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The Importance of Enterococci in the Monitoring of Fecal Pollution in River Water in Forests and Urban Areas

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Abstract: The aim of this study was to determine the spatial distribution of enterococci as indicators of fecal pollution in river water in forest reserves and urban areas. The biodiversity, multidrug resistance, and virulence of enterococci were monitored using conventional and molecular methods, including fluorescence in situ hybridization (FISH). Enterococcal (ENT) counts determined via the FISH method were several thousand times higher than those determined by the culture-based method. This observation points to the abundance of viable but non-culturable (VBNC) bacteria that are often more toxic. Water from the river source was characterized by the lowest number of multidrug-resistant and virulent enterococcal strains. The abundance of the analyzed bacteria was highest (more than 50% of the strains) in the area where treated wastewater was evacuated to the river. Statistical analysis confirmed the presence of relationships between these environments. Pathogenic enterococci, which are not effectively eliminated during wastewater treatment, spread in the river continuum, thus posing a health threat to humans and animals. A combination of conventional and molecular techniques for the identification of bacteria supports a rapid and reliable assessment of pollution sources in the examined environment and the implementation of protective measures.

Keywords: river; fecal pollution; enterococci; pathogenicity; forests reserves; urban areas; fluorescence in situ hybridization (FISH)

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1. Introduction

The quality of surface waters continues to deteriorate worldwide. The main causes of this decline are climate change and the anthropogenic pressures associated with rapidly growing economies that generate massive quantities of waste and wastewater. Global water consumption has increased six-fold in the last century, and two thirds of the world's population are experiencing water scarcity [1,2]. Therefore, urgent measures are needed to identify the types and sources of pollution to protect natural aquatic habitats and preserve their ecological identity. Microorganisms are robust and sensitive bioindicators of water quality and safety for users. Changes in the biodiversity of both allochthonous and native microorganisms provide reliable information about the quality and purity of flowing waters [3,4]. According to numerous research studies, the abundance of fecal bacteria such as *Escherichia coli* and *Enterococcus faecalis* continues to increase in rivers receiving treated wastewater in urban areas [5–9]. These commensal gut bacteria have been long regarded as indicators of fecal pollution in the environment [10]. In recent years, virulent and multidrug-resistant enterococci, in particular vancomycin-resistant enterococci (VRE), have attracted growing research interest due to their spread in the natural environment (Polish National Antibiotic Stewardship Program, NPOA; and the European Center for Disease Control and Prevention, ECDC). Enterococci reach surface waters mainly from point (hospital wastewater, wastewater treatment plants—WWTPs, food processing plants, livestock farms) and non-point sources of pollution (cultivated fields fertilized with manure and slurry), as well as runoffs in urban areas [11–16]. These pathogens are also shed in the

feces of wild animals, and contaminate environments with low levels of anthropogenic pressure, such as forests [17–19]. The overuse and misuse of antibiotics in agriculture and medicine exert selection pressure on bacteria and promote the transfer of drug-resistant and virulent strains of enterococci. Clinically significant strains include multidrug-resistant *E. faecalis* and vancomycin-resistant *E. faecium* (VRE) which have been classified as critical priority pathogens by the World Health Organization (WHO) [16,20,21]. In enterococci, mobile genetic elements (MGEs) such as plasmids and transposons promote the rapid acquisition and exchange of antibiotic resistance genes (ARGs) both within the same species and across native environmental bacteria. Consequently, ecological niches may become potential reservoirs of antibiotic-resistant bacteria (ARB) and ARGs, thus posing a serious sanitary and epidemiological threat to ecosystem users. Environmental monitoring methods involving the identification of enterococci and antibiotic-resistant strains have been approved by leading water conservation agencies around the world [20–22]. However, water monitoring procedures have not been standardized, and reliable data for determining the abundance of these microorganisms in the environment are not available. Only a small percentage of bacteria colonizing a given ecosystem (0.3% in soil and <0.1% in water) can be identified with the use of conventional culture-based methods [23–25]. The precise determination of microbial abundance requires a combination of methods, which is laborious and time-consuming [26,27]. In addition, conventional methods do not support the identification of viable but non-culturable (VBNC) bacteria whose populations continue to increase due to changing environmental conditions and exposure to physicochemical and biological factors (water temperature, pH, biogenic and mineral substances, solar radiation, predation, and competition) [28,29]. The above can lead to the underestimation and misinterpretation of monitoring results. Viable but non-culturable bacteria have a lower metabolic rate, and they synthesize new proteins in periods of starvation and heat shock (chaperones), which increases their resistance to unfavorable environmental conditions. These microorganisms are also more resistant to chemical and physical stress, and they are characterized by higher toxicity [30]. The formation of VBNC cells has been reported in various bacterial populations, including gut bacteria such as *Escherichia coli*, *Vibrio* spp., *Enterococcus faecalis*, *E. faecium*, and *E. hirae* [31,32]. Non-culturable bacteria, including pathogens, should be identified to evaluate biotope safety. Good laboratory practices and reliable indicators of microbial pollution are still being sought [27,33]. This problem could be addressed through molecular methods, such as fluorescence in situ hybridization (FISH) which involves the use of specific oligonucleotide probes labeled with fluorescent dyes. These probes target the 16S rRNA sequence in bacterial cells which are visualized under an epifluorescent microscope. The FISH technique opens new possibilities in research, and it supports real-time determination of the quantitative and qualitative composition, structure, and spatial distribution of bacterial populations in the environment with high precision and accuracy [24,25,34]. The FISH method is used in clinical studies to identify pathogenic microorganisms [35,36] and the microbiota present in soil [37], sediments, [38] and freshwater and marine water ecosystems [6,25]. This technique can also be used to study microbial metabolism because the target sites of hybridization are mainly intact ribosomes that are present in large numbers only in viable and active cells [24,39].

In the present study, the FISH technique and conventional analytical methods were used to determine the abundance of bacteria of the Enterococcaceae family (*E. faecium*, *E. faecalis*, and *E. gallinarum*) in samples of river water collected in forest reserves and urban areas downstream and upstream from the wastewater discharge point, as well as in samples of untreated wastewater and treated wastewater evacuated to the river. The multi-drug resistance and virulence profiles of the isolated strains of enterococci and the extent to which these bacteria are eliminated during wastewater treatment were analyzed to assess the microbiological safety of the examined river. The combination of conventional and molecular techniques used in this study supports a rapid and reliable assessment of microbial pollution in river water, its sources, and the potential spread of pollutants in the river continuum.

2. Materials and Methods

2.1. Study Site and Sampling

The study was conducted on the Łyna River (length—190 km, catchment area—5700 km^2, average depth—2 m) which is one of the longest rivers in north-eastern Poland that serves as a recreational outlet and a central part of many local economies (Figure 1). The study covered the southern part of the catchment area at the river source and urban areas. The southern and central parts of the catchment are extensively forested (68%), and the remaining parts are occupied by farmland and urban areas. The river source is characterized by headward erosion, and the Łyna River Source Reserve (153 m above sea level) has been created to protect this unique ecosystem. The reserve is subjected to minimal anthropogenic pressure. Along its course, the river intersects the Warmian Forest Reserve which is a part of the Natura 2000 network of protected habitats and bird species. The river that flows through Olsztyn, the largest city along its course (area—88.3 km^2, population—180,000) and the capital city of the Region of Warmia and Mazury (for a detailed description of the Łyna River, refer to Gotkowska-Płachta et al. [6]). A mechanical–biological WWTP with an average daily processing capacity of 32,000 m^3 is located on the Łyna River downstream from Olsztyn. Treated municipal wastewater from Olsztyn and the surrounding areas is evacuated directly to the river (the wastewater treatment process and the plant's operating parameters have been described in detail by Gotkowska-Płachta [5]).

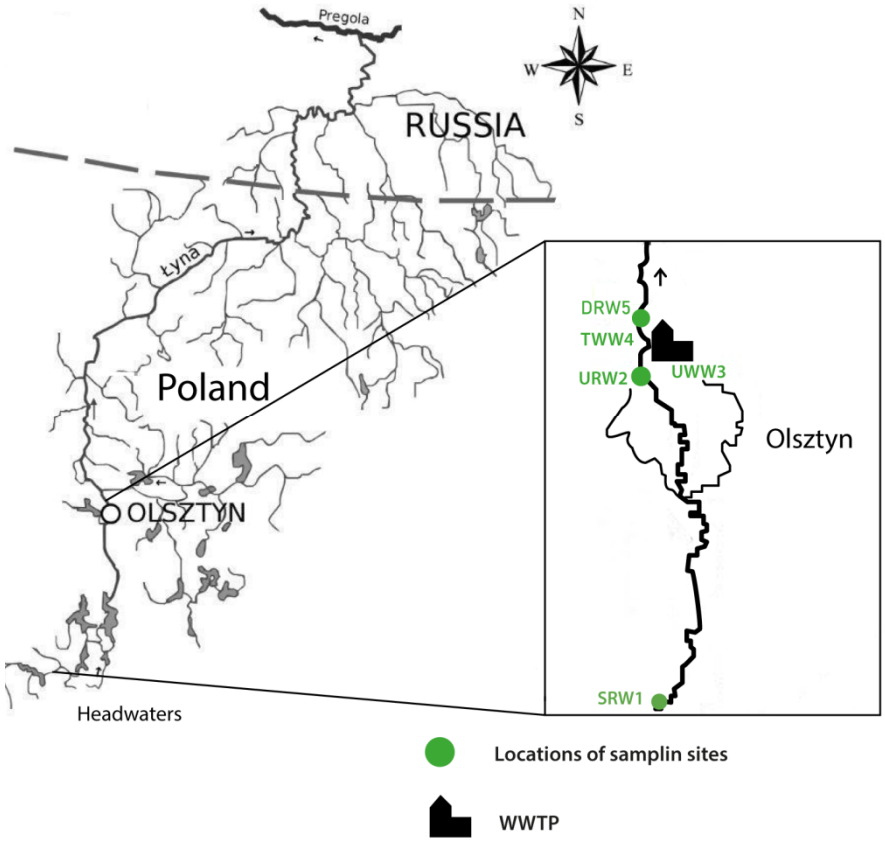


Figure 1. Location of sampling sites (green point) on the Łyna River.

