genes tested but bla_{CTXM} were detected in both DNA fractions. The free-floating exDNA template harbored a lower number of ARG and MGE copies than iDNA, with a significant difference (Δ) of 0.87 $\pm$ 0.32 $\log_{10}$ gene copies ($p<0.005$) across the gene panel.

At individual gene level, the free-floating exDNA displayed a significantly lower log-based gene copy number of both the bacterial *16S rRNA* gene (Δ = 1.2 $\pm$ 0.43 $\log_{10}$ gene copies) and the ARGs (Δ values given hereafter) *vs.* iDNA. Sulfonamides (Δ *sul1* = 0.94 $\pm$ 0.33; Δ *sul2* = 1.11 $\pm$ 0.32 $\log_{10}$ gene copies mL^{-1}) and β-lactamase (Δ bla_{CTXM} = 1.92 $\pm$ 0.25 $\log_{10}$ gene copies mL^{-1}) ARGs and the integrase type I (Δ *intI1* = 1.32 $\pm$ 0.38 $\log_{10}$ gene copies mL^{-1}) MGE were significantly lesser in the free-floating exDNA than in the iDNA. Conversely, the macrolides (Δ *ermB* = 0.27 $\pm$ 0.24 $\log_{10}$ gene copies) and fluoroquinolone (Δ *qnrS* <0.1 $\log_{10}$ gene copies) ARGs displayed similar copy numbers in both iDNA and exDNA.

Macrolide resistance genes (*ermB*) have been described to be embedded in transposon-like elements such as Tn551, Tn552, Tn4001, or Tn4003 conferring resistance to macrolide-lincosamide-streptogramin B (MLS) (Partridge et al., 2018). Fluoroquinolone resistance genes have been reported to be plasmid-borne since 1998 (Martínez-martínez et al., 1998). Most of the *qnr* genes have been detected in *Entereobacteriaceae*, with the *qnrS* gene prevalent both in environmental strains (Guillard et al., 2014) and in non-conjugative plasmids harbouring *mob* genes allowing their mobilization (Kehrenberg et al., 2007). These ARGs are commonly found in MGEs forming the principal subsystem component of the exDNA fraction (Fig. 6).

The genes encoding resistance to sulfonamides (*sul1* and *sul2*) were the most abundant ARGs in both iDNA and exDNA fractions from the selected panel. Sulfonamides account within the most systemically used antibiotics in hospitals in The Netherlands (National Institute for Public Health and the Environment, 2019). Such a high level of *sul* genes meets with other studies that have measured ARGs from total DNA extracted out of activated sludge biomasses and effluent water (Czekalski et al., 2012; Rocha et al., 2019). Lower number of ARGs copies have already been observed in the exDNA fraction when compared to the iDNA from sludge using a protocol for extracting exDNA from marine sediments (Corinaldesi et al., 2005; Zhang et al., 2013).

Here, we made a key contribution to enable the direct comparison of the molecular compositions of the free-floating exDNA and iDNA fractions of wastewater environments, as a relevant milestone within the problematic of ARGs and MGEs in environmental and public health protection. Lower copy numbers measured in the exDNA fraction can arise from different possible causes. It may be considered that DNA fragments of the genes are less released outside cells, thus interrogating whether *sul1/2* and bla_{CTXM} genes are

