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Prevalence of antimicrobial resistance in a Bulgarian drinking water supply system

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ABSTRACT

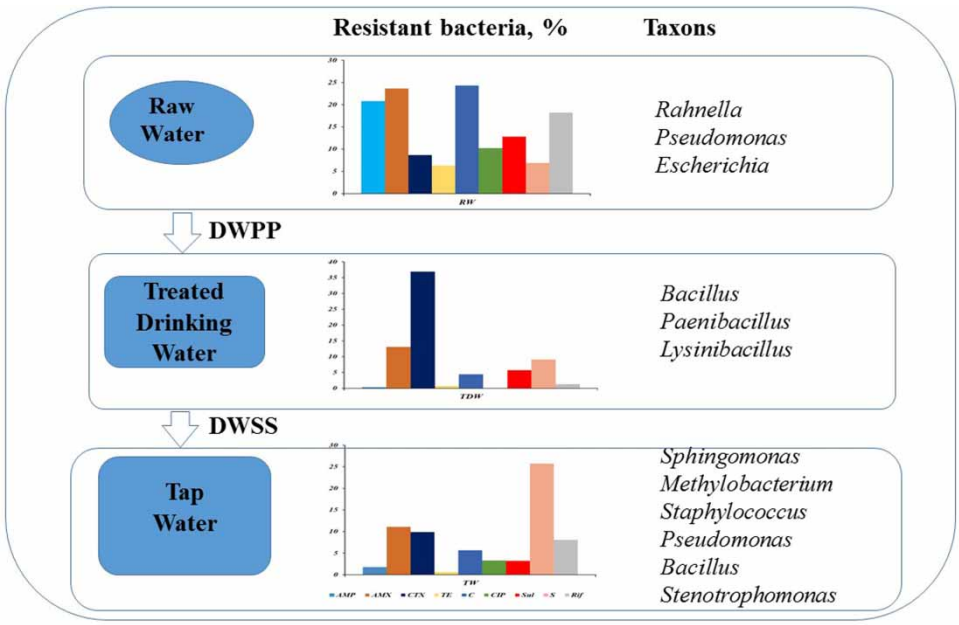
Drinking water quality and safety are of great concern for public health. The aim of this study was to assess the prevalence of antimicrobial resistance (AMR) among the heterotrophic bacteria in drinking water provided by a Bulgarian drinking water supply system (DWSS). Culture-dependent methods and conventional PCR assays were used to study drinking water sampled from six locations on its way from the water source to the consumers' taps. The populations of bacteria resistant to nine antibiotics (ABs) from different classes were quantified and the occurrence of seven antibiotic resistance genes (ARG) was determined. The species composition of the bacterial community and the AMR phenotype of isolated bacteria were determined. The AMR level underwent changes within the DWSS network and the population's proportion of bacteria resistant towards the tested ABs differed depending on the sampling site. The increased level of resistance towards some ABs in drinking water emphasize the role of DWSS as a reservoir of antibiotic-resistant bacteria (ARB) and ARGs that could pose a potential human health risk. Being focused on a Bulgarian DWSS, our study will contribute to establishing health hazards associated with ARB and ARGs in drinking water.

Key words: antibiotic resistance genes, antibiotic resistant bacteria, antimicrobial resistance, drinking water, heterotrophic bacteria

HIGHLIGHTS

- The study is the first one estimating the prevalence of AMR in a DWSS in Bulgaria.
- The populations of antibiotic-resistant bacteria significantly reduced in the finished water, and underwent changes in the tap water.
- Some resistant opportunistic pathogens, including *Stenotrophomonas maltophilia* were detected in tap water.
- The *cat* and *sull* resistance genes were most commonly detected in tap water.

GRAPHICAL ABSTRACT



INTRODUCTION

Antimicrobial resistance (AMR) continues to be a global health challenge and recently has been turning into an environmental challenge. AMR hotspots have been found not only in medical settings but also in environmental compartments, such as water resources that are subjected to anthropogenic pressure (Vaz-Moreira *et al.* 2014; Berendonk *et al.* 2015). There is increasing evidence for interrelation between the increasing AMR and the anthropogenic impact on water resources (Goñi-Urriza *et al.* 2000; Berglund *et al.* 2015). As a result of human activities such as drinking water supply for human consumption and industrial usage, and wastewater treatment and its release in water bodies, the bacteria, including those resistant to antibiotics, are able to move from polluted wastewater into treated wastewater and natural waters. In this way, this Urban Water Cycle contributes to dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) and is in the base for potential transmission routes of AMR from aquatic environment to human and vice versa (Vaz-Moreira *et al.* 2014; Manaia 2016).

The presence of ARB and ARGs in water sources and purified drinking water is an emerging health-related problem for water supply industry (Xi *et al.* 2009; Khan *et al.* 2016; Destiani & Templeton 2019). Despite that the conventional water treatment and disinfection can reduce the total number of bacteria in drinking water, there is evidence for increased AMR of some aquatic bacteria and increased levels of ARGs (Xi *et al.* 2009; Narciso-da-Rocha *et al.* 2013; Lu *et al.* 2018; Su *et al.* 2018). The prevalence of bacteria from the genera *Sphingomonas*, *Pseudomonas* and *Acinetobacter,* capable of withstanding the action of various antibiotics (ABs), have been proven in drinking water (Vaz-Moreira *et al.* 2011, 2012; Narciso-da-Rocha *et al.* 2013). The increased AMR can be related to the selective effect of chlorine and the bacterial community changes in a drinking water supply system (DWSS) (Xi *et al.* 2009; Khan *et al.* 2016). Thus, the DWSSs could serve as a reservoir and an incubator for growth of ARB and play role of an important source for dissemination of AMR (Xi *et al.* 2009; Vaz-Moreira *et al.* 2014; Destiani & Templeton 2019).

Different approaches are used to evaluate the status of AMR in aquatic environment each with its own limitations. One of them is based on culture-dependent methods for assessment of AMR, determining the ratio between the number of bacteria that can grow on culture media supplemented with the AB in doses close to minimal inhibitory concentrations and the number of bacteria growing on AB-free media (Xi *et al.* 2009; Berendonk *et al.* 2015; Destiani & Templeton 2019). The culture-dependent methods assess the total (intrinsic and acquired) AMR of culturable, but not of viable non-culturable bacteria, thus excluding from analyses significant part of the aquatic community (Vaz-Moreira *et al.* 2013).

