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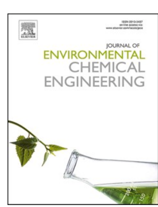

Treating domestic wastewater towards freshwater quality: Bacterial community and antibiotic resistance profiles highlight critical steps and improvement opportunities

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ABSTRACT

Ideally, wastewater treatment aims to produce water indistinguishable from freshwater, especially for reuse. This study evaluated bacterial community and antibiotic resistance variations throughout treatment and benchmarked these with freshwater sources. Samples collected from six points of a full-scale wastewater treatment plant, pilot-scale advanced treatment options (non-thermal plasma - NTP, ultrafiltration - UF, UF followed by reverse osmosis- UF+RO), two rivers and a borehole were analyzed for quality parameters (BOD5, TSS, turbidity, *Escherichia coli*), antibiotic resistance genes (quantitative PCR), class 1 integron variable region composition (Oxford Nanopore sequencing), and bacterial community composition (16S rRNA Illumina sequencing).

Secondary treatment followed by sand filters and coagulants caused the highest reduction (~2 log-unit/volume) of all analyzed parameters and the sharpest reduction of diversity of antibiotic resistance genes within class 1 integrons' variable region. Ultraviolet disinfection triggered minimal bacterial or genes reduction, while among advanced treatments, UF+RO caused the highest, and NTP the lowest.

Principal component analysis suggested significant associations between antibiotic resistance ($n = 32$) and genetic recombination elements ($n = 12$) and predominant bacterial families in raw wastewater (*Aeromonadaceae*, *Moraxellaceae*, *Campylobacteraceae*, *Lachnospiraceae*). For predominant freshwater families (*Comamonadaceae*, *Chitinophagaceae*, *Flavobacteriaceae*) no significant associations were observed. Freshwater differed from UF-treated water by a lower antibiotic resistance abundance, higher bacterial richness (~4000 vs.1200 operational taxonomic units) and distinct predominant families - *Alcaligenaceae*, *Sphingomonadaceae*, *Chitinophagaceae*, and *Microbacteriaceae* in UF water. The findings underscore the critical role of secondary/post-secondary treatments in shaping resistance and community profiles and suggest that advanced treatment should balance water quality with bacterial diversity preservation for sustainable reuse.

1. Introduction

Urban wastewater treatment plants (UWTPs) are among the major barriers to reduce the load of chemical and microbiological contaminants present in wastewater generated from domestic uses and human sewage [8,30]. Nonetheless, the limited capacity of UWTPs to reduce unwanted microorganisms and chemical contaminants to levels safe to humans and the environment has been consistently recognized [49,57]. Among the contaminants released by UWTPs are antibiotic resistant bacteria and antibiotic resistance genes [21,50]. Although these contaminants do not represent an environmental problem in itself, once spread in the environment, antibiotic resistant bacteria and their genes can persist, self-replicate, and may reach humans [34,35,42]. While, in general, the direct return of antibiotic resistant bacteria emitted by UWTPs to humans seems unlikely, the risk may increase when treated wastewater is used for irrigation of crops for human consumption [3,73].

Based on 16S rRNA gene quantification, influents and effluents from

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UWTPs worldwide have been reported to contain 10–12 and 8–10 log units of bacteria / 100 mL, respectively [43]. Despite only a small fraction of these bacteria harbor acquired antibiotic resistance genes, some can persist in downstream environments [72,75], raising public health concerns. This is one of the reasons why the reuse of treated wastewater for agricultural irrigation has poor public acceptance. Microbiological parameters to assess the suitability of treated effluent for agricultural irrigation in accordance with regulatory guidelines include biological indicators such as *Escherichia coli*, *Legionella* spp., nematode eggs, total and faecal coliforms [3,52]. The monitoring of antibiotic resistance has not been considered so far for water reuse purposes, although the recent revision of the Urban Wastewater Treatment Directive imposes the monitoring of antimicrobial resistance in wastewater for agglomerations exceeding 100 000 population equivalent (EU [19]). Some studies have suggested that antibiotic resistance genes may be taken up by crops when treated wastewater is used for irrigation [6,13]. Also, previous studies showed that edible plants hosted endophytes of the genera *Acinetobacter*, *Enterobacter* or *Pseudomonas*, groups with recognized potential to serve as antibiotic resistance genes carriers [59]. Unrelatedly to the intentional or accidental reuse of treated wastewater, it has been assumed that antibiotic resistance discharged by UWTPs may return back to humans in irrigated food products, bathing waters, or other [16,37]. Both regulatory frameworks and public perceptions have created an increasing pressure to improve the quality and safety of treated wastewater in what concerns antibiotic resistance [73]. Tertiary treatment processes with disinfection potential, i.e., of reducing the microbial load, such as membrane separation and advanced oxidation processes, have received much attention lately [25,44]. Nonetheless, the capacity of these processes to enrich the wastewater microbiota with fast growing bacterial populations with capacity to recover cellular damages caused by disinfection, such as those of the genera *Pseudomonas* or *Acinetobacter* [4,53] that frequently harbor acquired antibiotic resistance genes, has been largely debated [54]. As the reuse of treated wastewater for agricultural irrigation is generally poorly accepted, it is important to investigate how these compare to existing alternatives, such as freshwater sources, and whether these pose lower risks in terms of antibiotic resistance spread. This study was based on two major arguments: i) that raw wastewater is the major source of human commensal and antibiotic resistance determinants that are observed in treated effluents, and ii) that ideally wastewater treatment aims to produce water with quality close to freshwater sources. Considering the low public acceptance of the reuse of treated wastewater, it was considered that benchmarking treated wastewater and surface water is an adequate approach to relieve some misconceptions that may exist regarding the occurrence of pathogens or harmful bacteria. Accordingly, this study brings a new insight by aiming to track antibiotic resistance genes and the bacterial community through the treatment path in a full-scale UWTP, including UV disinfection, storage of treated wastewater and pilot-scale advanced treatment, and compare those with freshwater sources located in the same region. Ultrafiltration, ultrafiltration combined with reverse osmosis, and non-thermal plasma technology, an emerging advanced oxidation technology for wastewater treatment [33,64] were the pilot-scale processes tested. The investigation combined bacterial community (16S rRNA metabarcoding) and antibiotic resistance profiling (HT-qPCR and Nanopore long-read sequencing).

2. Material and methods

2.1. Urban wastewater treatment plant and pilot advanced treatment systems

Samples were collected from different points of a full-scale UWTP located in Northern Portugal, dimensioned to serve 300 000 population equivalents and with an effective average daily flow of 70,000 m^3. The treatment process comprises preliminary treatment and primary settling, with sand and grease removal, followed by secondary treatment with conventional activated sludge biological treatment and secondary settling (Fig. 1A). The clarified secondary effluent is discharged in the Atlantic Ocean at a depth of 30 m through a submarine outfall in environmentally suitable conditions. A fraction of ~3 % of the total volume of the secondary treated effluent is diverted into a tertiary treatment system. The tertiary treatment system has a capacity to treat 100 m^3/h and consists of two vertical sand filters in series (FV2B, Degrémont, France) with coagulation (100 mg/L aluminum sulphate) (SF+Cg), followed by UV-B disinfection in open channel with 40 (8 ×5 banks) horizontal low pressure mercury lamps, providing a UV dosage of 33 mJ/cm^2 (UV3000 Type B, Trojan Technologies Inc., Canada). The tertiary treated effluent is stored for about 3 days in a closed 180 m^3 cistern for further reuse at the UWTP. A small amount of the stored treated wastewater is recirculated to the UV channel to keep it full while it is not being used to produce wastewater for reuse.

As part of this study, secondary effluent was diverted to alternative tertiary treatment processes tested at pilot scale with ultrafiltration (UF), UF and reverse osmosis (RO) (UF+RO), and non-thermal plasma (NTP) (Fig. 1B). The NTP was applied to the secondary effluent after the sand filtration and coagulation (SF+Cg+NTP). Operation and technical details of the membrane pilot units have been previously described in [64]. In short, the UF pilot was a containerized unit of hollow-fiber membranes modules with average pore size of 20 nm, operated in outside-in mode, processing a flowrate of 9 m^3/h of effluent. The RO pilot was applied in series with the UF, equipped with spiral-wound polymeric membrane modules (Borum rejection > 90 %), and operating with a flow rate of 2.2 m^3/h. The NTP pilot unit consisted of a dielectric barrier discharge (DBD) plasma generator (model IXS-500-KISS, SFC Umwelttechnik GmbH, Austria) connected to a reactor tank with a working volume of 50 L, which was applied to the effluent after sand-filtration pre-treatment (SF+Cg+NTP). The NTP generator was operated with ambient air supplied at an average gas flow rate of 50 L/min, at a fixed frequency of 500 Hz, which corresponded to an applied power of 40 W. The plasma was generated in the gaseous phase and was distributed in the effluent tank through a 35 mm diameter diffuser hose (Wagner Vertriebs GmbH, Germany) installed at the bottom of the tank. The results shown for the NTP pilot unit in this study correspond to one-hour operation in batch mode.

