environments

MDPI

Article

Tracing Antimicrobial Resistance Across the Hospital–Wastewater–River Continuum: A Pre-COVID-19 and Pandemic-Era Study in Bucharest, Romania

Ecaterina Monica Sarbu [1,†], Marcela Popa [1,2,†], Corneliu Ovidiu Vrancianu [1,3,4,†], Luminita Gabriela Marutescu [1,5,†], Ilda Czobor Barbu [1,5], Irina Gheorghe-Barbu [1,5], Mihai Nita-Lazar [2], Madalina-Maria Muntean [6], Cerasella Cristiana Dragomirescu [6,7], Gabriela Loredana Popa [8,9], Mădălina Preda [10], Carmen Postolache [11,*], Alexandru Andrei Muntean [6,7], Adrian Streinu-Cercel [12,13] and Mircea Ioan Popa [6,7]

1 Research Institute of the University of Bucharest—ICUB, University of Bucharest, 050657 Bucharest, Romania; ecaterina-monica.sarbu@drd.unibuc.ro (E.M.S.); marcela.popa@bio.unibuc.ro (M.P.); ovidiu.vrancianu@yahoo.com (C.O.V.); luminita.marutescu@bio.unibuc.ro (L.G.M.); ilda.barbu@bio.unibuc.ro (I.C.B.); iryna_84@yahoo.com (I.G.-B.)
2 National Research and Development Institute for Industrial Ecology—ECOIND, 57-73 Drumul Podu Dambovitei, District 6, 060652 Bucharest, Romania; mihai.nita@incdecoind.ro
3 National Institute of Research and Development for Biological Sciences, 060031 Bucharest, Romania
4 Doctoral School, Carol Davila University of Medicine and Pharmacy, 020021 Bucharest, Romania
5 Microbiology-Immunology Department, Faculty of Biology, University of Bucharest, 050095 Bucharest, Romania
6 Department of Microbiology II, "Carol Davila" University of Medicine and Pharmacy, 020021 Bucharest, Romania; madalina.muntean@umfcd.ro (M.-M.M.); cristiana.dragomirescu@umfcd.ro (C.C.D.); alexandru.muntean@umfcd.ro (A.A.M.); mircea.ioan.popa@gmail.com (M.I.P.)
7 Department of Microbiology, Cantacuzino National Military Medical Institute for Research and Development, 050096 Bucharest, Romania
8 Department of Parasitology, "Carol Davila" University of Medicine and Pharmacy, 020021 Bucharest, Romania; gabriela.popa@umfcd.ro
9 Parasitic Disease Department, Colentina Clinical Hospital, 020125 Bucharest, Romania
10 Department of Microbiology, Parasitology and Virology, Faculty of Midwives and Nursing, "Carol Davila" University of Medicine and Pharmacy, 020021 Bucharest, Romania; madalina.preda@umfcd.ro
11 Doctoral School in Ecology, University of Bucharest, 91-95 Splaiul Independenței, 050095 Bucharest, Romania
12 Department of Infectious Diseases I, Faculty of Medicine, Carol Davila University of Medicine and Pharmacy, 020021 Bucharest, Romania; adrian.streinucercel@umfcd.ro
13 National Institute for Infectious Diseases "Prof. Dr. Matei Balş", Strada Doctor Calistrat Grozovici 1, 021105 Bucharest, Romania
* Correspondence: carmen.postolache@bio.unibuc.ro
† These authors contributed equally to this work.

Abstract

Hospital and municipal wastewaters can transport antimicrobial-resistant bacteria from human-associated sources into receiving aquatic environments, yet longitudinal evidence of their occurrence across the COVID-19 transition remains limited in Eastern Europe. We analyzed seven Bucharest-area sampling campaigns from June 2019 to March 2021 across hospital wastewater, the Glina municipal wastewater treatment plant (WWTP), and the Dâmbovița River. Culture-based abundances of extended-spectrum beta-lactamase (ESBL), carbapenem resistance (CARBA), OXA-48, and vancomycin-resistant enterococci (VRE) screening phenotypes were compared between four pre-COVID-19 and three pandemic-era campaigns. During the pandemic era, ESBL and several carbapenem-resistance screening phenotypes were detected in all three Glina effluent campaigns, whereas detection was generally intermittent before COVID-19. Under the baseline non-detect convention, median effluent abundances were 2.12–4.12 $\log_{10}$ units higher during the COVID-19 era for selected ESBL, CARBA, and OXA screening phenotypes. These descriptive differences were estimated from only four pre-COVID-19 and three COVID-19-era campaigns and were sensitive

Check for updates

Academic Editor: Petros A. Kokkinos

Received: 10 September 2026
Revised: 23 October 2026
Accepted: 27 October 2026
Published: 29 October 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

to assumptions regarding non-detects and sampling formats. Under the original non-detect substitution convention, median CARBA non-fermenter treatment-associated reduction decreased from 2.29 to 0.38 $\log_{10}$ units, while VRE showed inconsistent influent–effluent patterns, including a negative median concentration difference (−1.76 $\log_{10}$ units) during the pandemic era. Downstream river samples exceeded upstream levels for several phenotypes across most campaigns, whereas hospital effluent remained a high-AMR compartment without a uniform pandemic-era increase. These findings highlight municipal effluent and the receiving-water interface as important surveillance points and support integrated AMR monitoring across the urban wastewater continuum.

Keywords: antimicrobial resistance; hospital wastewater; wastewater treatment plant; COVID-19; receiving water; One Health

1. Introduction

Antimicrobial resistance (AMR) is a global health threat whose consequences extend beyond clinical settings. The 2021 global burden analysis estimated about 1.14 million deaths directly attributable to bacterial AMR and 4.71 million deaths associated with it, with attributable mortality projected to increase by 2050 [1]. Environmental pathways are increasingly recognized as part of this burden because resistant bacteria, resistance determinants, antimicrobial residues, disinfectants, and other co-selective agents can move between human populations, engineered wastewater systems, and natural aquatic environments [2–4].

Wastewater is particularly informative because it integrates biological material from large populations and connects healthcare facilities with municipal treatment infrastructure. Hospital wastewater may contain high densities of clinically relevant resistant organisms, whereas municipal wastewater treatment plants (WWTPs) receive biological material from a much broader community. Treatment can substantially reduce bacterial loads, but removal is neither complete nor uniform across resistant taxa and resistance determinants [2,3]. Sewage surveillance has therefore been proposed as a complementary AMR surveillance approach, although interpretation requires careful attention to sampling, normalization, treatment configuration, and links to clinical data [5].

Bucharest provides a relevant setting for such an analysis. Previous work demonstrated that multidrug-resistant *Klebsiella pneumoniae* ST101 could be recovered from patients, hospital wastewater influent, and treated hospital effluent, including clonally related isolates carrying the resistance genes $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}48}$, and other resistance determinants [6]. This established local evidence that clinically important resistant organisms can persist across the hospital wastewater barrier. Previous investigations in Bucharest have also examined microbiological contamination across hospital wastewater, municipal treatment systems, and the Dâmbovița River, including comparisons spanning the COVID-19 period [7]. Complementary research at the Glina WWTP and receiving aquatic environments has characterized antimicrobial resistance and the genomic diversity of *Acinetobacter baumannii* isolates collected in 2022 [8]. However, longitudinal evidence tracing broader culturable resistance phenotypes from hospital wastewater through municipal treatment and into receiving waters remains limited, particularly in Eastern European settings. Such integrated surveillance is needed to determine whether changes observed at hospital sources remain detectable after municipal treatment and at the receiving-water interface.

The COVID-19 pandemic introduced an additional temporal dimension to this surveillance framework. Infection-prevention measures, healthcare utilization, patient case mix, antimicrobial prescribing, and use of broad-spectrum or last-line antibiotics changed during 2020–2021. Hospital wastewater studies have reported temporal changes in resistance

genes during the pandemic [9,10], while wastewater-based epidemiology in community catchments has shown that antimicrobial and resistance-gene loads can vary substantially across pandemic phases [11]. At the EU/EEA level, total antibiotic consumption fell sharply from 2019 to 2020, mainly in the community, while the relative use of broad-spectrum and last-line agents increased in hospitals; by 2021, Romania was among the few countries in which community consumption exceeded the 2019 level [12,13]. These observations provide plausible context, but wastewater AMR patterns cannot be causally attributed to antimicrobial use without linked prescribing and operational data.

The present study therefore examined culture-based AMR across a hospital–municipal wastewater–river continuum in Bucharest using seven surveillance campaigns conducted between June 2019 and March 2021. The primary objectives were to (i) describe the distribution of ESBL, carbapenem-resistance screening, OXA-48 screening, and VRE phenotypes across sequential compartments; (ii) compare four pre-COVID-19 campaigns with three COVID-19-era campaigns; (iii) assess treatment-associated log reductions at the Glina WWTP; and (iv) quantify upstream-to-downstream changes in the Dâmbovița River. Because the time series is short, temporal comparisons are interpreted as exploratory effect-size patterns rather than causal evidence of a pandemic effect.

