Antibiotic Resistance of Heterotrophic Bacteria Isolated from Drinking Water – from the Water Source to the Consumers' Taps

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Abstract: *The aim of this study was to determine the antibiotic resistance (ABR) phenotype of heterotrophic bacteria isolated from a Bulgarian drinking water supply system. The disc diffusion method of Kirby-Bauer was used in the bacterial isolates susceptibility testing toward 13 antibiotics from 7 classes. The ABR phenotype of 233 bacterial strains was determined and the influence of drinking water treatment and its transportation in the water supply network on the ABR prevalence was assessed. Among the isolates, the ABR incidence was low or medium, only tetracycline resistance was very rare, and no gentamicin resistance was detected; 42% of the isolates were resistant to one class of antibiotics, and 11% exhibited multiple resistance. Water chlorination has led to restructuring of the aquatic community and changes of the predominant ABR phenotypes. The ABR phenotype of the isolates differed depending on the drinking water sampling point demonstrating the local influence of the water supply network. Although there is no regulation of the ABR of drinking water, the data demonstrated prevalence of bacterial resistance to different antibiotics and need of a greater concern the microbiological water quality.*

Keywords: *Antibiotic resistance, Heterotrophic bacteria, Drinking water, Drinking water distribution system.*

Introduction

Very soon after introduction of antibiotics in medical practice, it has been found that the target pathogens develop capability to resist and adapt to antibiotic action. Excessive and inappropriate use of antibiotics (AB) for therapeutic purposes in human and veterinary medicine, and additionally for stimulation of growth in animal husbandry, have leading role to acquisition of antibiotic resistance (ABR) in the wide variety of pathogenic bacteria [9, 21]. The increasing ABR among pathogens creates serious health risk, however, the residual amounts of ABs and a great mass of bacterial species exhibiting ABR to one or more classes of ABs detected in water resources and in wastewater, is turning it into an environmental problem [4, 5, 6, 8]. There is growing evidence that aquatic microbiota is a significant reservoir of ABR, and the contamination of water with ABs can facilitate the selection of

antibiotic resistant bacteria (ARB) and transfer of antibiotic resistance genes (ARGs) to the human-associated microbiome [17, 28].

There is a growing number of scientific proofs for the interrelation between the increasing ABR and the anthropogenic impact on the aquatic ecosystems [6, 8, 14]. As a result of human activities (drinking water supply for human consumption and industrial usage; wastewater treatment and its release in water bodies), bacteria, including the resistant ones, can move from the polluted wastewater into the treated wastewater and natural water, and reach human-associated microbiome. In this way, the urban water cycle contributes to the dissemination of ARB and ARG being in the potential transmission routes of ABR from environment to human and vice versa [17].

The presence of ARB and ARGs in water sources and purified drinking water is an emerging health related problem for water supply industry [10, 16, 29, 30]. In recent years, numerous studies have reported a higher dissemination of bacteria resistant toward some classes of ABs in drinking water compared to untreated source water, as well an occurrence of bacteria with multiple antibiotic resistance (MAR) [3, 30]. Despite the conventional water treatment and disinfection can reduce the total number of bacteria in drinking water there is evidence for increased ABR of some ubiquitous aquatic bacteria and increased levels of ARGs [11, 19, 25, 26]. The increased ABR can be related to the selective effect of chlorine [15, 30], or to the restructuring processes of aquatic communities in drinking water supply systems (DWSS) as a result of bacterial re-growth in water or biofilm dispersal. Thus, the DWSS could be an incubator for the growth of ARB and important reservoir for dissemination of ABR [17, 28].

In clinical practice, susceptibility of pathogenic bacteria toward ABs from different classes is determined by the Kirby-Bauer disc diffusion method, based on minimum inhibitory concentrations (MIC) defined for each particular pathogen. Few data are available for the phenotype of antibiotic susceptibility of bacteria isolated from drinking water. The difficulties in determining the antibiotic susceptibility of aquatic bacteria are related to: lack of defined MICs of environmental bacteria without health significance; the slow and difficult growth of many bacterial species inhabiting aquatic ecosystems, even in rich nutrient media; the psychrophilic and other specific requirements; evidenced need for identification of isolated bacterial species before the testing, etc. [22]. To assess the antibiotic susceptibility of bacteria isolated from drinking water, methods from clinical microbiology or their modifications are used, as well Mueller-Hinton agar is often replaced by other media suitable for growth of aquatic bacteria but under similar incubation conditions and interpretation of the results.

Among the aquatic bacteria, most often the ABR profile of the opportunistic pathogens having defined clinical breakpoints for ABR, have been analyzed [11, 19, 25, 26]. Due to very few data available on the ABR of the microbiome in Bulgarian drinking water [24], the aim of this study was to determine the profile of antibiotic resistance of heterotrophic bacteria isolated from drinking water by the classical disc diffusion method, taking into account the influence of important factors, as drinking water treatment and transportation in the DWSS network.