Samples of river water were collected at the river source (SRW1) in a forested area, around 500 m upstream from the WWTP (URW2), and 500 m downstream from the WWTP (DRW5). Untreated wastewater flowing into the WWTP (UWW3) and treated wastewater discharged to the river (TWW4) were also analyzed. Water and wastewater samples were collected into sterile 1 L containers every 8–10 weeks over a one year period. A total of 24 river water samples and 16 wastewater samples were collected in accordance with

standard PN-EN ISO 19458:2007 [40]. The samples were transported to the laboratory in refrigerated containers and the analysis was completed within 24 h of sampling.

2.2. Microbiological and Physicochemical Analyses

2.2.1. Physicochemical Parameters

Temperature (°C), pH, and dissolved oxygen (DO) concentration (mg O_2 L^{-1}) in water and wastewater samples were determined using the YSI 556 MPS (Multiprobe System, Xylem Analytics Germany Sales GmbH & Co.KG-WTW, Weilheim, Bavaria, Germany)-Handheld Multiparameter Instrument with an accuracy of ±0.1 °C, ±0.01 pH, and ±0.01 mg O_2/L, respectively. Chemical oxygen demand (COD), total dissolved solids (TDS), and the concentrations of NH_4-N, NO_2-N, NO_3-N, PO_4-P, and P_T (biogenic compounds) were determined with the use of the methods described by Hermanowicz et al. [41] and the results were expressed in standard hydrochemical units (mg/L).

2.2.2. Identification of Fecal Enterococci via the Culture-Based Method and Determination of Their Multidrug Resistance and Virulence Profiles

Enterococci were filtered through a membrane (Merck, KGaA, Darmstadt, Germany), cultured on the Slanetz-Bartley medium (Merck, KGaA, Darmstadt, Germany) at 36 ± 2 °C for 48 h, and enumerated on Bile Aesculin Azide Agar (Merck, KGaA, Darmstadt, Germany) at 44 ± 0.5 °C two hours after incubation according to standard PN-EN ISO 7899-2:2004 [42]. The resulting colonies were counted and expressed in colony forming units (CFU)/mL of water or wastewater [43]. Representative colonies (several to more than ten) were Gram-stained, and catalase-negative strains were plated on brain heart infusion (BHI) agar (Biocorp, Warsaw, Poland) to obtain pure isolates. A total of 300 strains were classified as *Enterococcus* spp. The isolates were frozen at −80 °C on LB broth (Miller) (Merck, KGaA, Darmstadt, Germany) containing 10% glycerol and stored until further analyses. DNA was isolated from the identified strains using the Genomic Mini AX Bacteria Spin Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. Enterococcal strains were identified based on the presence of the tuf gene that encodes the EF-Tu elongation factor and has a strongly conserved sequence in the examined bacteria. An internal primer targeting a fragment (sodA int) of the sodA gene that encodes manganese dismutase was designed. Bacterial species were identified by amplifying ddl genes that encode D–alanyl–D–alanine ligase (D–Ala–D–Ala) [44,45].

The DNA isolation procedure and the Polymerase Chain Reaction (PCR) protocol for identifying enterococci were previously described by Gotkowska-Płachta [5]. Based on the results of the PCR assay, a total of 225 strains were identified as *Enterococcus* spp., including 15 strains from SRW1 samples, 20 strains from URW2 samples, 20 strains from DRW5 samples, 70 strains from UWW3 samples, and 100 strains from TWW4 samples.

The distribution of virulence markers in the identified enterococci was determined based on the presence of cylA, hyl, ace, EfaA, gelE, as, esp, cpd, cob, and ccf genes encoding cytolysin, hyaluronidase, surface-associated collagen-binding protein, cell wall adhesin, gelatinase, aggregation substance, enterococcal surface protein, and sex pheromones, respectively [46–49], with the use of the PCR protocol previously described by Gotkowska-Płachta [5].

The isolates were analyzed for sensitivity to nine classes of antibiotics: beta-lactams (Bl)—ampicillin (2 µg); aminoglycosides (Am)—gentamycin (30 µg); glycopeptides (Gl)—vancomycin (5 µg); streptogramins (St)—quinupristin/dalfopristin (15 µg); glycyclines (Gc)—tigecycline (15 µg); oxazolidinones (Ok)—linezolid (10 µg); chemotherapeutics (Che)—nitrofurantoin (100 µg); fluoroquinolones (Fl)—ciprofloxacin (5 µg); tetracyclines (Te)—doxycycline (30 µg). Drug-sensitive isolates were identified in the disc diffusion test using Mueller-Hinton agar (bioMerieux, Warsaw, Poland) and Oxoid antibiotics (Basingstoke, Hampshire, UK). After incubation (37 °C/16–18 h), the strains were classified as susceptible or non-susceptible to the tested drugs by comparing the size of the growth inhibition zone with the guidelines of the European Committee on Antimicrobial Suscepti-

bility Testing (EUCAST 2012) [50] and the Clinical and Laboratory Standards Institute CLSI, (2012) [51]. The susceptibility of isolates from different sampling sites and different types of samples was compared by calculating the MAR index = a/b × c (where a is the number of antibiotics to which an isolate was resistant, b is the total number of tested antibiotics, and c is the number of isolates per sample) [52,53].

2.2.3. Identification of Enterococcus Strains via Fluorescence in Situ Hybridization (FISH)

Total enterococcal (ENT) counts (including VBNC counts), the species composition, and the percentage of *Enterococcus* spp. in total bacterial counts (TBC) in river water and wastewater samples were determined via the FISH method. The prepared and fixed samples (based on the protocol proposed by Korzeniewska and Harnisz [7], and Loy et al. [54]) were passed through polycarbonate membrane filters (0.2 µm, Millipore). Dried filters were incubated with lysozyme (1 mg·mL^{-1}; Sigma-Aldrich, St. Louis, MI, USA) at a temperature of 30 °C for 15 min (under experimentally determined conditions) to digest the bacterial cell walls in Gram-positive enterococci. The filter was rinsed with distilled water to stop the reaction [55]. The samples retained on the filters were hybridized with the use of fluorochrome-labeled oligonucleotide probes targeting conserved regions in 16S rRNA sequences or species-specific 23S rRNA sequences (ENC176 for *Enterococcus* spp., ENF 191 for *E. faecalis*, ENU140 for *E. faecium*, and EGAC 173 for *E. gallinarum*). The percentage of *Eubacteria* (defined as TBC) was determined using the EUB338I-III probe [56]. The NON338 nonsense probe was used as a negative control to determine autofluorescence and non-specific binding [34]. All probes were labeled with the CY3 dye [57] (Oligo.pl—DNA sequencing and synthesis, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences). After preliminary analyses, hybridization conditions were modified in several cases to optimize the results. The applied oligonucleotide probes and hybridization conditions are described in Table 1. Enterococcal cells retained on filters were enumerated via digital image analysis (Cell F) under the Olympus BX51 epifluorescent microscope equipped with an oil immersion lens (100×), a UV lamp, CY3 filters, and a CCD camera (Olympus). For each sample and probe (FISH), the number of cells was calculated in 20 random fields of view; bacterial counts were expressed per 1 mL (cells mL^{-1}) of river water or wastewater, and the percentage of *Enterococcus* spp. in TBC (*Eubacteria*) was determined.

Table 1. Oligonucleotide probes and hybridization conditions.

