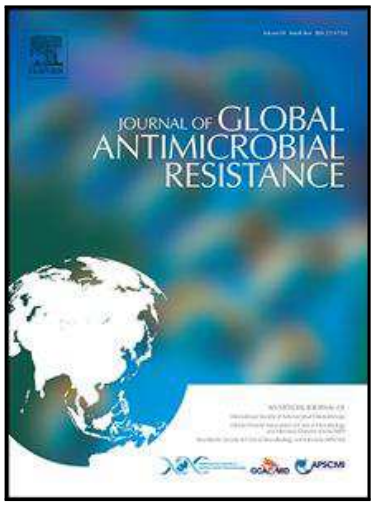

Screening for carbapenemase-producing Escherichia coli and Klebsiella pneumoniae in freshwater, bathing water and hospital continuum in Belgium

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Highlights

- Neither CP *E. coli* nor CP *K. pneumoniae* could be isolated from bathing water
- Surface water and hospital continuums contain CP *E. coli* and CP *K. pneumoniae*
- Hospital effluent plays an important role in the dissemination of CP bacteria
- A diversity of CP genes was found in the sequenced bacterial genomes

Screening for carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* in freshwater, bathing water and hospital continuum in Belgium

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Abstract

Objectives

This study focused on carbapenemase-producing (CP) *Escherichia coli* and *Klebsiella pneumoniae* in the aquatic environment in Belgium, based on the screening of three hospital continuums (hospital-WWTP-receiving river), 20 surface waters and 29 bathing waters in 2022.

Methods

The strains were enumerated and isolated from CHROMagar SuperCARBA medium. Phenotypic tests including disk combination tests to identify the No carbapenemase-producing (CP) *E. coli* nor CP *K. pneumoniae* was isolated from bathing waters, while 24 CP *E. coli* and 22 CP *K. pneumoniae* were isolated from 6 of the 20 surface water samples and from hospital continuums. Of the 24 CP *E. coli* strains, 20 had the bla_{KPC} gene (83.3%) and 4 the bla_{OXA} gene (16.6%), while of the 22 CP *K. pneumoniae* strains, the number of strains with carbapenemases genes was: 11 bla_{KPC} (50.0%), 9 bla_{OXA} (40.9%), one bla_{VIM} (4.5%), one bla_{NDM} (4.5%). The genomes of 11 CP *E. coli* and 14 CP *K. pneumoniae* were sequenced, and all possessed carbapenemase genes - bla_{KPC-3} (n=12), bla_{OXA-48} (n=10), $bla_{OXA-244}$ (n=1), bla_{VIM-1} (n=1) and bla_{NDM-5} (n=1) - mostly coupled to ESBL-encoding genes.

Conclusion This study demonstrates that CP *E. coli* and CP *K. pneumoniae* are disseminated in the Belgian aquatic environment and suggest the role of hospital effluents in this dissemination.

KEYWORDS (6 maximum)

Escherichia coli, *Klebsiella pneumoniae*, carbapenem resistance, freshwaters, hospital effluents

1. INTRODUCTION

The global rise in antibiotic resistance poses a significant threat, diminishing the efficacy of common antibiotics against widespread bacterial infections. Projections by the Organisation for Economic Co-operation and Development (OECD) indicate that resistance to antibiotics of last resort is expected to double by 2035, compared with 2005 levels, underlining the need to adopt robust antimicrobial stewardship practices and improve surveillance coverage worldwide [1].

Escherichia coli and *Klebsiella pneumoniae* are intestinal bacterium of human and warm-blooded animals belonging to the *Enterobacteriaceae* family. They can be released into the environment, particularly into aquatic systems, via hospital or municipal wastewater discharges and intensive livestock farming [2].

Klebsiella pneumoniae, included in the well-known ESKAPE, a group of multiresistant bacteria responsible of nosocomial infection, has also shown high levels of resistance to essential antibiotics [3]. Carbapenems, part of the β-lactam family, possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. As a result, they are often used as "last resort agents" when patients with bacterial infections become seriously ill or are suspected of harbouring resistant bacteria [4].

