



Survival of clinical and environmental carbapenem-resistant *Klebsiella pneumoniae* ST147 in surface water

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ABSTRACT

Carbapenem-resistant *Klebsiella pneumoniae* represents a healthcare threat, already disseminated in the environment. This study aimed to compare the behaviour of a clinical and an environmental *K. pneumoniae* strain (multilocus sequence type ST147) harbouring the gene *bla_{KPC-3}* in water. The abundance of the genes *phoE* (specific for *K. pneumoniae*) and *bla_{KPC-3}* was monitored by quantitative PCR in urban runoff water and sterile ultra-pure water microcosms, aiming to assess survival, *bla_{KPC-3}* persistence, and the effect of the native water microbiota. In sterile ultra-pure water, the abundance of cultivable *K. pneumoniae* and *bla_{KPC-3}* gene did not change over the incubation period (8 days). In contrast, in urban runoff, the *K. pneumoniae* and the genes *phoE* and *bla_{KPC-3}* genes decreased by up to 3 log-units. These results suggest that *K. pneumoniae* were outcompeted by the native microbiota of the urban runoff water and that the decay of *bla_{KPC-3}* gene was due to host death, rather than to gene loss. The study highlights that although native microbiota is essential to hamper the persistence of non-native bacteria, carbapenemase producing *K. pneumoniae* can survive in urban runoff water for at least one week.

1. Introduction

Klebsiella pneumoniae are ubiquitous bacteria that integrate the list of priority pathogens while having an important environmental distribution (Holt et al., 2015; Rocha et al., 2022b). The capacity of *K. pneumoniae* to acquire and spread novel antibiotic resistance genes (ARGs) has been demonstrated (Navon-Venezia et al., 2017; Yu et al., 2019; Zhou et al., 2020) and is the major driver for its clinical relevance (CDC, 2019; WHO, 2020; ECDC, 2022). The increasing rates of carbapenem resistance in *K. pneumoniae* are of special concern since non-native infections may lead to high mortality rates (up to 40–50% in some studies) (CDC, 2015; Xu et al., 2017; Lou et al., 2022). In Europe, while carbapenem resistance remained rare among *Escherichia coli*, 30% of the countries reported resistance percentage values equal to or above 25% in *K. pneumoniae* (ECDC, 2022). In the year 2020, the European countries with the highest carbapenem resistance prevalence in clinical *K. pneumoniae* were Greece (66.2% of 726 isolates), Romania (48.3% of 474), Italy (29.5% of 8293), Bulgaria (28.1% of 249), Cyprus (19.8% of

172), Croatia (19.1% of 267), and Portugal (11.6% of 2780) (Surveillance Atlas of Infectious Diseases). The widespread occurrence of carbapenem resistant *K. pneumoniae* can also be inferred from the public database Patric v3.6.12 ([https://www.bv-brc.org/view/GenomeList/?and\(keyword\(Klebsiella\),keyword\(pneumoniae\)\)#view_tab%20=%20genomes&filter%20=%20false](https://www.bv-brc.org/view/GenomeList/?and(keyword(Klebsiella),keyword(pneumoniae))#view_tab%20=%20genomes&filter%20=%20false), accessed March 27, 2023) that showed that among a total of 27 131 genome sequences of worldwide isolates, more than 10% were putatively carbapenem-resistant. At least in clinical settings, some *K. pneumoniae* genetic lineages are observed to predominate, probably due to their high fitness under diverse conditions (Holt et al., 2015). Among these lineages are members of the multilocus sequence types ST258, ST11, ST307, or ST147 the most frequent among the 32 642 genome sequences available in the Centre for Genomic Pathogen Surveillance public database Pathogen Watch (<https://pathogen.watch/genomes/all?speciesId=573>, accessed March 21, 2023), represented by 4895, 2790, 1855 and 1651 genomes, respectively. Although when compared with the clinical settings, the information about *K. pneumoniae* thriving in the natural environment is scant, the

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ubiquity of this species across water and wastewater, plants, soil, and animals has been consistently demonstrated (Holt et al., 2015; Rocha et al., 2022b; Thorpe et al., 2022). Carbapenem-resistant *K. pneumoniae* have been reported in aquatic environments such as wastewater and river water (Ekwanzala et al., 2019; Lepuschitz et al., 2019; Teixeira et al., 2020). And, in a recent study, Zou et al. (2023) demonstrated that the same lineages of carbapenem-resistant *K. pneumoniae* may be isolated from the hospital and associated aquatic environments (wastewater treatment plant and the receiving river).

In previous studies, we aimed to investigate if clinical and environmental *K. pneumoniae* could be differentiated based on some specific genetic features that might hint distinct fitness properties or if genes acquired as an advantage in clinical contexts, such as antibiotic resistance, might be lost once in the environment (Rocha et al., 2022a, 2022b). As no evidence of such differentiation was observed at the genomic level, the present work was designed to compare the survival of *bla*_{KPC-3} *K. pneumoniae* strains in urban runoff water microcosms. To control the experimental conditions, one clinical and one river sediment isolate, both belonging to the ST147 and harbouring the *bla*_{KPC-3} variant of the gene, were selected. The work aimed to test three hypotheses: i) that the elimination of the gene *bla*_{KPC-3} would depend on the bacteria host decay; ii) that the clinical isolate would decay faster than the environmental one; and iii) that the water microbiota would be able to outcompete non-native *K. pneumoniae*.

2. Material and methods

2.1. Carbapenem-resistant *Klebsiella pneumoniae*: origin, features, and genome analysis

A clinical and an environmental *K. pneumoniae* isolate were tested. Both belonged to the multilocus sequence type 147 (ST147), were resistant to carbapenem antibiotics, and harboured the gene *bla*_{KPC-3}. The clinical isolate (KP2-448) was collected from urine, and the environmental (SM1) from a river sediment, both characterized before (Ferreira et al., 2021, 2022; Rocha et al., 2022b). The genomes of both isolates were sequenced with Illumina HiSeq (151 bp, paired-end), *de novo* assembled using SPAdes v3.11.1 (Bankevich et al., 2012), and the absence of contamination validated both with ContEst16S (Lee et al., 2017) and CheckM (Parks et al., 2014). Assembled contigs were annotated with Prokka version 1.14.6 (Seemann, 2014) and RASTtk (Brettin et al., 2015). Antibiotic resistance genes were annotated with CARD-RGI (Alcock et al., 2020) and ResFinder 4.1 (Bortolaia et al., 2020), virulence factors, heavy metals resistance genes, and efflux systems were identified with the Bacterial Isolate Genome Sequence Database (BIGSdb available at <https://bigsdb.pasteur.fr/klebsiella/>) (Jolley and Maiden, 2010), and plasmid replicon types identified with PlasmidFinder 2.0 (Carattoli et al., 2014). SOS/stress response genes were compared based on RASTtk. The transposons were identified with the TETyper tool (Sheppard et al., 2018), and a comparative image was generated with Easyfig 2.2.5 (Sullivan et al., 2011). The Average Nucleotide Identity (ANI) between the two genomes was determined with the ANI calculator, available in the Enveomics collection (Rodriguez-R & Konstantinidis, 2016). The origin of replication and the genes presumably involved in the plasmid mobility were annotated with the web-based tool oriTfinder (Li et al., 2018). The assembled genome sequences are deposited under the accession numbers GCF_019093695.1 and GCF_024179105.1.