Probe	Sequence (5′–3′)	Bacteria	Time (h)/% FA [a]	Position	References
ENC176	CA GTT CTC TGC GTC TAC CTC	*Enterococcus* spp. [b]	1.5/30	23S rRNA	[36]
ENF191	GAA AGC GCC TTT CAC TCT TAT GC	*Enterococcus faecalis*	1.5/30	16S rRNA	[36]
ENU140	TTC ACA CAA TCG TAA CAT CCT	*Enterococcus faecium*	1.5/30	23S rRNA	[36]
EGAC183	CAA CTT TCT TCC ATG CGG AAA AT	*Enterococcus gallinarum* [c]	3/30	16S rRNA	[36]
EUB338	GCT GCC TCC CGT AGG AGT	Domain *Eubacteria*	1.5/35	16S rRNA	[58]
NON338	ACT CCT ACG GGA GGC AGC	Negative control	3/35	16S rRNA	[59]

Note(s): [a] Hybridization time and formamide concentration in the hybridization buffer (FA), [b] Including *Carnobacterium* spp. [c] Probe targeting *Enterococcus gallinarum*, *E. flavescens*, and *E. casseliflavus* (VanC group).

2.2.4. Statistical Analysis

Statistical analyses were conducted using the Statistica 13.2 software package (StatSoft Inc., 1984–2019, Tulsa, OK, USA) at a significance level of 0.05. Spearman's rank correlation coefficient was calculated to analyze the relationships between the examined bacterial groups (identified by the culture-based method and the FISH method), physicochemical parameters, and the number of multidrug-resistant and virulent strains. The data did not have normal distribution, and the Kruskal–Wallis (KW) test, a non-parametric version of a standard one-way analysis of variance (ANOVA), was performed to determine differences in ENT counts between samples of river water and wastewater collected in different sites and seasons of the year. A heatmap was compiled, and a hierarchical cluster analysis

(Ward's method) was conducted using Statistica 13.2 (StatSoft Inc., 1984–2019, Tulsa, OK, USA) software.

The effects of environmental and biogenic factors on ENT counts in the analyzed water and wastewater samples were evaluated via principal component analysis (PCA) in the CANOCO 5.0 program [60].

3. Results

3.1. Physicochemical Parameters of River Water and Wastewater Samples

Significant differences ($p < 0.001$) in the values of all physicochemical parameters (excluding temperature) were noted across sampling sites, but not across seasons. Due to the absence of significant seasonal differences, only the average values of physicochemical parameters in each sampling site were analyzed (Table 2).

Table 2. Physicochemical parameters of wastewater and river water.

Parameter	Sampling Sites Mean ± SD					Differences (*p*) between
	SRW1	**URW2**	**UWW3**	**TWW4**	**DRW5**	**Site/Seasons**
Temp	12.3 ± 2.8	14.15 ± 4.50	13.60 ± 5.36	14.94 ± 6.88	14.88 ± 7.06	0.96/0.001 *
pH	7.28 ± 0.43	7.40 ± 0.37	8.22 ± 0.22	8.36 ± 0.30	7.23 ± 0.33	0.007 */0.324
DO	10.30 ± 2.8	8.57 ± 0.98	0.50 ± 0.15	8.96 ± 0.96	8.18 ± 0.59	0.023 */0.178
NH_4-N	0.06 ± 0.05	0.07 ± 0.02	78.08 ± 12.56	5.94 ± 2.44	0.19 ± 0.04	0.002 */0.951
NO_2-N	0.01 ± 0.004	0.01 ± 0.004	1.01 ± 0.002	1.98 ± 0.50	0.05 ± 0.03	0.002 */0.854
NO_3-N	2.41 ± 0.27	0.38 ± 0.55	1.14 ± 0.05	2.03 ± 0.71	0.18 ± 0.03	0.005 */0.807
PO_4-P	0.26 ± 0.07	0.23 ± 0.07	33.79 ± 9.33	1.96 ± 1.03	0.29 ± 0.19	0.005 */0.550
P_T	0.48 ± 0.25	0.44 ± 0.31	40.14 ± 7.22	2.81 ± 1.69	0.79 ± 0.17	0.003 */0.730
COD	9.0 ± 8.11	17.50 ± 5.19	389.94 ± 216.31	35.41 ± 2.96	22.10 ± 3.88	0.002 */0.748
TDS	0.29 ± 0.06	0.22 ± 0.02	1.77 ± 0.20	1.66 ± 0.030	0.21 ± 0.05	0.004 */0.767

Note(s): SRW1—river source, URW2—upstream river water, UWW3—untreated wastewater, TWW4—treated wastewater, DRW5—downstream river water; DO—dissolved oxygen, NH_4-N—ammonia nitrogen, NO_2-N—nitrite nitrogen, NO_3-N—nitrate nitrogen, PO_4-P—orthophosphate, P_T—total phosphorus, COD—chemical oxygen demand, TDS—total dissolved solids. * Differences are statistically significant at $p < 0.001$. All parameters are expressed in mg L^{-1}, excluding temperature (°C) and pH. SD—standard deviation.

The average temperature of river water was determined at 14 °C, excluding at site SRW1, where it did not exceed 12.3 °C, which could be attributed to dense tree cover and shading effects. Dissolved oxygen levels were characteristic of well-oxygenated waters, and average DO values did not fall below 8.18 mg O_2 l^{-1}. The analyzed parameter was lowest (0.5 mg O_2 L^{-1}) in TWW4. pH ranged from 7.28 in river water to 8.36 in TWW4. The average COD ranged from 9.0 (SRW1) to 22.10 mg L^{-1} (DRW5). The total dissolved solids did not exceed 0.30 mg L^{-1} in river water and were more than five-fold higher in wastewater at 1.77 mg L^{-1} in UWW3 and 1.66 mg L^{-1} in TWW4. The average concentrations of biogenic compounds (NH_4-N, NO_2-N, NO_3-N, PO_4-P, and P_T) were lower in samples collected upstream (0.06–0.07, 0.01, 0.38–2.41, 0.23–0.26, and 0.44–0.48, respectively) than downstream from the WWTP (0.19, 0.05, 0.18, 0.29, and 0.79, respectively). The concentrations of NH_4-N, PO_4-P, and PT were reduced by more than 90% in samples of treated wastewater (TWW4) evacuated to the river relative to untreated water (UWW3). The values of the analyzed physicochemical parameters were generally characteristic of lotic environments belonging to quality class I and II (Regulation of the Minister of the Environment of 5 August 2016; Regulation of the Minister of Marine Economy and Inland Navigation of 12 July 2019) (Table 2).

3.2. Fecal Enterococci in River Water and Wastewater

In samples collected from the examined sites, significant differences ($p < 0.05$, ANOVA) were observed in the abundance of enterococcal species targeted by ENC176 ($p = 0.018$), ENF191 ($p = 0.017$), ENU140 ($p = 0.024$), EGAC183 ($p = 0.002$), and EUB338 ($p = 0.028$) probes

in the FISH assay and the ENT counts determined via the culture-based method ($p = 0.091$). Due to an absence of seasonal differences, only the average counts of bacterial species identified in each sampling site were analyzed (Table 3). The total counts of *Eubacteria* (EUB338 probe) ranged from several (2.0, 5.1, and 5.5 $\times$ 10^6 cells mL^{-1} in SRW1, URW2, and DRW5) and several dozen million cells (45.2 $\times$ 10^6 cells mL^{-1} in TWW4) to several hundred million cells (216.1 $\times$ 10^6 cells mL^{-1} in UWW3) across the examined sampling sites.

Table 3. Mean (±SD), range, and percentage of the analyzed microorganisms in river water and wastewater.

Method		Sampling Sites SRW1	URW2	UWW3	TWW4	DRW5
FISH ($\times 10^6$ cell 1 mL^{-1})	EUB338	2.0 ± 1.5 [a] (0.9–4.0) [b]	5.1 ± 2.3 (2.7–7.7)	216.1 ± 95.5 (124.26–304.9)	45.2 ± 6.9 (37.2–52.6)	5.5 ± 2.7 (2.1–7.9)
	ENC176	0.05 ± 0.01 (0.04–0.07) 2.4% [c]	0.11 ± 0.05 (0.07–0.17) 2.2%	3.20 ± 1.74 (1.75–5.54) 1.5%	0.88 ± 0.12 (0.70–0.97) 1.9%	0.15 ± 0.06 (0.058–0.196) 2.7%
	ENF191	0.006 ± 0.002 (0.003–0.008) 0.3%	0.017 ± 0.006 (0.01–0.02) 0.3%	1.36 ± 0.67 (0.67–2.26) 0.6%	0.38 ± 0.20 (0.27–0.69) 0.8%	0.020 ± 0.006 (0.012–0.027) 0.4%
	ENU140	0.002 ± 0.001 (0.001–0.002) 0.08%	0.004 ± 0.002 (0.002–0.006) 0.09%	0.74 ± 0.68 (0.15–1.42) 0.3%	0.091 ± 0.035 (0.064–0.14) 0.2%	0.006 ± 0.003 (0.002–0.007) 0.11%
	EGAC183	0.0003 ± 0.0001 (0.0002–0.0005) 0.02%	0.0009 ± 0.0004 (0.0003–0.0012) 0.02%	0.17 ± 0.16 (0.03–0.37) 0.1%	0.021 ± 0.0085 (0.012–0.032) 0.05%	0.0012 ± 0.0003 (0.001–0.002) 0.02%
Culture method ($\times 10^3$ CFU 1 mL^{-1}) [b]	ENT	0.0003 ± 0.0003 (0.0–0.0007)	0.0019 ± 0.0013 (0.0008–0.0034)	22.60 ± 15.86 (10.12–45.00)	0.61 ± 0.67 (0.19–1.60)	0.0039 ± 00.42 (0.0006–0.0101)

Note(s): [a] mean ± SD; [b] range; [c] relative to EUB338. SRW1—river source; URW2—upstream river water; UWW3—untreated wastewater; TWW4—treated wastewater; DRW5—downstream river water.