2.2. Sampling and processing of wastewater and freshwater

Wastewater samples collected throughout the treatment process and storage, treated wastewater samples subjected to pilot-scale advanced treatments and also freshwater samples were analyzed in this study. Grab UWTP samples were collected in three consecutive days at six points (1–6 as indicated in Fig. 1A) of the treatment/storage line. For pilot-scale assays, samples were collected in three different days of operation under the same conditions before (secondary effluent for UF and UF+RO and sand filters for NTP) and after the advanced treatment (Fig. 1B). To assess the stability of the bacterial parameters measured immediately after advanced treatment, samples were also examined after 7 days storage in the dark, in a 25 L container for UF and UF+RO or 1 L (performed at laboratory scale) for SF+Cg+NTP. The storage period was selected according to the time interval that may occur between effluent production and its reuse. Freshwater samples were collected at three different seasons (Table S1) from two river streams (Rv_1 and Rv_2) and a borehole (Bh), all within an 8 km radius from the UWTP. After collection, the samples were transported to the laboratory in refrigerated bags and analyzed within the following 12 h.

The volumes collected and processed for each type of sample for DNA extraction are indicated in Table S1. All samples were processed and analyzed in triplicate, unless indicated. Samples were filtered through polycarbonate membranes (0.22 µm porosity, Whatman, UK) using volumes that balanced the aimed DNA quantity and membrane filtration capacity (Table S1). For DNA extraction, the Dneasy® PowerWater® kit

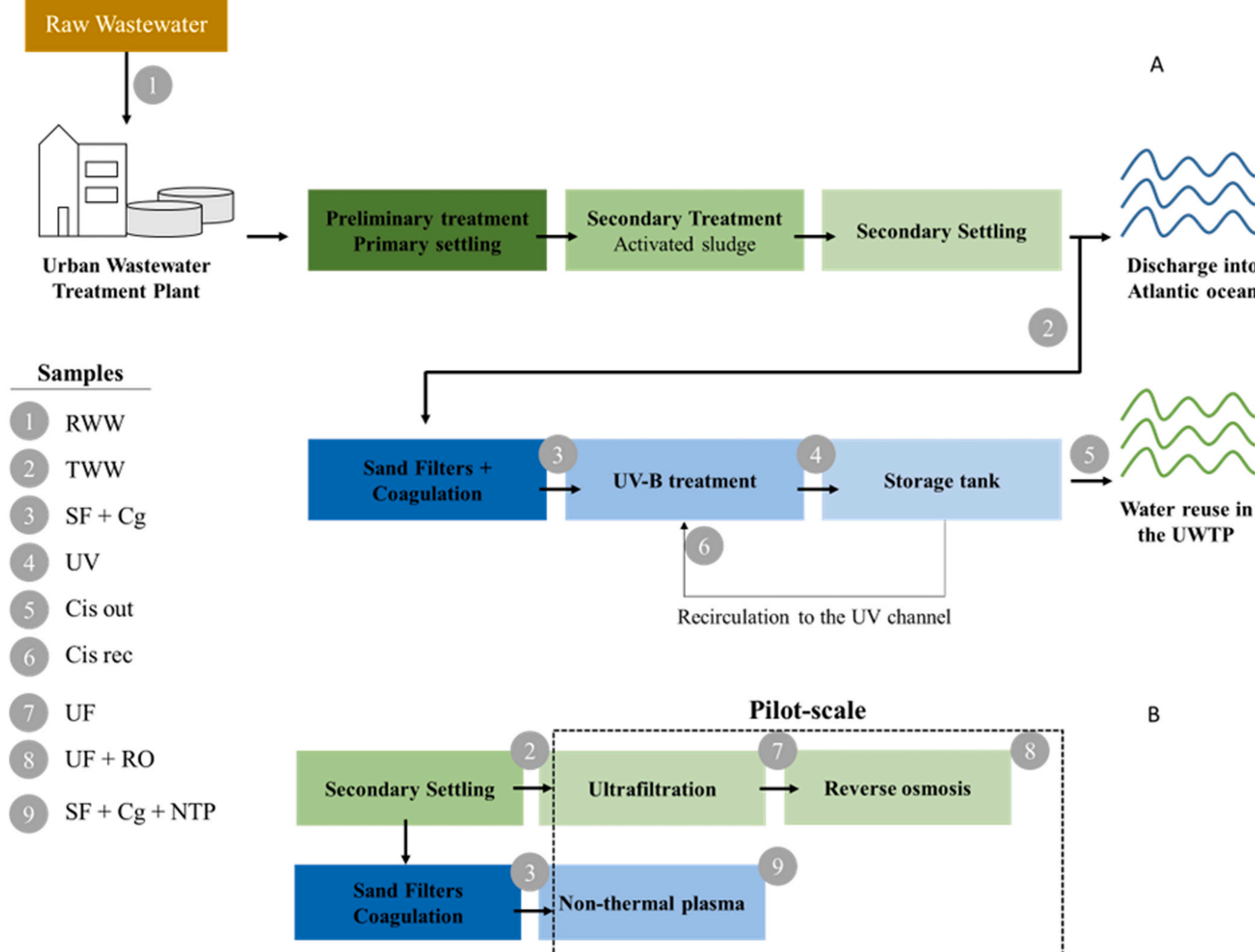


Fig. 1. Schematic representation of the UWTP sampled for this study. A) full-scale system and B) advanced treatments (at pilot-scale) implemented for this study. RWW – raw wastewater; TWW – secondary effluent after secondary settling; SF+Cg – effluent after sand filtration and coagulation; UV – UV-B effluent; Cis out – treated wastewater stored; Cis rec – treated wastewater stored and recirculated to the UV channel; UF – ultrafiltration effluent; UF + RO – effluent after ultrafiltration and reverse osmosis; SF+Cg+NTP – effluent after sand filtration and non-thermal plasma.

(Qiagen, Hilden, Germany) was used according to manufacturer instructions, except the cell lysis step that was extended for 10 minutes, and the pre-warming of the DNA elution buffer at 55 °C to increase the elution efficiency. DNA extracts' concentration was determined with Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific, USA). DNA extracts were stored at -20 °C for further analysis.

2.3. Sample analysis

Wastewater, storage and freshwater samples were characterized based on standard physical, chemical and microbiological analysis, quantitative polymerase chain reaction (qPCR), and 16S rRNA metabarcoding, as described in Table S1. For NTP, a single sample was processed for DNA extraction and further qPCR analysis. Standard parameters listed in the water reuse regulation [52] were determined by an accredited laboratory for water and wastewater analyses, and included biological oxygen demand (BOD5), total suspended solids (TSS), turbidity, and *Escherichia coli*. (Tables S3). For the storage assays performed at laboratory, these parameters were not evaluated.

Total bacteria (16S rRNA gene), faecal contamination indicators (*crAssphage* and *uidA*), a genetic recombination indicator (class 1 integron integrase - *intI1*), and an antibiotic resistance gene (*aph(3′)-ib*) were measured by quantitative PCR (StepOne™ Real-Time PCR System; Life Technologies, Carlsbad, CA, USA) using the standard curve method [9] (Table S2). Gene quantification was determined according to the quality criteria described before [56,66]. A subset of samples (Table S1) was also screened for the relative abundance of 48 genes (Table S4), mostly associated with antibiotic resistance and genetic recombination, using a qPCR array, SmartChip system (Resistomap Oy, Helsinki, Finland). The genes quantification was determined by the ΔC_t method, following the quality criteria described before [45]. Briefly, the array included the 16S rRNA gene, mobile genetic elements (n = 11), integrons (n = 1), genes conferring resistance to the antibiotic classes trimethoprim (n = 1), phenicols (n = 1), beta-lactams (n = 11), tetracyclines (n = 2), macrolide-lincosamide-streptogramin B (MLSB) (n = 3), aminoglycosides (n = 5), multidrug-resistance (n = 3), quinolones (n = 2), sulphonamides (n = 3), vancomycin (n = 1), others (n = 3).

To investigate potential genetic recombination events throughout the wastewater treatment line, amplicons of the variable region of class 1 integrons of total DNA extracts of samples of RWW, TWW, SF + Cg, UV, Cis out and Cis rec, collected in the same day (Table S1), were sequenced using long-read Oxford Nanopore (ONT). The PCR products obtained with the primers CS3 and CS5 (Table S2) and with sizes

between 800 and 3000 base pairs (bp) (1.5 % w/v agarose gel with TRIS-Acetate-EDTA buffer as eluent at 90 V) were purified using a commercial kit (GRS PCR Purification Kit, GRISP, Portugal). A total of 18 PCR products, with DNA concentrations ranging 6.9–39.9 μg/mL were multiplexed in a single DNA library using the ONT ligation Sequencing amplicons and Native barcoding kit 24 V14 (SQK-NDB114.24) following the manufacturer's instructions (protocol NBA_9168_v114_revH_15-Sep2022, updated on 24.04.2023). DNA library was sequenced using a MinION MK 1B sequencing device (Oxford Nanopore Technologies) with a FLO-MIN114 R10 flow cell. Sequencing ran for 8 hours, generating a total of 4 M reads. Guppy (version 6.5.7) (Oxford Nanopore Technologies) was used to perform base-calling and demultiplexing. Reads quality control was performed with Nanoplot (Galaxy Version 1.41.0 + galaxy0). Using the filter Fasta tool (Galaxy Version 4.8.1 +galaxy0), sequences were filtered to a length of 400–3000 bp. Unassembled reads were annotated for antibiotic resistance genes and mobile genetic elements using ABRicate with the ResFinder database (Galaxy Version 1.0.1) with default parameters. To assess the different gene and antibiotic resistance associations on the integron, sequences with 1000–3000 bp were analysed, and the combinations of genes compiled. For determination of the geneś relative abundance a stricter inclusion criteria was used, being considered only annotations obtained from a coverage ≥ 90 %, ID ≥ 95 % and gene length > 400 bp. The genes relative abundance was calculated by dividing the number of reads annotated to each gene by the total number of reads. The 16S rRNA gene and class I integron sequences were deposited in the NCBI SRA archive under BioProject number PRJNA1152317 and PRJNA1152510.