Both isolates were tested for tolerance (survival) to temperature, UV-C radiation, or H₂O₂. Briefly, fresh bacterial suspensions (10⁸ colony forming units (CFU)/mL) were prepared in saline solution (0.85%, w/v) and immediately exposed to one of the studied conditions (50 °C for 30 min; UV-C radiation for 0.5 min; 400 mM H₂O₂ for 15 min). A detailed description of the procedures is given in the Supplementary Material.

2.2. Microcosm assays

The water used for microcosm assays was collected on four different dates from an urban runoff (SW) that discharges into a beach with an area of 115.000 m², capacity for 10.000 people, and water temperature ranging 9–19 °C (Winter-Summer) (<https://www.cm-matosinhos.pt/servicos-municipais/ambiente/praias/matosinhos>). This beach is used throughout the year for surfing. For each strain, microcosms were prepared in triplicate with SW collected on three different dates (Table S1), representing at least 9 measurements for each (3 assays in triplicate). Each assay was prepared in 120 mL flasks containing 89.1 mL of SW water or sterile ultra-pure water (UP) and 0.9 mL of a *K. pneumoniae* bacterial suspension to obtain a final density of around 10⁵ CFU/mL. The controls consisted of non-inoculated SW, or UP inoculated with each of the test strains. The microcosms were incubated at 30 °C, for up to 8 days, and sacrificed for enumeration of total heterotrophs and total DNA extraction, immediately after being prepared (time 0, T0) and after 1, 3, or 8 days (T1, T3, T8) of incubation for SW assays. The UP controls were sampled at T0 and T8 (Table S1).

2.3. Microcosms assay analyses

Total heterotrophs were enumerated on serially diluted samples (from an initial volume of 1 mL) collected from each microcosm assay by spread plating on Luria-Bertani agar (LA), incubated at 37 °C for 24 h. Total DNA was extracted from each microcosm assay (from a volume of 89 mL), after filtration through a polycarbonate membrane (0.22 µm porosity, Whatman, Brentford, UK), using the NZY Tissue gDNA Isolation kit (NZYTech, Portugal) according to the manufacturer's instructions with the alterations described by Rocha and Manaia (2020). DNA concentration was measured using the Qubit fluorometer (Thermo Fisher Scientific, USA). The 16S rRNA gene was measured in the total DNA extracts, using universal primers (total bacteria), or specific for *Pseudomonadota* classes (*Alpha*-, *Beta*-, *Gammaproteobacteria*). In addition, the genes *phoE* (amplicon specific for *K. pneumoniae*) and *bla*_{KPC} genes were quantified. Gene quantification used Real-Time PCR (qPCR, StepOnePlus™ Real-Time PCR System, Life Technologies, Carlsbad, CA, USA), based on the standard curve method as described by Brankatschk et al. (2012) with the conditions described in Table S2. Possible qPCR inhibition was assessed as suggested by Bustin et al. (2009), by the quantification of the target genes in serially diluted samples.

Aiming the profiling of the SW bacterial community and its variation over the incubation period, the non-inoculated stream water microcosm (M3_SW_C) was analysed in DNA pools prepared from triplicates of T0, T1, T3, or T8 samples, based on (300 bp) amplicon sequencing of the 16S rRNA gene variable regions V3–V4 (paired-end Illumina MiSeq Personal Sequencer). The raw reads were processed, normalized to the lowest number (77349), and the bacterial diversity was analysed using the EzBioCloud 16S-based MTP pipeline (Yoon et al., 2017), and the method CL_OPEN_REF_UCLUST_MC2 with a cut-off of 97% similarity for OTUs definition.

2.4. Statistical analysis

Gene abundance and gene prevalence were expressed as gene copy number per mL of sample or per 16S rRNA gene copy number, respectively. The enumeration of total heterotrophic bacteria was expressed as log units of CFU/mL of sample. The one-way analysis of variance (ANOVA) and Tukey's and Bonferroni's post-hoc tests (SPSS Statistics for Windows v.24.0; IBM Corp., Armonk, NY, USA) and t-student test (PAST v4.0) (Hammer et al., 2001) were used to assess statistically significant differences (*p* < 0.01) in cultivable bacteria counts, abundance and prevalence of measured genes over time, and decay rates between the microcosms inoculated with the clinical or environmental strain, or in comparison with the controls.

3. Results

3.1. Genomic- and phenotypic-based comparison of the environmental and clinical strains

As expected, the clinical and the environmental *K. pneumoniae* strains shared a high Average Nucleotide Identity (99.98%), although with slightly distinct genome sizes, number of RNA coding regions, and proteins (Table S3). The genomes of both strains shared the same set of genes associated with plasmid replicon types, virulence factors, and efflux systems and shared almost all the SOS/stress response encoding genes (Table 1). In contrast, the profile of antibiotic resistance genes was

Table 1

Genetic determinants associated with resistance to different antibiotics, virulence factors, resistance to heavy metals, efflux systems, SOS/stress response, and mobile genetic elements in the *Klebsiella pneumoniae* clinical isolate KP2-448 and environmental isolate SM1. More detailed information is available in Table S4.