Carbapenem-resistant *Enterobacteriaceae* (CRE) are increasing worldwide, and the most dangerous are known to produce enzymes called carbapenemases [5]. Among the four classes of β-lactamases defined by the Ambler classification system, the carbapenemases produced by carbapenemase-producing (CP) *Enterobacteriaceae* belong to three of them: Class A (*K. pneumoniae* carbapenemases, KPC), Class B (metallo-b-lactamases, MBL including New Delhi metallo-β-lactamases, NDM) and Class D (OXA-48-like carbapenemases) [6].

Environmental reservoirs of AMR, such as aquatic environments, are a growing concern that are gathering more attention as potential sources for human infection [7–9].

The aims of this study were therefore (i) to assess the CP *E. coli* and CP *K. pneumoniae* prevalence in surface waters, bathing waters and hospital effluents in Belgium (ii) to provide insights on the putative

impact of hospital effluents and WWTPs on the CP *E. coli* and *K. pneumoniae* dissemination (iii) to characterize the CP *E. coli* and *K. pneumoniae* by whole genome sequencing.

Material and methods

2.1 Water sampling

A screening of several types of water was carried out in Belgium in 2022. A total of 29 official bathing waters and 20 freshwaters were sampled in Wallonia (Figure 1). The bathing sites were selected during the bathing season in order to cover as much of the Wallonia region as possible. Surface waters were selected on the basis of previous ISSeP analysis, which showed high faecal contamination or high oestrogenic activity often linked to the presence of hospital effluents as these contain steroid hormones (natural and synthetic oestrogens) from medicines and patient excretions.

Three hospital continuums (hospital effluent – input/output of wastewater treatment plant (WWTP)-upstream/downstream from the WWTP in the river) in three watersheds (Meuse, Sambre, Ourthe) were sampled (not shown in the Figure 1 for confidential reason, Supplementary Table 1).

Water samples were collected in 1-L sterile polyethylene bottles without any preservative, transported at 4°C, stored at 5±3°C and analyzed within 24 h.