In the FISH assay, the average counts of bacteria hybridized with probes ENC176, ENU140, ENF191, and EGAC183 were lowest (0.05–0.0003 $\times$ 10^6 cells mL^{-1}) in SRW1 samples collected in a forested area without anthropogenic pressure. Enterococcal counts were somewhat higher at 0.0009 and 0.15 $\times$ 10^6 cells mL^{-1} on average in URW2 (EGAC183) and DRW5 (ENC176) samples, respectively. In SRW1 and DRW5 samples, ENT counts were several orders of magnitude lower in culture-based analyses (0.0003 $\times$ 10^3 and 0.0039 $\times$ 10^3 CFU mL^{-1}, respectively) than in the FISH assay. In UWW3 and TWW4 samples, ENT counts were determined at 22.60 and 0.61 CFU mL^{-1}, respectively.

In the FISH assay, ENT counts were several or more than ten times higher in wastewater samples (UWW3 and TWW4) than in river water. In UWW3 samples, ENT counts determined with the use of ENC176, ENU140, ENF191, and EGAC183 probes reached 3.20 $\times$ 10^6, 1.36 $\times$ 10^6, 0.74 $\times$ 10^6, and 0.17 $\times$ 10^6 cells mL^{-1}, respectively. Enterococcal counts in TWW4 samples were significantly reduced (ENC176 by 73%, ENF191 by 72%, ENU140 and EGAC183 by 88%) in comparison with UWW3 samples. Despite the above, several to several thousand bacterial cells were evacuated to the river with every mL of treated wastewater (TWW4).

Enterococcal species determined with the use of ENC176, ENF191, and ENU140 probes accounted for 0.08–2.7% of TBC (*Eubacteria*), and their abundance generally increased downstream. The only exception was *E. gallinarum* (EGAC183) which accounted for 0.02% of TBC in river water and treated wastewater (TWW4) and for 0.05% of TBC in UWW3 samples (Table 3).

3.3. Analysis of the Relationships between Enterococcal Counts in Sampling Sites and the Physicochemical Parameters of River Water and Wastewater

The calculated values of Spearman's rank correlation coefficients revealed highly significant positive correlations ($p \leq 0.05$) between the ENT counts determined with the

use of ENF191, ENU140, and EGAC183 probes in URW2 and UWW3 samples. Enterococcal counts enumerated with ENC176 and ENU140 probes in SRW1 samples were positively correlated with the ENT counts enumerated with EGAC183 and ENU140 probes in river water and wastewater from the remaining sampling sites. Enterococcal counts determined via the culture-based method were significantly correlated ($p \leq 0.05$) with most enterococci enumerated in TWW4 and DRW5 samples (Figure 2).

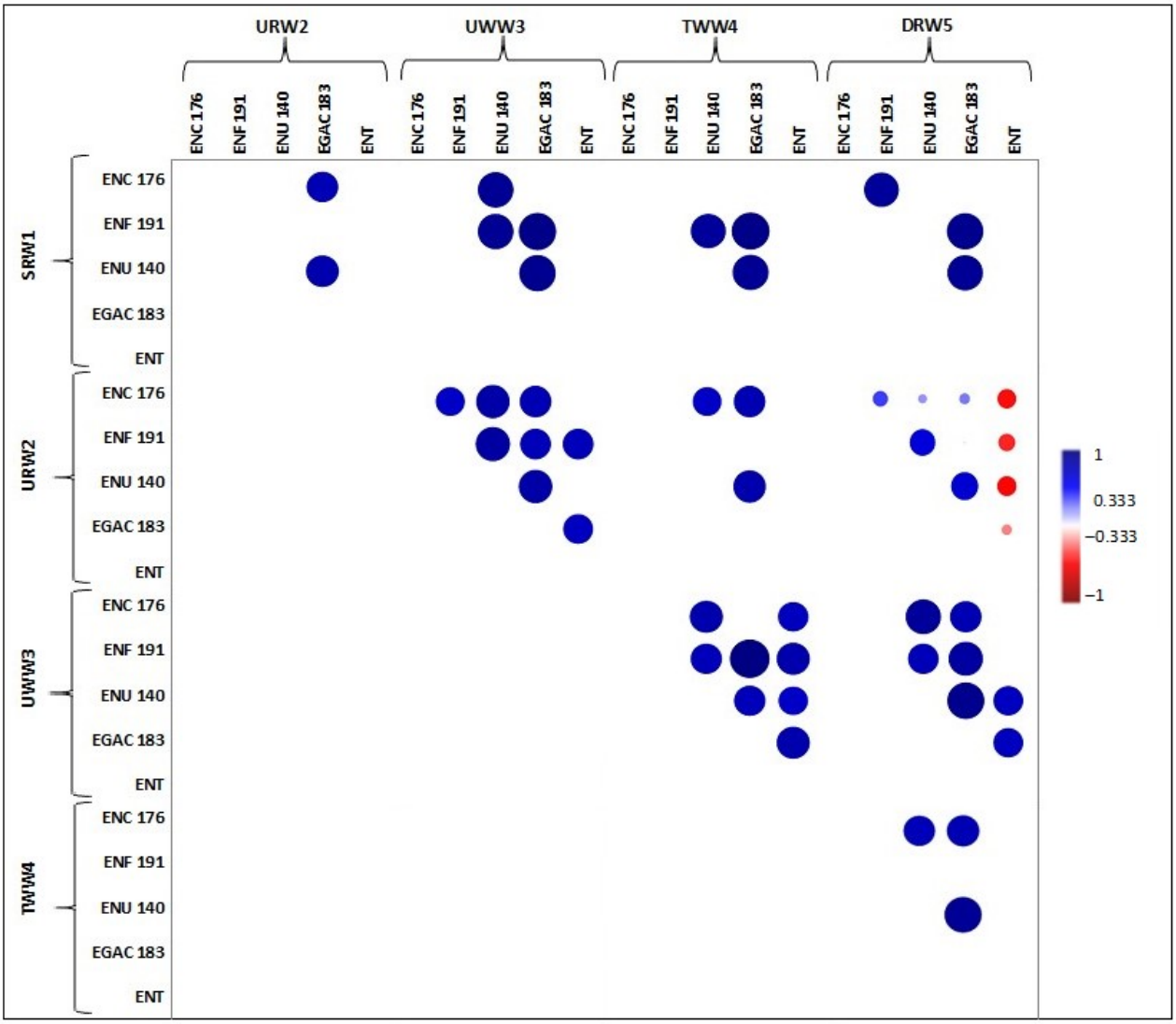
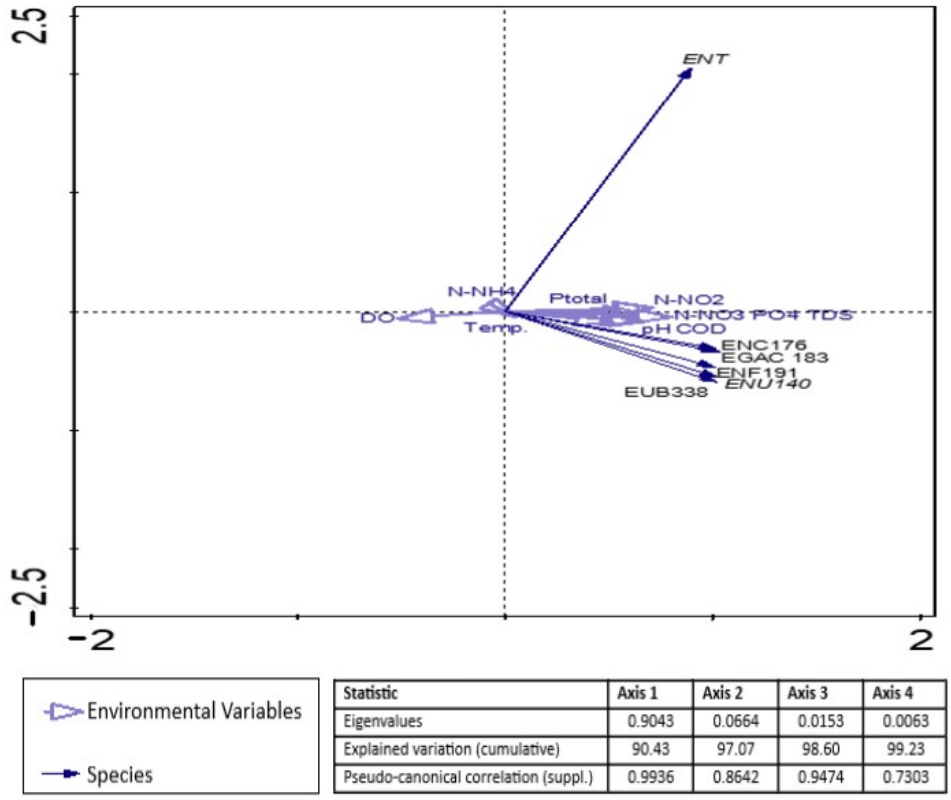


Figure 2. Spearman's rank correlation coefficients denoting the relationships between bacterial counts determined via FISH and culture-based methods in river water and wastewater samples from different sites.