The bacterial communities of RWW, TWW, SF+Cg, UV, Cis out, Cis rec, UF, Riv_1, Riv_2 and Bh samples (Table S1) were characterized based on the sequencing of the 16S rRNA gene hypervariable regions V3-V4 amplicons (paired-end Illumina Miseq, Novogene, Cambridge, UK). All samples were analyzed in triplicate, except UF samples for which only two replicas met quality control parameters due to low concentration of DNA. The raw reads were merged using FLASH (version 1.2.11), quality filtered, chimeras removed, and operational taxonomic units (OTUs) defined at 97 % sequence similarity defined with UCLUST [18]. The taxonomic annotation was performed with 16S database using the 16S rRNA gene-based microbiome taxonomy profiling (MTP) pipeline from EZBioCloud (CJ Bioscience, Inc., South Korea).

2.4. Statistical analysis

Quantitative PCR, qPCR array, bacterial community composition and class I integron amplicon sequencing data were analyzed based on descriptive statistics. One-way ANOVA and the post-hoc Tukey test were used for comparative assessments (IBM SPSS Statistics version 28), with a significance level established at 0.01. qPCR array and amplicon sequencing data (class I integron and 16S rRNA gene) results were interpreted with the aid of Canoco 5.01 software [62] by Principal Component Analysis (PCA). Standard physical and chemical analysis results are presented in Table S3 as the average ± standard deviation of 3 samples collected in 3 different days of operation.

3. Results

3.1. Wastewater treatment, storage and suitability for reuse

The first goal of this study was to assess the effect of secondary treatment on wastewater quality and its microbiota. The physicochemical characterization of all samples is presented in Table S3. Throughout this study the UWTP was operating under normal conditions, discharging a secondary effluent (TWW) with an average quality of 10.3 mgO_2/L BOD5 and 20.8 mg/L TSS. Concerning the microbiological reductions between raw influent (RWW) and TWW, these ranged ~1 log-unit/volume for *Escherichia coli*, for total bacteria assessed based on the16S rRNA gene, as well as for other genes (Fig. 2). According to the minimum requirements for water reuse (Regulation EU 2020/741), *E. coli* abundance after the secondary treatment (5.2 log-units CFU/ 100 mL) was above the least stringent requirements (<4 log-units CFU / 100 mL, class D for industrial, energy and seeded crops), as well as above solids content, rendering it unfitting for reuse. Post-secondary treatment implemented on site to enable internal reuse from a storage cistern, combining sand filtration with coagulation (SF+Cg) and ultraviolet radiation (UV), provided a quality improvement of physicochemical parameters, with reduction in BOD5, TSS and turbidity down to average values of 4.2 mgO_2/L, 5.8 mg/L and below quantification, respectively (Table S3). Furthermore, after SF+Cg, *E. coli was* reduced in ~1.5 log-units / 100 mL and 16S rRNA (total bacteria) and the other genes (*intI1, uidA, crAssphage, aph(3*′′*)-ib*) were reduced in about 1.2 log-units. With this additional treatment, wastewater met the class D minimal requirements for reuse [52], with *E.coli* levels of 3.7 log-units / 100 mL. After UV radiation and cistern recirculation/outlet, no significant variations were observed, except for the genes *aph(3'')-ib* and *crAssphage* which significantly increased (≤1 log-unit/volume) after UV and cistern storage or during storage, respectively (Fig. 2).

3.2. Inferring genetic recombination throughout the treatment process

The analysis of the variable region of class 1 integrons aimed to infer genetic recombination events and rearrangements (e.g. gene acquisition or loss). The analysis of the nucleotide sequences of amplicons of the variable region of class 1 integrons, using strict parameters, permitted the annotation of 14 genes (*aac3*, *aac6, aadA*, *ant(2*′′*)*, *ant(3*′′*), ARR,* bla_{BEL}, bla_{CARB}, bla_{GES}, bla_{IMP}, bla_{OXA}, *catB*, *dfrA*, *qnrVC6*) and 128 gene variants (114 in RWW, 62 in TWW, 55 in SF+Cg, 54 in UV, 65 in Cis rec, and 35 in Cis out), being RWW and Cis out the samples with highest and lowest richness of variants, respectively. Most of the annotated genes were associated with beta-lactams, trimethoprim and aminoglycoside resistance (Fig. 3, Table S4). The dynamics of class 1 integron variable region was suggested by the observation of distinct alleles prevailing at different stages, being after secondary treatment, sand filters and coagulants and storage that the most prominent variations were observed. Nevertheless, considering genes with a relative abundance higher than 1 %, the profile of genes inserted in variable regions of class 1 integrons was fairly stable (Fig. 3). A total of 32 alleles was observed in RWW but not further throughout the process (11 aminoglycoside; 8 beta-lactams and 13 trimethoprim) (Table S5). In contrast, in TWW were detected 4 alleles not observed in RWW (*aac(6*′*)-IIc_1*, bla_{IMP-24_1}, bla_{IMP-8_1}, $bla_{OXA-520_1}$, *dfrA1_13*) (Table S5). Other alleles became detectable only in further stages - *aadA2_2* and *dfrA33_1* after SF+Cg, *ant(2'')-Ia_16* after UV, *aadA15_1* after storage, and $bla_{CARB-11_1}$, $bla_{OXA-147_1}$, bla_{OXA-19_1}, bla_{OXA-56_1} and *dfrA1_5* after recirculation to the UV line (Table S5). Significant differences in the frequency of alleles were observed for the gene *aadA2_1* (decreased after SF+Cg, increased after UV and after storage), for the gene *ant(2*′′*)-Ia_1* (decreased after SF+Cg and after storage), for the gene $bla_{OXA-205_1}$ (decreased along the line of treatment), for the gene *dfrA12_10* (decreased after SF+Cg until after storage), for the gene *dfrA22_2* (decreased after TWW until after storage), for the gene *dfrA32_1* (increased after SF+Cg and decreased after storage), for the gene dfrA5_1 (decreased after SF+Cg, after UV and after storage) and for the gene *dfrA7_5* (decreased after SF+Cg until after storage).

The association of gene cassettes within the same class I integron variable region was examined in sequences with 1000–3000 bp, being observed 275 linkage profiles. The diversity of linkage profiles decreased along the pipeline (average values for each sample: RWW – 164; TWW – 35; SF + Cg – 27; UV – 23; Cis rec – 39; Cis out – 16). Most of the linkage profiles were observed between genes that conferred resistance to aminoglycosides and trimethoprim (n = 84) or beta-lactam and trimethoprim (n = 69) (Table S7). The most frequent combinations in the RWW (bla_{OXA-10}|*dfrB4*, *aadA2*|*dfrA7*, ant(3'')-Ia|*dfrA7*, *aadA2*|*ant* (3'')-Ia, *ant(3'')-Ia*|*dfrA1*, *dfrA7*|*dfrB2*, bla_{OXA-10}|*dfrA7*, *aadA2*|*dfrB2*,