2. Materials and Methods

2.1. Study Area and Surveillance Continuum

The surveillance design encompassed successive compartments of the urban wastewater continuum in Bucharest, Romania. Hospital wastewater was collected from the influent and effluent of the local wastewater treatment system at the National Institute for Infectious Diseases "Prof. Dr. Matei Balș", a tertiary infectious diseases referral center in Bucharest (680 beds for continuous inpatient care and 20 beds for day hospitalization).

The hospital's local wastewater treatment system included a tank equipped with an intermittent chlorination process prior to discharge into the municipal sewer network. As previously documented at the same hospital, disinfection involved the intermittent application of an active chlorine solution (0.06 g/L) according to the hospital's standard operating procedure [6]. Previous investigations also demonstrated the persistence of multidrug-resistant *Klebsiella pneumoniae* in hospital effluent following chlorine treatment [6].

Municipal wastewater was sampled at the Glina wastewater treatment plant (WWTP), located southeast of Bucharest, at three treatment stages: influent, activated sludge, and treated effluent. The Glina WWTP receives predominantly domestic wastewater, together with industrial and commercial contributions and stormwater inputs, and applies mechanical and biological activated-sludge treatment. Treated effluent from the WWTP is discharged into the Dâmbovița River. Surface-water samples were collected approximately 200 m upstream and 200 m downstream of the discharge point. A comparable upstream–downstream sampling configuration at approximately 200 m from the WWTP discharge was previously reported for the Dâmbovița River by Banciu et al. [7] and was also used in a subsequent investigation of *A. baumannii* conducted in August 2022 by Gheorghe-Barbu et al. [8]. These sampling locations were selected to characterize local upstream–downstream differences in the receiving water near the WWTP discharge, consistent with a previously reported monitoring configuration in the same river system. The sampling distance was not established through site-specific hydrodynamic or effluent-mixing assessments. Thus, the surveillance continuum comprised the hospital wastewater source, the municipal wastewater treatment system, and the upstream and downstream receiving-water compartments, as detailed in Table 1.

The geographical setting of the Glina WWTP, its discharge location, and the approximate upstream and downstream sampling areas along the Dâmbovița River are illustrated in Figure 1.

Table 1. Sampling design used in the Bucharest longitudinal analysis.

Compartment	Role in Continuum	Pre-COVID Campaigns	COVID-Era Campaigns	Analytical Use
Hospital influent	Untreated hospital wastewater	4	3	Source signal
Hospital effluent	Post-local-treatment hospital wastewater	4	3	Persistence after hospital barrier
Glina WWTP influent	Municipal influent	4	3	City-scale input
Activated sludge	Biological treatment compartment	4	3	Treatment ecology; descriptive
Glina WWTP effluent	Treated municipal effluent	4	3	Primary temporal endpoint
Dâmbovița upstream	Receiving-water reference	4	3	Upstream baseline
Dâmbovița downstream	Receiving water after discharge	4	3	Environmental increment

Note: Seven sampling campaigns were included for each principal compartment. When multiple municipal wastewater samples were available within the same campaign, their concentrations were summarized by the median to obtain one campaign-level estimate. Sampling-format availability differed between campaigns and periods, as detailed in Section 2.2.

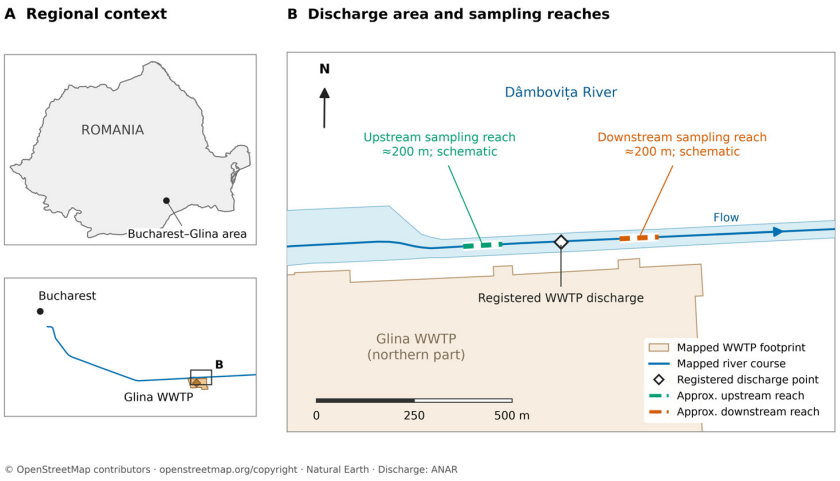


Figure 1. Geographical overview of the Bucharest wastewater surveillance area: (**A**) Regional location of Bucharest and the Glina wastewater treatment plant (WWTP). (**B**) Glina WWTP and the Dâmbovița River, showing the mapped discharge location and schematic representations of the upstream and downstream sampling areas approximately 200 m from the discharge. The sampling positions are indicative rather than independently verified GPS coordinates. Geographical data: OpenStreetMap contributors and Natural Earth; discharge-location data: Romanian National Administration "Romanian Waters" (ANAR).

2.2. Sampling Campaigns and Period Definition

Seven sampling campaigns were included in the longitudinal analysis. The pre-COVID-19 period comprised four campaigns conducted in June, September, and November 2019 and February 2020, whereas the COVID-19 period comprised three campaigns conducted in June and September 2020 and March 2021. The temporal boundary was defined as 26 February 2020, corresponding to the first confirmed COVID-19 case in Romania. The sampling compartments and their analytical roles across the surveillance continuum are summarized in Table 1.

Hospital wastewater, municipal wastewater, and river samples were collected on the same calendar date in six of the seven campaigns. The only exception was June 2019, when hospital influent and effluent samples were collected on 24 June, five days after municipal wastewater and river samples were collected (19 June). Within-system hospital influent–effluent, Glina WWTP influent–effluent, and river upstream–downstream samples shared the same recorded sampling date in all campaigns. However, matching calendar dates do not establish simultaneous collection or account for hydraulic retention and transport times.

Sampling formats at the Glina WWTP varied across campaigns. Both instantaneous and averaged/composite-labeled samples were explicitly identified in September 2019, February 2020, and June 2020. The June and November 2019 campaigns included one sample per treatment compartment without an explicit sampling-format designation, whereas September 2020 and March 2021 included composite-labeled samples alongside samples of unspecified format. For both influent and effluent, the pre-COVID-19 period included two explicitly instantaneous, two composite-labeled, and two unspecified samples, compared with one, three, and two samples, respectively, during the COVID-19 era. Thus, the documented sampling formats were not balanced between periods.

When multiple municipal wastewater samples were available within the same campaign, concentrations were summarized using the median to obtain a single site–campaign estimate, thereby avoiding pseudoreplication. In addition to the primary comparison between four pre-COVID-19 and three COVID-19-era campaigns, two supplementary sensitivity analyses were performed. First, approximately seasonally matched comparisons were conducted using June 2019 versus June 2020, September 2019 versus September 2020, and February 2020 versus March 2021. Second, sampling-format-restricted comparisons were performed using only samples explicitly identified as instantaneous or composite-labeled, without assigning a format to unspecified samples. Campaigns remained the independent temporal units throughout the analyses.

2.3. Culture-Based Quantification of Resistant Bacteria

Water samples were processed on the day of collection or within 24 h after storage at 4 °C. Defined sample volumes were filtered through 47-mm mixed-cellulose-ester membranes with 0.45 µm pores. Membranes were transferred to selective chromogenic media, including TBX agar for *Escherichia coli*, chromID ESBL for ESBL screening phenotypes, chromID OXA-48 and chromID CARBA for carbapenem-resistance screening phenotypes, and chromID VRE for vancomycin-resistant enterococci (chromID media: bioMérieux, Marcy-l'Étoile, France).

The longitudinal analysis included the quantitative culture variables consistently available across the Bucharest surveillance continuum: ESBL *E. coli*, ESBL KESC, ESBL non-fermenting Gram-negative bacilli (NFGNB), CARBA *E. coli*, CARBA KESC, CARBA NFGNB, OXA *E. coli*, OXA KESC, OXA NFGNB, and VRE. KESC denotes the *Klebsiella–Enterobacter–Serratia–Citrobacter* complex. CARBA and OXA designations indicate growth on the respective selective screening media and are therefore reported as screening phenotypes rather than definitive molecular carbapenemase assignments.