	KP2-448	KP2-448 and SM1	SM1
Antibiotic resistance genes ^(a)	<i>aadA16</i> , <i>aadA1</i> , <i>aac(3)-IIa</i> , <i>aac(6')-Ib-cr</i> , <i>bla_{OXA-1}</i> , <i>bla_{SHV-1}</i> , <i>qacE</i> , <i>qnrB6</i> , <i>catB3</i> , <i>arr-3</i> , <i>sul1</i> , <i>dfrA27</i> , <i>dfrA1</i>	<i>bla_{KPC-3}</i> , <i>fosA</i> , <i>oqxA</i> , <i>oqxR</i>	<i>bla_{SHV-11}</i> , <i>bla_{SHV-67}</i>
Virulence factors ^(b)		<i>wzi</i> , <i>fyuA</i> , <i>glxK</i> , <i>irp1</i> , <i>irp2</i> , <i>iutA</i> , <i>mrkA</i> , <i>mrkB</i> , <i>mrkC</i> , <i>mrkD</i> , <i>mrkF</i> , <i>mrkH</i> , <i>mrkI</i> , <i>mrkJ</i> , <i>ybtA</i> , <i>ybtE</i> , <i>ybtP</i> , <i>ybtQ</i> , <i>ybtS</i> , <i>ybtT</i> , <i>ybtU</i> , <i>ybtX</i>	
Heavy metals resistance genes ^(c)	<i>arsA</i> , <i>arsD</i> , <i>pcoA</i> , <i>pcoB</i> , <i>pcoC</i> , <i>pcoD</i> , <i>pcoE</i> , <i>pcoR</i> , <i>pcoS</i> , <i>silB</i> , <i>silC</i> , <i>silF</i> , <i>silG</i> , <i>silP</i> , <i>copG</i>	<i>arsB</i> , <i>arsC</i> , <i>arsR</i> , <i>silA</i> , <i>silE</i> , <i>silR</i> , <i>silS</i>	
Efflux systems ^(d)		<i>acrA</i> , <i>acrB</i> , <i>acrR</i> , <i>envR</i> , <i>fis</i> , <i>marA</i> , <i>marR</i> , <i>oqxR</i> , <i>oqxX</i> , <i>oqxY</i> , <i>ramA</i> , <i>ramR</i> , <i>rarA</i> , <i>rob</i> , <i>sdiA</i> , <i>soxR</i> , <i>soxS</i>	
SOS/stress response ^(e)	<i>mepM</i>	<i>tehA/tehB</i> , <i>dedA/cysA</i> , <i>betA/betB/betT</i> , <i>yehW/yehX/yehY/yehZ</i> , <i>aqpZ/osmY</i>	
Mobile genetic elements ^(f)	<i>incFIA(pBK30683)</i> , <i>intI1</i>	<i>incFIB(K)</i> , <i>incFIB(pKPHS1)</i> , <i>incFII(K)</i> , <i>incN</i>	

^a **Antibiotic resistance genes:** *aadA16*, *aadA1*, *aac(3)-IIa* (resistance to aminoglycosides); *aac(6')-Ib-cr* (aminoglycoside and fluoroquinolone); *qnrB6* (fluoroquinolone); *bla_{OXA-1}*, *bla_{SHV-11}*, *bla_{SHV-67}*, *bla_{KPC-3}* (beta-lactams); *sul1* (sulfonamides); *catB3* (phenolics); *arr-3* (rifamycin); *dfrA1*, *dfrA27* (diaminopyrimidine); *fosA* (fosfomycin); *qacE*, *oqxR*, *oqxX* (multidrug).

^b **Virulence factors:** *wzi* (capsule assembly protein); *fyuA* (pesticin receptor); *glxK* (glycerate 2-kinase); *irp1* (Yersiniabactin biosynthetic protein), *irp2* (Yersiniabactin non-ribosomal peptide synthetase HMWP2); *iutA* (ferric aerobactin receptor); *mrkA-mrkJ* (type 3 fimbriae); *ybtA-ybtX* (iron transport).

^c **Heavy metals resistance genes:** *arsA-arsR* (arsenic resistance); *pcoA-pcoS*, *copG* (copper resistance); *silA-silS* (copper/silver resistance).

^d **Efflux systems:** *acrA-acrR* (multidrug efflux RND transporter); *envR* (multidrug efflux pump *acrAB* operon transcription repressor); *fis* (DNA-binding protein); *marA* (AraC family transcriptional regulator); *marR* (DNA-binding transcriptional repressor); *oqxR-oqxX* (multidrug efflux RND transporter); *ramA* (transcriptional activator), *ramR* (transcriptional regulator); *rarA* (replication-associated recombination protein); *rob* (MDR efflux pump *AcrAB* transcriptional activator); *sdiA* (regulatory activator); *soxR* (redox-sensitive transcriptional activator); *soxS* (AraC family transcriptional regulator).

^e **SOS/stress response:** *mepM* (cell wall endopeptidase); *tehA/tehB* (tellurite resistance); *dedA/cysA* (uptake of selenate and selenite); *betA/betB/betT* (choline and betaine uptake and betaine biosynthesis); *yehW/yehX/yehY/yehZ* (osmoprotectant ABC transporter); *aqpZ/osmY* (osmoregulation).

^f **Mobile genetic elements:** *inc* groups (plasmid replicon types); *intI1* (class I integrons integrase).

distinct with genes associated with aminoglycosides and quinolones resistance observed only in the clinical isolate (Table 1), which is consistent with the antibiotic resistance phenotypes (Table S3). The *intI1* gene, encoding an integrase associated to class 1 integrons was also present only in the clinical isolate. The genome of the environmental isolate presented two variants of the beta-lactamase encoding genes *bla_{SHV}* (SHV-11 and SHV-67) distinct from that observed in the clinical isolate (SHV-1). In addition, the *pco* operon (associated with copper resistance) was observed only in the genome of the clinical isolate. The genes that were common to both strains shared a high sequence identity (100–99.95%). The gene *bla_{KPC-3}* integrated in an *incN* plasmid was inserted in a *Tn4401d* transposon with the 10 Kbp upstream and downstream regions sharing sequence identity values ranging 81–100% in both strains (Fig. 1). The *Tn4401d* flanking regions included genes with homology to those involved in mobility, such as the relaxase (*traI* F), T4CP (*traG*), and those of the T4SS genetic region (*trwN-D*), corroborating the conjugative phenotype previously described for the 55 Kbp *incN* plasmid of the clinical strain (Ferreira et al., 2021). Phenotypically, both strains differed in stress tolerance, as was confirmed after exposure to sublethal conditions of 50 °C (higher for the environmental strain), UV-C radiation, and hydrogen peroxide (both higher for the clinical strain) (Table S3).