Another culture-dependent method, the antimicrobial susceptibility testing, is widely applied for the AMR assessment of the fecal indicator bacteria, *Pseudomonas aeruginosa* and *Aeromonas spp.,* used in monitoring of the microbiological

water quality. The phenotypic AMR of environmental bacteria is interpreted based on clinical standards and recommended breakpoints developed for pathogenic bacteria by CLSI and EUCAST. Numerous studies have determined the AMR phenotype of bacteria isolated from drinking water and the incidence of multiple drug resistance (MDR) (Armstrong *et al.* 1981; Vaz-Moreira *et al.* 2011, 2012; Khan *et al.* 2016a). The widespread in drinking water of members of the family *Sphingomonadaceae* has demonstrated a rich and diverse profile of AMR, including prevalence of MDR (Vaz-Moreira *et al.* 2011), while among the *Acinetobacter* isolates, intrinsic AMR and low levels of acquired AMR have been predominant (Narciso-da-Rocha *et al.* 2013). Resistant coagulase-negative staphylococci have been also detected in DWSS network (Faria *et al.* 2009), as well as *Pseudomonas* isolates exhibiting a low degree of AMR (Vaz-Moreira *et al.* 2012).

Application of culture-independent approaches in the AMR assessment, in particular quantitative qPCR, can give a good estimate of the level of water contamination with known ARGs, although careful standardization of gene copy number is needed. The changes in bacterial community and ARGs abundance in the processes of drinking water treatment and chlorination, and the water distribution, have been documented through qPCR assays, high-throughput sequencing and metagenomic approaches (Xi *et al.* 2009; Shi *et al.* 2013; Lu *et al.* 2018; Su *et al.* 2018). Significant diversity of ARGs has been detected in drinking water on its way from the water sources to consumers (Lu *et al.* 2018; Su *et al.* 2018), and a catalogue of antibiotic resistome in drinking water from a wide range of regions have been established (Ma *et al.* 2017). Some studies identified residual chlorine as the key factor driving the bacterial community shift and resistome profile. There is data, that chlorination could increase the total abundance of ARGs, while reducing their diversity in opportunistic pathogenic bacteria (Jia *et al.* 2015). Shi *et al.* (2013) have reported quantitative changes of different ARGs in the course of drinking water treatment and distribution: chlorination could concentrate various ARGs, while transportation tended to reduce their number.

Drinking water quality and safety are of great concern for public health. Access to clean and safe drinking water is a fundamental human right, and in the European countries, the quality of water intended for human consumption, including microbiological quality, is regulated (Drinking Water Directive 98/83/EC). Water treatment and disinfection in drinking water purification plants (DWPPs) provide microbiological quality, evaluated by using fecal indicator bacteria. However, the number of the heterotrophic plate count bacteria (HPC) is not regulated, and the assessment of AMR of aquatic microbiota still remains outside the scope of the microbiological monitoring of drinking water.

Despite the significant number of AMR studies of drinking water through cultural and molecular methods, not many have made an overall assessment of the prevalence of AMR in real DWSSs (Xi *et al.* 2009; Destiani & Templeton 2019). Some contradictory results for abundance and diversity of ARB and ARGs in drinking water on its way from water source to consumers have been reported, as well (Bergeron *et al.* 2015; Xi *et al.* 2009).

The risk of ARB reaching the end-users, as well the absence of data on the prevalence of ARB and ARGs in the drinking water supplied to Bulgarian consumers, determined the goals of our study, namely: (*a*) to assess the AMR of heterotrophic bacteria in drinking water from selected Bulgarian DWSS by culture-dependent methods and qualitative PCR assays; (*b*) to evaluate the impact of the purification and disinfection processes and the DWSS network on the abundance of different types of ARB in drinking water; (*c*) to determine the species composition of the bacterial community and to assess the AMR phenotype of bacterial strains isolated from drinking water on its way from the water source to end-users.

MATERIALS AND METHODS

Study area and sample collection

The object of the study is the DWSS supplying drinking water to the population of the region of Veliko Tarnovo, Bulgaria. The source of raw water is the Yovkovtsi Dam (42.946044N, 25.760527E). After raw water treatment in a DWPP (42.975911N, 25.734135E), the finished water is distributed to many settlements in the region, supplying more than 300,000 inhabitants. The water treatment in the DWPP includes processes of pre-chlorination, settlement, fast sand filtration and chlorine disinfection. The finished water is transported gravimetrically to the villages in surrounding area and to water tanks in the town of Veliko Tarnovo (43.0734341 N, 25.6037837E), and then, by pumping, to the end consumers.

The water samples were collected from six locations: the DWPP entrance (raw water, RW), the DWPP exit (treated drinking water, TDW), one public building (tap water, TW-2), one public fountain (TW-3) and two residential buildings (TW-1 and TW-4). The sampling locations were selected to cover sections of the DWSS network in which drinking water differs in residual chlorine content. The sampling points TW-1 and TW-4 were selected to collect low residual chlorine-containing

tap water (respectively, 0.34 ± 0.06 mg L^{-1} for TW-1 and 0.32 ± 0.03 mg L^{-1} for TW-4; n=18), while the TW-2 and TW-3 – water samples with higher residual chlorine content (respectively, 0.71 ± 0.13 mg L^{-1} for TW-2 and 0.74 ± 0.09 mg L^{-1} for TW-3; n=22), analyzed by *o*-tolidine method. A total of four duplicate water samples were collected from each DWSS location to cover the four seasons. The only exception was TW-2 because the public fountain is being stopped in winter.

Enumeration of total cultivable heterotrophic bacteria and antibiotic resistant bacteria

The number of culturable heterotrophic bacteria in drinking water was analyzed by membrane filtration through a 0.45 μm pore size, 47 mm diameter sterile membrane filter (Sartorious AG). Then, the membrane filters were placed on the surface of R2A agar (HiMedia, India), that is AB-free or is supplemented with given AB. The incubation was for 7 days at a temperature of 25 °C. Each water sample was analyzed in duplicate for enumeration of total HPC bacteria or ARB.

By filtering certain volumes of drinking water (depending on the putative number of the analyzed type of ARB) and incubating the filters on standard R2A agar or R2A agar with addition of AB, the total number of HPC bacteria and the number of bacteria resistant to each individual AB were determined in parallel. Based on the obtained HPC data pairs (each measured as CFU/100 ml), the percentage of bacteria resistant to individual ABs was calculated.