The volume filtered varied from 10 to 400 mL depending on the sample matrix, anticipated microbial load, and target microorganism group. Smaller volumes were generally filtered for highly contaminated wastewater samples and less selective culture media, whereas larger volumes were used for samples with lower expected bacterial concentrations or when highly selective media were employed. For TBX, chromID ESBL, chromID CARBA, and chromID OXA-48, membrane filters were subjected to an initial incubation at 44 °C for 4–5 h, followed by incubation at 37 °C for 18–24 h. For chromID VRE, incubation was performed at 37 °C for 18–24 h.

After incubation, characteristic colonies were enumerated, and representative colonies were subcultured on the corresponding selective media. Bacterial densities were expressed as CFU/100 mL according to the laboratory calculation framework based on SR EN ISO 8199:2018 [14]. Up to six resistant isolates per environmental sample were retained for downstream characterization within the RADAR project (PN-III-P4-ID-PCCF-2016-0114), a national research initiative investigating the selection and dissemination of antibiotic resistance determinants across wastewater treatment plants, aquatic environments, and clinical settings. The present study focuses specifically on the longitudinal culture-based screening data collected from the Bucharest surveillance compartments.

Colonies displaying the expected morphology and chromogenic characteristics were presumptively assigned to the corresponding screening categories and enumerated. Counts obtained on chromID CARBA, chromID OXA-48, and chromID VRE were treated as presumptive resistance screening phenotypes. No systematic isolate-level confirmatory testing was performed for the colonies included in the quantitative longitudinal analysis; therefore, growth on these media was not interpreted as definitive confirmation of species identity or specific resistance mechanisms.

2.4. Data Harmonization and Statistical Analysis

Statistical analyses were conducted using Python (version 3.12.14), with pandas (version 3.0.1), NumPy (version 2.3.5), and SciPy (version 1.18.1). Analyses were performed at the sampling-campaign level, with four pre-COVID-19 and three COVID-19-era campaigns treated as independent temporal observations. Values reported as below the detection limit ("<") were classified as non-detects and assigned zero on the concentration scale for the primary descriptive analysis, without implying confirmed biological absence. Missing or invalid measurements were excluded. When multiple municipal wastewater samples were available within a campaign, their concentrations were summarized using the median to obtain a single site–campaign estimate, thereby avoiding pseudoreplication. Campaign-level estimates were subsequently transformed as $\log_{10}(\text{CFU}/100\ \text{mL} + 1)$. Period-level distributions were summarized using medians, interquartile ranges (25th–75th percentiles), and positive/evaluable campaign counts.

Temporal differences in Glina WWTP effluent were primarily characterized as differences between COVID-19-era and pre-COVID-19 period medians. As a complementary descriptive effect estimate, the two-sample Hodges–Lehmann (HL) estimator was calculated as the median of all pairwise differences between campaign-level observations from the two periods. These pairwise differences were not treated as independent observations, and the HL estimator was distinguished from the difference between period medians. Given the small number of independent campaigns, extensive ties arising from non-detect substitution, and potential differences in distributional shape, a common location-shift model was not assumed. Conventional exact two-sided 95% confidence intervals for a location-shift parameter were unbounded at the available sample sizes; therefore, finite confidence limits are not reported. Statistical interpretation focused on descriptive effect magnitudes, observed between-campaign variability, detection frequencies, and sensitivity

analyses rather than hypothesis-testing thresholds. Interquartile ranges were interpreted as measures of observed variability, not confidence intervals.

Treatment-associated concentration contrasts at the Glina WWTP were calculated within each campaign as $\log_{10}(\text{influent} + 1) - \log_{10}(\text{effluent} + 1)$. Similarly, upstream-to-downstream differences in the Dâmbovița River were calculated as $\log_{10}(\text{downstream} + 1) - \log_{10}(\text{upstream} + 1)$, with positive values indicating higher downstream concentrations. Campaign-level contrasts were subsequently summarized using medians and interquartile ranges.

To assess the influence of non-detect handling, additional sensitivity analyses replaced non-detect observations with alternative hypothetical concentrations before within-campaign aggregation, while retaining measured positive values. Scenarios included substitutions from 0.1 to 100 CFU/100 mL, with higher concentrations evaluated as extended stress tests. Temporal differences, treatment-associated contrasts, and river contrasts were recalculated using the same analytical framework. Sampling-format-restricted comparisons were also performed using explicitly identified instantaneous or composite-labeled samples. These analyses were interpreted descriptively and were not considered corrections for potential seasonal or sampling-format confounding. Hypothetical substitutions were not interpreted as experimentally validated detection limits or LOD/2 estimates.

3. Results

3.1. Resistance Screening Phenotypes Were Detected Across the Hospital–Wastewater–River Continuum

Resistance screening phenotypes were recovered from hospital wastewater, municipal influent, activated sludge, treated municipal effluent, and Dâmbovița River samples. Median abundances varied markedly across compartments and phenotypes (Figure 2). Hospital wastewater showed some of the highest median abundances, particularly for ESBL KESC, CARBA KESC, and VRE. Median abundances were generally lower in Glina WWTP effluent than in influent, although the magnitude of this difference varied among phenotypes. Upstream river samples showed low or non-detectable median abundances for most screening phenotypes, whereas several phenotypes were detected at higher median abundances downstream.

Resistance screening phenotypes were also recovered from the activated sludge compartment of the Glina WWTP across the surveillance period. The original measurements demonstrated the presence of ESBL, CARBA, OXA, and VRE screening phenotypes, with heterogeneous detection patterns across campaigns and phenotypic groups. These findings provide descriptive evidence of resistant bacterial populations within the biological treatment compartment. Individual activated sludge measurements for all seven campaigns are provided in Supplementary Table S2.

3.2. Hospital Wastewater Remained Enriched in Resistance Screening Phenotypes Without a Uniform COVID-19-Era Increase

Hospital effluent remained enriched in clinically relevant resistance screening phenotypes in both periods. Pre-COVID-19 median abundances were 6.74 $\log_{10}$ (CFU/100 mL + 1) for ESBL KESC, 6.90 for CARBA KESC, 6.11 for OXA KESC, and 6.93 for VRE. Corresponding COVID-19-era medians were 6.13, 5.42, 3.52, and 4.15 $\log_{10}$ (CFU/100 mL + 1), respectively. ESBL NFGNB was the main phenotype showing a higher hospital-effluent median during the COVID-19 era (5.65 vs. 5.21 $\log_{10}$ (CFU/100 mL + 1)). Hospital influent similarly showed no uniform COVID-19-era increase, with lower median abundances for several KESC-associated phenotypes and VRE, but a higher median abundance for ESBL NFGNB.

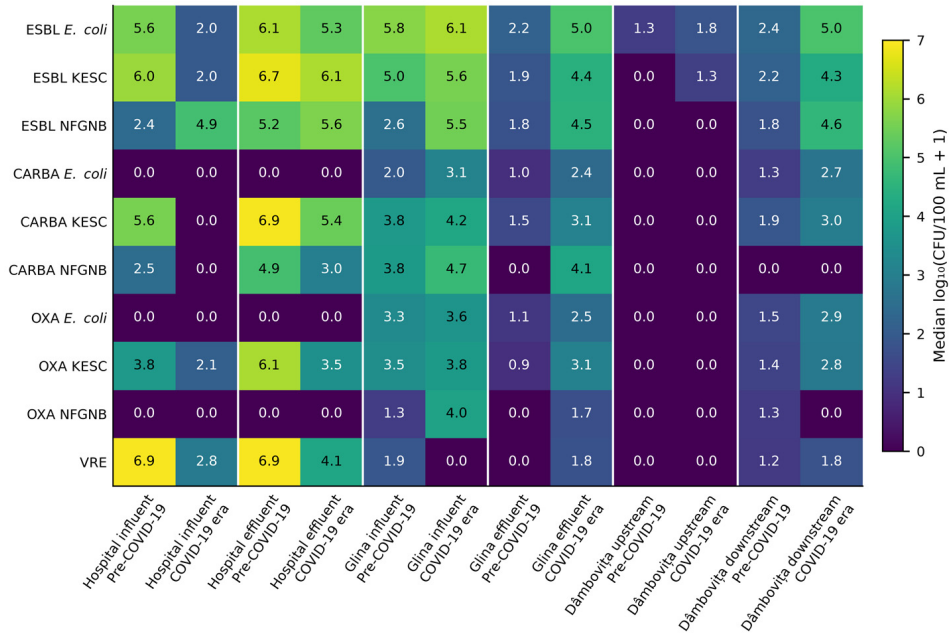


Figure 2. Median abundances of culture-based antimicrobial resistance screening phenotypes across the Bucharest hospital–WWTP–river continuum during the pre-COVID-19 and COVID-19 periods. Values represent medians of campaign-level estimates expressed as $\log_{10}(\text{CFU}/100\ \text{mL} + 1)$, based on four pre-COVID-19 and three COVID-19-era campaigns, except for VRE in Glina WWTP effluent, for which three evaluable pre-COVID-19 campaigns were available. Within-campaign municipal wastewater concentrations were aggregated before logarithmic transformation. Non-detect observations were assigned zero for the baseline summaries; therefore, displayed zero values do not necessarily indicate confirmed bacterial absence.