3.2. Survival of *bla_{KPC-3}* + *K. pneumoniae* in urban runoff microcosm assays

The survival of the clinical and the environmental *K. pneumoniae* isolates in urban runoff water was assessed by comparing the abundance over time of the total and cultivable bacteria and the *phoE* and *bla_{KPC-3}* genes in inoculated microcosms of urban runoff water (SW) and the control consisting of sterile ultra-pure water (UP) (Figs. 2 and 3). Cultivable heterotrophic bacteria decreased 2.2 to 2.8 log units CFU/mL after 8 days, with non-significantly different decay rates (-1.51 ± 0.59 d⁻¹, -1.43 ± 0.27 d⁻¹, and -1.15 ± 0.03 d⁻¹ for the microcosms inoculated with the clinical and the environmental isolate, and non-inoculated microcosms, respectively) in all runoff water-based microcosms (Fig. 2A and S1). The abundance of total bacteria, assessed by the quantification of the 16S rRNA gene in the same samples, followed the same pattern (Fig. 2B). At T0, the abundance of total bacteria was about 2 log-units higher than of cultivable bacteria abundance (6.9 log-units vs. 4.7 log-units), which may be justified by the presence of multiple 16S rRNA gene copies in some genomes; for example *K. pneumoniae* have on average 8 copies (<https://rrndb.umms.med.umich.edu/>). The decrease of total bacteria was approximately half of what was observed for cultivable bacteria (~1 log-unit vs. >2 log-units, respectively) over the 8 days incubation period. Since free DNA was not expected to be retained in the filtering membrane, these results suggest that the loss of cultivability may be superior to the effective cell decay in both inoculated and non-inoculated microcosms. In contrast, in UP microcosms, no variation was observed for both isolates suggesting the lasting viability of these bacterial strains in clean water, where no microbial competition exists (Fig. 2A and B).

The quantification of the genes *phoE* and *bla_{KPC-3}* aimed to assess the occurrence of the acquired gene (*bla_{KPC-3}*) or cell death (*phoE*). In UP microcosms, for both isolates the abundance of the *phoE* and *bla_{KPC-3}* genes did not vary over the 8 days incubation period. These results indicate that *K. pneumoniae* cells maintained their viability and did not lose the acquired carbapenem-resistance gene, suggesting that it did not represent a fitness cost. In urban runoff water-based inoculated microcosms, for both isolates, both *phoE* and *bla_{KPC-3}* genes decreased significantly over T0-T8. The *phoE* gene presented a total reduction of 1.8–3.6 log-units, in line with the *bla_{KPC-3}* decrease in a total of 2.8–3.0 log-units reduction (Fig. 3A). In addition, the paired *t*-test showed that the rate of decay of the genes *phoE* (-2.10 ± 0.52 d⁻¹ and -2.07 ± 0.29 d⁻¹, respectively) and *bla_{KPC-3}* (-1.92 ± 0.15 d⁻¹ and -1.61 ± 0.77 d⁻¹, respectively) genes was non-significantly different for the clinical and

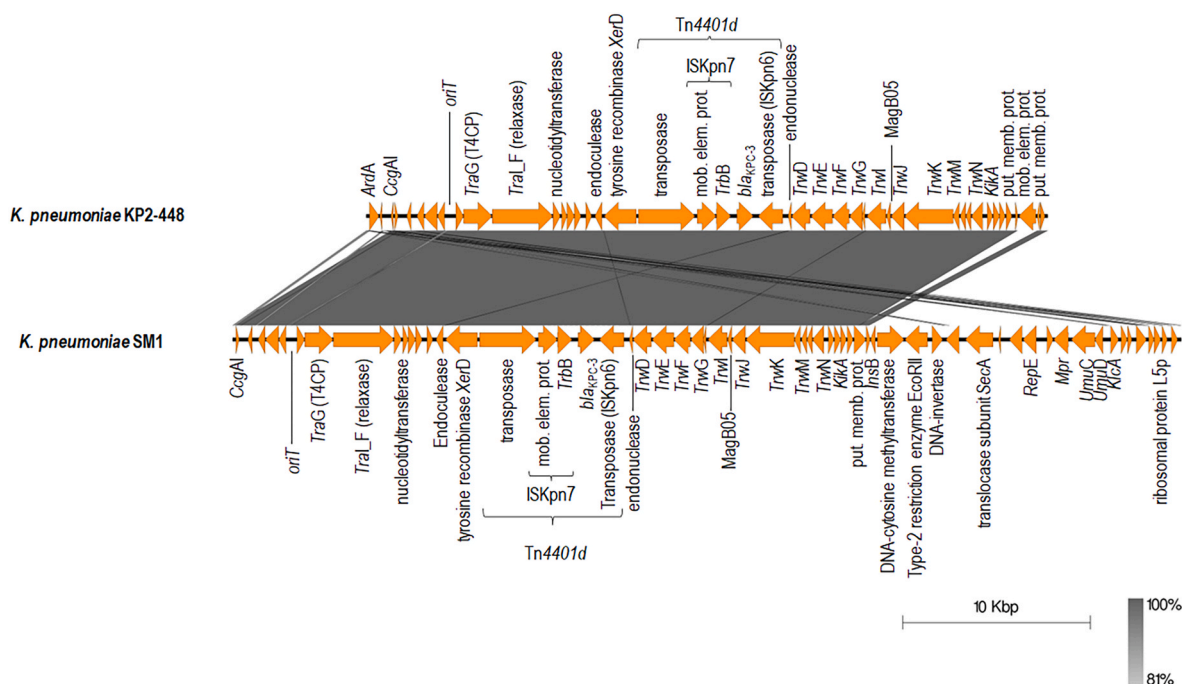


Fig. 1. Mapping of the *bla*_{KPC-3} gene genetic vicinity in isolates *K. pneumoniae* KP2-448 (clinic) and *K. pneumoniae* SM1 (environmental). In both isolates, the *bla*_{KPC-3} gene is located in a transposon, Tn4401d. Image generated by Easyfig 2.2.5. Note: The size of the contigs is 36341 bp for KP2-448 and 50595 bp for SM1.

the environmental isolate (Figure S2). The control assays, run in non-inoculated urban runoff water samples, showed the occurrence of low levels (up to 3 log-units/mL), sometimes below the limits of quantification, of the *bla*_{KPC-3} and *phoE* genes (Fig. 3). In these microcosms, the abundance of the *phoE* and *bla*_{KPC-3} genes did not vary significantly over the incubation period and their abundance was deduced in the test (inoculated) assays since we suspect, at least for the *bla*_{KPC-3} gene, that it can be a residual contamination of the lab. In general, the log reduction values, as well as the rate of decay of the genes *phoE* and *bla*_{KPC-3} were not significantly different from those of total heterotrophic bacteria for both inoculated and non-inoculated microcosms (Figures S3). These results showed that non-native (exogenous) *bla*_{KPC-3+} *K. pneumoniae*, regardless of clinical or environmental origin, can survive and the carbapenem-resistance gene can persist in water, with the native microbiota being an important limiting factor. These observations motivated the analysis of the SW native microbiota.