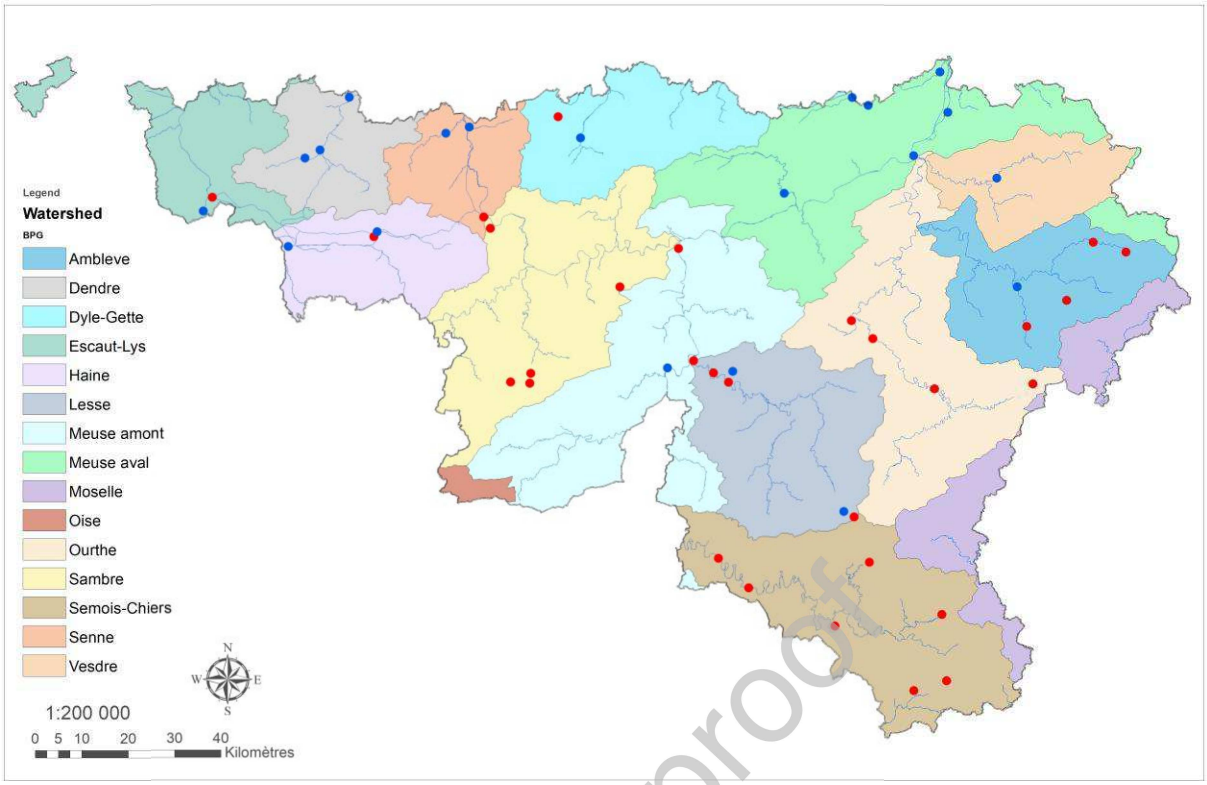

Figure 1: Map showing the 29 bathing waters (red) and 20 surface waters (blue) sampled in Belgium

2.2 CP *E. coli* and CP *K. pneumoniae* isolation

From each sample, different volumes or 10-fold dilutions of untreated water were membrane filtered through 0.45-µm pore size filters (Millipore Corporation, USA) which were placed on CHROMagar mSuperCARBA for the CP *E. coli* and CP *K. pneumoniae* enumeration (Supplementary Tables 2–5). The petri dishes were incubated overnight at 37°C. On this medium, CP *E. coli* are dark pink to reddish colonies while CP *K. pneumoniae* are part of the KESC (*Klebsiella, Enterobacter, Serratia, Citrobacter*) which are metallic blue colonies. To discriminate *K. pneumoniae*, the MIO (motility, indole and ornithine negative) Medium test (Condalab, Spain) was used. The specie identifications were confirmed with API 20E gallery.

2.3 Carbapenemase typing

2.3.1 Antimicrobial Susceptibility Testing

A susceptibility test was performed on Mueller-Hinton (MH) agar (Bio-Rad, Marnes-la-Coquette, France) using the disk diffusion assay and plates were incubated for 18 ± 2 h at 35 ± 1°C according to the European Committee on Antimicrobials with 2020 V.10.0 clinical breakpoints (EUCAST, 2020) [10]. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, US) was included in each assay as negative control. The antimicrobial disks (Bio-Rad, Marnes-la-Coquette, France) were placed on MH agar with automatic disk dispenser (16-disks per plate) (Bio-Rad, Marnes-la-Coquette, France). The KPC&MBL&OXA-48 disk kit (Liofilchem, Italy) was incorporated to the antibiogram to deduce the Ambler class of the carbapenemase according to the supplier's recommendations (Supplementary Table 6).

A total of 16 antibiotics were tested based on their belonging to the β-lactam family and their usefulness in discriminating beta-lactamase type : ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), cefepime (FEP, 30 µg), cefotaxim (CTX, 5 µg), ceftazidim (CAZ, 10 µg), cefuroxime (CXM, 30 µg), cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 µg), ertapenem (ETP, 10 µg), piperacillin/tazobactam (TZP, 30/6 µg), temocillin (TEM, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), meropenem/EDTA, meropenem/phenylboronic acid, meropenem/cloxacillin.