The PCA revealed that four principal components explained more than 90% of the variance between the analyzed physicochemical parameters of river water and wastewater and the ENT counts determined via FISH and culture-based methods (Figure 3). Significant positive correlations ($p \leq 0.05$) were noted between ENT counts enumerated with EUB338, ENC176, ENF191, ENU140, and EGAC183 probes in the FISH assay, and between ENT counts and selected physicochemical parameters (pH, COD, and TDS), and the concentrations of NH_4-N, NO_2-N, NO_3-N, PO_4-P, and P_T (Figure 3).



Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.9043	0.0664	0.0153	0.0063
Explained variation (cumulative)	90.43	97.07	98.60	99.23
Pseudo-canonical correlation (suppl.)	0.9936	0.8642	0.9474	0.7303

Figure 3. Principal component analysis (PCA) based on the distribution of enterococcal counts determined via FISH and culture methods (black) and physicochemical parameters (blue), (DO—dissolved oxygen, NH_4-N—ammonia nitrogen, NO_2-N—nitrite nitrogen, NO_3-N—nitrate nitrogen, PO_4-P—orthophosphate, P_T—total phosphorus, COD—chemical oxygen demand, TDS—total dissolved solids).

3.4. Antibiotic Resistance and Virulence Factors of Enterococci Isolated from River Water and Wastewater

A total of 225 fecal enterococcal strains were identified in samples of river water and wastewater, including 15 in SRW1, 20 in URW2, 70 in UWW3, 100 in TWW4, and 20 in DRW5. Their resistance to nine classes of antibiotics (Bl—beta-lactams, Am—aminoglycosides, Gl—glycopeptides, St—streptogramins, Gc—glycyclines, Ok—oxazolidinones, Che—chemotherapeutics, Fl—fluoroquinolones, and Te—tetracyclines) was determined, and their pathogenicity was analyzed based on the presence of genes encoding virulence factors. The MAR index was calculated (Table 4), and resistance profiles (Figure 4) were determined to assess the spread of resistant enterococci in the examined environments.

Table 4. Multiple antibiotic resistance (MAR) index of enterococcal isolates.

No. of Antibiotics to Which an Isolate Was Resistant (a)	No. of Tested Antibiotics (b)	MAR Index (a/b)	No. of MAR Isolates (%)				
			SRW1 (n = 15)	URW2 (n = 20)	UWW3 (n = 70)	TWW4 (n = 100)	DRW5 (n = 20)
9	9	1.0	0	0	1 (1.4)	6 (6.0)	0
8	9	0.8	0	0	2 (2.9)	4 (4.0)	0
7	9	0.7	0	1 (5.0)	5 (7.1)	14 (14.0)	2 (10.0)
6	9	0.6	1 (6.7)	0	6 (8.6)	18 (15.0)	1 (5.0)
5	9	0.5	1 (6.7)	1 (5.0)	17 (24.3)	8 (8.0)	2 (10.0)
4	9	0.4	2 (13.3)	2 (10.0)	11 (15.7)	4 (4.0)	2 (10.0)
3	9	0.3	4 (26.6)	1 (5.0)	4 (5.7)	7 (7.0)	6 (30.0)
2	9	0.2	1 (6.7)	4 (20)	9 (12.6)	18 (18.0)	3 (15.0)
		0.56	9 (60)	10 (50)	55 (78.6)	79 (79.0)	16 (80.0)

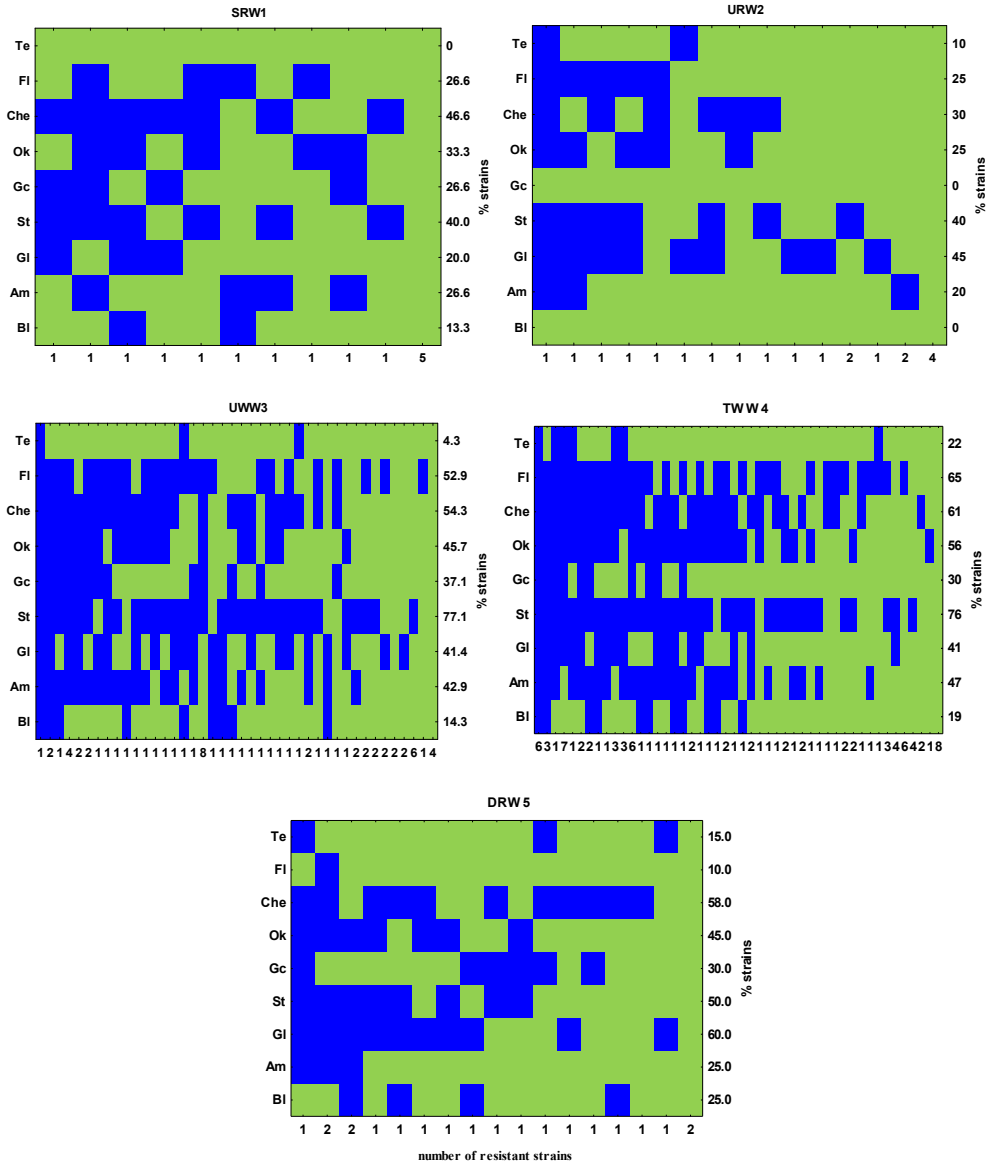


Figure 4. Resistance profiles, number, and percentage of resistant enterococcal strains in river water (SRW1, URW2, DRW5) and wastewater samples (UWW3, TWW4).

The MAR index ranged from 0.2 to 1.0 in samples collected from different sites. The MAR index was highest (1.0) in the UWW3 (1.4%) and TWW4 (6.0%) samples, which contained extensively drug-resistant (XDR) strains that were not susceptible to any of the tested antibiotics. In UWW3 samples, the greatest number of strains (17/24.3%) were resistant to five antibiotics (MAR = 0.5), whereas in TWW4 samples, the greatest number of strains (18/15.0%) were resistant to six antibiotics. Strains resistant to two and three classes of antibiotics were predominant in river water samples and accounted for 20%, 26.6%, and 30% of all strains isolated from URW2, SRW1, and DRW5 samples, respectively (Table 4).

The highest percentage of enterococci isolated from UWW3 and TWW4 samples (54–77% and 65–76%, respectively) were resistant to chemotherapeutics (Che), streptogramins (St), and fluoroquinolones (Fl). The strains isolated from UWW3 and TWW4 samples had a similar resistance profile, but TWW4 strains were resistant to a higher number of antimicrobials. The percentage of strains resistant to different classes of antibiotics was much smaller in river water sampled upstream from the WWTP. The highest proportion of strains isolated from SRW1 and URW2 samples (approx. 40%) were resistant to glycopeptides (Gl), oxazolidinones (Ok), and chemotherapeutics (Che). Similarly to

TWW4 samples, in DRW5 samples collected downstream from the WWTP, the highest percentage of isolates were resistant to glycopeptides (Gl, 60%), streptogramins (St, 50%), and chemotherapeutics (Che, 58%). In all sampling sites, the smallest percentage (0–22%) of strains were resistant to tetracyclines (Te) (Table 4, Figure 4).