Overall, the hospital compartment remained a persistent source of high abundances of resistance screening phenotypes across the surveillance period, but the direction and magnitude of temporal changes varied among phenotypes.

3.3. Treated Municipal Effluent Showed a Consistent COVID-19-Era Increase Across Multiple Resistance Screening Phenotypes

In contrast to the heterogeneous temporal pattern observed in hospital wastewater, Glina WWTP effluent showed higher COVID-19-era median abundances across all ten resistance screening phenotypes under the baseline non-detect convention. Median ESBL *E. coli* abundance increased from 2.21 to 5.02 $\log_{10}(\text{CFU}/100\ \text{mL} + 1)$, ESBL KESC from 1.87 to 4.43, ESBL NFGNB from 1.76 to 4.49, CARBA NFGNB from 0.00 to 4.12, and OXA KESC from 0.94 to 3.07. Across all ten phenotypes, differences between period medians ranged from +1.32 to +4.12 $\log_{10}$ units, while the corresponding Hodges–Lehmann pairwise-median contrasts ranged from +1.36 to +4.04 $\log_{10}$ units (Table 2). These descriptive estimates reflect a limited number of independent campaigns and do not establish a causal pandemic-related effect.

Detection frequencies showed a similar directional pattern. ESBL *E. coli*, ESBL KESC, ESBL NFGNB, CARBA *E. coli*, CARBA KESC, OXA *E. coli*, and OXA KESC were detected in all three COVID-19-era Glina effluent campaigns, compared with two of four pre-COVID-19 campaigns for each phenotype. CARBA NFGNB increased from one of four to three of three campaigns, whereas OXA NFGNB and VRE increased from one of four and zero of three evaluable pre-COVID-19 campaigns, respectively, to two of three COVID-19-era campaigns (Figure 3).

Table 2. Glina WWTP effluent before COVID-19 and during the COVID-19 era.

Phenotype	Pre-COVID Median (IQR), *n*	Pre-COVID Detection	COVID-Era Median (IQR), *n*	COVID-Era Detection	Difference of Medians	HL Contrast
ESBL *E. coli*	2.21 (0.00–4.47), 4	2/4	5.02 (4.88–5.17), 3	3/3	+2.81	+2.82
ESBL KESC	1.87 (0.00–3.77), 4	2/4	4.43 (4.22–4.48), 3	3/3	+2.56	+2.40
ESBL NFGNB	1.76 (0.00–3.63), 4	2/4	4.49 (4.12–4.87), 3	3/3	+2.73	+2.73
CARBA *E. coli*	0.95 (0.00–1.94), 4	2/4	2.42 (2.18–2.58), 3	3/3	+1.47	+1.38
CARBA KESC	1.54 (0.00–3.11), 4	2/4	3.09 (2.96–3.47), 3	3/3	+1.55	+1.81
CARBA NFGNB	0.00 (0.00–0.76), 4	1/4	4.12 (4.04–4.44), 3	3/3	+4.12	+4.04
OXA *E. coli*	1.14 (0.00–2.36), 4	2/4	2.46 (2.31–2.66), 3	3/3	+1.32	+1.36
OXA KESC	0.94 (0.00–2.08), 4	2/4	3.07 (3.00–3.08), 3	3/3	+2.12	+2.08
OXA NFGNB	0.00 (0.00–0.40), 4	1/4	1.72 (0.86–2.45), 3	2/3	+1.72	+1.65
VRE	0.00 (0.00–0.00), 3	0/3	1.76 (0.88–2.46), 3	2/3	+1.76	+1.76

Values are expressed as $\log_{10}$(CFU/100 mL + 1). Medians (IQRs) and sample sizes (*n*) refer to evaluable campaigns; detection frequencies indicate positive/evaluable campaigns. The difference of medians represents the COVID-19-era median minus the pre-COVID-19 median, whereas HL denotes the Hodges–Lehmann pairwise-median contrast. Non-detects were assigned zero for the baseline analysis. The June 2019 VRE effluent measurement was invalid and excluded.

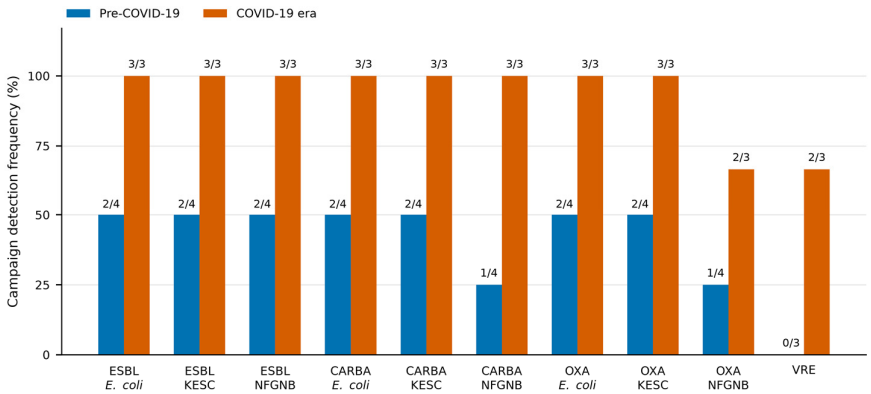


Figure 3. Campaign-level detection frequencies of culture-based antimicrobial resistance screening phenotypes in Glina WWTP effluent during the pre-COVID-19 and COVID-19 periods. Bars indicate the percentage of evaluable campaigns in which each phenotype was detected, and the labels above bars show the corresponding positive/evaluable campaign counts. Four pre-COVID-19 and three COVID-19-era campaigns were available for most phenotypes; for VRE, one pre-COVID-19 effluent measurement was invalid, resulting in three evaluable pre-COVID-19 campaigns. Detection frequencies are shown descriptively and do not imply statistical significance.

The seasonally matched sensitivity analysis showed the same overall direction of change. Across the three approximately matched campaign pairs (June 2019 vs. June 2020,

September 2019 vs. September 2020, and February 2020 vs. March 2021), the median COVID-19-era minus pre-COVID-19 difference was positive for all ten phenotypes. The largest matched differences were observed for CARBA NFGNB (+3.97 $\log_{10}$), VRE (+2.46), ESBL NFGNB (+1.72), OXA NFGNB (+1.58), and OXA KESC (+1.22). Given the limited number of campaign pairs and the approximate seasonal correspondence of February 2020 and March 2021, these findings provide only a descriptive assessment of directional consistency and cannot exclude seasonal confounding.

An additional sensitivity analysis examined the influence of non-detect substitution on the observed temporal differences in Glina WWTP effluent. When non-detects were replaced with hypothetical concentrations ranging from 0 to 100 CFU/100 mL, COVID-19-era median abundances remained higher than pre-COVID-19 medians for all ten screening phenotypes, although the magnitude of these differences varied substantially. For CARBA NFGNB, the median difference decreased from +4.12 $\log_{10}$ units under zero substitution to +3.08 and +2.11 $\log_{10}$ units under substitutions of 10 and 100 CFU/100 mL, respectively. The ESBL *E. coli*, KESC, and NFGNB differences remained positive and exceeded 1.5 $\log_{10}$ units at 100 CFU/100 mL. These analyses demonstrated that the magnitude of the descriptive temporal contrasts depended on the assumed concentrations assigned to non-detect observations. The hypothetical substitution scenarios were used to evaluate this sensitivity rather than to establish inferential uncertainty or statistical significance.

Sampling-format-restricted comparisons showed that seven of the ten Glina-effluent screening phenotypes retained higher COVID-19-era median abundances under both instantaneous-only and composite-only selections. However, several estimates were sensitive to sample selection. For CARBA NFGNB, the COVID-19-era minus pre-COVID-19 median difference decreased from +4.12 $\log_{10}$ units in the primary analysis to +2.86 under composite-only selection and −1.46 under instantaneous-only selection. OXA NFGNB and VRE also showed attenuated or reversed differences under format restrictions. The instantaneous-only comparison included only one COVID-19-era campaign and therefore cannot support a period-wide temporal inference. Detailed phenotype-specific differences under the primary and format-restricted analyses are provided in Supplementary Table S1.

3.4. Treatment-Associated Reductions Varied Across Resistance Screening Phenotypes

Glina WWTP treatment was associated with positive median log reductions for most resistance screening phenotypes in both periods (Figure 4). During the COVID-19 era, median reductions were 1.22 $\log_{10}$ for ESBL *E. coli*, 1.23 for ESBL KESC, 1.65 for ESBL NFGNB, 1.07 for CARBA KESC, 0.90 for OXA *E. coli*, and 0.85 for OXA KESC. CARBA NFGNB showed the largest decrease in treatment-associated reduction between periods, from a median of 2.29 $\log_{10}$ pre-COVID-19 to 0.38 $\log_{10}$ during the COVID-19 era. In contrast, OXA NFGNB showed a higher median reduction during the COVID-19 era (2.47 vs. 1.23 $\log_{10}$ units).