2.3.2 Carbapenemase genotyping

Total DNA was extracted by using the boiling method [11]. All the CP *E. coli* and CP *K. pneumoniae* isolates were tested for the presence of $bla_{\text{OXA-48}}$, bla_{KPC}, bla_{NDM}, bla_{VIM} et bla_{IMP} with specific primer described by Hooban *et al.* (2021) [12] (Supplementary Table 7).

For amplification on a CFX96 thermocycler (Bio-Rad, Marnes-la-Coquette, France), 5 µl of DNA extract was mixed with 1,5 µl of each primer (final concentration : 0.6 µM), 12 µl Takyon No ROX SYBR 2x MasterMix blue dTTP (Eurogentec, Liège, Belgium) and 5 µl of DNase free water in a final

volume of 25 µl. The temperature profile was as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation for 15 s, annealing for 1 min at 59,4°C.

2.3.3 Immunoassay tests

A rapid in vitro diagnostic test (O. K. N. V. I. RESIST-5, Coris Bioconcept, Belgium) for the detection of carbapenemases (*bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$ *and bla*$_{\text{IMP}}$) from bacterial cultures was performed on each isolate [13].

2.4 Whole-genome sequencing

The genomes of a selection of 11 CP *E. coli* and 14 CP *K. pneumoniae* were sequenced. They were chosen on the basis of the resistance profiles, but also based on their location in order to cover as many locations as possible. DNA extraction was carried out using the NucleoSpinR Microbial DNA (Macherey-Nagel). The genomic libraries were prepared using the Nextera XT library preparation kit (Illumina). Sequencing was carried out using the Illumina MiSeq platform to generate 300 bp paired-end reads by the GIGA Institute (ULiège). Raw read sequences obtained in this study were deposited to GenBank/SRA under the BioProject ID **PRJNA1300363**.

Assembly of the Illumina sequence reads was performed using the SPAdes genome assembler (v3.13.0). The assembled *E. coli* genomes were uploaded to the *Escherichia* database on PubMLST to confirm the species (https://pubmlst.org/organisms/escherichia-spp/). Bioinformatic analysis were carried out using the Center for Genomic Epidemiology (CGE) pipelines (Serotype Finder v2.0, MLST v2.0, cgMLST Finder v1.2, ResFinder v4.1).

2. RESULTS AND DISCUSSION

3.1 CP E. coli and CP K. pneumoniae isolation

No isolation of CP *E. coli* and *K. pneumoniae* was possible in the bathing water. These 29 bathing sites (10 rivers and 19 lakes or ponds) were official bathing sites analyzed in the laboratory during the bathing season. An assessment of the risk of human exposure to antibiotic-resistant bacteria in bathing waters was carried out, demonstrating their relatively good quality in Belgium, even though ESBL-EC have been found in good classified bathing areas [14].

In surface water, isolation was possible in 6 of the 20 surface water samples taken: in the Hain at Tubize, the Haine canalised at Hensies, the Meuse at Liège, the Geer at Bassenge, the Senne at Rebecq and the Sainte-Julienne stream at Visé.

These watercourses were located in densely populated areas rather than rural areas, selected on the basis of ecotoxicological data.

CP *E. coli* and CP *K. pneumoniae* were also isolated from the three hospital continuums. A total of 24 CP *E. coli* and 22 CP *K. pneumoniae* were isolated, of which 16 CP *E. coli* and 7 CP *K. pneumoniae* came from hospital effluents (Supplementary Table 5).

3.2 Carbapenemase typing

Of the 24 isolated CP *E. coli*, 20 isolates possessed the bla_{KPC} gene and 4 the bla_{OXA-48} gene. Most of the 20 strains containing the bla_{KPC} gene (16/20 strains) came from the effluent of hospital A. Of the 22 CP *K. pneumoniae*, 11 strains had the bla_{KPC} gene, 9 the bla_{OXA-48} gene, one bla_{NDM} and one bla_{VIM}.

Three techniques (antibiogram, PCR and immunochromatographic test) were used to identify the main genes (bla_{NDM}, bla_{KPC}, bla_{OXA-48}, bla_{VIM}, bla_{IMP}) involved in carbapenemase production.