Twenty-eight of the 55 strains isolated from river water (50.9%) harbored various virulence genes. The greatest number of strains contained ccf and hyl genes. These genes were identified in 40% and 45% of the strains isolated from URW2 samples and in 50% and 65% of the strains isolated from DRW5 samples, respectively. Around 30% of the strains identified in SRW1 samples (collected from a site characterized by minimal anthropogenic pressure) carried genes associated with virulence. Six strains (40%) harbored efaA genes, and only two strains (13%) harbored cylA genes. A much higher number of virulent strains (54 and 60) was noted in UWW3 (77.1%) and TWW4 (60%) samples. The largest percentage of isolates (51–77%) carrying efaA, cob, cpd, and ccf genes was found in UWW3 samples. The strains isolated from DRW5 samples most frequently harbored efaA (45%), ccf (50%), and hyl (65%) genes. The percentage of isolates carrying esp genes was smallest in UWW3 and TWW4 samples (14% and 22%, respectively), (Figure 5).

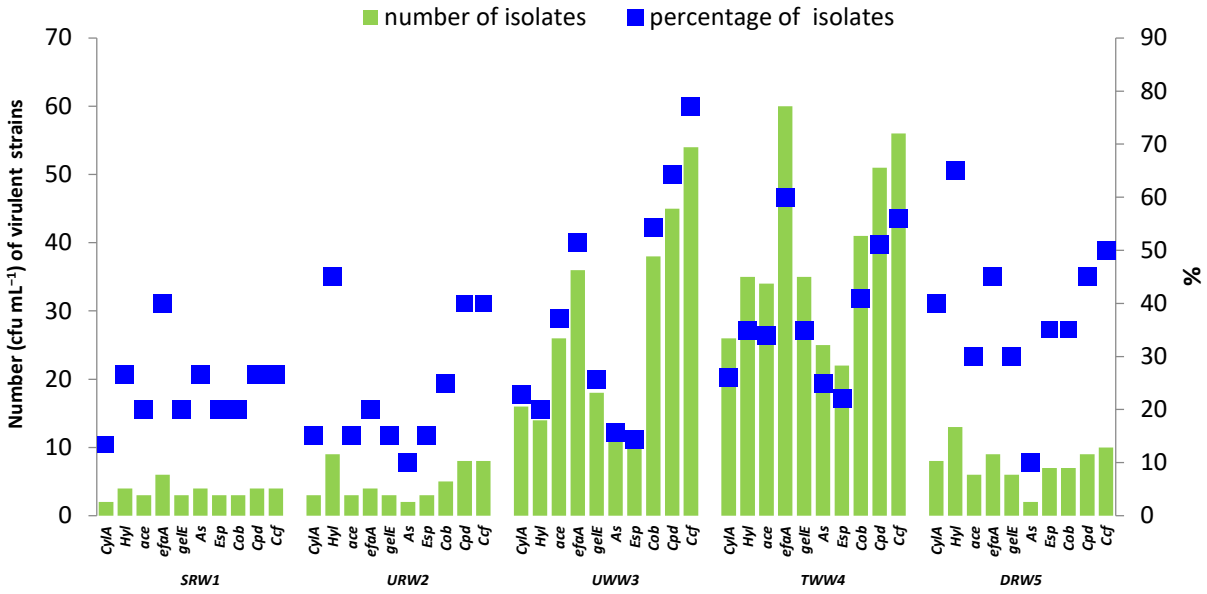


Figure 5. Number (CFU mL^{-1}) and percentage of virulence genes in enterococcal strains isolated from SRW1, URW2, UWW3, TWW4, and DRW5 samples.

3.5. Removal of Microorganisms during Wastewater Treatment

An analysis of treated wastewater samples (TWW4) revealed that 97% of ENT identified in untreated wastewater (UWW3) were removed during the wastewater treatment process. However, the number of antibiotic-resistant strains increased by more than ten or several dozen percent in TWW4 samples. The virulence of strains harboring cylA, esp, As, efaA and hyl genes also increased by 3–15% in TWW4 samples. Despite the fact that the examined WWTP effectively reduced the counts of antibiotic-resistant and virulent strains (>90%), billions of resistant and virulent enterococcal cells are evacuated to river water with treated wastewater each day. TWW4 samples were most abundant in strains containing ccf and efaA virulence genes and resistant to streptogramins and fluoroquinolones (6.08 and 5.02×10^{11} cells 24 h^{-1}), and least abundant in strains harboring esp genes and resistant to beta-lactams (Bl), but their numbers did not decrease below 1.5×10^{11} cells 24 h^{-1} (Figure 6).

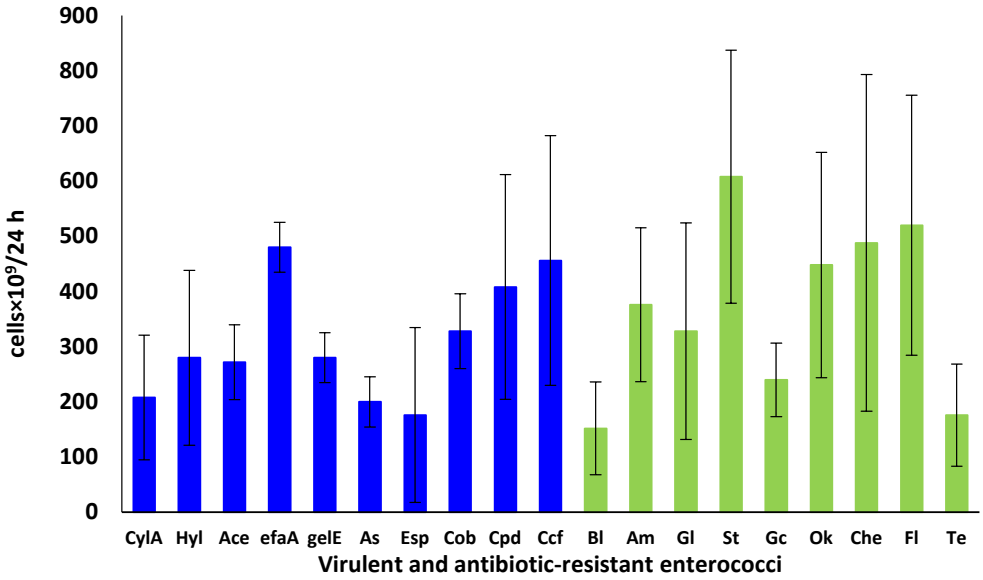


Figure 6. Virulent and antibiotic-resistant enterococci (cells/24 h) evacuated to the river with treated wastewater (TWW4) from the municipal mechanical–biological wastewater treatment plant.

The correlation analysis revealed significant ($p \leq 0.05$) correlations between the counts of most ENT determined via FISH and culture-based methods. Significant ($p \leq 0.05$) correlations were also noted between the groups of these bacteria and the majority of antibiotic-resistant strains and strains harboring virulence genes. Strains resistant to different classes of antibiotics were also significantly correlated with strains harboring virulence genes (Figure 7).

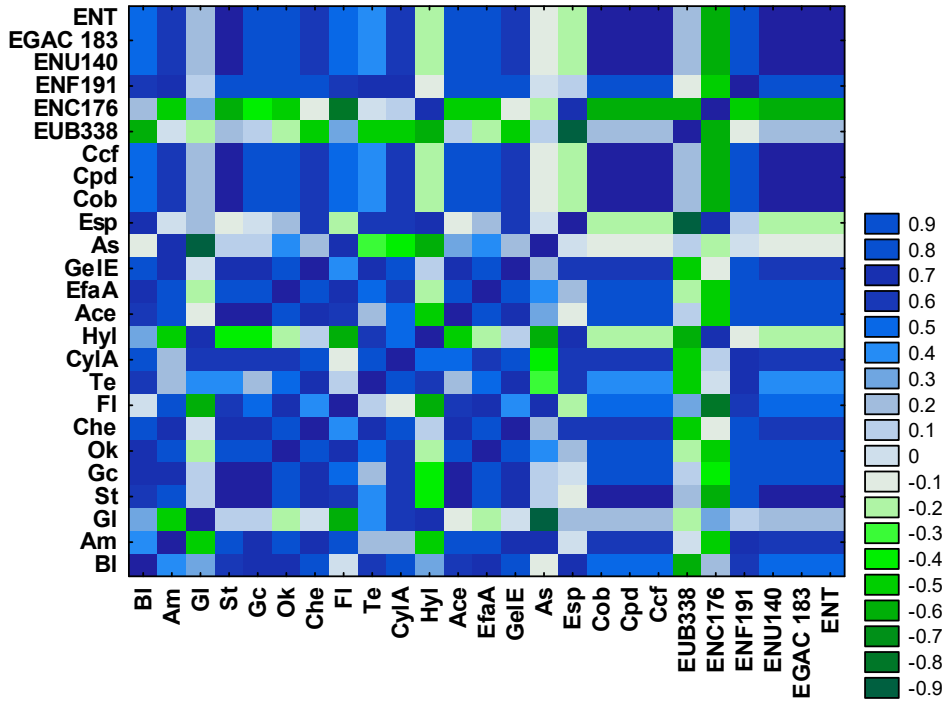


Figure 7. Heatmap presenting the relationships between enterococcal counts determined via FISH and culture-based methods, virulence genes, and the antibiotic resistance of the isolated strains.