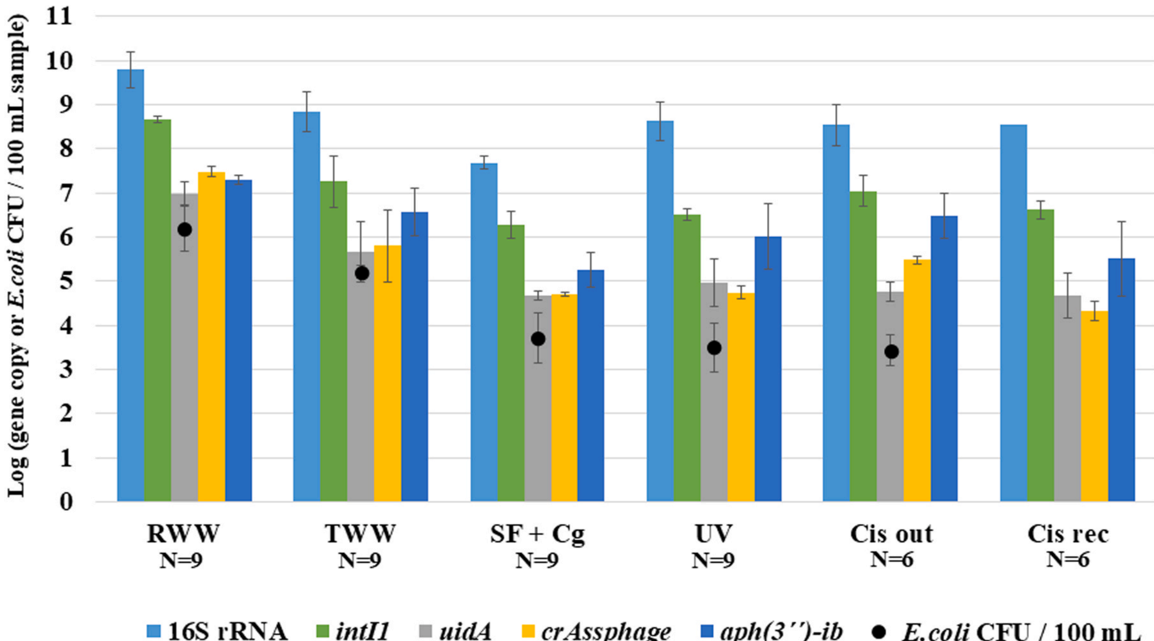


Fig. 2. Abundance of total bacteria (16S rRNA gene), and of the genes *intI1*, *uidA*, *crAssphage* and *aph(3*′′*)-ib* expressed as log-units of gene copy per 100 mL of sample, and *Escherichia coli* colonies forming units (CFU) per 100 mL of sample. RWW – raw wastewater; TWW – secondary effluent after secondary settling; SF+Cg – effluent after sand filtration and coagulation; UV – UV-B effluent; Cis out – treated wastewater stored; Cis rec – treated wastewater stored recirculated to the UV channel. *E. coli* was not quantified in Cis rec sample. A significative decrease ($p < 0.01$) was observed between RWW and TWW for the *intI1*, *crAssphage*, *uidA* and *aph(3*′′*)-ib*. This significant decrease was also observed for the *E.coli* load ($p < 0.001$). Significative differences between TWW and the SF+Cg were observed for the total bacteria load (16S rRNA) and the genes *crAssphage* and *aph(3*′′*)-ib* (p value < 0.001), and for the genes *intI1* and *uidA* ($p = 0.003$). Cis rec and Cis out samples presented different abundances of the genes *crAssphage* ($p = 0.009$) and *aph(3*′′*)-ib* ($p < 0.001$).

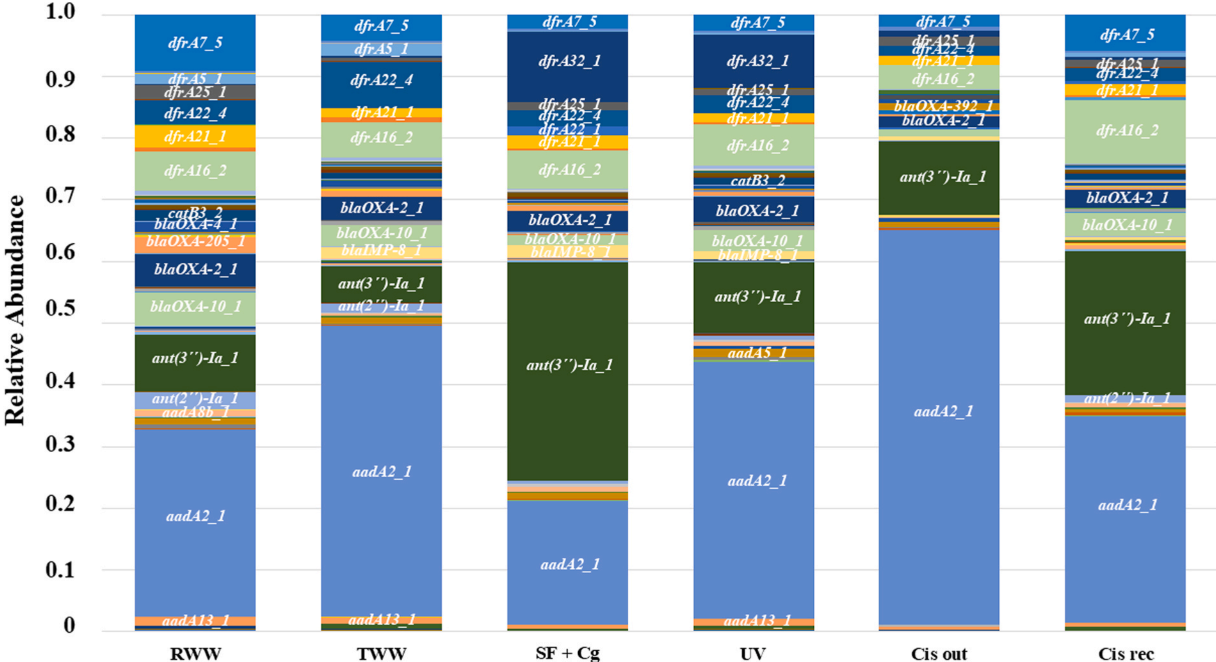
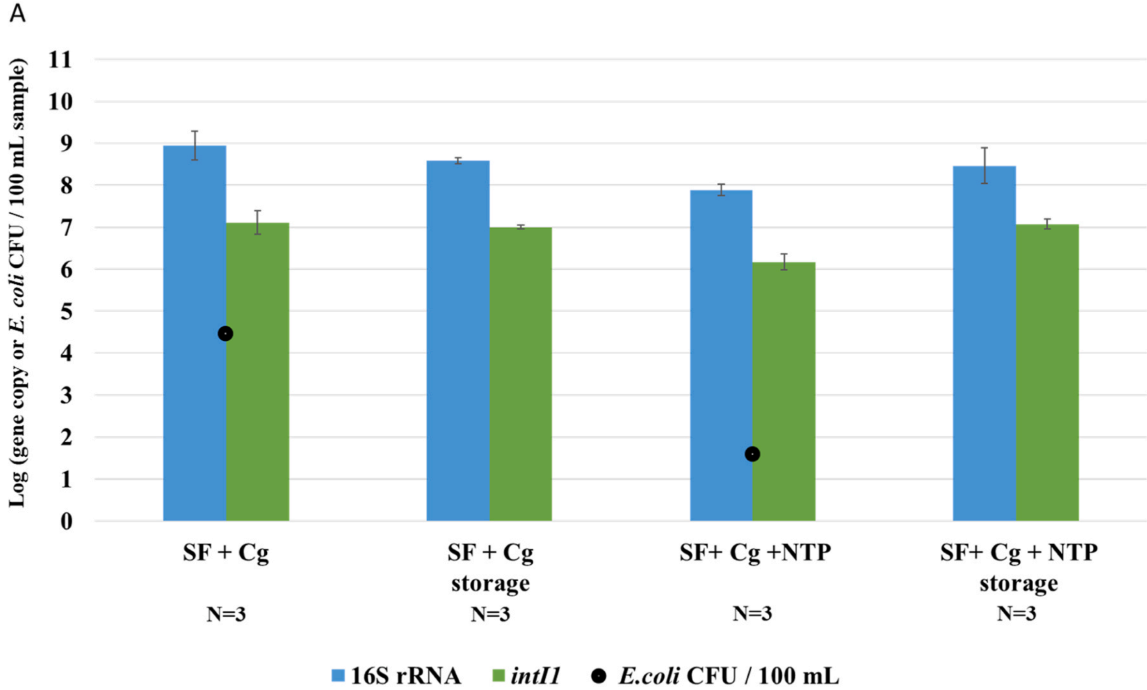


Fig. 3. Antibiotic resistance genes profile across the wastewater treatment process, obtained from the sequencing of class I integrons variable region. RWW – raw wastewater; TWW – secondary effluent after secondary settling; SF+Cg – effluent after sand filtration and coagulation; UV – UV-B effluent; Cis out – treated wastewater stored; Cis rec – treated wastewater stored recirculated to the UV channel. Detail about the number of reads obtained for each sample are available on Table S6. The sample Cis out presented the highest relative abundance for the gene *aadA2_1*, being significantly different from all the other samples except TWW (RWW: $p < 0.001$; SF+Cg: $p < 0.001$; UV: $p = 0.003$; Cis rec: $p < 0.001$). Only being identified in the sample from RWW, the relative abundance of gene *dfrA22_2* in this sample, was significantly different from all the other samples ($p < 0.001$). The sample RWW also differed significantly from SF+Cg and Cis out on the relative abundance of the gene *ant(2*′′*)-Ia-1* ($p = 0.007$ and $p = 0.002$), presenting the highest relative abundance in the sample RWW. In the sample SF+Cg, the gene *dfrA32_1* had the highest relative abundance when compared to the other samples analysed, being significantly different from RWW ($p = 0.005$), TWW ($p = 0.006$), Cis rec ($p = 0.007$) and Cis out ($p = 0.009$). The relative abundance in the sample TWW of the gene *dfrA5_1,* was significantly different from the relative abundance in all the samples in study except for RWW (SF+Cg: $p < 0.001$; UV: $p = 0.001$; Cis rec: $p = 0.003$; Cis out: $p < 0.001$).

ant(3'')-Ia|dfrB2) decreased their prevalence along the treatment, being the combinations (*aadA2|dfrA7*, *ant(3'')-Ia|dfrA7*, *aadA2|ant(3'')-Ia*, *ant(3'')-Ia|dfrA1*) the ones that most prevailed after the treatment (Table S7).