The heterotrophic bacteria resistant to nine AB substances from seven classes were enumerated: β-lactams (ampicillin, *AMP* – 32 mg L^{-1}; amoxicillin, *AMX* – 8 mg L^{-1}; cefotaxime, *CTX* – 4 mg L^{-1}; all as sodium salts); aminoglycosides (streptomycin sulphate, *S* – 32 mg L^{-1}); tetracyclines (tetracycline hydrochloride, *TE*, 8 mg L^{-1}); amphenicols (chloramphenicol, *C* – 16 mg L^{-1}); fluoroquinolones (ciprofloxacin hydrochloride monohydrate, *CIP* – 4 mg L^{-1}); antifolates (sulphamethoxazole, *Sul* – 256 mg L^{-1}), and rifampicin, Rif – 4 mg L^{-1} (HiMedia, India).

Isolation of heterotrophic bacteria

Pure bacterial cultures were isolated from selected colonies growing on R2A agar. All bacterial colonies from the membrane incubated on R2A agar were isolated when their number was below 10. When their number was above 10, representatives of all morphologically different colonies and a minimum of 5 colonies with similar characteristics were isolated.

Selective culture media were used for isolation of *Escherichia coli*/coliform bacteria (Lactose TTC agar with tergitol®7; Merck, Germany), enterococci (*Enterococcus* selective agar acc. Slanetz-Bartley; Merck, Germany) and pseudomonads (Cetrimide agar; Merck, Germany) from the RW samples. The typical colonies isolated through the selective culture media were confirmed in accordance with the current standards for microbiological quality of water: EN ISO 9308-1: 2014 – for detection of *E. coli* and coliform bacteria; EN ISO 7899-2:2000 – for detection and enumeration of intestinal enterococci; EN ISO 16266:2008 – for detection and enumeration of *P. aeruginosa*. The purity of sub-cultured bacterial strains was tested by soybean casein digest agar (HiMedia, India) and culturing at 35 °C for 24–48 hours. The pure bacteria cultures were stored at −20 °C.

Biochemical identification of bacterial isolates

The isolates were identified by the MICROLATEST® tests (Erba Lachema s.r.o., Czech Republic) as follows: NEFERMtest 24 was used for non-fastidious, non-enteric, Gram-negative bacteria, and ENTEROtest 24N was applied for coliforms. The resulting ID score indicates the extent to which the taxon can be distinguished from other taxa. The strain can be distinguished perfectly when ID ≥99% or very well – at ID ≥95%, and cannot be sufficiently distinguished without additional tests at ID <90%. The strains were considered identified at ID ≥90%.

The biochemical identification of bacterial strains with established MDR and those representing the largest populations in DW was verified using BD Phoenix™ M50 Automated Microbiology System (Becton, Dickinson and Company, USA). The instrument BD Phoenix™ M50 simultaneously identifies the isolates and assesses their antimicrobial susceptibility by antibiotic dilution tests in combined panels. NMIC/ID-76 panels were used for identification and antimicrobial susceptibility testing of Gram-negative bacteria, and PMIC/ID-88 were used for Gram-positive bacteria. The procedure, as described by the manufacturer, involves preparing bacterial inoculum (0.5 McFarland) in BD Phoenix ID Broth (for identification) and transferring of 25 μl to the BD Phoenix AST Broth (for antimicrobial susceptibility testing) with a pre-added drop of BD Phoenix AST indicator solution. After both suspensions of the tested strain were poured into the separate entrances of the appropriate panel, it was loaded into the instrument at 35°C for 24 ± 4 h. The obtained data were analyzed by EpiCentre™ software.

Determination of antibiotic resistance pattern of bacterial isolates

Antimicrobial susceptibility of the bacterial isolates that could not be assessed using the BD Phoenix™ M50 system, due to absence of appropriate panels, was evaluated only by the disc diffusion method. The susceptibility was tested to: *β*-lactams (*AMP 10* μg – ampicillin; *AMC 20/10* μg – amoxicillin/clavulanic acid; *CF 30* μg – cefalothine; *CX 30* μg – cefoxitin; *CTX 30* μg – cefotaxime); aminoglycosides (*S 10* μg – streptomycin, *GEN 10* μg – gentamicin); quinolones (*CIP 5* μg – ciprofloxacin; *NA 30* μg – nalidixic acid); antifolates (*SXT 1.25/23.75* μg – trimethoprim/sulfamethoxazole); tetracycline, *TE 30* μg; chloramphenicol, *C 30* μg, and macrolides (*E 15* μg – erythromycin) (CLSI 2017).

The tested strain was inoculated on the surface of Mueller Hinton agar (HiMedia, India) as a calibrated suspension (0.5 MacFarland). Disks with the tested ABs (HiMedia, India) were placed on the surface of the inoculated agar. After 18 h of incubation at 35°C, the inhibition zone diameter around each AB disk was measured (in *mm*). The strains were classified as S – sensitive or R – resistant. According to EUCAST, AMR towards at least one AB from at least three different classes was defined as multidrug resistance. Since there are no guidelines for the AMR breakpoints of most environmental bacteria, the results for Gram-positive bacteria were interpreted according to CLSI breakpoints for *Staphylococcus spp*., and those for Gram-negative bacteria according to the AMR breakpoints for the family *Enterobacteriaceae*.

DNA extraction and purification

Forty-one bacterial strains with antibiotic resistance patterns were selected for genetic analyses. Genomic DNA from Gram-negative and Gram-positive bacteria was isolated and purified by GENE MATRIX Bacterial/Yeast Genomic DNA purification kit (EURx® Molecular Biology Products, Poland).

PCR assays for detection of ARGs

Qualitative PCR assays were performed to determine the presence of seven targeted ARGs in the isolated bacteria. For PCR amplification of the required ARGs, primers with known oligonucleotide sequence were used. The characteristics of the primers for all the targeted genes (synthesized by Microsynth AG, Switzerland), and the corresponding references are presented in Table 1.