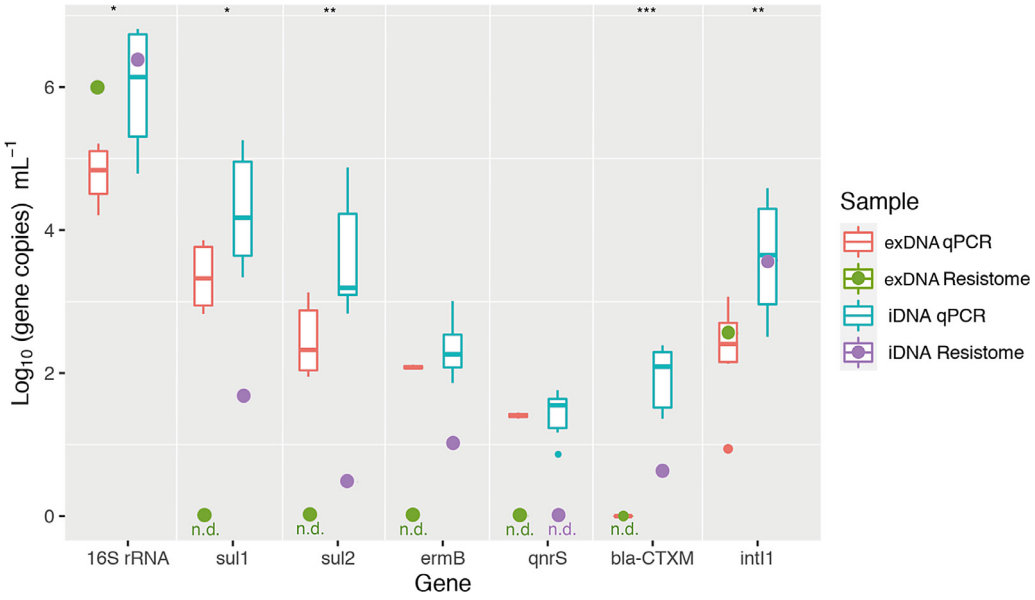

Fig. 5. Comparison of the qPCR analysis of the selected panel of ARGs and MGEs from the free-floating exDNA and the iDNA fractions of effluent water and of the number of primary aligned metagenomic reads for these genes present in the resistome of exDNA and iDNA of activated sludge. Values are displayed as Log_{10} gene copies mL^{-1} of the *16S rRNA* gene, five antibiotic resistance genes (*sul1, sul2, ermB, qnrS and* bla_{CTXM}), and one mobile genetic element (*intI1*). The significance levels of the differences between exDNA and iDNA levels are displayed as $p<0.05$ (*), $p<0.005$ (**), $p<0.0005$ (***). The n.d. labels in the resistome analyses indicate that the primary aligned reads were not detected by metagenomics.

less transferred on plasmids thus less mobile. It can also suggest that iDNA, when released because of cell decay or active release mechanisms, may be degraded by nucleases present in both DNA fractions. Another possibility is the degradation of DNA fragments that carried these genes by microorganisms, but DNA degradation by microbes is likely not specific at gene level. All in all, the reason for this difference between compositions of iDNA and free-floating exDNA fractions needs to be investigated in more detail. Our isolation method provides the analytical key to investigate it.

What it is highly remarkable is that ARGs can still be detected in significant amounts (100–1000 gene copies mL^{-1}) in the exDNA fraction that is fully exposed to the environment. Surface water is considered as an indicator for low ARG level and hospitals effluent water as high ARG level. An ARG level of less than 1000 gene copies mL^{-1} is considered low and an ARG level of more than 10^4 gene copies mL^{-1} is considered high (Le et al., 2016; Wu et al., 2020). However, all these indicatory ARG levels are based on analyses of intracellular DNA. Obtaining 100–1000 gene copies mL^{-1} in the free-floating extracellular DNA sample, where DNA is exposed to all kind of nucleases and environmental hazards, would be considered as high. A plausible explanation of why exDNA persists in aqueous environments is that it is clothed with a mesh of extracellular proteins (demonstrated here with the proteinase K post-treatments). Other causes of protection against degradation by nucleases can relate to exDNA being locked within organic and inorganic aggregates, adsorbed onto mineral matrices, or integrated into viral genomes (Torti et al., 2015).