VRE differed from the other phenotypes, with the median influent–effluent difference changing from 0.00 $\log_{10}$ pre-COVID-19 to −1.76 $\log_{10}$ during the COVID-19 era. The negative value indicates that the median effluent abundance exceeded the corresponding influent abundance under the campaign-level comparison and should not be interpreted as evidence of within-treatment microbial amplification.

3.5. Resistance Screening Phenotypes Were Frequently Higher Downstream than Upstream in the Dâmbovița River

Across all seven campaigns, the median downstream-minus-upstream difference was positive for eight of the ten resistance screening phenotypes (Figure 5).

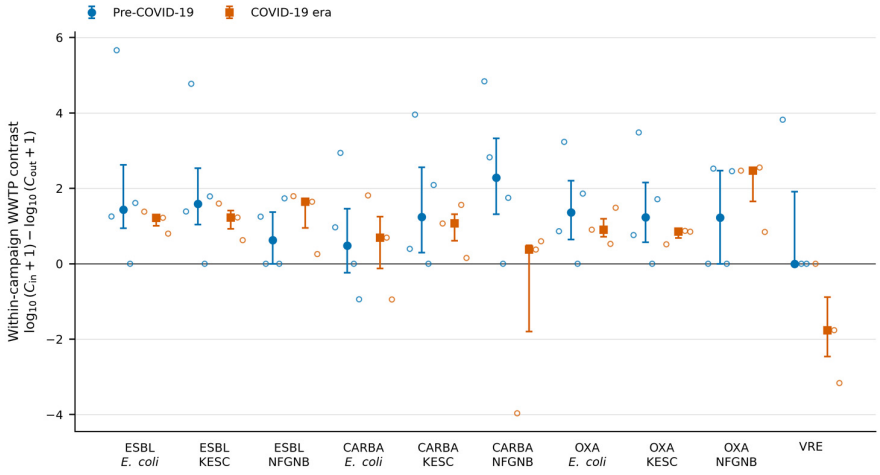


Figure 4. Treatment-associated influent–effluent concentration contrasts for antimicrobial resistance screening phenotypes at the Glina wastewater treatment plant during the pre-COVID-19 and COVID-19 periods. Contrasts were calculated within each campaign as $\log_{10}(C_in + 1) - \log_{10}(C_out + 1)$, where concentrations are expressed in CFU/100 mL. Filled symbols represent period medians, error bars indicate interquartile ranges, and open circles represent individual campaign-level observations. Four pre-COVID-19 and three COVID-19-era campaigns contributed to most phenotype comparisons; for VRE, only three pre-COVID-19 campaigns were evaluable. Positive values indicate lower effluent concentrations relative to influent concentrations, whereas negative values indicate higher effluent concentrations. Non-detect observations were assigned zero for the baseline calculations. These differences represent treatment-associated concentration contrasts rather than flow-normalized removal efficiencies.

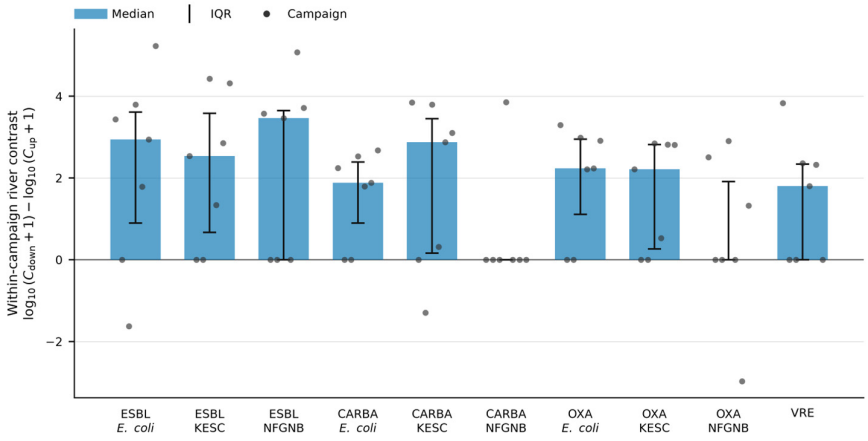


Figure 5. Campaign-level downstream–upstream concentration contrasts for antimicrobial resistance screening phenotypes in the Dâmbovița River across seven sampling campaigns (June 2019–March 2021). Contrasts were calculated within each campaign as $\log_{10}(C_down + 1) - \log_{10}(C_up + 1)$, where concentrations are expressed in CFU/100 mL. Bars represent median contrasts, error bars indicate interquartile ranges, and grey points represent individual campaign-level observations ($n = 7$ per phenotype). Individual points were slightly displaced horizontally to improve visibility without altering their values. Positive values indicate higher downstream concentrations, whereas negative values indicate higher upstream concentrations. Non-detect observations were assigned zero for the baseline calculations. These spatial contrasts do not establish exclusive attribution to the Glina WWTP discharge.

The largest median differences were observed for ESBL NFGNB (+3.47 $\log_{10}$), ESBL *E. coli* (+2.94), CARBA KESC (+2.88), ESBL KESC (+2.54), OXA *E. coli* (+2.23), OXA KESC (+2.21), CARBA *E. coli* (+1.88), and VRE (+1.80). For ESBL *E. coli*, ESBL KESC, CARBA *E. coli*, CARBA KESC, OXA *E. coli*, and OXA KESC, downstream abundance exceeded upstream

abundance in five of the seven campaigns. CARBA NFGNB and OXA NFGNB had median downstream-minus-upstream differences of zero, reflecting frequent non-detection at both river sites.

The predominantly positive downstream–upstream contrasts represent a descriptive spatial pattern observed across several resistance screening phenotypes, although the magnitude and direction of individual campaign differences varied (Figure 5). These contrasts do not establish a consistent population-level effect or permit definitive attribution to the Glina WWTP discharge. Treatment-associated reductions at the Glina WWTP and downstream–upstream river differences are summarized across all phenotypes in Table 3.

Table 3. Treatment and receiving-water effect-size summary.

Phenotype	WWTP Reduction Pre	WWTP Reduction COVID-Era	River Δ Downstream − Upstream	River Positive Campaigns
ESBL *E. coli*	1.43 (4)	1.22 (3)	+2.94 (7)	5/7
ESBL KESC	1.59 (4)	1.23 (3)	+2.54 (7)	5/7
ESBL NFGNB	0.62 (4)	1.65 (3)	+3.47 (7)	4/7
CARBA *E. coli*	0.48 (4)	0.69 (3)	+1.88 (7)	5/7
CARBA KESC	1.24 (4)	1.07 (3)	+2.88 (7)	5/7
CARBA NFGNB	2.29 (4)	0.38 (3)	+0.00 (7)	1/7
OXA *E. coli*	1.36 (4)	0.90 (3)	+2.23 (7)	5/7
OXA KESC	1.24 (4)	0.85 (3)	+2.21 (7)	5/7
OXA NFGNB	1.23 (4)	2.47 (3)	+0.00 (7)	3/7
VRE	0.00 (3)	−1.76 (3)	+1.80 (7)	4/7

Values are expressed in $\log_{10}$(CFU/100 mL + 1) units. The reported *n* represents the number of evaluable sampling campaigns contributing to each median, rather than the number of individual samples. Missing or invalid measurements were excluded. For VRE, the June 2019 Glina effluent measurement was invalid, resulting in three evaluable pre-COVID-19 campaigns for the corresponding effluent and treatment-associated comparisons.

4. Discussion

4.1. COVID-19-Era Changes Differed Between Hospital and Municipal Wastewater

The most important finding was not a generalized increase in hospital wastewater AMR during the COVID-19 era. Hospital effluent already represented a high-resistance compartment before the pandemic, and median abundances of several KESC-associated phenotypes and VRE were lower during the three COVID-19-era campaigns. The high resistance burden observed in hospital wastewater is consistent with previous Romanian surveillance demonstrating carbapenemase-producing *A. baumannii* and *P. aeruginosa* across clinical, hospital-wastewater, and WWTP-associated samples, including genetic relatedness between clinical and wastewater isolates [15]. In contrast, the clearest descriptive temporal pattern was observed in Glina WWTP effluent, where median abundances were higher across all ten resistance screening phenotypes during the COVID-19 era. Several ESBL, CARBA, and OXA screening groups were also detected in a greater proportion of campaigns, and the approximately seasonally matched comparisons showed the same overall direction. However, these patterns were based on a limited number of campaigns and were sensitive to non-detect handling and sampling-format selection. They therefore do not support a

simple interpretation in which pandemic-associated AMR changes originated uniformly at the hospital source and subsequently spread throughout the wastewater continuum.