3.3. Pilot-scale advanced treatment

Advanced treatment processes by NTP, UF or UF+RO were tested at pilot scale. Improvement in water reuse quality parameters was observed for the 3 pilot units (Table S3), with further reduction in CBO5,



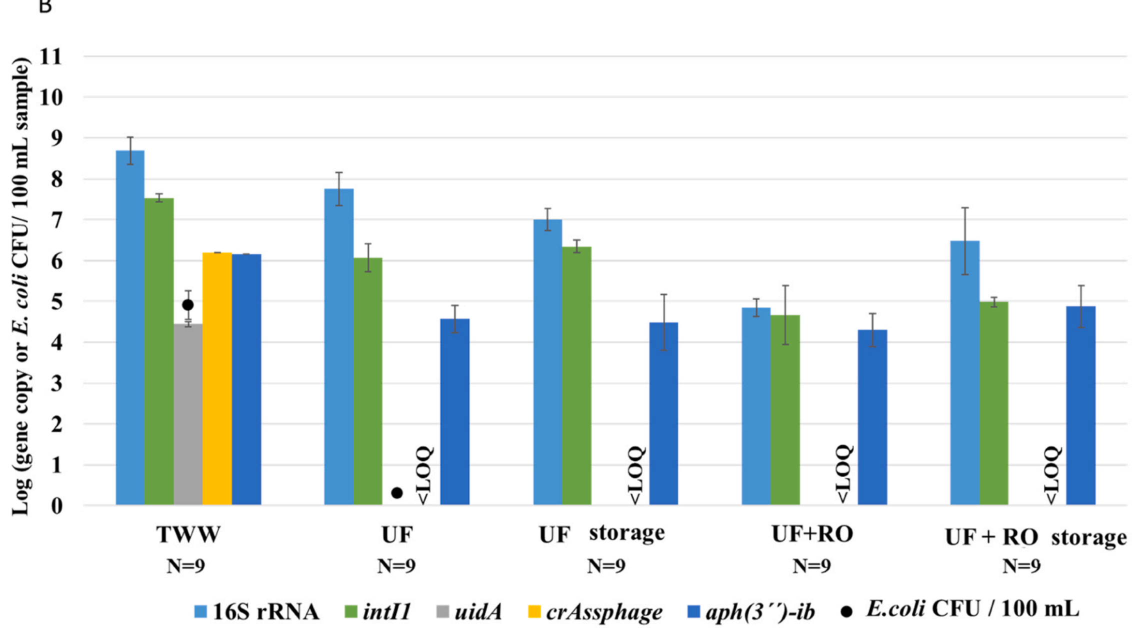
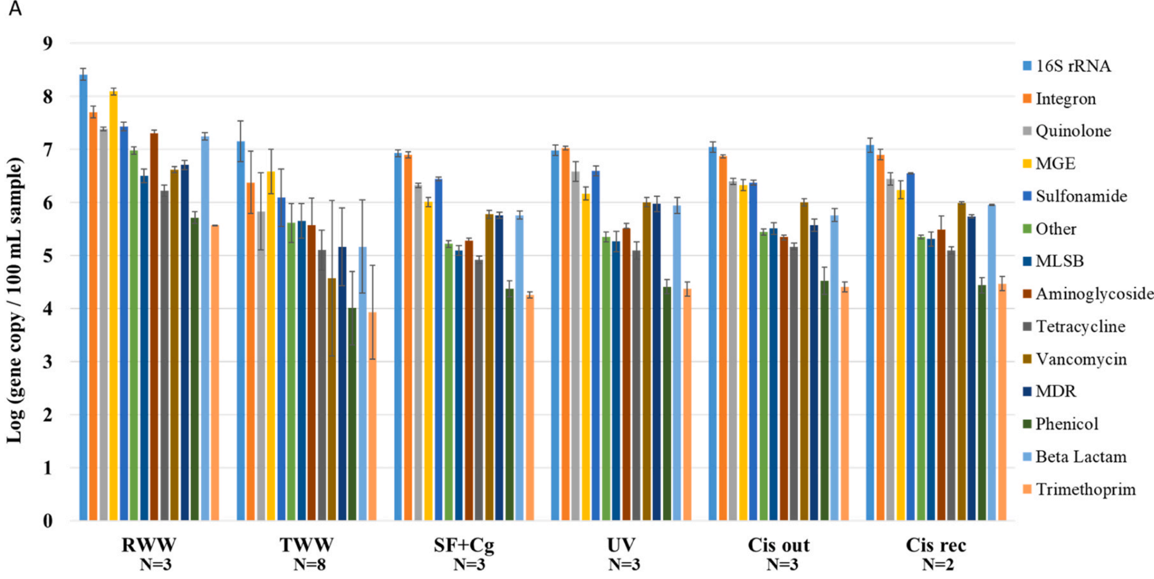


Fig. 4. Abundance of total bacteria (16S rRNA gene), *intI1, uidA, crAssphage* and *aph(3´)-ib* genes expressed as log-units of gene copy per 100 mL of sample and *Escherichia coli* colonies forming units per 100 mL (*E.coli* CFU / 100 mL). A) Advanced treatment using non-thermal plasma (NTP). B) Advanced treatment using ultrafiltration (UF) and reverse osmosis (UF+RO). SF+Cg – effluent after sand filtration and coagulation; SF+Cg storage - effluent after sand filtration and coagulation with 7 days of storage; SF+Cg+NTP – non-thermal plasma effluent; SF+Cg+NTP storage - non-thermal plasma effluent after 7 days of storage. TWW – secondary effluent after secondary settling; UF – effluent after ultrafiltration; UF storage – effluent from ultrafiltration after 7 days of storage; UF + RO – effluent after reverse osmosis; UF + RO storage – effluent after reverse osmosis after 7 days of storage. Fig. 4A A: No significant differences were oberved, except for the *E.coli* load that decreased significantly ($p = 0.005$) between SF+Cg and SF+Cg+NTP. Fig. 4B B: The total bacteria load was significantly decreased between the sample TWW and UF storage, UF+RO and UF+RO storage ($p < 0.001$). The total load of bacteria also decreased significantly between UF and UF+RO ($p < 0.001$). A significative decrease ($p < 0.001$) was observed between TWW and UF+RO for the *intI1* ($p < 0.001$) gene. In terms of *E. coli* load, TWW effluent presented a load significantly higher than all the other samples̓ load ($p < 0.001$). The gene *uidA* was not quantified in the samples UF, UFstorage, UF+RO and UF+RO storage because of the results obtained for the *crAssphage* gene. The *crAssphage* gene was below the limit of quantification in UF/ UF + RO / UF storage / UF+RO storage samples.

and values below the limit of detection for TSS and turbidity. These processes also led to *E. coli* average reduction (log-units/volume) values of 3.5, 4.6 or to values below the quantification limits, respectively. Lower reduction values were observed for the examined biomarkers, specifically for NTP, UF and UF+RO were 1.1, 0.9 and 3.9 for the 16S rRNA gene, and 0.9, 1.5 and 2.9 for the *intI1* gene. Storage tests did not reveal significant increases of the genes analysed for any of the treatment processes tested (Fig. 4A, B). According to the *E.coli* enumeration, and considering the physicochemical characterization, treated wastewater with UF (0.3 log-units) and UF+RO (non-detected) would meet the class A minimum quality requirements (≤10 CFU /100 mL) for irrigation (Regulation EU 2020/741) with both treatments exhibiting a *E. coli* reduction above 4.5 log-units/100 mL.Based on these findings, UF, but not UF+RO samples, given the limitations imposed by the extremely low DNA concentration, was selected for further comparison with freshwater. Under the conditions tested, NTP produced class B water for irrigation reuse, demonstrating its potential for tertiary treatment.

3.4. Benchmarking between wastewater and freshwater

Samples collected throughout the conventional wastewater treatment (RWW-TWW-SF+Cg-UV-Cis), pilot UF and from freshwater sources, including two rivers and a borehole, were compared based on their genetic profiles obtained by qPCR array (16S rRNA gene and 47 other genes) (Table S4). The most abundant (log-units/100 mL) genetic



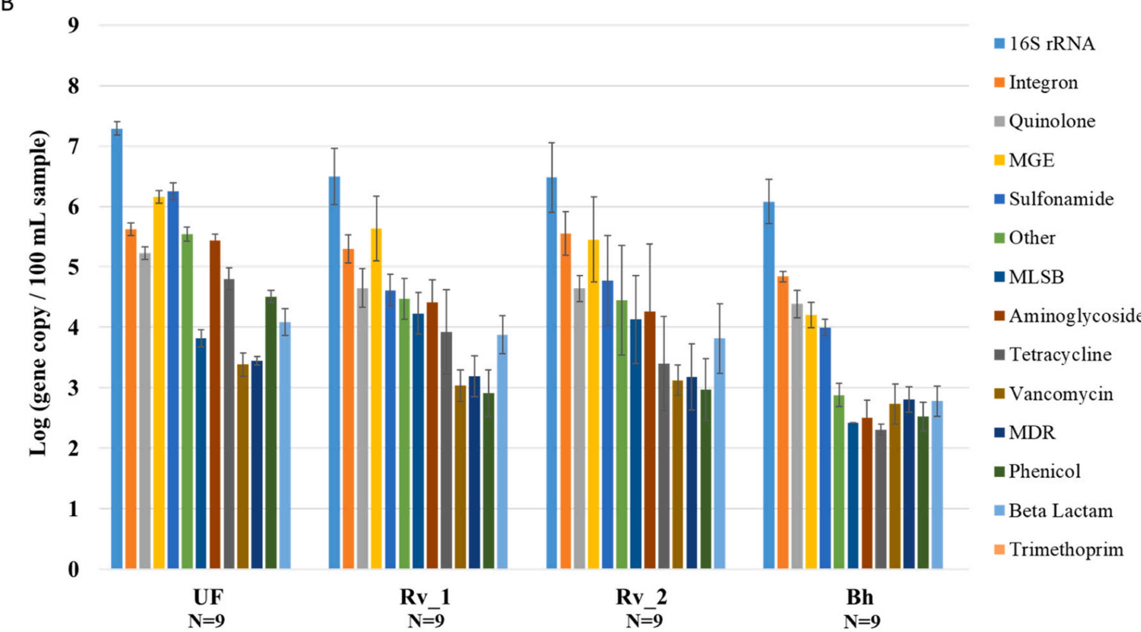


Fig. 5. Resistome profile assessed by qPCR array – A) Resistome in UWTP samples; B) Resistome in wastewater treated with ultrafiltration (UF) and freshwater samples from river (Rv_1 and Rv_2) and a borehole (Bh). RWW – raw wastewater; TWW – secondary effluent after secondary settling; SF+Cg – effluent after sand filtration and coagulation; UV – UV-B effluent; Cis out – treated wastewater stored; Cis rec – treated wastewater stored recirculated to the UV channel; UF – effluent after ultrafiltration; Rv_1 – river water 1; Rv_2 – river water 2; Bh – borehole.