The qPCR data obtained on the selected panel of ARGs and MGEs measured from effluent water are displayed in Fig. 5 together with the number of primary metagenomic reads retrieved for these genes from the resistome of free-floating exDNA isolated from activated sludge (see next section 3.5). Although the two types of analyses cannot be directly compared since made on effluent water and activated sludge, respectively, this comparison was performed to verify if there was a quantitative tendency between DNA fractions followed by both detection methods (qPCR and metagenomics). The resistome dataset showed a lower log_{10} number of ARG reads when compared to qPCR data. This is due to lower sensitivity of metagenomics when compared to PCR-based methods on wastewater samples (Manaia et al., 2018). The integration of the results presented here with previous studies highlights the need to combine molecular methods for an accurate analysis of ARGs and MGEs in wastewater. Metagenomics provides high resolution on the diversity of genes and their alleles. qPCR provides high sensitivity for the detection of ARGs and MGEs. Strong new insight on the separation and comparison of free-floating exDNA fractions from complex environmental biological samples is therefore provided here.

Genes displayed in Fig. 5 (*sul1, sul2, ermB, qnrS* and bla_{CTXM}) corresponded to less than 2% of the total primary ARGs reads found in the iDNA resistome. From the activated sludge resistome, only the number of reads of the universal *16S rRNA* and ubiquitous *intI1* genes were in line with the ones measured by qPCR in both DNA fractions. When the trend in gene copies per mL was compared, iDNA fractions contained a higher number of primary aligned reads from the selected panel of genes than the exDNA fraction. This was similar to qPCR results. Differences on the comparison results can be explained by the databases used as the resistome analysis fully depends on the databases selected for gene mapping. The resistome analysis was also done with ResFinder (**Table S5**). Results suggested that using a curated database did not modify the results obtained. ResFinder is also not fully complete as *sul1, sul2, ermB, qnrS* nor bla_{ctxm} genes were not found. ARG sequences that are highly dissimilar to reference sequences deposited in databases cannot be detected, thus not resulting in hits and not appearing in final results (Willmann and Peter, 2017). Harmonization of both molecular methods and databases are needed to advance the field.

3.5. Metagenomics allowed the comparison of the resistome, mobilome, and microbiome between iDNA and exDNA

The quantity and quality of the exDNA and iDNA templates that were separately retrieved from the complex activated sludge enabled a functional analysis of the different DNA fractions (Fig. 6) and a high-throughput metagenomics analysis of their mobilome, resistome, and microbiome compositions (Fig. 7). The number of