The dendrogram illustrating the relationships between the presence of virulence genes, the antibiotic resistance of the analyzed strains, and their quantitative and qualitative composition determined via the FISH method, revealed three main clusters. The first cluster

contained strains that were resistant to Che, Ok, and Gc, and strains that carried efaA, gelE, and ace genes. The second cluster was composed of bacteria identified via FISH and culture-based methods (ENT176, ENU140, EGAC183, EUB338, ENT), strains carrying virulence genes (ccf, cpd, cob, as), and antibiotic-resistant strains (Fl, St, and Am). The third cluster grouped bacteria determined with the use of probe ENC176, strains harboring hyl, esp, and cylA genes, and antibiotic-resistant strains (Te, Gl, and Bl) (Figure 8). The hierarchical clustering analysis revealed the presence of correlations between the biodiversity, multidrug resistance, and virulence of enterococci isolated from UWW3 samples and those isolated from TWW4 and DRW5 samples. SRW1 and URW2 samples constituted a separate group in this respect (Figure 9).

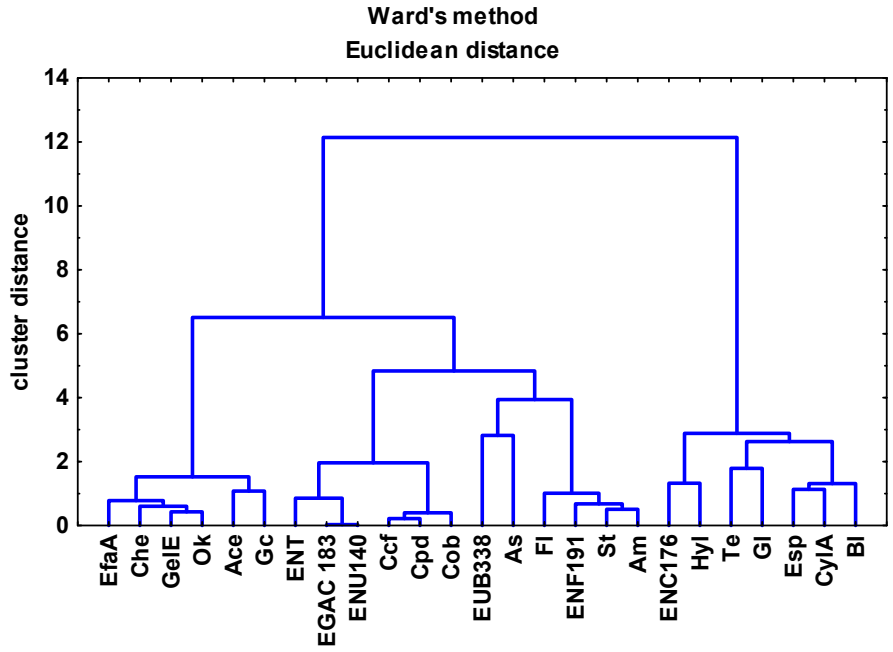


Figure 8. Dendrogram from the agglomerative clustering analysis, presenting the hierarchical relationships between enterococcal counts determined via FISH and culture-based methods and antibiotic-resistant and virulent strains in the analyzed samples of river water and wastewater.

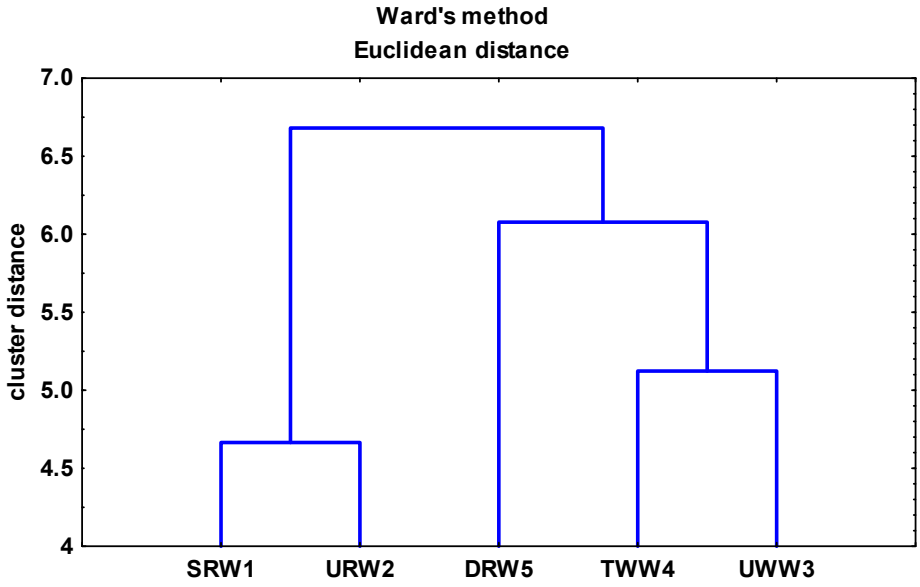


Figure 9. Dendrogram presenting the hierarchical relationships between enterococci identified in samples collected in different sites.

4. Discussion

4.1. Characterization of Bacteria Identified in River Water and Wastewater in the FISH Assay

Rivers flowing through vast areas with varying degrees of anthropopression are carriers of microbial pollution including various pathogenic and multidrug-resistant microorganisms. The use of such waters might be hazardous to public health and requires monitoring. Good bioindicators of sanitary water quality are fecal enterococci and Escherichia coli, referred to as FIB (fecal indicator bacteria) [6,12,22]. They can be found in large numbers in the intestines and feces of humans and animals; they are not found in waters not contaminated with feces; they survive longer than pathogens and their numbers are proportional to the level of contamination. Furthermore, along with the presence of FIB in fecal-contaminated waters, there is a high probability of the presence of other pathogenic bacteria, such as Listeria monocytogenes, salmonella, campylobacter, enterotoxigenic *E. coli* or *Vibrio* spp. [22] In the present study, particular attention was paid to fecal enterococci, which have spread very rapidly in surface waters in recent years and often correlate with the occurrence of disease, gastrointestinal and skin diseases in bathers, and display the presence of virulence genes, or resistance to various antibiotics [5,22].

The biological markers of the type and source of microbial pollution, the abundance of microbial pollutants, and the potential health threat are difficult to identify because they require the use of multiple, time-consuming analytical techniques [26,27]. The development and application of molecular methods including FISH offer the possibility of counting both the culturable and non-culturable so-called VBNC cells (viable but non-culturable). The biodiversity of fecal enterococci as indicators of river water (SRW1, URW2, DRW5) and wastewater (UWW3 and TWW4) quality was assessed with the use of the FISH method. Labeled oligonucleotide probes (EUB338, ENC176, ENF191, ENU140, and EGAC183) targeting specific DNA sequences in the analyzed material using fluorescence microscopy supported the determination of ENT counts in near real-time. The size of *Enterococcus* spp., *E. faecalis*, *E. faecium*, and *E. gallinarum* populations differed by several orders of magnitude depending on the sampling sites, bacterial type, anthropogenic pressure, and environmental conditions. The observed relationships were confirmed by the results of the statistical analysis. The PCA revealed strong ($p \leq 0.05$) correlations between bacterial species, and between bacterial species and most physicochemical and biogenic parameters in river water and wastewater (Figure 3). These correlations indicate that the analyzed microorganisms were sensitive to changes in the type and availability of organic matter. Enterococci that reach water bodies with feces are exposed to different environmental conditions (temperature, pH, oxygen, nutrients, light). These environmental stressors often increase bacterial virulence, which poses a considerable health threat [22]. In the present study, the average ENT counts determined via the FISH technique were lowest (0.0003–2.0 $\times$ 10^6 cells mL^{-1}) in the samples collected at the river source (SRW1) in a forest reserve with very low levels of anthropogenic pressure. In this sampling site, the presence of *E. faecalis*, *E. faecium*, and *E. gallinarum* (targeted using ENF191, ENU140, and EGAC183 probes, respectively) in river water could be attributed to wild animals and birds inhabiting the forest. The average ENT counts in animal feces have been determined at 10^5–10^6 CFU/g, and some of the identified strains are resistant to antibiotics [17,19,61,62]. In the Łyna River, the abundance of the examined bacteria increased downstream, proportionally to pollution, and peaked (0.0012–5.5 $\times$ 10^6 cells mL^{-1}) in samples collected downstream from the WWTP. Research studies examining rivers in Poland and in other countries indicate that an increase in Escherichia coli, *E. faecalis*, and *E. faecium* counts is indicative of microbial pollution and changes in the trophy levels. In the largest Polish rivers (Vistula, Brda, Drwęca), *Eubacteria* counts were determined at 10^5–10^7 cells and were highly similar to the values noted in other lotic environments [5,6,12].