Published studies similarly indicate that wastewater AMR responses during the COVID-19 pandemic were context-dependent. Wang et al. reported temporal increases in genes associated with resistance to macrolides, sulfonamides, tetracyclines, and selected beta-lactams in untreated wastewater from a hospital treating COVID-19 patients [9]. Zhao et al. observed changes in antibiotics, ARGs, and microbial communities in hospital wastewater after the COVID-19 outbreak, with seasonal variation also contributing to the observed patterns [10]. At the community scale, Xu et al. reported higher antimicrobial and antimicrobial resistance gene (ARG) loads during 2021–2022 than during 2020–2021 and identified changes associated with the relaxation of pandemic restrictions [11]. Together, these studies support a context-dependent rather than uniform pandemic-associated AMR response. The Bucharest data add an Eastern European culture-based perspective, showing that the most coherent temporal change was observed in treated municipal effluent rather than as a generalized increase at the hospital sampling points.

4.2. Changes in Antibiotic Use Provide Plausible Context but Not a Causal Explanation

The COVID-19 period altered antimicrobial use in complex and partly opposing ways. Across the EU/EEA, total antibiotic consumption declined sharply between 2019 and 2020, with a particularly marked reduction in the community sector [12]. Although overall hospital antibiotic consumption also decreased, the relative use of broad-spectrum and last-line agents increased during 2020–2021 [13]. Romania remained among the countries with the highest overall antibiotic consumption in the EU/EEA, with total consumption in 2021 returning close to its 2019 level [13]. These changes could plausibly influence the mixture of resistant bacteria entering Bucharest sewers, but the present dataset contains no linked hospital prescribing, community dispensing, patient census, SARS-CoV-2 burden, or antimicrobial-residue exposure data for each campaign. Accordingly, the term "COVID-19-era" is used as a temporal descriptor rather than as a proven causal exposure.

The approximately seasonally matched comparisons provide limited descriptive support for the direction of the Glina-effluent differences but cannot adequately control for seasonal variation. Higher COVID-19-era median abundances were observed across all ten screening phenotypes in these comparisons, which involved June 2019 versus June 2020, September 2019 versus September 2020, and February 2020 versus March 2021. The final comparison was only approximately aligned by season. Seasonal variability is particularly relevant in the Romanian wastewater context, as a recent multi-WWTP study reported seasonal differences in antibiotic residues and bacterial loads, together with compound-specific variation in treatment removal [16]. Rainfall and variations in wastewater inflow may influence bacterial concentrations through dilution, increased runoff, changes in hydraulic loading, and altered retention times within the treatment system. Similarly, variations in WWTP effluent discharge and Dâmbovița River flow may affect downstream concentrations through changes in dilution and mixing conditions. Because campaign-specific precipitation records, WWTP influent and effluent flow measurements, and river discharge data were not integrated into the present analysis, these potential influences could not be quantified or controlled for. Moreover, the reported CFU/100 mL measurements represent concentrations rather than flow-normalized bacterial loads. With only three approximately seasonally matched pairs, the observed differences cannot be reliably separated from hydrological, seasonal, or operational variability. More frequent sampling combined with concurrent meteorological, hydraulic, and treatment-operational measurements would be required to evaluate these influences adequately.

4.3. Glina Functioned as an Important but Incomplete AMR Barrier

Positive median influent–effluent concentration contrasts for most phenotypes indicate that municipal treatment substantially decreased the abundance of culturable resistant bacteria. However, the persistence of ESBL and carbapenem-resistance screening phenotypes in treated effluent shows that removal was incomplete. This is consistent with broader evidence that conventional and biological wastewater treatment can reduce bacterial and resistance-associated loads without uniformly eliminating resistant bacteria or resistance determinants [17–20]. This interpretation is also consistent with the broader assessment by Marutescu et al. [21], which identified wastewater treatment plants as important interfaces in the environmental dissemination of clinically relevant resistant pathogens and emphasized the need for surveillance of resistant bacteria and resistance determinants across treatment systems. Their fate during treatment depends on microbial hosts, treatment conditions, community dynamics, and the genetic context of resistance [17,18,20]. Brown et al. showed that resistance-gene fate during activated-sludge treatment can vary at the microdiversity level and reported associations between fluctuating antibiotic concentrations, horizontal gene transfer, and microbial ecology [17]. Genome-resolved analyses have further demonstrated that wastewater ARGs occur across diverse microbial hosts and mobile genetic contexts, including organisms with pathogenic potential [18]. Recent metagenomic evidence following a hospital–WWTP–receiving-water continuum similarly showed progressive reductions in antibiotics and high-risk ARGs during treatment, while clinically relevant resistance determinants and potential bacterial hosts remained detectable in receiving waters [22].

The marked change in CARBA NFGNB treatment-associated concentration contrasts and the inconsistent VRE pattern are particularly relevant as descriptive surveillance findings. However, the available data cannot establish whether these patterns reflect selection or microbial amplification within the treatment plant. Alternative explanations include hydraulic retention and sampling-time mismatches, release from sludge-associated communities, matrix-dependent culturability, and differences in microbial recovery efficiency. In particular, the negative COVID-19-era median influent–effluent contrast observed for VRE indicates higher measured concentrations in the effluent than in the influent under the baseline non-detect convention but does not establish the underlying mechanism. Moreover, these concentration contrasts cannot be interpreted as flow-normalized removal efficiencies. The observed phenotype-specific variability highlights the limitations of relying on a single generic bacterial indicator to characterize AMR dynamics across treatment stages. Future surveillance should therefore integrate culture-based resistant-bacteria counts with molecular resistance targets, host-resolved genomic data, and flow-normalized loads.

The apparent decrease in CARBA NFGNB treatment-associated concentration contrasts was sensitive to non-detect handling. Under the original zero-substitution convention, the median influent–effluent contrast decreased from 2.29 $\log_{10}$ units before COVID-19 to 0.38 $\log_{10}$ units during the COVID-19 era. With a hypothetical substitution of 100 CFU/100 mL, the corresponding medians were 1.30 and 0.38 $\log_{10}$ units, respectively. Thus, the direction of the period contrast was retained under this scenario, but its magnitude was substantially attenuated. These findings should be interpreted as censoring-sensitive descriptive concentration contrasts rather than direct estimates of biological removal efficiency.

4.4. Downstream Enrichment Supports Surveillance Beyond the WWTP Outlet

The Dâmbovița River results extend the surveillance framework beyond the treatment plant. For several major resistance screening phenotypes, downstream abundance exceeded upstream abundance in five of the seven campaigns, with median downstream-minus-upstream differences of approximately 2–3.5 $\log_{10}$ units. This pattern is consistent

with recent studies showing that substantial AMR signals can persist beyond wastewater treatment and influence receiving-river resistomes. Read et al. reported that, despite substantial reductions in normalized ARG abundance and richness during wastewater treatment, receiving-river sediments showed marked increases in both measures downstream of WWTP discharges [23]. Similarly, Lee et al. found that river water was the habitat most strongly affected by WWTP effluent across nine Swiss river systems, with several resistance determinants, including class A beta-lactamase genes, significantly enriched downstream relative to upstream sites [24].

The present findings should nevertheless be interpreted conservatively. Unlike studies incorporating multiple environmental matrices, molecular markers, or stronger source-discrimination designs [25,26], our culture-based observations cannot attribute the higher downstream abundances exclusively to the Glina WWTP. The Dâmbovița is an open environmental system, and the analysis did not account for river and effluent flow, travel time, tributary inputs, sediment exchange, or other point and diffuse sources. Campaign-specific measurements of river discharge, flow velocity, and WWTP effluent flow were not available in the analytical dataset, precluding hydrodynamic modelling or flow-normalized estimates of bacterial transport. Consequently, the study was restricted to comparing measured bacterial concentrations at the upstream and downstream sampling locations rather than quantifying pollutant fluxes or effluent-plume dispersion. Complete mixing of the WWTP effluent within the approximately 200 m downstream reach was not independently verified. In addition, potential inputs between the sampling locations could not be excluded. Therefore, upstream–downstream differences should be interpreted as local spatial contrasts rather than definitive estimates of the WWTP-specific contribution. Hydrodynamic conditions are particularly relevant because recent experimental evidence indicates that water-flow regimes can shape ARG trajectories and persistence in wastewater-impacted river biofilms [27]. The predominantly positive downstream-minus-upstream pattern therefore provides evidence of spatial enrichment rather than definitive source attribution and supports the value of paired upstream/downstream monitoring within integrated urban AMR surveillance.