The PCR assays were carried out using the onTaq PCR Master Mix 2x (EURx® Molecular Biology Products, Poland) in a 25 μl volume reaction, with primers in final concentration of 0.4 μM. The PCR amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratory, USA), as follows: initial denaturation at 95 °C for 10 min, followed by 35 cycles

Table 1 | Primer sets used in PCR analyses

Targeted gene	Primer name	Sequence	Annealing T°C	Reference
tetA	tetA F	GGTCATTTTCGGCGAGGATC	60.5	Destiani & Templeton (2019)
	tetA R	GAAGGCAAGCAGGATGTAGC		
$bla_{\text{TEM-1}}$	blaTEM1 F	GCGCCAACTTACTTCTGACAACG	64.9	Xi *et al.* (2009)
	blaTEM1 R	CTTTATCCGCCTCCATCCAGTCTA		
sulI	sulI F	CGCACCGGAAACATCGCTGCAC	67.9	Pei *et al.* (2006)
	sulI R	TGAAGTTCCGCCGCAAGGCTCG		
cat	cat F	ATGGCAATGAAAGACGGTGAGC	62.1	Xi *et al.* (2009)
	cat R	TGCCGGAAATCGTCGTGGTATT		
qrnS	qnrSrtF11	GACGTGCTAACTTGCGTGAT	55.7	Marty & Balcazar (2012)
	qnrSrtR11	TGGCATTGTTGGAAACTTG		
dfrA7	dfrA7 F	CAACGATGTTACGCAGCAGG	61.5	Destiani & Templeton (2019)
	dfrA7 R	GGACCACTACCGATTACGCC		
intI	intI F	GGG TCA AGG ATC TGG ATT TCG	60.7	Mazel *et al.* (2000)
	intI R	ACA TGC GTG TAA ATC ATC GTCG		

F, forward primer; R, reverse primer.

(denaturation 30 s at 94 °C; annealing 30 s at specific temperature (Table 1); extension 1 min at 72 °C), a final extension step (7 min at 72 °C) and cooling at 4 °C.

The PCR products were visualized in 1.5% agarose gels in Tris-Acetate-EDTA (TAE) solution at pH 8.3. As positive controls *E. coli* ATCC 35218 carrying *blaTEM*, *E. coli* NBIMCC 1164 carrying *tet* and *cat* genes, and *E. coli* NBIMCC 1223 exhibiting trimethoprim-resistance were used.

Statistical analysis of the data

One-way analysis of variance (ANOVA) was performed to assess the significance of differences between the sampling locations on the percentage of ARB to individual ABs as the dependent variable, and sampling location as the factor. $P<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Microbiological characteristics of water on its way from the water source to the consumers' tap

The data show that the total number of HPC bacteria in DW depended on the DWSS sampling point (Table 2). The treatment processes in the DWPP reduced the number of HPC bacteria in the finished water by 96.6%. The HPCs in the drinking water from the consumers' tap ranged from 5.2×10^1 to 1.7×10^3 CFU/100 ml, and most HPC values were higher compared to the TDW from the DWPP exit, indicating the influence of the distribution network. The exception was the TW-3 from continuously running public fountain. The number of HPCs in drinking water showed seasonal fluctuations, with predominantly higher values in summer (no data presented).

In total, 292 bacterial strains were isolated (Table 2), and 40% of them were Gram-positive. The untreated RW contained the lowest number of Gram-positive bacteria, while the highest one was detected in the freshly chlorinated TDW at the DWPP outlet. The number of Gram-positive bacteria was higher in tap water TW-2 and TW-3 (containing residual chlorine of 0.7 mg L^{-1}) compared to TW-1 and TP-4 (residual chlorine of 0.4 mg L^{-1}). These data are in accordance with the reported findings of changes in bacterial community resulting from drinking water chlorination (Armstrong *et al.* 1981; Norton & LeChevallier 2000; Vaz-Moreira *et al.* 2013). In addition, bacterial number in water decreased during the spring-winter season, but an increase in the relative proportion of Gram-positive bacteria was recorded with the highest values in February and March.

The AMR of HPC bacteria in drinking water from the water source to the consumers' tap

The population proportion of different type of ARB in drinking water represents the ratio of the bacterial population that survived in the presence of each particular AB to the total HPCs deriving from all analyzed samples of each single location (Table 3). The data show that up to one third of the heterotrophic bacteria population in drinking water was resistant to the individual ABs in the tested concentrations. In the RW, the bacteria resistant to the beta-lactams ampicillin (20.8%) and amoxicillin (23.6%), and to chloramphenicol (24.8%) and rifampicin (18.2%) were predominant. The water treatment and disinfection process significantly reduced the population density of the bacteria resistant to individual ABs. The

Table 2 | HPC and number of isolates from drinking water samples

Sampling point	Denotation	HPC[a], CFU/100 ml	Number of isolates	Number of Gram (–) isolates	Number of Gram (+) isolates	% of Gram (+) isolates
Raw water (at the entrance of DWPP)	RW	$8.4 \pm 0.05 \times 10^3$	48	39	9	18.8
Treated drinking water (at the DWPP exit)	TDW	$2.8 \pm 0.7 \times 10^2$	35	3	32	91.4
Tap water 1	TW-1	$6.5 \pm 1.8 \times 10^2$	90	66	24	26.7
Tap water 2	TW-2	$6.9 \pm 4.7 \times 10^2$	69	38	31	44.9
Tap water 3	TW-3	$5.2 \pm 3.0 \times 10^1$	20	5	15	75.0
Tap water 4	TW-4	$1.7 \pm 0.3 \times 10^3$	30	23	7	23.3
Total number of isolates, *n*			292	174	118	40.4

[a]average value of the samples for a year-long test period.

Table 3 | Percentage of heterotrophic bacteria resistant to individual antibiotics in the drinking water of the selected sampling points

	Heterotrophic bacteria resistant to individual ABs. %					
Type of ABs	**RW**	**TDW**	**TW-1**	**TW-2**	**TW-3**	**TW-4**
AMP	20.8 (2.0)	0.3 (0.2)	1.8 (1.4)	3.3 (2.8)	1.8 (1.8)	0.1 (0.1)
AMX	23.6 (4.4)	13.1 (2.0)	27.6 (19.1)	7.7 (6.8)	7.5 (5.6)	1.8 (1.3)
CTX	8.7 (1.5)	36.9 (5.3)	9.4 (8.2)	7.7 (4.9)	20.4 (15.7)	2.1 (1.7)
TE	6.3 (0.7)	0.7 (0.4)	0.5 (0.4)[a]	1.2 (1.6)[a]	0.5 (0.5)[a]	0.2 (0.2)
C	24.3 (1.4)	4.4 (2.5)	12.0 (5.2)	7.5 (3.4)	2.0 (2.0)	1.2 (1.0)
CIP	10.2 (6.8)	0.0	6.3 (4.3)	5.1 (5.2)	1.7 (1.1)	0.2 (0.2)
Sul	12.8 (3.5)	5.7 (2.2)	2.8 (2.7)	4.1 (2.9)[a]	4.3 (4.0)[a]	1.6 (1.4)
S	6.9 (4.5)	9.1 (4.3)[b]	33.3 (10.0)	27.1 (16.6)	11.1 (10.3)[a]	31.5 (19.9)
Rif	18.2 (4.2)	1.3 (0.2)	8.0 (3.8)	14.7 (11.4)	3.7 (2.0)	6.0 (5.6)