3.3. Urban runoff microbiota

The composition of the bacterial community of the urban runoff microcosms and its variation over 8 incubation days was assessed based on 16S rRNA gene metabarcoding analysis. The community was represented by > 1500 operational taxonomic units (OTUs, >97% sequence identity), mostly of the phylum *Pseudomonadota* (>67%) (Fig. 4A). Among these, members of the classes *Beta*- (16–31%), *Gamma*- (23–28%), and *Epsilonproteobacteria* (up to 31%) predominated, being the latter replaced mostly by *Alphaproteobacteria* over the incubation period (1% at day 0–26% at day 8) (Fig. 4B). The shift observed in the community during the 8 days of the microcosm incubation was mostly between *Epsilonproteobacteria* and *Alphaproteobacteria*, while the relative abundance of *Gammaproteobacteria*, which includes the family *Enterobacteriaceae* to which belongs the species *K. pneumoniae*, was stable over the incubation period. The class *Gammaproteobacteria* was mostly represented by members of the families *Aeromonadaceae* (0.6–10.3% of the total number of OTUs), *Moraxellaceae* (1.1–8.1%), and *Pseudomonadaceae* (0.4–4.0%), while *Enterobacteriaceae* constituted 0.7–3.4% of the total relative abundance (Figure S4). *Klebsiella* spp. were detected at

very low relative abundance values, representing 0.003–0.02% of the total number of reads (data not shown). Based on these results, *Alpha*-, *Beta*-, and *Gammaproteobacteria* were selected to further assess variations in their total and relative abundance in both non-inoculated and inoculated microcosms.

The quantitative analysis of *Alpha*-, *Beta*- and *Gammaproteobacteria* revealed that the abundance of members of these classes was similar in both non-inoculated and inoculated microcosms, as well as its rate of variation over the incubation period (Fig. 5 and S5). The abundance of *Gammaproteobacteria* per volume of water significantly decreased in 1.2–1.5 log-units/mL from 6.6 to 6.8 at T0 to 5.3–5.4 at T8 in all the microcosms (Fig. 5A). However, as observed in the bacterial community analysis (based on 16S rRNA gene sequence) of the non-inoculated microcosms, the prevalence of members of the class *Gammaproteobacteria* determined based on qPCR and normalized by total bacteria (log gene copy number/16S rRNA gene copy number), did not differ significantly over the 8 days in all the microcosms (Fig. 5B). A similar variation was observed for the abundance of *Betaproteobacteria*, which decreased in 1.3 log-units/mL from approximately 6.0 at T0 to 4.4–4.8 at T8, while the respective relative abundance, determined based on qPCR, was stable over the incubation period (Fig. 5). In contrast, the abundance of *Alphaproteobacteria* was similar over the incubation period, although the relative abundance shifted after T0, with significant increases of approximately 1 log gene copy/16S rRNA gene in all microcosms (Fig. 5). In the non-inoculated microcosms, this value corresponded to >20% increase in the relative abundance of amplicon reads after 8 days of incubation (Fig. 4B). In these microcosms, the *Alphaproteobacteria* families that contributed the most to the increase were *Zavarziniaceae* (increase of 5.9%), *Rhodospirillaceae* (increase of 5.8%), *Sphingomonadaceae* (increase of 3.5%), *Parvibaculaceae* (increase of 1.7%), *Acetobacteraceae* (increase of 1.1%) and *Caulobacteraceae* (increase of 1.1%) (Figure S4B).

3.4. Persistence of exogenous versus native microbiota

The log reduction and the rate of decay values of the *bla*_{KPC-3} and *phoE* genes (3.0–3.6 log-units and –2.35 to –2.67 d⁻¹, respectively)