This perspective is increasingly relevant from a policy standpoint. The recast EU Urban Wastewater Treatment Directive introduces AMR-related surveillance requirements for large agglomerations and emphasizes harmonized monitoring approaches [28]. WHO guidance similarly recognizes wastewater and environmental surveillance as a potential complement to clinical AMR surveillance while emphasizing the need for standardized methods and actionable interpretation [29].

4.5. Strengths and Limitations

An important strength of this study is its integrated sampling design, which followed culturable resistance screening phenotypes from hospital wastewater through a major municipal WWTP and into paired upstream and downstream receiving-water sites across both pre-COVID-19 and COVID-19-era campaigns. The use of a consistent selective-culture platform provided a biologically interpretable endpoint based on viable organisms capable of growth under resistance-selective conditions. Recent culture-based genomic surveillance has likewise demonstrated widespread and genetically diverse ESBL-producing *E. coli* across wastewater-associated and aquatic environments, highlighting the complementary value of viable-isolate recovery and genomic characterization for environmental AMR surveillance [30]. The comparison of three approximately seasonally matched campaign pairs provided a supplementary descriptive assessment of temporal direction, although its limited size and imperfect seasonal alignment precluded a rigorous evaluation of seasonal confounding.

Several limitations define the appropriate scope of inference. First, the temporal analysis included only seven independent campaigns (four pre-COVID-19 and three COVID-19-era), with three evaluable pre-COVID-19 campaigns for Glina-effluent VRE. This small sample size severely limits inferential precision. Conventional exact two-sided 95% confidence intervals for a common location-shift parameter were unbounded, while the assumption of a common distributional shift could not be established. Accordingly, temporal findings were interpreted through descriptive differences between period medians, complementary Hodges–Lehmann pairwise-median contrasts, observed campaign variability, and sensitivity analyses rather than statistical significance thresholds. The reported interquartile ranges characterize observed variability and should not be interpreted as confidence intervals. Second, campaign-specific precipitation, WWTP influent and effluent flow, and Dâmbovița River discharge measurements were not incorporated into the analysis, preventing assessment of hydrological influences, flow-normalized bacterial loads, and potential dilution effects. Detailed WWTP operational data, antimicrobial consumption, hospital occupancy, and COVID-19 case burden were also unavailable for integrated analysis. Third, sampling-format availability differed between periods, with fewer explicitly instantaneous samples and more composite-labeled samples during the COVID-19 era. Although within-campaign aggregation avoided pseudoreplication, format-restricted comparisons showed that several temporal estimates, particularly CARBA NFGNB, OXA NFGNB, and VRE, were sensitive to sample selection and changes in campaign coverage. Fourth, non-detect observations were assigned zero in the primary analysis because numerical culture-specific detection limits were not documented in the available consolidated dataset. This convention may influence estimated effect magnitudes and does not imply confirmed bacterial absence. Fifth, growth on selective chromogenic media represents presumptive resistance screening phenotypes rather than definitive phenotypic or molecular confirmation of specific resistance mechanisms. Additionally, the five-day offset between hospital and municipal/river sampling in June 2019 limits contemporaneous cross-compartment interpretation for that campaign, while matching sampling dates in the remaining campaigns do not establish hydraulic or microbial-flow correspondence between compartments. Finally, the available series ended in March 2021 and supports a descriptive comparison between pre-COVID-19 and COVID-19-era campaigns, rather than causal attribution to the pandemic or evaluation of post-pandemic trends. Longer and more densely sampled studies integrating culture-based surveillance with genomic, operational, epidemiological, and environmental data are needed to clarify these patterns and their underlying drivers.

5. Conclusions

Longitudinal surveillance in Bucharest identified resistance screening phenotypes across the hospital–wastewater–river continuum before and during the COVID-19 era. Hospital wastewater remained a high-abundance compartment throughout the study but did not exhibit a uniform temporal increase. In contrast, Glina WWTP effluent showed higher median abundances across all ten evaluated screening phenotypes during the COVID-19 era, accompanied by increased detection frequencies for several ESBL, CARBA, and OXA screening groups. These descriptive differences were based on a limited number of independent campaigns and were sensitive to non-detect handling and sampling-format selection. Treatment-associated influent–effluent concentration contrasts were positive for most phenotypes but varied considerably among groups, while several resistance screening phenotypes showed higher downstream than upstream abundances in the Dâmbovița River. Together, these findings support integrated AMR surveillance extending from wastewater sources and treatment systems into receiving aquatic environments.

These observations do not establish that the COVID-19 pandemic caused the temporal differences observed in Bucharest, nor do the downstream patterns permit definitive source attribution to the Glina WWTP. Rather, they demonstrate the potential value of integrated longitudinal surveillance for identifying temporal and spatial variation that may not be evident from hospital wastewater alone. Future studies incorporating more frequent and seasonally balanced sampling, genomic characterization, antimicrobial-residue measurements, flow-normalized loads, WWTP operational parameters, and linked antimicrobial-consumption and epidemiological data are needed to clarify the observed patterns and their underlying drivers, strengthening wastewater-based AMR surveillance within a One Health framework.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/environments13110591/s1, Table S1: Sensitivity of Glina WWTP effluent temporal differences to sampling-format restriction. Table S2: Individual culture-based antimicrobial resistance screening measurements across seven sampling campaigns in the Bucharest hospital–WWTP–river continuum (2019–2021).

Author Contributions: Conceptualization, E.M.S., M.P. (Marcela Popa), L.G.M. and A.S.-C.; methodology, M.P. (Marcela Popa), L.G.M., I.C.B., I.G.-B. and M.N.-L.; validation, I.C.B. and I.G.-B.; formal analysis, E.M.S. and M.P. (Marcela Popa); investigation, M.P. (Marcela Popa), L.G.M., I.C.B., I.G.-B., M.N.-L. and G.L.P.; resources, M.N.-L., G.L.P., M.I.P., C.C.D., A.A.M., M.-M.M. and M.I.P.; data curation, E.M.S., C.P. and M.P. (Mădălina Preda); writing—original draft preparation, E.M.S., M.P. (Marcela Popa), C.O.V. and L.G.M.; writing—review and editing, E.M.S., M.P. (Mădălina Preda), C.O.V., L.G.M., M.I.P., C.P. and A.S.-C.; visualization, E.M.S.; supervision, M.I.P. and A.S.-C.; project administration, M.I.P., C.P. and A.S.-C.; funding acquisition, A.S.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by two grants from the Romanian National Authority for Scientific Research, respectively PN-III-P4-ID-PCCF-2016-0114/Contract 10 (7 February 2018)—Selection and dissemination of antibiotic resistance genes from wastewater treatment plants into the aquatic environment and the clinical sector (RADAR) and PN-IV-P6-6.1CoEx-2024-0196, Acronym ARES, Contract no. 17/2.03.2026.

Data Availability Statement: The original contributions presented in this study are included in the article and Supplementary Material. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors acknowledge the support of the Core Program within the National Research, Development and Innovation Plan 2022–2027, carried out with support of MRID, project no. 2302101 (SIA-PRO), contract no. 7N/2022, and the Ministry of Investments and European Projects, through the Managing Authority for the Health Program, PS/272/PS_P5/OP1/RSO1.1/PS_P5_RSO1.1_A9—ROGEN Project (MySMIS 324809). During the preparation of this manuscript, the authors used ChatGPT Plus (OpenAI; GPT-5.6 and GPT-6) to assist with English-language editing, including improvements in grammar, clarity, and textual coherence. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
WWTPs	Wastewater treatment plants
ESBL	Extended-spectrum beta-lactamase
VRE	Vancomycin-resistant enterococci

CARBA	Carbapenem-resistance screening phenotype
NFGNB	Non-fermenting Gram-negative bacilli
IQR	Interquartile range
CFUs	Colony-forming units
EU/EEA	European Union/European Economic Area
COVID-19	Coronavirus disease 2019
KESC	*Klebsiella–Enterobacter–Serratia–Citrobacter* complex
OXA-48	Oxacillinase-48
HL	Hodges–Lehmann estimator