elements in all samples were integron, mobile genetic elements (MGE) and sulfonamide, ranging from 4.8 to 7.7, 4.2–8.1 and 4.0–7.4, respectively (Table S8). The least abundant genes belonged to the classes phenicol and trimethoprim, ranging from 2.5 to 5.7 log-units/100 mL and <LOQ-5.6 log-units/100 mL, respectively. This analysis confirmed the secondary treatment as the most important stage for antibiotic resistance reduction (Fig. 5A). In addition, the results showed that antibiotic resistance and related genes were > 2 log-units/100 mL more abundant in TWW treated with sand filters and coagulation and UV than in freshwater (Fig. 5). While the 48 genes screened were detected in all the wastewater types, a lower number was above the detection limit in freshwater (n = 41 in Rv_1; n = 44 in Rv_2; n = 24 in Bh). In UF effluent, 31 genes were detected out of 48 present in RWW, suggesting the efficacy of that treatment. On average, UF samples contained (log-units/100 mL) 4.6 of antibiotic resistance genes, 6.2 of mobile genetic elements and 5.6 of *intI*, higher values than those obtained in river samples (3.9, 5.6, 5.3 in Rv_1 and 3.7, 5.5, 5.5 in Rv_2, respectively) or in the borehole (2.9, 4.2, 4.8, respectively) (Fig. 5B, Table S8). Compared with river samples, UF contained significantly higher abundance (log-units/100 mL) of sulfonamide resistance genes (6.3 ± 0.1 vs. 4.6 ± 0.3 Rv_1 or 4.8 ± 0.7 Rv_2*)*, specifically the genes *sul1* and *sul2.* Nevertheless, UF contained significantly lower abundance of the genes *qnrS2* (Rv_1), *crAss64*, *InuC*, bla_{GES} and bla_{OXA-58} (Rv_1). Compared with borehole samples, UF contained significantly higher abundance (log-units/100 mL) of different genes, specifically the 16S rRNA gene, *intI1,* multiple mobile genetic elements (6.2 ± 0.1 vs. 4.2 ± 0.2*) (ISPps, IS6100, IS26_1, TnpA_1, TnpA_5, trfA)* and distinct resistance groups, namely sulfonamides (6.3 ± 0.1 vs. 4.0 ± 0.1*)* (*sul1_1* and *sul2_1),* *a*minoglycosides (5.4 ± 0.1 vs. 2.5 ± 0.3) (*aadA_1, strB, aadA5_2),* MLSB (*ermF),* tetracyclines (4.8 ± 0.2 vs. 2.3 ± 0.1) (*tetX),* phenicols (4.5 ± 0.1 vs. 2.5 ± 0.1) (*cmxA), beta-lactams* (4.1 ± 0.2 vs. 2.8 ± 0.3) *(*bla_{CTX-M} *and* bla_{VEB}) and *qacEdelta* (5.5 ± 0.1 vs. 2.9 ± 0.2*)* (Fig. 5B).

Bacterial community analysis based on 16S rRNA gene metabarcoding allowed the comparison of UWTP treatment stages, UF and freshwater samples, with the identification of 13 phyla that presented relative abundance values higher than 1 % (Figure S1). In the wastewater samples, the highest number of OTUs was identified in TWW and the lowest in UF. The average wastewater OTU numbers (RWW- 1743 ± 172, TWW- 3240 ± 517, SF+Cg– 2076 ± 244, UV- 2238 ± 236, Cis rec- 2741 ± 37, Cis out- 2481 ± 356, UF- 1244 ± 4) were always lower than in the freshwater samples (Rv_1- 4402 ± 814, Rv_2- 4120 ± 818, Bh- 3838 ± 181), which suggest a higher species richness in freshwater (Chao1 index, Table S9). The overall diversity expressed by the Shannon diversity index reached the highest values in TWW and Cis out (5.6) and in river water (5.8) (Table S9). At the phylum level, *Pseudomonadota* were observed to predominate in all samples, with an average relative abundance ranging from 45.4 % in Cis rec samples to 76.7 % (*Gamma-* and *Betaproteobacteria*) in the borehole. At the family level, wastewater treatment led to three important shifts. The first referred to the extensive removal of members of the *Aeromonadaceae, Campylobacteraceae* and *Moraxellaceae* during biological treatment, presenting significantly higher relative abundance in RWW than in TWW (13.0 %, 20.6 %, 27.6 % in RWW *vs.* 1.7 %, 7.9 %, 5.9 % in TWW, respectively) (Fig. 6). The second, consisted of the significant increase ($p < 0.01$) of the relative abundance of the non-validly named families GU199451_f and CP011215_f after SF+Cg and UV (8.0 % and 0.3 % in TWW *vs.* 40.8 % and 1.3 % in SF+Cg, 36.9 % and 3.0 % in UV). Both taxa, GU199451_f and CP011215_f, decreased after storage, with the significant increase of members of the families *Azonexaceae*, *Intrasporangiaceae*, *Nocardiaceae* (UV to cistern - 1.8–5.1 %; 0.9–5.3 %, and 1.1–7.5 %). The third shift was observed after UF, consisting of a significant increase of *Alcaligenaceae*, *Comamonadaceae*, *Chitinophagaceae*, *Flavobacteriaceae,* and *Sphingomonadaceae* (0.5 %, 9.3 %, 0.3 %, not detected and 0.5 % in TWW vs. 5.6 %, 40.5 %, 12.1 %, 10.1 % and 6.6 % in UF, respectively). These families showed a significantly lower relative abundance in freshwater (*Alcaligenaceae* < 1 %, *Chitinophagaceae* < 3.3 %, *Flavobacteriaceae* <2 %, *Sphingomonadaceae* <2.2 %) (Fig. 6). River and borehole freshwater samples were significantly different in the relative

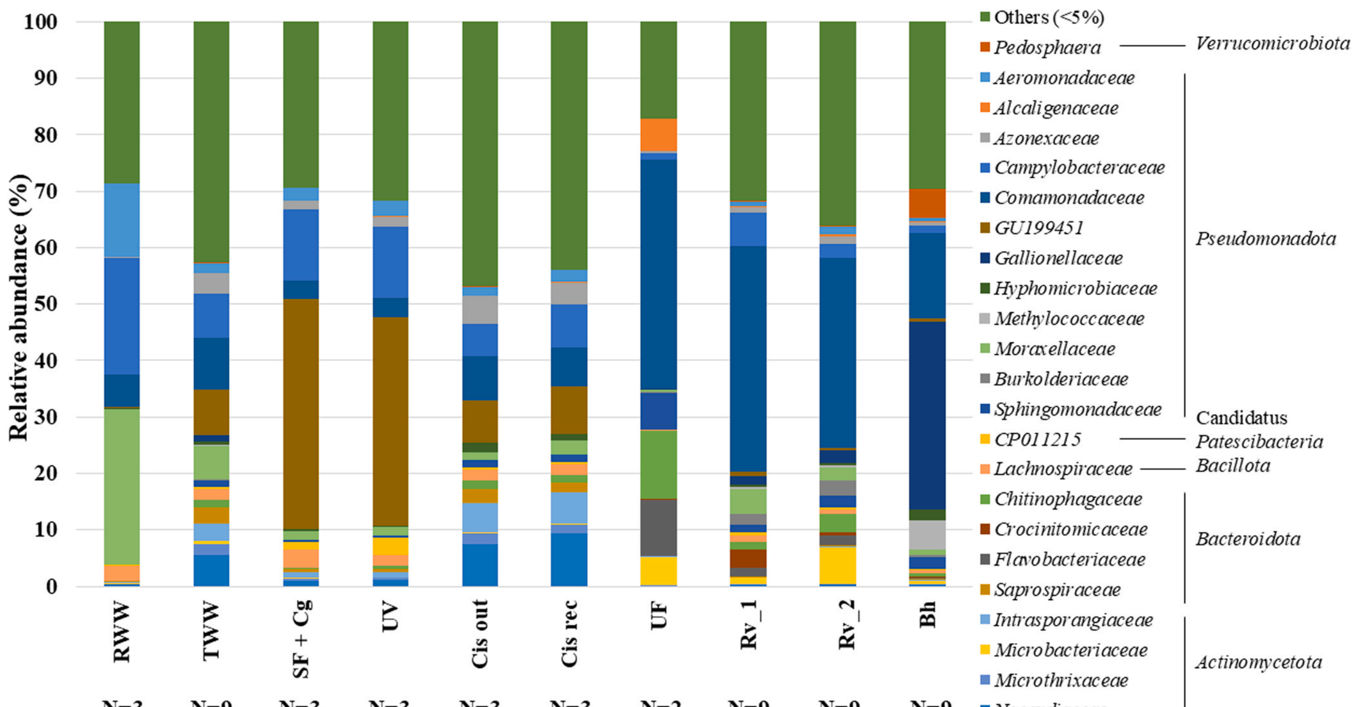


Fig. 6. Bacterial diversity at the family level (based on an average number of reads: RWW – 61291; TWW-70892; SF + Cg – 51357; UV- 65051; Cis rec – 74906; Cis out – 64076; UF – 78291; RV_1 – 70840; RV_2- 66508; Bh –72840). RWW – raw wastewater; TWW – secondary effluent after secondary settling; SF+Cg – effluent after sand filtration and coagulation; UV – UV-B effluent; Cis out – treated wastewater stored; Cis rec – treated wastewater stored recirculated to the UV channel; UF – effluent after ultrafiltration; Rv_1 – river water 1; Rv_2 – river water 2; Bh – borehole.

abundance of members of the families *Comamonadaceae* and *Gallionellaceae* (15.2 % *vs.* >33.5 % and 33.3 % *vs.* <2.4 %).