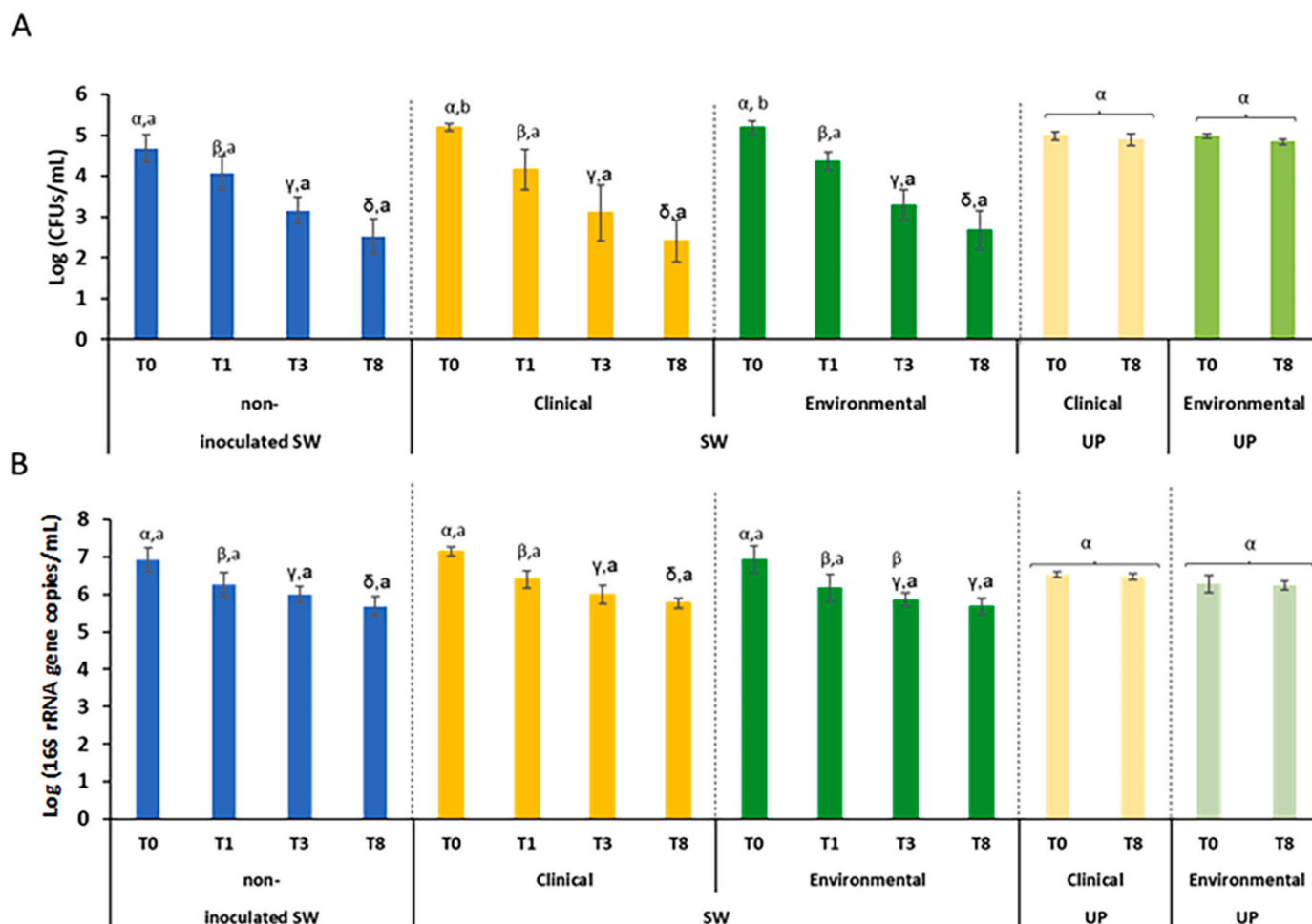


Fig. 2. Colony forming units (CFU) per volume (mL) of sample of total heterotrophs (A) and absolute abundance (Log gene copies/mL of sample) of 16S rRNA gene as a measurement of total bacteria (B) in microcosm assays. Microcosms were composed of non-inoculated urban runoff water (SW) and inoculated SW or ultra-pure water (UP) with *Klebsiella pneumoniae* isolate KP2-448 (clinical) or *K. pneumoniae* isolate SM1 (environmental). Microcosms with urban runoff water were incubated for 1, 3, and 8 days at 30 °C in triplicate, and microcosms with UP were incubated for 8 days at 30 °C in duplicate.

α, β, γ, δ, represent significant differences ($p < 0.01$) among incubation times, for each microcosm. a, b, represent significant differences ($p < 0.01$) between microcosms, non-inoculated and inoculated with clinical or environmental isolate, for the same incubation time.

were significantly higher ($p < 0.01$) than those of Gammaproteobacteria and Betaproteobacteria (1.2–1.5 log-units and -0.63 to -0.72 d $^{-1}$, respectively) in inoculated microcosms (Figure S6), suggesting the comparative lower fitness of the exogenous bacteria. Nevertheless, altogether, the results suggest that the *K. pneumoniae* survived and the gene *bla*_{KPC-3} was not lost as long as the host was viable. Moreover, it was suggested that the fitness of the *K. pneumoniae* isolates did not differ from that of the cultivable urban runoff native heterotrophs. The role of the native microbiota was also evidenced by the fact that neither the *K. pneumoniae* (gene *phoE*) nor the *bla*_{KPC-3} gene suffered any measurable decrease when incubated in ultra-pure water, suggesting that the decrease of the exogenous (non-native) *K. pneumoniae* results from bacteria dynamics and competition.

4. Discussion

In the last years, the spread of carbapenemase producing bacteria in the environment, mainly in aquatic environments has been frequently reported (Hooban et al., 2020; Cherak et al., 2021). Recently, Zou et al. (2023) reported the dissemination of clonal lineages of carbapenem-resistant *K. pneumoniae* from a hospital to urban aquatic environments (the urban wastewater treatment plant and the adjacent river), including of hypervirulent lineages belonging to the ST11 and

carrying the *bla*_{KPC-2} gene (Zou et al., 2023). Indeed, the gene *bla*_{KPC} has been one of the most reported carbapenemase encoding determinants in aquatic environments (Hooban et al., 2020). In the present work, we compared the behaviour of *bla*_{KPC-3} clinical and environmental *K. pneumoniae* strains belonging to the clinically relevant ST147, in urban runoff water and ultra-pure water. The aim was to study the influence of the origin of the isolates as well as the role of the water microbiota in the survival of these strains. This knowledge is a relevant contribution to predict the impact of anthropogenic contamination in aquatic environments in the future. Besides having different origins (clinical and environmental), the strains selected for the study were isolated in non-related areas (more than 50 km apart). Nevertheless, both were closely phylogenetically related, belonging to the multilocus sequence type ST147 and harbouring the same variant of the *bla*_{KPC} gene, as the passenger gene of Tn4401d, located in an incN plasmid, previously shown to be mobile in the clinical (KP2-448) strain (Ferreira et al., 2021). Though, both strains could be differentiated based on phenotypic and genotypic traits; namely the plasmid replicon type FIA (pBK30683) was detected only in the clinical strain. Curiously, this incN plasmid was previously described in a *K. pneumoniae* strain isolated from river water in the center of Portugal (Teixeira et al., 2020). Other differences between the two isolates examined in this study referred to the antibiotic and metals resistance genetic determinants, more diverse in