In the current study, ENT counts determined via the FISH technique were 1000–100,000 times higher than those determined via the culture-based method. In the culture-based method, the abundance of enterococci did not exceed several dozen CFU in 100 mL of the samples collected at the river source, several hundred CFU in the samples

collected downstream from the WWTP, and several hundred to several dozen thousand CFU in wastewater samples. Only a small percentage of bacteria colonizing a given environment can be identified with the use of culture-based methods [23,24]. Standard laboratory methods do not support the identification of VBNC bacteria, either. Therefore, culture-based methods can lead to the underestimation of bacterial abundance in a given biotope and the misinterpretation of the results. Bacterial counts were many-fold higher in the FISH assay than in the culture-based analysis, which indicates that many *E. faecalis* and *E. faecium* (ENF191, ENU140) strains found in river water were non-culturable. Similar observations were previously made by Gotkowska-Płachta et al. (2016) [6], who analyzed the abundance of Enterobacteriaceae spp. and Escherichia coli in the Łyna River with the use of the FISH method.

In this study, enterococci were also highly abundant (0.17–216.1 $\times$ 10^6 cells mL^{-1}) in UWW3 samples analyzed using the FISH technique. Enterococcal counts were reduced by 70–80% (depending on species) during the wastewater treatment process. Despite the above, several thousand to several hundred thousand enterococcal cells/mL were evacuated to the river with treated wastewater (WWT4 samples), which indicates that microbial pollutants are not completely eliminated in WWTPs. According to Council Directive 91/271/EEC [63] (OJ, item 1800), treated wastewater does not have be disinfected before it is discharged to a river. Chlorine disinfection can reduce bacterial counts, but it can also contribute to the transmission of genetic determinants of antimicrobial resistance. Rolbiecki et al. [8] found that chlorine disinfection of hospital wastewater promoted the survival of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae and increased their abundance in treated wastewater. Therefore, both chlorination and the absence of disinfection can accelerate the spread of antibiotic resistance in the natural environment. Multidrug-resistant ARB can be transported across considerable distances with flowing waters, and their ARGs can be transferred to native environmental bacteria, which poses a serious threat to public health [5,64].

4.2. Antibiotic Resistance and Virulence Factors of Enterococci Isolated from River Water and Wastewater

Enterococci were identified via the FISH technique, and the resistance profile and virulence of 225 fecal ENT strains were also determined. Significant ($p \leq 0.05$) correlations were observed between most microorganisms identified in the FISH assay and resistant and virulent ENT strains (Figure 7). The MAR index was calculated for the identified strains. A MAR index greater than 0.2 points to the high risk of spread of antibiotic resistance. The MAR index of the analyzed strains was high, at 0.2–1.0, and drug resistance profiles differed across sampling sites. Around 30% of the strains isolated from URW2, SRW1, and DRW5 samples were resistant to two or three classes of antibiotics. In river water samples collected upstream from the WWTP, most of the isolated strains were resistant to glycopeptides (Gl), oxazolidinones (Ok), and chemotherapeutics (Che). The strains isolated from wastewater were characterized by the highest levels of antibiotic resistance (MAR = 0.7–1.0). More than 15% of these strains were resistant to five or six classes of antibiotics, and around 50–70% of the isolates were resistant to chemotherapeutics (Che), fluoroquinolones (Fl), and streptogramins (St). The smallest proportion of strains (0–22%) isolated from river water and wastewater were resistant to doxycycline (tetracycline class). In a study by Monteiro and Santos [53], more than 50% of enterococci isolated after different stages of wastewater treatment were resistant to ciprofloxacin, tetracycline, and linezolid in three WWTPs deploying various treatment methods. In the work of Gotkowska-Płachta [5], *E. faecalis* and *E. faecium* isolated from hospital wastewater and from treated and untreated municipal water were characterized by similar resistance profiles. In the present study, and in previous research, VRE were also identified in river water and wastewater. According to the WHO, vancomycin-resistant *E. faecium* is a high-priority pathogen (WHO, 2017) [20]. A report from the European Center for Disease Prevention and Control [65] revealed that the abundance of vancomycin-resistant *E. faecium* (VRE-fm) has increased by more

than 20% throughout Europe in the last 10 years. In the current study, XDR enterococci accounted for 6% of the strains isolated from TWW4 samples. These isolates were resistant to all tested antibiotics, including vancomycin. This observation indicates that antibiotic resistance mechanisms are acquired by enterococci during wastewater treatment, and it was confirmed by similarities in the resistance profiles of the strains isolated from UWW3 and TWW4 samples (Figure 4). Extensively drug-resistant strains, including VRE, are increasingly identified in natural and contaminated environments [17,22,37,66]. In samples collected downstream from the WWTP (DRW5), the greatest proportion of the isolated enterococci were resistant to the same classes of antibiotics as the strains isolated from TWW4. The presence of drug-resistant enterococci in treated wastewater evacuated to the Łyna River and their spread in the river continuum can promote horizontal gene transfer and the potential selection of ARB. Numerous studies have shown that treated wastewater is a major source of ARB and ARGs in lotic environments [5,17,66–68].

In addition to a high percentage of antibiotic-resistant strains, enterococci harboring virulence genes that point to the toxicity of the analyzed strains were also isolated from river water and wastewater samples. Virulence genes were determined in more than 50% of the analyzed isolates. Virulence genes, mostly the efaA gene encoding cell wall adhesin and the cylA gene encoding cytolysin, were least prevalent in SRW1 samples (approx. 30%). In URW2 and DRW5 samples, around 40% and 50% of the isolates, respectively, harbored hyl (hyaluronidase) and ccf (sex pheromones) genes that participate in the exchange of genetic material and induce virulence. The highest proportion (45–77%) of isolates carrying efaA, cob, cpd, ccf, and hyl genes that induce virulence and trigger inflammatory responses [69] was noted in the UWW3 and TWW4 samples. Isolates carrying genes that encode cytolysin (cylA), enterococcal surface protein (esp), and aggregation substance (as) were the least prevalent in all samples. Virulent enterococci are isolated mainly from hospital environments, animals, and humans, and they pose significant therapeutic and epidemiological challenges [70–72]. The horizontal transfer of virulence genes leads to the spread of these pathogenic bacteria in the natural environment. Their main sources include human activities (wastewater management, agriculture, tourism) and environmental factors (wild animals and birds) [17,67,71].

The dendrograms revealed the presence of correlations between the biodiversity of bacteria identified in the FISH assay and the multidrug resistance and virulence of the isolated enterococci (Figure 8). The agglomerative clustering analysis confirmed that UWW3, TWW4, and DRW5 samples were mostly closely related in terms of the biodiversity, multidrug resistance, and virulence of the isolated enterococci. SRW1 and URW2 samples constituted a separate group in this respect (Figure 9). The results of this study indicate that anthropogenic pressure exerts a significant influence on river microbiota and microbial pollution in lotic environments.

5. Conclusions

Fluorescent in situ hybridization supported the rapid and accurate identification of fecal enterococci as indicators of the water quality and microbial pollution in river water. In most cases, ENT counts were several thousand times higher in the FISH assay than in the culture-based analysis. These results indicate that the examined biotope was colonized by numerous VBNC bacteria (mainly *E. faecalis* and *E. faecium*) that could not be enumerated with the use of culture-based methods. The underestimation of fecal enterococci in the studied environment can lead to the misinterpretation of the results, which poses a particular risk in analyses of multidrug-resistant and virulent strains. Around 30–50% of enterococcal strains isolated from river water and wastewater samples were multidrug-resistant and harbored virulence genes. Samples collected at the river source in a forest reserve with minimal anthropogenic pressure were characterized by the lowest levels of microbial pollution, but microbial counts increased several-fold in urban areas downstream from the WWTP. The statistical analysis revealed that samples of untreated wastewater, treated wastewater, and river water collected downstream from the WWTP

were mostly closely related in terms of the biodiversity, multidrug resistance, and virulence of enterococci. Samples of river water collected at the source and upstream from the WWTP formed a separate group in this respect. In addition, the strains isolated from river water downstream from the WWTP and the isolates identified in wastewater had similar virulence and antibiotic resistance profiles, which suggest that wastewater management exerts a considerable influence on the river biotope. The exchange of genetic material between antibiotic-resistant enterococci and native river bacteria promotes the spread of antibiotic resistance in the entire river ecosystem, and poses a health threat to humans and animals. A combination of conventional and molecular techniques, including the FISH method, supports the determination of the actual abundance of enterococci in the examined biotope as well as a rapid and reliable assessment of water quality with the use of these sensitive bioindicators of pollution.

Author Contributions: Conceptualization, writing—original draft preparation, and methodology, A.G.-P.; writing—review and editing, supervision, and data curation A.G.-P. and I.G. All authors have read and agreed to the published version of the manuscript.

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