Water sampling locations of the studied DWSS: RW- raw water from the DWPP inlet; TDW – treated drinking water from the DWPP outlet; TW-1. TW-2. TW-3 and TW-4 - tap water from the distribution network; *Target antibiotics: AMP* – ampicillin; *AMX* – amoxicillin; *CTX* – cefotaxime; *S* – streptomycin; *TE* – tetracycline; *C* – chloramphenicol; *CIP* – ciprofloxacin; *Sul* – sulfamethoxazole. and *Rif*– rifampicin;.

[a]insignificant difference between TDW and individual TW ($p>0.5$).

[b]insignificant difference between RW and TDW.

ciprofloxacin-resistant bacteria were fully eliminated in the TDW and the decrease of the populations of bacteria resistant to some beta-lactams, tetracycline, chloramphenicol and rifampicin was also significant. Only the percentage of cefotaxime-resistant bacteria significantly increased, and the population of bacteria resistant to streptomycin underwent insignificant change ($p>0.05$). These data demonstrate the effectiveness of the treatment process in the DWPP, while for other DWPPs, diverse effects have been reported – from complete elimination of ARB (Bergeron *et al.* 2015) to significant increasing of the bacteria resistant to amoxicillin, chloramphenicol and rifampicin, but decreasing of sulfisoxazole resistance (Xi *et al.* 2009).

All tap water data (TW-1÷TW-4) reveal that the populations of the bacteria resistant towards individual ABs underwent appreciable changes in the DWSS network. Compared to TDW, the abundance of bacteria resistant to ampicillin, ciprofloxacin, rifampicin and streptomycin significantly increased in all TW sampling locations, while the populations of bacteria resistant to amoxicillin (with one exception), cefotaxime and chloramphenicol (in two TW locations) significantly decreased. The populations of bacteria resistant to tetracycline or sulfamethoxazole only insignificantly altered in most TW locations compared to TDW.

The population data on the prevalence of ARB in TW sampling locations revealed:

– the lowest abundance of tetracycline-resistant bacteria, and the highest of streptomycin-resistant ones;
– predominantly low proportion of bacteria resistant to ciprofloxacin and sulfamethoxazole;
– the population share of the bacteria resistant to the individual ABs was in ascending order:

$$TE < Amp < CIP/Sul < C < Rif/CTX/Amo < S$$

The populations' abundance of bacteria resistant to individual ABs, as well as the predominant ARB types, differed in the tap water samples depending on the sampling location. In TW-1, predominant were the bacteria resistant to amoxicillin, chloramphenicol and streptomycin, while, in TW-2 and TW-4, streptomycin-resistant bacteria prevailed. In TW-3, the resistance to cefotaxime was particularly high. The differences in the prevalence of the bacteria resistant to individual ABs in the TW sampling sites can be related to the water chlorination disinfecting effect, but also to potential impact of the biofilms existing in the water supply network that could emit bacteria into the water passing through the pipelines (Flemming *et al.* 2002; Xi *et al.* 2009). The fluctuations in the number of bacteria resistant to individual ABs in tap water from a single sampling location can be related to the seasonal dynamics of the total HPC during the a year-long test period.

Despite the incomplete agreement of the tested ABs, a comparison of our AMR results with the data reported for other DWSS was made. In drinking water of London, Destiani & Templeton (2019) also reported the lowest occurrence of

tetracycline- and ciprofloxacin-resistant bacteria (from 1.5% to 14%), while Xi *et al.* (2009) found 0.04–3.78% tetracycline resistance and 10–13% ciprofloxacin resistance in American tap waters. The amoxicillin-resistant bacteria in London drinking water ranged from 8 to 43%, while in American tap waters – from 3 to 15.2%. As may be assumed, the abundance of the different ARB types in the individual DWSSs could be related to natural and/or contaminant AMR in water source, as well to the effect of water purification and disinfection. The preferences in ABs usage in human and veterinary medicine in each particular country/region could predetermine the various residual amounts of ABs in water resources and their selective effect on microbial community. The various water treatment technologies differing in their efficiency and the applied disinfectants would also contribute to the populations differences of the bacteria resistant to corresponding ABs (Hoefel *et al.* 2005; Jia *et al.* 2015; Khan *et al.* 2016; Su *et al.* 2018).

The conducted study on the municipal DWSS found out that the aquatic bacterial community was subjected to restructuring processes as a result of the water treatment and disinfection, and during its transportation in the DWSS network. The populations' density of the bacteria resistant to most of the tested ABs was significantly reduced in the finished water at the DWPP exit and varied in the tap water depending on the sampling location. The increased levels of bacterial resistance to ciprofloxacin, streptomycin and rifampicin found in tap water compared to finished water suggest that the DWSS network could have influence on the prevalence of AMR through bacterial regrowth or biofilm impact. The high proportion of bacteria resistant to amoxicillin, cefotaxime, rifampicin or streptomycin in tap water could pose a human health risk. The low abundance of bacteria resistant to tetracycline, ciprofloxacin or sulfamethoxazole established among the heterotrophic bacteria do not mitigate the role of drinking water as a reservoir of AMR.

Identification and AMR phenotype of bacteria isolated from water source to tap

In total, 91 of the isolated bacteria were identified to species or genus level (Table 4) and their AMR phenotype was assessed. The data on bacteria resistant to at least two classes of ABs are presented in Table 5.

Among the RW isolates, 28 strains were identified (Table 4), mainly from the family *Enterobacteriaceae* and genus *Pseudomonas*. *Rahnella aquatilis* were the most abundant *Enterobacteriaceae* members, isolated from untreated RW. This species is rarely reported as human pathogen, being mainly associated with infections in immuno-compromised patients (Stock *et al.* 2000). All *Rahnella* strains were resistant to *AMP* and some of them to *CF*, *CTX* and *CX*. It is known that *R. aquatilis* is naturally resistant to amoxycillin, ticarcillin, fosfomycin and to ABs to which other species of *Enterobacteriaceae* are also intrinsically resistant (Stock *et al.* 2000). The *Serratia fonticola* isolates were susceptible to the tested ABs and naturally resistant to *AMP*, *CX* and *CTX*. The isolated species *Aeromonas* were susceptible toward the tested ABs. The identified bacilli from the genera *Lysinibacillus* and *Bacillus* were resistant up to two groups of ABs, and *S. aureus* expressed MDR (Table 5).