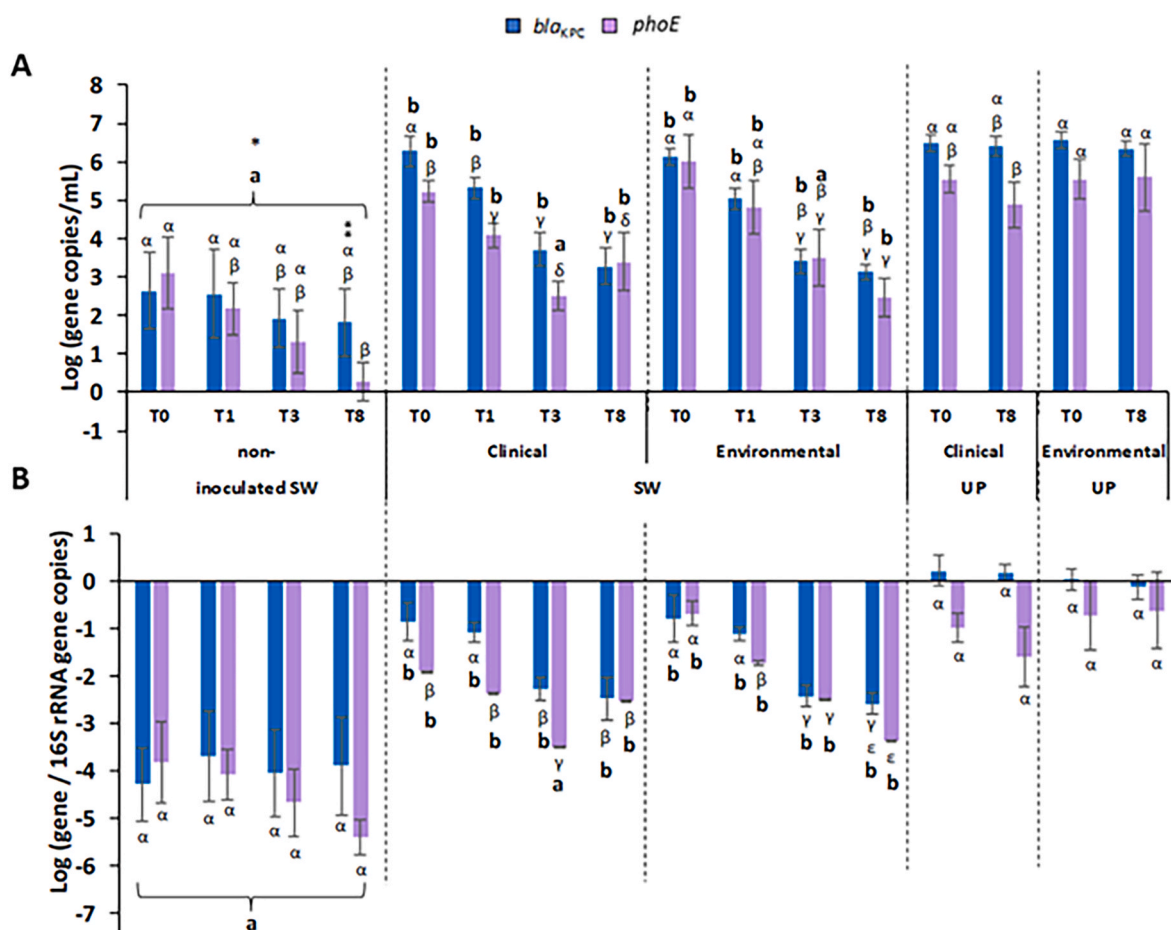


Fig. 3. Absolute (Log gene copies/mL of sample) (A) and relative abundance (Log gene copies/16S rRNA gene copy number) (B) of *bla*_{KPC} gene in microcosm assays composed of non-inoculated urban runoff water (SW) and inoculated SW or ultra-pure water (UP) with *Klebsiella pneumoniae* isolate KP2-448 (clinical) or *K. pneumoniae* isolate SM1 (environmental). Microcosms with urban runoff water were incubated for 1, 3, and 8 days at 30 °C in triplicate, and microcosms with UP were incubated for 8 days at 30 °C in duplicate.

*, indicate the presence of values below the limit of quantification (LOQ = 1 log (gene copies/mL)) obtained in one replica out of the three replicas of non-inoculated microcosms. In these cases, a value corresponding to half of the LOQ was used for the calculation of the average values and statistical analyses. **, indicate the omission of 1 replicate from 1 microcosm assay.

α, β, γ, δ, ε represent significant differences ($p < 0.01$), simultaneous among genes and incubation times (T0, T1, T3, T8), for each microcosm. a, b, represent significant differences ($p < 0.01$) between microcosms, non-inoculated and inoculated with clinical or environmental isolate, for the same incubation time and target gene.

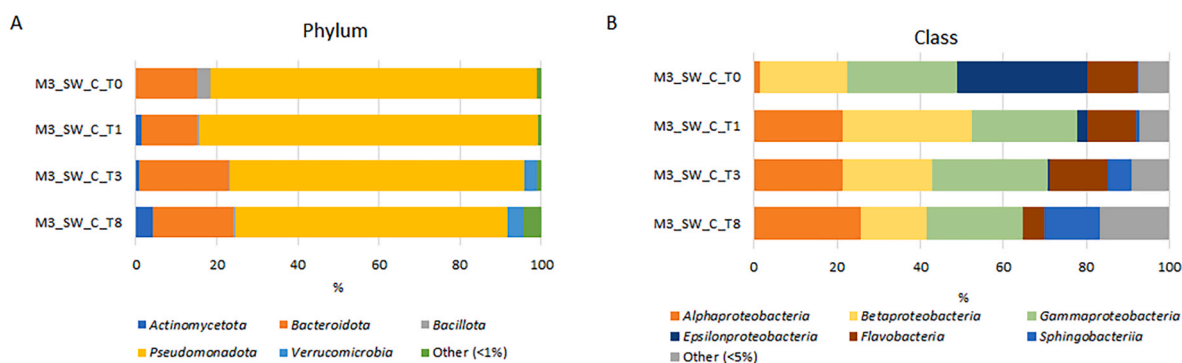


Fig. 4. Bacterial community composition of the non-inoculated urban runoff water (M3_SW_C) over time, at the Phylum (A) and Class (B) ranks, based on the analysis of the 16S rRNA gene amplicons. Microcosms were incubated for 1, 3, and 8 days at 30 °C in triplicate.

the clinical (KP2-448) than in the environmental isolate (SM1) (Table S4) and on sublethal conditions tolerance (temperature – strain SM1 vs. oxidative stress and UV-C radiation – strain KP2-448) (Table S3). However, these differences, contrary to the original

hypothesis of the study, did not explain distinct behaviours in surface water. Nevertheless, it is important to note that this similar behaviour may be observed only in the absence of strong selective pressure, such as the presence of heavy metals and antibiotics at high concentrations.