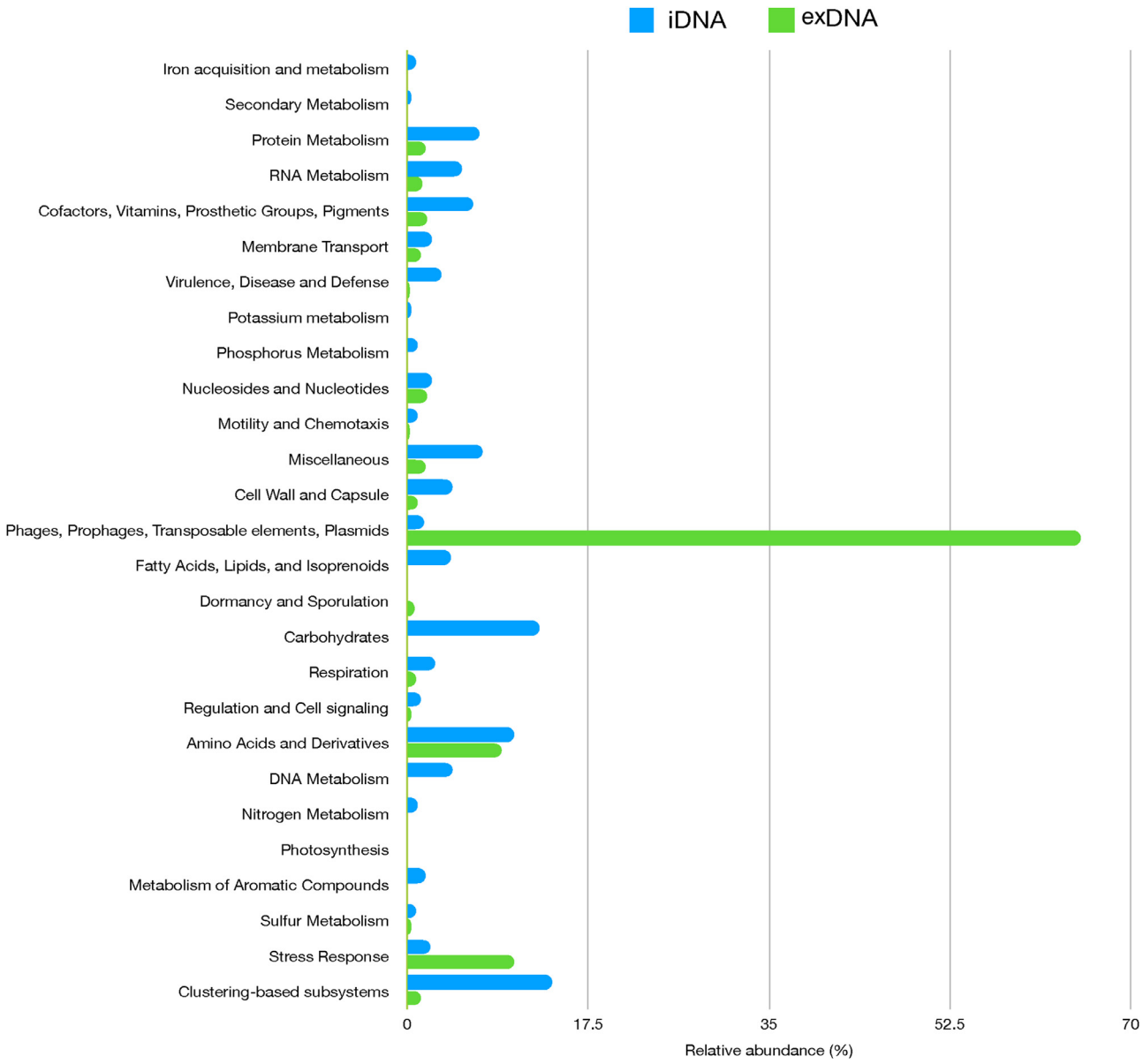

Fig. 6. Subsystem **classification of the functional genes identified from the metagenome of the free-floating extracellular DNA fraction (exDNA, *green*) fraction and the intracellular DNA (iDNA, *blue*) fraction using MG-RAST for mapping and computation of relative abundances (%).**

reads used for metagenomic analysis are summarized in **Table S6 - S7**. The absolute read counts of specific genes were then translated into relative abundances when normalized by the total number of primary aligned reads after samclip filtration per database selected.

3.5.1. Functional analysis and mobilome profiling of exDNA and iDNA fractions

Fig. 6 displays the functional assignment (level 1 of subsystem) of metagenomics sequences. From the exDNA and iDNA metagenomes, approximately 40% and 50% of the total predicted proteins with known functions (808′680 and 6′771'570), respectively, matched with the subsystems database on the MG-RAST server (Meyer et al., 2008).

Interestingly, the patterns in the data confirmed that the metagenome of exDNA was predominantly composed of MGEs with phages, prophages, transposable elements and plasmids making 65.1% (526′534 hits) of the sequencing dataset. The same category corresponded to a residual 1.6% (108′713 hits) on the iDNA metagenome. The iDNA fraction showed a heterogeneous distribution, classified mainly by clustering-based subsystems (13.94%, 944′394 hits), carbohydrates (12.8%, 865′220 hits), and amino acids and derivatives (9.13%, 701′175 hits) sublevels, among others. Clustering-based subsystems contain functions such as proteosomes, ribosomes, and recombination-related clusters (Delmont et al., 2012), which are localized in the cytoplasm of microorganisms (*i.e.*, iDNA).

Mobilome results showed that both the free-floating exDNA and the iDNA fractions contained a higher relative abundance of plasmids (58% and 74%, respectively) than viruses and prophages (41% and 26%, respectively) (Fig. 7a). In the exDNA fraction, the relative abundance of plasmids was significantly lower than the viruses. This might be explained by extracellular plasmids exposure to and degradation by environmental nucleases. The presence of microorganisms may also enhance the degradation rate of free-floating extracellular DNA plasmids (Zhu, 2006); thus, increasing the relative abundance of viral units in the free-floating extracellular DNA fraction.