4. Discussion

Water stress, due to scarcity, climate change and pollution, and the urgent need of protecting natural resources has encouraged circular economy practices, in which the reuse of treated wastewater arises as a priority (European [15,39]). In parallel, the holistic perception of factors influencing human health highlights the need to integrate human, animal and environmental health into One Health [55]. Antibiotic resistant bacteria and antibiotic resistance genes interlink circular economy and One Health. The first, because these contaminants can be spread through circulated materials, being wastewater/water among the most important candidates [26]. The second, because as self-replicating entities these contaminants can spread in the environment and be transmitted to humans, through water or the food-chain [23,48]. These One Health issues negatively affect the public acceptance of the reuse of treated wastewater for agricultural irrigation and therefore water circularity. A common misconception is that freshwater sources are safer for irrigation than treated wastewater. However, this is not necessarily true, as important levels of river water contamination with antibiotic resistant bacteria and antibiotic resistance genes has been reported worldwide [11,60].

This study was designed to make this benchmarking, focused on bacterial community and antibiotic resistance determinants, by comparing wastewater collected throughout a conventional UWTP process, after advanced disinfection, and then comparing these samples with freshwater. The study of the complete UWTP treatment line was important to trace some resistance determinants, mobile genetic elements and bacterial populations. The analysis of the variable region of class 1 integrons shed some light on the dynamic of acquired antibiotic resistance genes throughout the process. Genes belonging to the aminoglycoside, beta-lactam and trimethoprim resistance classes were among the most frequent inside the variable regions of integrons across the complete treatment line, confirming previous studies [5,22,63,65,70]. However, due to bacterial community rearrangement or genetic recombination events, the proportions of these genes varied throughout the process, suggesting that treatment conditions may be tuned to promote more extensive reduction of unwanted genes. As it could be expected, secondary treatment was observed to be the most effective barrier to reduce antibiotic resistance determinants [21,43,46] and caused an important shift in the bacterial population with the reduction of important opportunistic pathogens and antibiotic resistance carriers, specifically members of the family *Aeromonadaceae, Campylobacteraceae,* and *Moraxellaceae* [51,58]. Interestingly, the analysis of the variable regions of integrons showed that also the number genetic linkages decreased sharply after secondary treatment, suggesting that excision of antibiotic resistance gene may occur at this stage (Table S7). The conventional treatment also had a significant impact on reducing the effluent organic matter and solids, however, the TWW did not meet the minimum quality requirements for reuse [52], as it exceeded the defined *E. coli* and solids (TSS and turbidity) thresholds. Confirming previous studies, it was observed that TWW contained a load of antibiotic resistance determinants capable of negatively impact the surrounding environment [17,27], although in the current study the discharge is made in the ocean at a 30 m depth.

Four different advanced treatment solutions were tested to improve the chemical and microbial quality of the TWW and produce treated wastewater suitable for reuse in irrigation. Although TWW with post-treatment of SF+Cg+UV complied with the physicochemical quality requirements for reuse (Table S3), because of *E. coli* it met exclusively class D [52] minimum quality requirements for reuse that has a very limited application for crop irrigation. Sand filtration and coagulation contributed to reduce fecal contamination and caused a dramatic shift in the bacterial community, although it showed a limited capacity to reduce most of the 48 genetic determinants examined by PCR array (Fig. 2 and Fig. 5A). The events occurring after coagulants and sand filtration seem to impose important changes in the resistome and community, however it is not possible to determine what can be attributed to one or the other. It is suspected that the aggregation of biomass may cause some of the variations observed, including the significant reduction of the total bacteria load, *crAssphage*, *intI1*, *uidA* and *aph(3')-ib* genes, 16S rRNA, *qepA*, *tnpA_5*, *IS26_1*, *tnpA_1*, *sul1* and *sul2*, *folA*, *crAssphage64*, *InuC*, *ereB_2*, *tetM*, *ars2*, *mexA*, bla_{NDM}, bla_{VIM} and bla_{OXA-48} (Fig. 2 and Fig. 3) and in the community a sharp increase in the relative abundance of *Azonexaceae, Saprospiraceae, Intrasporangiaceae, Microthrixaceae* and *Nocardiaceae*. These observations may suggest that the aggregation was not homogenous within the whole community. The UV disinfection step also failed to provide any significant reduction in microbial contamination. For the applied UV dosage, reductions in *E. coli* from 1 to 3 log units have been reported for real effluents, which is highly dependent on the suspended solids content in the effluent, as the presence of particles can obstruct and disperse UV light [14]. The poor results obtained could be due to the low TWW transmittance that was below the accepted threshold of 50 % to make UV disinfection practical [2,68]. After UV disinfection, the abundance of some genes was observed to significantly increase. Previous studies have suggested that extensive removal of antibiotic resistance determinants may require a UV dose higher than that normally used in UWTPs, [31,43,67]. Also, previous studies suggested that the application of UV treatment may trigger the occurrence of horizontal gene transfer [28,31]. However, we did not detect relevant indications that the variable regions of class 1 integrons were rearranged after UV exposure, except for the *aadA2_1* gene, which relative abundance increased significantly ($p < 0.01$) (Fig. 3). Storage did not significantly affect the antibiotic resistance load in treated wastewater (Fig. 2). However, some taxa, mainly of environmental bacteria (*Azonexaceae*, *Intrasporangiaceae*, *Nocardiaceae*) [7,47] significantly increased in relative abundance at this stage (Fig. 6). These results differ from the previous study by Leão et al. [36] that demonstrated that the storage of wastewater treated by membrane-aerated bioreactor in an open reservoir led to reductions between 1.0 and 1.5 log-units per mL of sample of *E. coli*, antibiotic resistance genes and mobile genetic elements. Discrepant results may be due to the use of different treatment pipelines and mainly to the fact that in this study the reservoir was a closed container, while in Leão et al. [36] it was an open tank, with sunlight and air exposure. Sand filtration with coagulation followed by storage (Fig. 1) was the post-treatment that most contributed to reduction of the *E. coli* load (values of 1.5 log-units / 100 mL), without raising risks related with antibiotic resistance (Fig. 2 and Supplementary Table S5), as can be assumed based on the decrease in the richness of the genetic elements measured in these samples.

All pilot-scale treatment processes tested improved the physicochemical TWW quality within the EU reuse regulations standards, in particular UF+RO was able to reduce BOD5, TSS and turbidity below detection limits. Although for irrigation purposes this configuration might be too costly, its application has been expanding for potable reuse in areas with severe water scarcity, where a very high level of water purification is demanded, especially of biological contaminants [29]. In our study UF+RO reached the highest reduction values for 16S rRNA, *intI1* genes and *E. coli* (3.9 log-units/100 mL, 2.9 log-units/100 mL, and 5 log CFU/100 mL (Figs. 4 and 7). Comparatively, for UF alone, *E. coli* reduction was also high (4.6 log CFU/100 mL),but 16SrRNA and *intI1* gene reductions were lower, with 0.9 and 1.5 respectively, which demonstrates the superior retention RO membranes offer to biological contaminants [29]. This agrees with previous studies that reported *E. coli* removal over 4 log CFU/100 mL with UF [25], although higher reduction of 16S sRNA and *intI1* gene were reported in that study (>4 log-units). This could be due to different UF membrane characteristics or operating conditions, such as the addition of precipitating agents. NTP achieved an *E. coli* reduction of 3.5, which agrees with the few studies