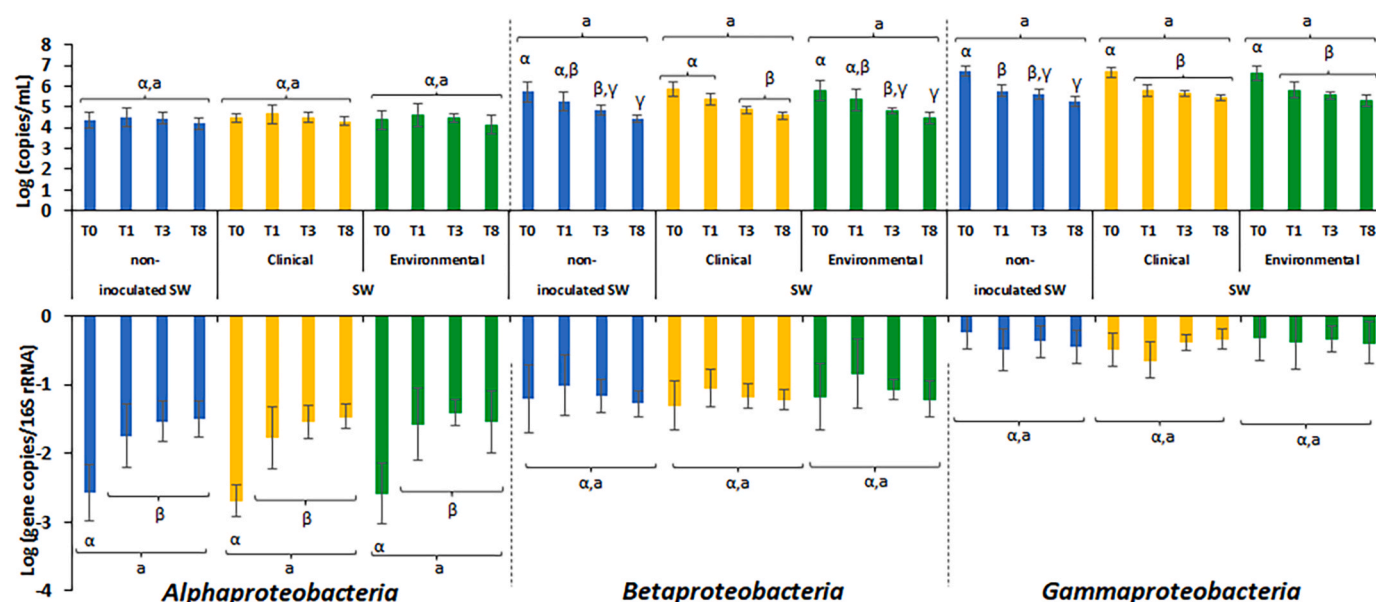


Fig. 5. Absolute abundance (Log gene copies/mL of sample) and relative abundance (Log gene copies of the taxa amplicon/16S rRNA gene copies) of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* in microcosm assays. Microcosms were composed of non-inoculated urban runoff water (non-inoculated SW, blue bars) and urban runoff water inoculated with *Klebsiella pneumoniae* isolate KP2-448 (Clinical, yellow bars) or *K. pneumoniae* isolate SMI (Environmental, green bars). Microcosms were incubated for 1, 3, and 8 days at 30 °C in triplicate. α, β, γ, represent significant differences ($p < 0.01$) among incubation times for each *Pseudomonadota* class, for each microcosm. a, represent non-significant differences ($p > 0.01$) between non-inoculated and inoculated microcosms for each *Pseudomonadota* class, for the same incubation time.

Previous studies (Korajkic et al., 2019) have shown that the survival of enterobacteria in water can be influenced by factors such as temperature, water type (marine/freshwater), or sunlight exposure, suggesting that bacterial properties that determine the degree of tolerance to these factors will be the major drivers for persistence. Another interesting finding of this study was related with the initial hypothesis that the elimination of the gene *bla_{KPC-3}* would depend on the decay of the bacterial host. In the absence of a bacterial community, *K. pneumoniae*, assessed based on the enumeration of the total bacteria, total heterotrophs, and the genes *phoE* and *bla_{KPC}* were unaltered over the 8 days of incubation. This confirmed not only the cell viability but also the stability of the resistance gene *bla_{KPC-3}* in pure water. Similarly, in urban runoff water, the decay rate of the *bla_{KPC-3}* gene was similar to that of the *phoE* gene. These observations suggest that the *bla_{KPC-3}* gene is stable in the genomes of these bacteria and that no selective pressure is required to maintain it. The absence of fitness costs of acquired antibiotic resistance may explain the emerging, although underestimated, spread of multidrug resistant *K. pneumoniae* ST147, which was originally endemic in India, Italy, Greece, and regions of North Africa, and expanding worldwide (Peirano et al., 2020). The fact that the absence of fitness costs will favour the stabilization and dissemination of acquired antibiotic resistance genes even in the absence of selective pressures has been discussed in the literature. Compensatory mutations, genetic linkage or co-selection or gene interaction are examples of mechanisms that may reduce fitness costs and therefore contribute to the stabilization of acquired resistance determinants (Hernando-Amado et al., 2017; Durão et al., 2018; Christaki et al., 2020). Yet, non-native bacteria may tend to have overall lower fitness than their native counterparts, highlighting the importance of robust microbiomes in the environment to avoid the proliferation and persistence of clinically relevant bacteria (Van Elsas et al., 2012; Ribeirinho-Soares et al., 2022).

However, the current knowledge is still insufficient to dictate what can be considered a robust microbiome capable of outcompeting with invasive bacteria. By studying the variation of the microbial community composition in this study, it was aimed a better understanding about the taxa that might explain the elimination of *bla_{KPC-3}* *K. pneumoniae*. Apparently, it could not be explained based on the decrease of

Gammaproteobacteria, but possibly on maintenance of *Alphaproteobacteria*. The shift in *Alphaproteobacteria* relative abundance and constancy of its abundance over time, which may be associated with a microcosm effect, might have had a relevant role to outcompete the exogenous strains. Interestingly, this phylum has been associated with pristine aquatic environments and the capacity of these bacteria to survive in water and outcompete other bacterial groups mostly related with anthropogenic activity may deserve further investigation (Schiaffino et al., 2016; Hördt et al., 2020).

In summary, this study supported four main conclusions.

- The *bla_{KPC-3}* gene was lost due to host death;
- Strains from environmental and clinical origin, harbouring distinct antibiotic and metal resistance genes and stress tolerance profiles, had similar behaviour in urban runoff water microcosms;
- The native microbiota of the urban runoff water was determinant to reduce the abundance of non-native carbapenem resistant *K. pneumoniae*, highlighting the importance of competitive microbiota;
- Non-native carbapenem resistant *K. pneumoniae* had lower fitness than other members of the classes *Gammaproteobacteria* or *Betaproteobacteria* in microcosm assays.
- *Alphaproteobacteria* had higher fitness than *Gammaproteobacteria* or *Betaproteobacteria* in microcosm assays.

This study contributed to elucidating the mechanisms explaining the fate of clinically relevant bacteria and antibiotic resistance genes in the environment and, therefore, how these can be controlled in the future. Considering the reduced number of strains tested, it would be important to validate these results with a higher number of strains.

Author contribution statement

Catarina Ferreira: Conceptualization, Data curation, Formal analysis, Investigation; Methodology; Writing – original draft, Lara Luzietti: Investigation; Methodology; Sara Ribeirinho-Soares: Investigation; Methodology; Writing – review & editing, Olga C. Nunes: Data curation;

Supervision; Writing – original draft, Writing – review & editing, Ivone Vaz-Moreira: Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Célia M. Manaia: Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision; Writing – original draft, Writing – review & editing.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2023.116928>.

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