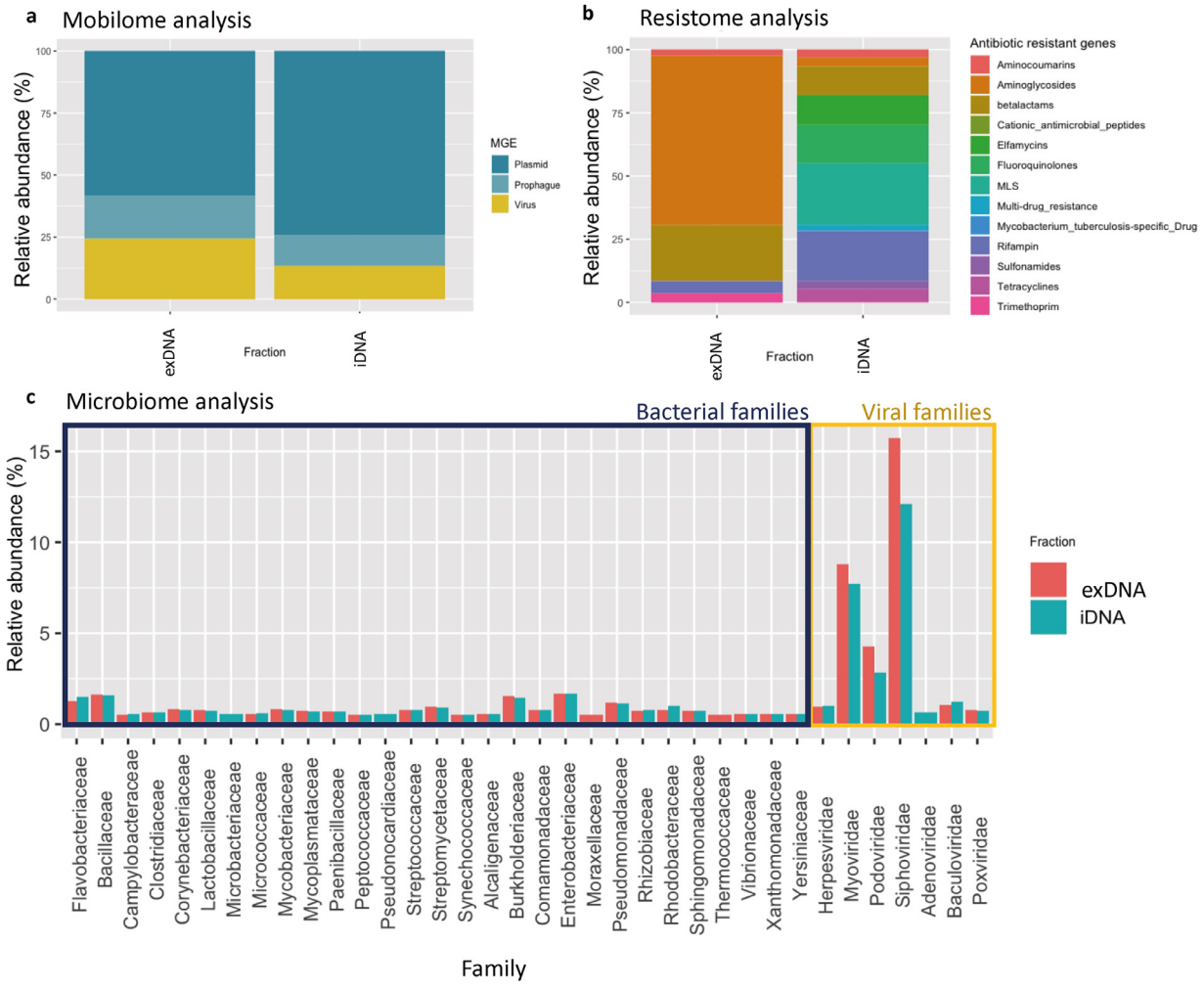

Fig. 7. Metagenome analysis from exDNA and iDNA fractions obtained from an activated sludge sample **(a)** Mobilome relative abundance. **(b)** Resistome relative abundance. **(c)** Microbiome relative abundance showing bacterial and viral families, whose population composition was >0.5%. Relative abundance (%) states as the ratio between the number of gene target hits divided by the number of total hits from a specific analysis category (mobilome, resistome, or microbiome).