References

1. GBD 2021 Antimicrobial Resistance Collaborators. Global burden of bacterial antimicrobial resistance 1990–2021: A systematic analysis with forecasts to 2050. *Lancet* **2024**, *404*, 1199–1226. [CrossRef]
2. Berendonk, T.U.; Manaia, C.M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Bürgmann, H.; Sørum, H.; Norström, M.; Pons, M.-N.; et al. Tackling antibiotic resistance: The environmental framework. *Nat. Rev. Microbiol.* **2015**, *13*, 310–317. [CrossRef]
3. Larsson, D.G.J.; Flach, C.-F. Antibiotic resistance in the environment. *Nat. Rev. Microbiol.* **2022**, *20*, 257–269. [CrossRef]
4. United Nations Environment Programme. *Bracing for Superbugs: Strengthening Environmental Action in the One Health Response to Antimicrobial Resistance*; UNEP: Nairobi, Kenya, 2023.
5. Larsson, D.G.J.; Flach, C.-F.; Laxminarayan, R. Sewage surveillance of antibiotic resistance holds both opportunities and challenges. *Nat. Rev. Microbiol.* **2023**, *21*, 213–214. [CrossRef]
6. Popa, L.I.; Gheorghe, I.; Barbu, I.C.; Surleac, M.; Paraschiv, S.; Măruțescu, L.; Popa, M.; Pîrcălăbioru, G.G.; Talapan, D.; Niță, M.; et al. Multidrug Resistant *Klebsiella pneumoniae* ST101 Clone Survival Chain from Inpatients to Hospital Effluent After Chlorine Treatment. *Front. Microbiol.* **2021**, *11*, 610296. [CrossRef]
7. Banciu, A.R.; Pascu, L.F.; Radulescu, D.M.; Stoica, C.; Gheorghe, S.; Lucaciu, I.; Ciobotaru, F.V.; Novac, L.; Manea, C.; Nita-Lazar, M. The COVID-19 Pandemic Impact of Hospital Wastewater on Aquatic Systems in Bucharest. *Water* **2024**, *16*, 245. [CrossRef]
8. Gheorghe-Barbu, I.; Dragomir, R.-I.; Gradisteanu Pircalabioru, G.; Surleac, M.; Dinu, I.A.; Gaboreanu, M.D.; Czobor Barbu, I. Tracing *Acinetobacter baumannii*'s Journey from Hospitals to Aquatic Ecosystems. *Microorganisms* **2024**, *12*, 1703. [CrossRef]
9. Wang, C.; Mantilla-Calderon, D.; Xiong, Y.; Alkahtani, M.; Bashawri, Y.M.; Al Qarni, H.; Hong, P.-Y. Investigation of Antibiotic Resistome in Hospital Wastewater during the COVID-19 Pandemic: Is the Initial Phase of the Pandemic Contributing to Antimicrobial Resistance? *Environ. Sci. Technol.* **2022**, *56*, 15007–15018. [CrossRef]
10. Zhao, L.; Lv, Z.; Lin, L.; Li, X.; Xu, J.; Huang, S.; Chen, Y.; Fu, Y.; Peng, C.; Cao, T.; et al. Impact of COVID-19 pandemic on profiles of antibiotic-resistant genes and bacteria in hospital wastewater. *Environ. Pollut.* **2023**, *334*, 122133. [CrossRef]
11. Xu, L.; Ceolotto, N.; Jagadeesan, K.; Standerwick, R.; Robertson, M.; Barden, R.; Kasprzyk-Hordern, B. Antimicrobials and antimicrobial resistance genes in the shadow of COVID-19 pandemic: A wastewater-based epidemiology perspective. *Water Res.* **2024**, *257*, 121665. [CrossRef]
12. European Centre for Disease Prevention and Control. *Antimicrobial Consumption in the EU/EEA (ESAC-Net)—Annual Epidemiological Report for 2020*; ECDC: Stockholm, Sweden, 2021.
13. European Centre for Disease Prevention and Control. *Antimicrobial Consumption in the EU/EEA (ESAC-Net)—Annual Epidemiological Report for 2021*; ECDC: Stockholm, Sweden, 2022.
14. *ISO 8199:2018*; Water Quality—General Requirements and Guidance for Microbiological Examinations by Culture. International Organization for Standardization (ISO): Geneva, Switzerland, 2018.
15. Gheorghe-Barbu, I.; Barbu, I.C.; Popa, L.I.; Pîrcălăbioru, G.G.; Popa, M.; Măruțescu, L.; Niță-Lazar, M.; Banciu, A.; Stoica, C.; Gheorghe, Ș.; et al. Temporo-spatial variations in resistance determinants and clonality of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains from Romanian hospitals and wastewaters. *Antimicrob. Resist. Infect. Control* **2022**, *11*, 115. [CrossRef]
16. Polianciuc, S.I.; Ciorîță, A.; Soran, M.L.; Lung, I.; Kiss, B.; Ștefan, M.G.; Leucuța, D.C.; Gurzău, A.E.; Carpa, R.; Colobațiu, L.M.; et al. Antibiotic Residues and Resistance in Three Wastewater Treatment Plants in Romania. *Antibiotics* **2024**, *13*, 780. [CrossRef]
17. Brown, C.L.; Maile-Moskowitz, A.; Lopatkin, A.J.; Xia, K.; Logan, L.K.; Davis, B.C.; Zhang, L.; Vikesland, P.J.; Pruden, A. Selection and horizontal gene transfer underlie microdiversity-level heterogeneity in resistance gene fate during wastewater treatment. *Nat. Commun.* **2024**, *15*, 5412. [CrossRef]
18. Abdulkadir, N.; Saraiva, J.P.; Zhang, J.; Stolte, S.; Gillor, O.; Harms, H.; Rocha, U. Genome-centric analyses of 165 metagenomes show that mobile genetic elements are crucial for the transmission of antimicrobial resistance genes to pathogens in activated sludge and wastewater. *Microbiol. Spectr.* **2024**, *12*, e02918-23. [CrossRef]
19. Matesun, J.; Petrik, L.; Musvoto, E.; Ayinde, W.; Ikumi, D. Limitations of wastewater treatment plants in removing trace anthropogenic biomarkers and future directions: A review. *Ecotoxicol. Environ. Saf.* **2024**, *281*, 116610. [CrossRef]

20. Lu, Z.; Liu, G.; Xie, H.; Zhai, Y.; Li, X. Advances and solutions in biological treatment for antibiotic wastewater with resistance genes: A review. *J. Environ. Manag.* **2024**, *368*, 122115. [CrossRef]
21. Marutescu, L.G.; Popa, M.; Gheorghe-Barbu, I.; Barbu, I.C.; Rodríguez-Molina, D.; Berglund, F.; Blaak, H.; Flach, C.F.; Kemper, M.A.; Spießberger, B.; et al. Wastewater treatment plants, an "escape gate" for ESCAPE pathogens. *Front. Microbiol.* **2023**, *14*, 1193907. [CrossRef]
22. Chi, T.; Liu, Z.; Zhang, B.; Zhu, L.; Hu, B. Risk assessment of the spread of antibiotic resistance genes from hospitals to the receiving environment via wastewater treatment plants. *Ecotoxicol. Environ. Saf.* **2025**, *306*, 119264. [CrossRef]
23. Read, D.S.; Gweon, H.S.; Bowes, M.J.; Anjum, M.F.; Crook, D.W.; Chau, K.K.; Shaw, L.P.; Hubbard, A.; AbuOun, M.; Tipper, H.J.; et al. Dissemination and persistence of antimicrobial resistance (AMR) along the wastewater-river continuum. *Water Res.* **2024**, *264*, 122204. [CrossRef]
24. Lee, J.; Ju, F.; Beck, K.; Bürgmann, H. Differential effects of wastewater treatment plant effluents on the antibiotic resistomes of diverse river habitats. *ISME J.* **2023**, *17*, 1993–2002. [CrossRef]
25. Rodríguez-Molina, D.; Berglund, F.; Blaak, H.; Flach, C.F.; Kemper, M.; Marutescu, L.; Gradisteanu, G.P.; Popa, M.; Spießberger, B.; Weinmann, T.; et al. Carriage of ESBL-producing Enterobacterales in wastewater treatment plant workers and surrounding residents—The AWARE Study. *Eur. J. Clin. Microbiol. Infect. Dis.* **2021**, 1–16. [CrossRef]
26. Berglund, F.; Rodríguez-Molina, D.; Pircalabioru, G.G.; Blaak, H.; Chifiriuc, M.C.; Barbu, I.C.; Flach, C.F.; Gheorghe-Barbu, I.; Măruțescu, L.; Popa, M.; et al. The resistome and microbiome of wastewater treatment plant workers—The AWARE study. *Environ. Int.* **2023**, *180*, 108242. [CrossRef]
27. Teban-Man, A.; Erdem, E.D.; Berendonk, T.U.; Klümper, U.; Coman, C.; Szekeres, E. Hydrodynamics Shape Antibiotic Resistance in Wastewater-Impacted River Biofilms. *Microb. Ecol.* **2026**, *89*, 82. [CrossRef]
28. European Parliament and Council of the European Union. Directive (EU) 2024/3019 of the European Parliament and of the Council of 27 November 2024 concerning urban wastewater treatment (recast). *Off. J. Eur. Union* **2024**, *L 2024*, 3019.
29. World Health Organization. *Wastewater and Environmental Surveillance—A Global Landscape Analysis*; WHO: Geneva, Switzerland, 2026.
30. Maric, L.; Rupnik, M.; Janezic, S. Diversity of ESBL-producing *E. coli* in various water and sediment types. *PLoS ONE* **2025**, *20*, e0338703. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.