There was only one discrepancy in the results for CP *E. coli* for the isolate 1440/1r (Hain in Tubize) (Table 1a).

3.3 Genomic analysis

11 CP *E. coli* and 14 CP *K. pneumoniae* were sequenced. Bacterial species revealed with API 20 E gallery were confirmed by SpeciesFinder. All these strains possessed CP genes - bla_{KPC-3} (n=12), bla_{OXA-48} (n=10), $bla_{OXA-244}$ (n=1), bla_{VIM-1} (n=1) and bla_{NDM-5} (n=1) - mostly coupled to ESBL-encoding genes. The genes sequenced were consistent with the phenotypic profiles obtained (antibiogram) and with the PCR and immunochromatographic tests performed (Table 1a–1b).

Different serotypes and sequence types were detected. For *E. coli*, three strains of serotype O18:H7 and ST 1463 were found, one in hospital A effluent, as it was the case in a previous study [15], another at the output of the Tilff WWTP and the third in the Meuse at Liège. These results suggest that this clone would be originated from the Hospital A and was disseminated, via WWTP, to the downstream in the Ourthe and the Meuse. In Sweden, Khan and colleagues (2018) [16] compared the profile of two CP *Klebsiella* isolated from the aquatic environment with hospital isolates and suggested their dispersal from the hospital environment into the aquatic environment.

For *K. pneumoniae,* four strains belonged to the serotype O1 which is associated with multidrug resistance while no hypervirulent strains (serotype K1 and K2) were isolated [17]. One ST11 strain was found in the Sambre river. A ST16 strain (code /-3/2b) was isolated from the effluent of hospital A. This emerging clone has the potential to become a dual-risk global epidemic clone and a major threat to public health as for ST11 [18].

This strain /-3/2b was the strain with the highest number of *bla* genes (11 *bla* genes). It had an MBL phenotype and was resistant to all antibiotics tested in the antibiogram, including imipenem and meropenem. Other strains (code INt/7b, OUTt/13m/3b, 1440/1b) containing fewer *bla* genes nevertheless have the same phenotype, with resistance to all antibiotics tested (Supplementary Table 8 and 9).

Two *K. pneumoniae* ST512 with the bla_{KPC-3} gene were detected at the input/output of the Tilff WWTP and one *K. pneumoniae* ST101 was detected in surface water in the Hain at Tubize. In Spain, 5 clones of KPC-producing *Enterobacteriaceae* increased : ST11 and ST512 which have caused interregional spread, ST101 regional spread and ST1961 and ST678 independent hospital outbreaks [19].

The genetic resistance profile of the sequenced bacteria showed a wide range of antimicrobial families such as quinolones (ciprofloxacin; 23 resistant bacteria out of 25 sequenced), chloramphenicol (18/25), sulfamethoxazole (14/25), trimethoprim (18/25) and aminoglycosides (gentamicin (13/25), tobramycin (17/25)). In addition, several resistances to quaternary ammoniums used as biocides or disinfectants have been detected (cetylpyridium chloride (22/25), benzylkonium chloride (22/25)). Biocides contribute to antibiotic resistance through co-resistance mechanisms or cross-resistance.

Carenco (2017) [20] suggested that resistance to disinfectants does not arise in healthcare or community environments, where concentrations of disinfectants far exceed bacterial defense capacities. Instead, they develop rather in sewage systems and in the environment.

CONCLUSION

This study has shown that CP *E. coli* and CP *K. pneumoniae* are present in the aquatic environment in Belgium and suggests contamination of the aquatic environment.

In Europe, carbapenems are currently prescribed in human medicine for hospital use only. The origin of the CP bacteria isolated in this study is certainly human, as the *E. coli* O18:H7 ST1463 strain, given that they showed great similarities with human clinical isolates and were isolated along the hospital continuum. That is why WWTP should be installed at hospital outlets (which is not currently the case everywhere). The integration of disinfection technologies such as ozonation, chlorination and UV disinfection at the outlet of WWTP would be beneficial in reducing the levels of antibiotic-resistant bacteria and genes discharged into the aquatic environment.