MGEs are made of DNA segments that are capable to jump randomly to new locations within a single cell or multiple microorganisms (Jørgensen et al., 2014). They include viruses, plasmids, and associated elements such as insertion sequences, transposons, and integrons. Detailed mobilome compositions are summarized in the supplementary material **Table S7** and **Figure S4** for both free-floating exDNA and iDNA. Integrons, integrative conjugative elements (ICE), and insertion sequences (IS) were analyzed from the exDNA fraction: *intI1* (92%) **(Figure S4b)**, actinomycete conjugative integrative element (AICE) (35%) and SXT/R391 (30%) **(Figure S4c)**, and IS200 (38%) **(Figure S4d)** were the most abundant components in their category, respectively.

Microorganisms use MGEs via HGT as a mechanism to evolve and adapt to new environmental conditions by being subjected to natural selection. Consideringthat antibiotic concentrations in effluent wastewater and surface waters have increased up to 1 μg L^{-1} in the last years (Sanseverino et al., 2018), microorganisms can take profit from these mobile genes as an indirect tool to increase their resistance towards antimicrobials. Further research needs to be conducted on the relationship between the MGEs present in the free-floating exDNA fraction of biological systems like wastewater environments and the molecular mechanisms conferring antibiotics resistance.

Overall, the free-floating exDNA was confirmed as a pool of potentially transferrable DNA via HGT mechanisms, since mainly composed of MGEs. These MGEs do not necessarily mobilize ARGs in their structural composition (Jørgensen et al., 2014) but can contribute to antibiotic resistance acquisition.

3.5.2. Resistome profiling of exDNA and iDNA fractions

We are showing the profile of the resistome of the free-floating exDNA from an activated sludge sample. Resistome results showed more distinct patterns among DNA fractions (Fig. 7b). The free-floating exDNA fraction harbored a total of 12 different ARGs from 5 different antibiotic resistance families. There was a clear relative dominance of aminoglycosides (67%) and β-lactams (22%) resistant genes on the exDNA.

A total of 289 different ARGs from 15 different antibiotic resistance families were found in the iDNA fraction. This matched with the latest work of Jia et al. (2020) who detected 297 different genes from 17 different families in water and water filters from a drinking water treatment plant. MLS dominated (24%), followed by rifampin (20%), fluoroquinolones (15.1%), and elfamycins (11.7%) among other resistance genes on the iDNA.

Most of the ARGs are enclosed inside bacterial cells, while some specific types may endure floating or are bound to matrices in activated sludge samples, even if in low quantities. The low amount of ARGs on the free-floating exDNA suggests that natural transformation may not be the main mechanism through which ARG transfer and exchange does occur (Zhang et al., 2013). However, this smaller

amount of extracellular ARGs does not preclude any possibility to be taken up by naturally competent bacteria in complex cultures via transformation processes. It could be discussed whether the occurrence rate is lower in the exDNA fraction because of preferential uptake of exDNA fragments with ARGs.