Table 4 | Taxonomic characteristics of the isolated bacterial strains

Sampling point	Biochemically identified bacterial taxons
RW (28)	*Pseudomonas spp.* (8)[a]; *Enterococcus spp* (2); *Escherichia coli* (1); *Escherichia hermannii* (2); *Enterobacter cloacae* (1); *Serratia fonticola* (2); *Yersinia intermedia* (2); *Rahnella aquatilis* (5); *Aeromonas veronii bv sobria* (1); *Aeromonas caviae* (1); *Lysinibacillus sphaericus* (1); *Bacillus thuringiensis* (1); *Staphylococcus aureus* (1)
TDW (5)	*Lysinibacillus sphaericus* (1); *Bacillus pumilus* (2); *Paenibacillus alvei* (2)
TW-1 (11)	*Pseudomonas putida* (1); *Stenotrophomonas maltophila* (1); *Methylobacterium exorquens* (2); *Sphingomonas paucimobilis* (2); *Embedobacter brevis* (2); *Micrococcus luteus* (1); *Staphylococcus cohnii spp. cohnii* (1) *Brevibacterium spp.* (1)
TW-2 (16)	*Pseudomonas fluorescens* (4); *Pseudomonas putida* (2); *Pseudomonas spp.* (2); *Stenotrophomonas maltophila* (3); *Moraxella spp* (1); *Staphylococcus epidermidis* (1) *Bacillus megaterium* (1) *Bacillus licheniformis* (2)
TW-3 (11)	*Acinetobacter lwoffii (1); Sphingomonas paucimobilis* (1); *Staphylococcus spp.* (1); *Bacillus pumilus* (4); *Bacillus cereus* (1); *Paenibacillus alvei (2); Lysinibacillus sphaericus (1);*
TW-4 (20)	*Sphingomonas paucimobilis* (9); *Embedobacter brevis* (2); *Chryseobacterium indologenes* (2); *Myroides spp.* (2); *Methylobacterium exorquens* (1); *Staphylococcus epidermidis* (1); *Staphylococcus lugdunensis* (1); *Staphylococcus warneri* (1); *Streptococcus pneumoniae* (1).

[a]Number of identified strains – in brackets.

Table 5 | Total AMR (intrinsic and acquired) of the bacterial isolates

Sample location	Strain №	Biochemical identification	AMR phenotype	AMR to classes of ABs
RW	3-49	*Staphylococcus aureus*	AMP, CX, CIP, FF, MUH, OX, P	4
RW	*P-1*	*Pseudomonas fluorescens*	AMC, CAZ, C, TE, SXT	4
RW	*P-9*	*Pseudomonas sp.*	AMP, CAZ, C, TE, SXT	4
RW	*P-11, P-12*	*Pseudomonas sp.*	AMP, AMC, CTX, CAZ, TE, SXT	3
RW	*E-4,E-10*	*Rahnella aquatilis*	AMP, CF, CX, CTX, TE	2
RW	*E-15*	*Escherichia hermanii*	AMP, S	2
TDW	3-21	*Lysinibacillus sphaericus*	S, NA, E	3
TW-1	17	*Stenotrophomonas maltophilia*	AMP, AMC, AN, ATM, CN, CTX, CXM, CZ, ETP, FF, FM, GEN, IPM, MEM, NN, TMP, TZP	5
TW-1	2-47, 2-51	*Sphingomonas paucimobilis*	AMC, CN, CXM, CZ, FM	2
TW-1	2-31	*Brevibacterium spp.*	AMP, AMC, SXT, C	3
TW-2	63, 57, 58	*Stenotrophomonas maltophilia*	AMP, AMC, AN, ATM, CN, CTX, CXM, CZ, ETP, FF, FM, GEN, IPM, MEM, NN, TMP, TZP	5
TW-2	50, 51, 56	*Pseudomonas fluorescens*	AMC, CN, CXM, CZ, FM	2
TW-2	64	*Pseudomonas sp.*	AMP, CF, CTX, C, SXT	3
TW-2	65, 66	*Pseudomonas putida*	AMP, AMC, CZ, CAZ, C, CIP, FM	4
TW-2	67	*Vibrio metschnikovii*	AMP, CF, CTX, C, SXT	3
TW-2	2-101	*Staphylococcus epidermidis*	CIP, FA	2
TW-3	124	*Sphingomonas paucimobilis*	AMC, CN, CXM, CZ, FM	2
TW-3	3-64	*Bacillus pumilus*	CTX, NA	2
TW-3	3-65	*Bacillus cereus spp. cereus*	AMP, AMC, CTX, CX, SXT	2
TW-3	3-68	*Paenibacillus alvei*	CTX, CX, NA	2
TW-3	3-70	*Lysinibacillus sphaericus*	S, NA	2
TW-4	2-55, 2-80, 2-84, 2-85, 2-86, 2-87, 2-88	*Sphingomonas paucimobilis*	AMC, CN, CXM, CZ, FM	2
TW-4	2-4, 2-5, 2-90, 2-91	*Embedobacter brevis*	AMC, CZ, CN, CXM, FM	2
TW-4	2-102	*Staphylococcus epidermidis*	CIP, FA	2
TW-4	2-103	*Paenibacillus alvei*	CTX, C	2
TW-4	2-105	*Staphylococcus lugdunensis*	AMP, P, CIP	2
TW-4	2-118	*Staphylococcus warneri*	AMP, P, CIP, FA	3
TW-4	2-50	*Streptococcus pneumoniae*	FA, GEN	2

AMP, ampicillin; *AMC*, amoxicillin/clavulanic acid; *AN*, amikacin; *ATM*, aztreonam; *C*, chloramphenicol, *CAZ*, ceftazidime; *CF*, cephalothin; *CN*, cephalexin; *CTX*, cefotaxime; *CZ*, cefazolin; *CX*, cefoxitin; *CXM*, cefuroxime; *CIP*, ciprofloxacin, *E*, erythromycin; *FA*, fusidic acid; *FF*, fosfomycin; *FM*, nitrofurantoin; *GEN*, gentamicin; *IPM*, imipenem; *MEM*, meropenem; *MUH*, mupirocin; *NA*, nalidixic acid; *NN*, tobramycin; *OX*, oxacillin; *P*, penicillin; *S*, streptomycin; *STX*, trimethoprim/sulfamethoxazole; *TE*, tetracycline; *TMP*, trimethoprim; *TZP*, piperacillin-tazobactam.