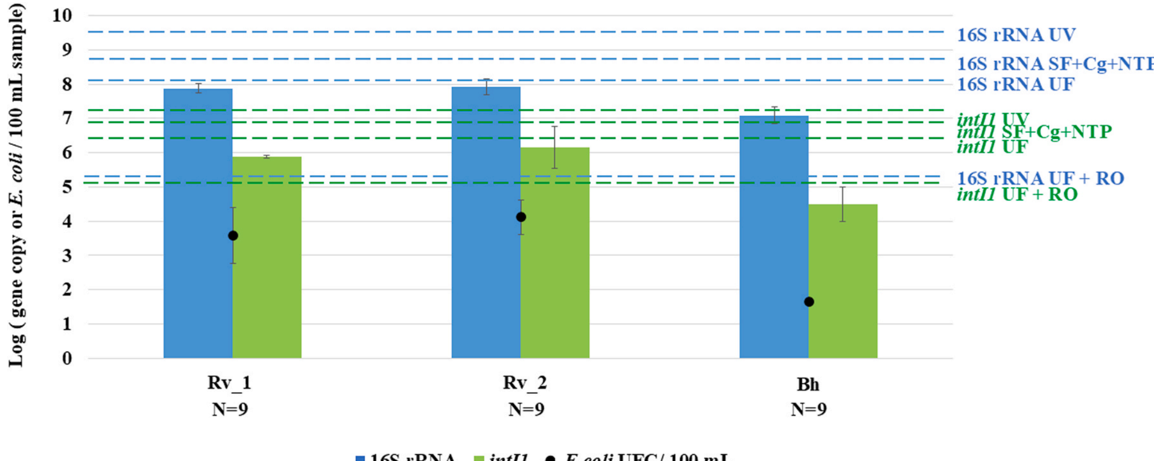


Fig. 7. Comparison of the total bacteria abundance (16S rRNA gene) and the genetic recombination indicator (*intI1* gene) with the loads obtained in the advanced treatments. UV – UV-B treated effluent; UF – effluent after ultrafiltration; UF + RO – effluent after reverse osmosis; SF+Cg+NTP, non-thermal plasma; Rv_1 – river water 1; Rv_2 – river water 2; Bh – borehole.

that reported this technique for *E. coli* removal from wastewaters (3–4 log-units) [24,38] and demonstrates the potential of this technology, that is still at an early stage of development in batch operation [64], for tertiary treatment. However, observed reductions in 16S rRNA and *intI1* genes with NTP were more limited, thus further development of this emerging technology should consider improving these determinants removal.

The next and central questions of this study were how wastewater treatment affects the microbiota, including antibiotic associated resistance determinants, and if treated wastewater quality compares to that of freshwater sources. The answers to these questions are summarized in the constrained analysis presented in Fig. 8. Most of the resistance classes and mobile genetic elements were significantly associated with wastewater, mainly with bacterial families that predominate in RWW (*Aeromonadaceae, Moraxellaceae, Campylobacteraceae, Lachnospiraceae*). All these four families are associated with humans and human activities

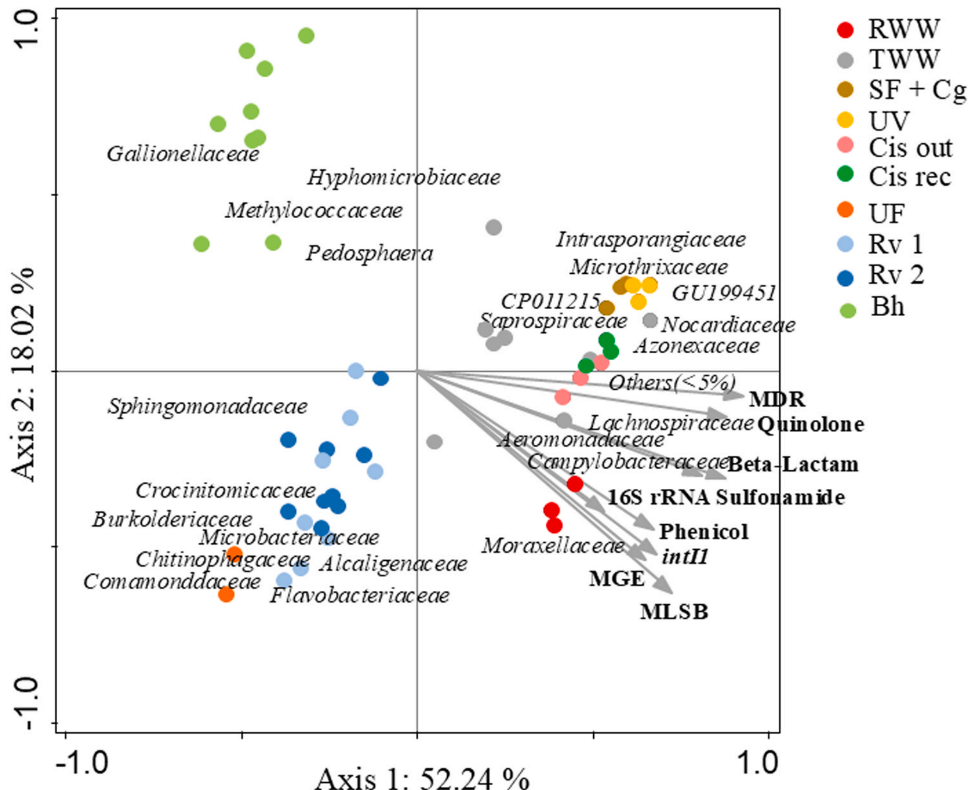


Fig. 8. Redundancy analysis of the bacterial community and the resistome profile. RWW – raw wastewater; TWW – secondary effluent after secondary settling; SF+Cg – effluent after sand filtration and coagulation; UV – UV-B effluent; Cis out – treated wastewater stored; Cis rec – treated wastewater stored recirculated to the UV channel; UF – effluent after ultrafiltration; Rv_1 – river water 1; Rv_2 – river water 2; Bh – borehole. This analysis showed a total variation of 525.49296, where the explanatory variables account for 73.7 %. The explanatory variables were MDR (33.6 % and $p = 0.001$); MLSB (10.7 % and $p = 0.001$); 16S rRNA (9.1 % and $p = 0.001$); Beta-Lactam (5.7 % and $p = 0.001$); Sulfonamide (3.8 % and $p = 0.001$); *intI1* (3.7 % and $p = 0.001$); MGE (2.5 % and $p = 0.001$); Quinolone (2.5 % and $p = 0.002$) and Phenicol (2.0 % and $p = 0.002$).

[20,69,71,74]. These results highlight the crucial importance of secondary treatment to remove bacteria and genes of human origin and potential clinical relevance. Indeed, a specific genetic pool of wastewater bacteria and genes seems to persist from RWW to UV, and it can be argued that improving secondary treatment could be pivotal to smooth and make less energy demanding the post-secondary and advanced treatment processes. As described by Cachetas et al. [12], wastewater has a characteristic core resistome with genes that are not found in freshwater samples, the removal of these resistome components at the earliest possible stage of wastewater treatment should be the aim. Although UF water was dominated by bacterial populations that are not associated with human commensal bacteria (Fig. 8), or key antibiotic resistance harbors, it did not meet the quality criteria of freshwater. Indeed, compared to freshwater, UF water contained a much lower species richness, with some groups – *Alcaligenaceae, Sphingomonadaceae, Chitinophagaceae* and *Microbacteriaceae*, becoming predominant and with higher abundance of resistance and mobile genetic elements. These results showed that despite the advanced treatment, wastewater that is disinfected at the end of the line may deviate considerably of the microbiological quality standards of freshwater. It has been consistently shown that the competition of native microbiota in water is essential to hamper the dissemination of antibiotic resistant bacteria and genes [32, 53]. Of note, is the fact that in this study the freshwater sources were not exempt of acquired antibiotic resistance genes (Fig. 5), exemplifying a real-world freshwater scenario, as has been shown in numerous publications [1,10–12,61]. This benchmark also showed that if the same quality criteria for water reuse for direct irrigation were to be applied to the freshwater samples, RV_1 would be classified as class D and RV_2 could not be used for irrigation purposes, due to the high *E. coli* content, while UF produced class-A water for reuse. This fact highlights that the common misconception that freshwater sources are safer for irrigation than treated wastewater might not hold in some contexts. On the other hand, it was shown that UF had higher abundance of resistance and genetic recombination elements than freshwater, which can present a higher risk from a One Health perspective. Remarkably, also among freshwater samples differences were observed in the bacterial community (Fig. 6), possibly suggesting the isolation of the borehole from local anthropogenic sources associated with the wastewater/pollution. Borehole community, although sharing part of its bacterial community members with river water, was characterized by a dominance of members of the families *Gallionellaceae*, *Comamonadaceae, Pedosphera* and *Methylomonas*, associated mainly with pristine water sources [40,41].

5. Conclusion

This study has shown that secondary treatment is critical in determining the quality of treated wastewater. As well as contributing to the most extensive removal of bacteria of human origin and antibiotic resistance determinants, it was also shown to be critical in reducing the degree of linkage of antibiotic resistance genes within the variable region of class 1 integrons, suggesting excision and loss of these genes. Advanced treatment by pilot-scale UF successfully removed *E. coli*, fecal contamination biomarkers and antibiotic resistance genes from the effluent, but the microbiological quality did not match that of freshwater sources. A higher load of antibiotic resistance genes and a distinct and poorer bacterial community in UF wastewater contrasted with the quality of the freshwater source, even though the latter was also of poor quality. These results show that in some cases, treated wastewater may be preferable to freshwater for irrigation purposes. However, the same results raise the important challenge of how to treat wastewater to achieve the healthy microbiological quality expected in freshwater sources. Improving the quality of treatment at the earliest possible stage, during secondary treatment, could help to reduce the load of human commensal bacteria and antibiotic resistance genes, producing a final effluent that may be smothered by disinfection and therefore less aggressive to native microbiota.

CRediT authorship contribution statement

Leão Inês: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Antunes Jorge:** Writing – review & editing, Investigation, Formal analysis. **Manaia Célia:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Jorge Ruben:** Resources, Project administration, Funding acquisition, Conceptualization. **Baptista Inês:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Löblich Stefan:** Resources, Conceptualization. **Marinheiro Luís:** Resources, Conceptualization. **Vaz-Moreira Ivone:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.116172.

Data availability

Data will be made available on request.

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