3.5.3. Microbiome profiling of exDNA and iDNA fractions

Microbiome profiles were more conserved (*i.e.*, both as populations and their relative abundances) between free-floating exDNA and iDNA (Fig. 7c). Operational taxonomic units (OTUs) found in the extracellular fraction are genomic fragments that at some point have been released from lysed or necropsied cells. These genes are easier to be degraded than plasmid or viral DNA, due to the potential for fragmented double-stranded DNA to be exposed to nucleases. Thus, the exDNA is more likely originating from cell lysis inside the activated sludge rather than immigrating from the influent. Moreover, some studies have suggested that DNA adsorbed to soil or EPS matrices are protected from degradation by environmental nucleases, indicating that genes on plasmid may be better preserved than genomic DNA (Corinaldesi et al., 2008; Grande et al., 2011). In this case, it can be observed that only some specific bacterial and viral families are exclusive from one or another DNA fraction if we consider the 0.5% relative abundance cutoff. Examples are *Microbacteriacae, Pseudonocardiacae*, and *Adenoviridae* for the iDNA fraction and *Moraxellaceae* and *Thermococcaceae* for the exDNA (Fig. 7c). However, the *Moraxellaceae* family is known as an inductor of activated sludge flocculation (Shchegolkova et al., 2016), being also in the iDNA fraction (0.48%). The same situation was observed with the other bacterial and viral families that were found only in one of the DNA fraction. This meant that no significant differences in populations could be observed at the bacterial family level between the exDNA and iDNA fractions from activated sludge samples. We, therefore, hypothesize that exDNA may originate to some extent from the influent water, but mainly from microbial populations that decayed in the activated sludge tank. Our combined exDNA isolation and characterization method can become an efficient way to track microbial decay phenomena in activated sludge at the population resolution level.

4. Conclusions

We aimed to develop a method to isolate and differentially characterize the genetic composition of free-floating exDNA fractions from wastewater. Their xenogenetic elements, namely ARGs and MGEs, were characterized from molecular quantification at high sensitivity with qPCR to profiling at high throughput and resolution with metagenome sequencing. This work led to the following key conclusions:

- The implementation of DEAE chromatography promoted an efficient isolation of free-floating exDNA at high yield and quality from complex matrices of WWTP environments from influent wastewater to activated sludge and effluent water samples.
- qPCR analyses of a panel of selected genes highlighted the presence of ARGs from 1.8 to 3.8 $\log_{10}$ copies in the free-floating exDNA fraction. Significant differences were detected between the exDNA and iDNA fractions for the predominant sulfonamides ARGs (*sul1* and *sul2* were 0.93–1.11 $\log_{10}$ more abundant in iDNA).
- The metagenomes displayed similar microbiome compositions between exDNA and iDNA fractions, suggesting that a big fraction of free exDNA found in activated sludge may derive from the flocs.
- The mobilomes and resistomes from the exDNA were less rich than on iDNA, but still form a source of MGEs for natural transformation. Interestingly, subsystems classifications showed that the exDNA fraction was mainly composed of MGEs (65.1%). This confirmed the hypothesis that free-floating exDNA is a pool of stable MGEs that can drive HGT and antibiotic resistance.
- Within the field of environmental ecology, free-floating exDNA can serve as a target matrix for biodiversity survey studies (Nagler et al., 2018a). It can help to elucidate the ecological relevance and persistence of pathogenic microorganisms throughout the water sanitation process or to estimate the microbial activity from specific environments (Levy-Booth et al., 2007), among others.

New physical-chemical technologies can be studied for the removal of xenogenetic pollution (specifically ARGs and MGEs) removal from wastewater (Calderón-Franco et al., 2020b). High removal of exDNA from effluent water by, *e.g.*, adsorption may allow the discharge of water streams deprived of xenogenetic elements in receiving bodies, thus potentially contributing to reducing the development antibiotic resistant bacteria by transformation processes (Gillings et al., 2018).

Further studies on the conditions promoting HGT in complex biological systems are needed to elucidate the actual natural transformation rate for free-floating exDNA components. Their systematic chromatographic isolation, molecular quantification, and metagenomics profiling will be powerful to generate data useful for xenogenetic risk assessments on ARGs, MGEs, but also residual GMO materials, across urban and natural water systems. It will support measures by water authorities to remove them from wastewater to safeguard environmental and public health.

Data availability

Metagenome sequencing data were deposited in the NCBI database with the BioProject ID PRJNA632452. Intracellular DNA BioSample ID: SAMN14909743. Free-floating extracellular DNA BioSample ID: SAMN14909742.

Authors' contributions

DCF designed the study with inputs of MvL, TA and DGW. DCF performed the experimental investigations. DCF wrote the manuscript with direct contribution, edits, and critical feedback by all authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116592.

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