The data on the TDW showed that the water treatment and disinfection in the DWPP ensure complete elimination of enterobacteria and enterococci, and no indicator bacteria were detected in all TW sampling locations. As a result of the high degree of TDW chlorination, 91.4% of the isolates were Gram-positive bacilli. Spore-forming bacilli predominated also in tap water

TW-2 and TW-3, containing 0.7 mg L^{-1} residual chlorine, and their abundance contributed to the high resistance level to beta-lactams in the TW from those sampling points. The detected high levels of resistance to *AMP, CTX* and CX are in consistence with the data reported for clinical isolates of *Bacillus spp*. (Weber *et al.* 1988). *Bacillus pumilus* and *Paenibacillus alvei* were the more common bacilli in TW-3, which like *B. cereus* were resistant to two classes of ABs. In TW-4, coagulase-negative *Staphylococcus* species resistant to two classes of ABs were the predominant Gram-positive bacteria. The occurrence of resistant coagulase-negative *Staphylococcus* species in tap water, after its transportation in the water supply network, was not uncommon, as it already has been reported by Faria *et al.* (2009) for resistant *S. epidermidis* and *S. pasteuri*.

Different species of pseudomonads were found in 3 out of 6 sampling locations – in RW and tap water TW-1 and TW-2. It was not surprising, as *Pseudomonas spp.* are normal residents of drinking water, water distribution systems, and premise plumbing. Destiani & Templeton (2019) have identified *Pseudomonas* in London tap water, detecting *P. fluorescens* in most sampling locations, and *P. aeruginosa* in only one location. Other studies also indicated *Pseudomonas* among the dominant species in finished water (Hoefel *et al.* 2005; Lu *et al.* 2018; Su *et al.* 2018), despite there is also findings that *Pseudomonas* were not as widespread in drinking water as is commonly thought (Vaz-Moreira *et al.* 2012). The *Pseudomonas* isolates from RW, in addition to their intrinsic resistance to some beta-lactams, tetracycline, chloramphenicol and trimethoprim (EUCAST 2021) exhibited resistance toward 3rd generation cephalosporins. The strains *P. fluorescens* and *P. putida* isolated from TW-2 were resistant to beta-lactams and fosfomycin, and *P. putida* were resistant to chloramphenicol and ciprofloxacin, as well.

The opportunistic pathogen *Stenotrophomonas maltophilia* was found in the sampling locations TW-1 and TW-2. The isolated strains were resistant to beta-lactams, aminoglycosides, fosfomycin, fusidic acid and trimethoprim, but their AMR was mainly intrinsic (EUCAST 2021). As an opportunistic premise plumbing pathogen, it shares a number of traits impacting on its spread in DWSS, as growth at microaerophilic and oligotrophic conditions, biofilm formation, and resistance to disinfectants (Norton & LeChevallier 2000; Hoefel *et al.* 2005).

Among the bacterial isolates from TW-1 and TW-4, the yellow pigment-forming flavobacteria from the family *Sphingomonadaceae* had high abundance. Some of them, as *Sphingomonas paucimobilis*, *Embedobacter brevis* and *Chryseobacterium indologenes* are opportunistic pathogens that can cause nosocomial, non-life-threatening infections. The increased number of sphingomonads in TW-1 and TW-4 is probably due to the biofilm impact on the plumbing of residential buildings, where water stagnation and intermittent daily consumption could have effect on biofilm formation and bacterial dispersal. It is also related to widespread of sphingomonads in water environment, due to their ability to survive at low temperatures and low nutrient concentrations and metabolize a wide variety of carbon sources (Koskinen *et al.* 2000; Vaz-Moreira *et al.* 2011). As in our study, members of different genera of the family *Sphingomonadaceae* have been found in Spanish DWSS (Vaz-Moreira *et al.* 2011) and isolated from biofilms or pipeline precipitates from municipal DWSS in Finland and Sweden (Koskinen *et al.* 2000).

In our study, the observed streptomycin resistance of sphingomonads and their great abundance in TW-1 and TW-4 could explain the higher population proportion of streptomycin resistant bacteria in these sampling locations. The natural streptomycin resistance together with the yellow pigmentation were used to facilitate recovery of *Sphingomonas* from environmental samples using streptomycin as a selective agent (Vanbroekhoven *et al.* 2004). It was also found that some *Sphingomonas* isolates were resistant to ciprofloxacin or co-trimoxazole that is in line with the finding of Vaz-Moreira *et al.* (2011). The *S. paucimobilis* strains were resistant to beta-lactams and fosfomycin, as well those of *E. brevis*.

The representatives of the genus *Methylobacterium,* resistant to *SXT* or completely sensitive, mainly were detected in TW-1 and TW-4. *Methylobacterium* are slow-growing, pink colony-forming organisms exhibiting resistance to chlorination, to whom being reported to be opportunistic pathogens in immuno-compromised patients. They have been isolated from tap water in various clinical settings and water supply systems (Rice *et al.* 2000; Szwetkowski & Falkinham 2020). *Methylobacterium* species exhibiting strong drug resistance, including MDR have been isolated from tap water in Japanese hospitals (Furuhata *et al.* 2006).

The discussed data show that the isolated bacteria belong mainly to environment species often found in drinking water, although some opportunistic pathogens were identified among the isolates, including multidrug resistant *S. maltophilia*. The most common HPC bacteria in drinking water were *Pseudomonas, Sphingomonas, Staphylococcus, Bacillus* and *Methylobacterium*. The number of bacterial species manifesting MDR varied between the DWSS sampling locations. The incidence of MDR-strains in the RW decreased in the TDW due to the effectiveness of the water treatment and chlorination processes in the DWPP. Bacterial species with MDR toward three and more classes of ABs more often were detected in TW-1 and TW-2, while, in TW-3 and TW-4, prevailed the isolates resistant to up to two classes of ABs. The opportunistic pathogen *S. maltophilia* expressing the highest MDR was detected in TW-1 and TW-2.