This study demonstrates the need to monitor aquatic environments to ensure public health, especially those where there is a risk of human exposure to AMR. Although no CP bacteria were found in the 29 bathing waters, a single screening cannot guarantee the absence of contamination especially since some surface waters are used for bathing like, as is the case in Belgium. Bathing water legislation should include this type of enumeration, which poses a potential risk to human health.

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Declaration of competing interests

The authors declare no conflict of interest.

Ethical approval

Not required

Table 1a: strain code, biosample number, localizations, carbapenemase genes identified by the 3 detection tests, serotype, MLST, cgMLST and *bla* genes identified for the 24 *Escherichia coli* isolated

Strain Code	Biosample	Localization	Phenotypic test	Genotypic test	Immunochromatographic test	Genomic analysis			
			Carbapenemase			Serotypes	MLST	cgMLST	*bla* Genes
/-2/6r /-2/10r /-2/7r	**SAMN50341134**	Hospital A effluent (Ourthe continuum – WWTP Tilff)	KPC	KPC	KPC	H25	635	67548	bla_{KPC-3}, bla_{TEM-1B}, $bla_{SHV-182}$
/-2/8r /-2/9r /-2/1r /-2/2r /-2/3r /-2/4r	**SAMN50341135**					O18:H7	1463	103730	bla_{KPC-3}, $bla_{SHV-182}$
/-2/5r /-3/3r /-3/2r /-3/4r /-3/5r /-3/6r /-3/7r	**SAMN50341136**					O18:H8	1463	103730	bla_{KPC-3}, $bla_{SHV-182}$
	SAMN50341137					O21:H25	401	97525	bla_{KPC-3}, bla_{TEM-1B}, $bla_{SHV-182}$
OUTt/1ml/4r	**SAMN50341139**	WWTP output Tilff	KPC	KPC	KPC	O18:H7	1463	103730	bla_{KPC-3}, $bla_{SHV-182}$
Bt/1r	**SAMN50341138**	Ourthe downstream WWTP Tilff	KPC	KPC	KPC	O25:H12	607	149	bla_{KPC-3}, $bla_{CTX-M-9}$, $bla_{SHV-182}$
Bt/5r			KPC	KPC	KPC				
Am-c/1r	**SAMN50341140**	Surface water	Sambre upstream	OXA	OXA	OXA	O86:H30	38	155495	bla_{OXA-48}, bla_{TEM-1B}, $bla_{SHV-182}$
12161/1r	**SAMN50341141**		Sainte-Julienne stream at Visé	OXA	OXA	OXA	O86:H18	38	180160	$bla_{OXA-244}$
1440/1r	**SAMN50341142**		Hain at Tubize	KPC (ou OXA)	OXA	OXA	O105:H10	607	100707	bla_{OXA-48}, bla_{SHV-12}
3294/1r	**SAMN50341143**		Meuse at Liège	KPC	KPC	KPC	O18:H7	1463	103730	bla_{KPC-3}, $bla_{SHV-182}$
4722/1r	**SAMN50341144**		Geer at Bassenge	OXA	OXA	OXA	H21	10	184324	bla_{OXA-48}

Table 1b: strain code, biosample number, localizations, carbapenemase genes identified by the 3 detection tests, serotype, MLST and *bla* genes identified for the 22 *Klebsiella pneumoniae* isolated