Prevalence of ARGs in bacterial isolates

Qualitative PCR assays were performed to determine which of the examined ARGs are present in the isolates expressing AMR phenotype (Figure 1). The obtained data show that the *cat* gene encoding chloramphenicol resistance was most commonly found among the isolates (in 11 of them, 27.5%). The *sulI* gene was harbored in 6 bacterial isolates (15%), while the *tetA* gene – in 3 isolates, only from the TW-2 (7.5%).

Only one isolate was positive for *qnrS* gene, and all isolates were negative for *drfA7* gene encoding resistance to trimethoprim and for the inhibitor susceptible beta-lactamase encoding gene *blaTEM-1*. Only one strain harbored the class 1 integrase gene *intI*. Despite the class 1 integrons have been primarily associated with AMR in clinical isolates, they were detected in water microbiome and water-associated biofilms (Farkas *et al.* 2013; Khan *et al.* 2016). The *sulI*, as a part of class 1 integron, can be disseminated and transferred horizontally within and between bacterial species (Farkas *et al.* 2013) but in our study the *sulI* gene was not associated with the *intI* gene.

The diversity and occurrence of the examined resistance encoding genes in the bacterial strains depended on the water sampling location, from which they were isolated. The results show an increase in the diversity and amount of ARGs in the TW-1 and TW-2 isolates compared to those isolated from TDW, among which only *sulI* and *cat* genes were detected. The TW-2 isolates demonstrated the greatest genetic diversity – 4 out of 7 targeted ARGs were detected, with prevalence of *cat* and *tetA* genes. Only one ARG type (*cat* gene) was detected among the isolates from TW-3 and TW-4. The increased diversity and incidence of ARGs among the TW-1 and TW-2 isolates may be related to the bacterial communities' changes in the individual sampling points as a result of bacterial regrowth and/or biofilm impact in the water supply network.

In many studies, it was indicated that the bacterial communities' shift is a key factor for change of ARGs (Xi *et al.* 2009; Su *et al.* 2018). Xi *et al.* (2009) have related the increased levels of most ARGs in tap water in comparison with the treated drinking water to either water treatment or bacterial re-growth within distribution systems. Our data on the most common resistant genes *cat, sulI*, and *tetA* are consistent with the data on the same ARGs established by other researchers (Xi *et al.* 2009; Khan *et al.* 2016a; Destiani & Templeton 2019). The genes *tetA* and *sulI* have been detected in all sampling locations of London tap water, and *sulI* been the most abundant gene. Unlike our results, the *drfA7* gene encoding trimethoprim resistance has been detected in all sampling locations (Destiani & Templeton 2019). Xi *et al.* (2009) have determined significant increase in the proportion of *cat* genes following the water treatment, suggesting ineffective removal/inactivation of the chloramphenicol-resistant bacteria, despite of the reduction of the *sulI* gene.

The PCR data support the already discussed differences between the tap water in all DWSS sampling locations, established at the population level or through the phenotypic AMR profile of ARB. However, a relationship between the phenotype pattern of ARB and the detected ARGs could not be established in our study. Despite the presence of bacteria resistant to

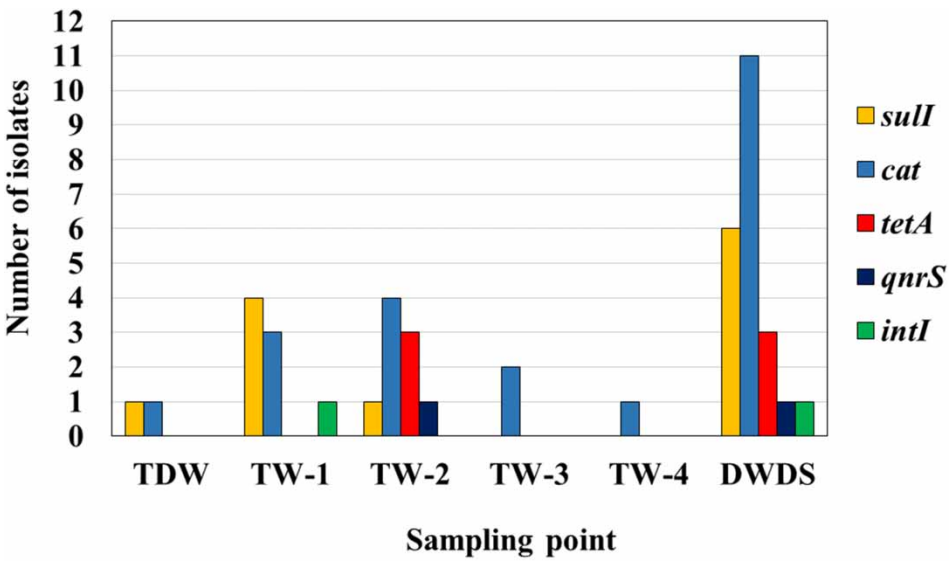


Figure 1 | Incidence of the examined encoding genes among the bacterial strains isolated from different drinking water sampling locations, as well for whole DWSS. The results are presented as number of isolates with proved target ARG.

beta-lactams, quinolones and tetracycline in the bacterial community in all drinking water samples (Table 3), as well the phenotypic AMR of the isolates to same ABs (Table 5), the examined genes, providing resistance to these antibiotics, were not frequently detected or generally absent. This may indicate that other ARGs, not tested in this study, confer the exhibiting AMR phenotype, as well that large groups of ARGs may also confer resistance to given antibiotic class.

CONCLUSIONS

As far as we know, our study is the first one estimating the prevalence of AMR among the heterotrophic bacteria in the Bulgarian drinking water. Thus, the beginning of studies on AMR in Bulgarian DWSSs was set up, which is an opportunity for enriching the published data on the prevalence of ARB and ARGs in real DWSSs.

It was found that the aquatic bacterial community underwent a restructuring processes in drinking water purification and disinfection, and its transportation in the DWSS network. As a result of those alterations in the abundance and the species diversity, the populations' proportion of bacteria resistant towards the tested ABs was different among the DWSS drinking water sampling sites. The increased level of resistance towards some ABs in drinking water emphasizes the role of the DWSS as a reservoir of ARB and ARSs that could pose potential human health risk. The data on the prevalence of AMR in drinking water and the occurrence of resistant opportunistic pathogens demonstrates the need of comprehensive monitoring of DWSSs and a greater concern for the microbiological water quality.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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