Strain Code	Biosample	Localization	Phenotypic test	Genotypic test	Immunochromatographic test	Genomic analysis		
			Carbapenemase			Serotypes	MLST	*bla* Genes
/-3/2b	SAMN50341145	Hospital A effluent (Ourthe continuum – WWTP Tilff)	MBL	NDM	NDM	K51/O3b	16	*bla*CTX-M-15, *bla*TEM-1B, *bla*OXA-1, *bla*NDM-5, *bla*SHV-26, *bla*SHV-78, *bla*SHV-98, *bla*SHV-145, *bla*SHV-179, *bla*SHV-194, *bla*SHV-199
INt/7b	SAMN50341146	Input WWTP Tilff	KPC	KPC	KPC	O2afg	512	*bla*TEM-1B, *bla*TEM-104, *bla*KPC-3,*bla*OXA-9, *bla*SHV-182
OUTt/1ml/3b	SAMN50341147	Output WWTP Tilff	KPC	KPC	KPC	O2afg	512	*bla*TEM-1B, *bla*TEM-104, *bla*KPC-3, *bla*OXA-9, *bla*SHV-182
OUTm-c/10b	SAMN50341148	Output WWTP Montignies	OXA	OXA	OXA	K10/O3/O3a	147	*bla*DHA-1, *bla*OXA-1, *bla*OXA-48, *bla*SHV-11, *bla*SHV-67
INm-c/-1/1b		Input WWTP Montignies	KPC	KPC	KPC			
INm-c/-1/2b			KPC	KPC	KPC			
HMCf/-2/1b			OXA	OXA	OXA			
HMCf/-2/2b	SAMN50341150	Hospital D aile F effluent (Sambre continuum – WWTP Montignies)	KPC	KPC	KPC	K38/O3b	1486	*bla*KPC-3, *bla*OXA-9, *bla*SHV-26, *bla*SHV-78, *bla*SHV-98, *bla*SHV-106, *bla*SHV-145, *bla*SHV-179, *bla*SHV-194, *bla*SHV-199
HMCf/2/5b	SAMN50341151		OXA	OXA	OXA	K10/O3/O3a	147	*bla*CTX-M-15, *bla*TEM-1B, *bla*OXA-1, *bla*OXA-48, *bla*SHV-11, *bla*SHV-67
HMCf/-3/3b			KPC	KPC	KPC			
/1b	SAMN50341153	Hospital C effluent (Meuse continuum)	OXA	OXA	OXA	K13/O1	540	*bla*CTX-M-15, *bla*TEM-1C, *bla*OXA-1, *bla*OXA-48, *bla*SHV-42
/-1/5b			OXA	OXA	OXA			
1440/1b	SAMN50341154	Surface water	Hain at Tubize	OXA	OXA	OXA	K17/O1	101	*bla*CTX-M-15, *bla*TEM-1A, *bla*OXA-1, *bla*OXA-48, *bla*SHV-28, *bla*SHV-106
2280/9b	SAMN50341155		Haine canalisée at Hensies	MBL	VIM	VIM	K46/O1	461	*bla*VIM-1,*bla*SHV-187
3294/3b	SAMN50341156		Meuse at Liège	KPC	KPC	KPC	O5	3318	*bla*TEM-1A, *bla*KPC-3, *bla*OXA-9
4722/1b	SAMN50341157		Geer at Bassenge	OXA	OXA	OXA	K24/O1	15	*bla*OXA-48, *bla*SHV-28, *bla*SHV-106
1395/2b				OXA	OXA	OXA			
1395/3b	SAMN50341158		Senne at Rebecq	OXA	OXA	OXA	K27/O4	392	*bla*CTX-M-15, *bla*OXA-48, *bla*SHV-11, *bla*SHV-67

Ag,b,/3b	**SAMN50341149**	Ourthe upstream WWTP Angleur	KPC	KPC	KPC	/	1770	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{OXA-48}$, *bla*$_{SHV-11}$, *bla*$_{SHV-67}$
Bt/3b		Ourthe downstream WWTP Tilff	KPC	KPC	KPC			
Bm-c/3b		Sambre downstream	KPC	KPC	KPC			
Bm-c/7b	**SAMN50341152**		KPC	KPC	KPC	K38/O3b	11	*bla*$_{TEM-1A}$, *bla*$_{KPC-3}$, *bla*$_{OXA-9}$, *bla*$_{SHV-182}$

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