Materials and methods

Study area and sample collection

The object of this study is the drinking water microbiome inhabiting the municipal water supply system that supplies drinking water to the population of the Veliko Tarnovo region. After raw water treatment in Drinking Water Purification Plant (DWPP), the disinfected drinking water is distributed to many settlements in the region. The water treatment in the DWPP includes pre-chlorination, settlement, fast sand filtration and chlorine disinfection. The finished water is transported gravimetrically to the settlements in the surrounding area and to water tanks in the town of Veliko Tarnovo, 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 distribution network, where 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 for TW-1 and 0.32 ± 0.03 mg/L for TW-4), while the TW-2 and TW-3 – for water samples with higher residual chlorine content (0.71 ± 0.13 mg/L for TW-2 and 0.74 ± 0.09 mg/L for TW-3).

Isolation of bacteria from drinking water samples

Water samples were analyzed by filtration through 0.45 μm pore size, 47 mm diameter sterile membrane filters (Sartorious AG, Germany) and subsequent cultivation of the filters on R2A agar (HiMedia, India) for 7 days at a temperature of 25 °C.

Pure bacterial cultures were isolated from selected colonies growing on R2A agar. From the membrane incubated on R2A agar, all bacterial colonies were isolated when their number was below 10. When they were more then 10, representatives of all morphologically different colonies, and a minimum of 5 colonies with similar characteristics were isolated. The purity of sub-cultured bacterial strains was tested by using soybean casein digest agar (HiMedia, India) and culturing at 35 °C for 24-48 hours. Gram staining of isolates was applied. Pure bacteria cultures were stored at -20 °C.

Biochemical identification of bacterial isolates

The biochemical identification of the isolates was performed with the MICROLATEST® tests (Erba Lachema s.r.o., Czech Republic): a NEFERMtest 24 was used for non-fastidious, non-enteric, Gram-negative bacteria and an ENTEROtest 24 – for coliforms. The resulting identification (ID) score indicates the extent to which the taxon can be distinguished from other taxa: the strain can be distinguished very well at ID ≥ 95%, and cannot be sufficiently distinguished without additional tests at ID < 90%. Strains with ID ≥ 90% were considered identified.

The identification and antibiotic susceptibility of the bacterial strains with established multidrug resistance and ones representing the largest population part was confirmed by BD Phoenix™ M50 Automated Microbiology System (Becton, Dickinson and Company, USA) by laboratory procedure, as described by the manufacturer. NMIC/ID-76 panels for Gram-negative bacteria or PMIC/ID-88 panels for Gram-positive bacteria were used.

Determination of the antibiotic resistance pattern of bacterial isolates

Disc diffusion method was used for assessing the antimicrobial susceptibility of the bacterial isolates to 13 antibiotic substances belonging to the following 7 classes [1, 2]:

- β-lactams (*Amp* (ampicillin) – 10 μg; *Aug* (amoxicillin/clavulanic acid) – 20/10 μg); *CEP* (cephalothin) – 30 μg; *Cx* (cefoxitin) – 30 μg; *CTX* (cefotaxime) – 30 μg);
- aminoglycosides (*S* (streptomycin) – 10 μg; *GEN* (gentamicin) – 10 μg);
- quinolones (*CIP* (ciprofloxacin) – 5 μg; *NA* (nalidixic acid) – 30 μg);
- antifolates (*COT* (trimethoprim/sulfamethoxazole) – 1.25/23.75 μg);
- *TE* (tetracycline) – 30 μg;
- *C* (chloramphenicol) –30 μg; and
- macrolides (*E* (erythromycin) – 15 μg).

Each tested strain was inoculated on 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 millimeters, mm). The strains were classified as sensitive (S) or resistant (R), taking into account the species belonging of bacteria and the corresponding interpretative criteria. Since there are no guidelines for ABR breakpoints of most environmental bacteria, the results for unidentified Gram-positive bacteria were interpreted according to CLSI breakpoints for *Staphylococcus spp*., and for Gram-negative ones – according to breakpoints for the family *Enterobacteriaceae* [1].

Results and discussion

In total, 292 bacterial strains were isolated from water samples (Table 1) and 60% of them were Gram-negative. As it is known Gram-negative bacteria dominate in natural water environments, and the highest value of 82% detected in the untreated source water (RW) is in agreement with this finding [19, 28]. Water treatment and disinfection in the DWPP is the reason behind the changes in species diversity of aquatic bacterial community and the predominance of Gram-positive bacteria [20, 27].

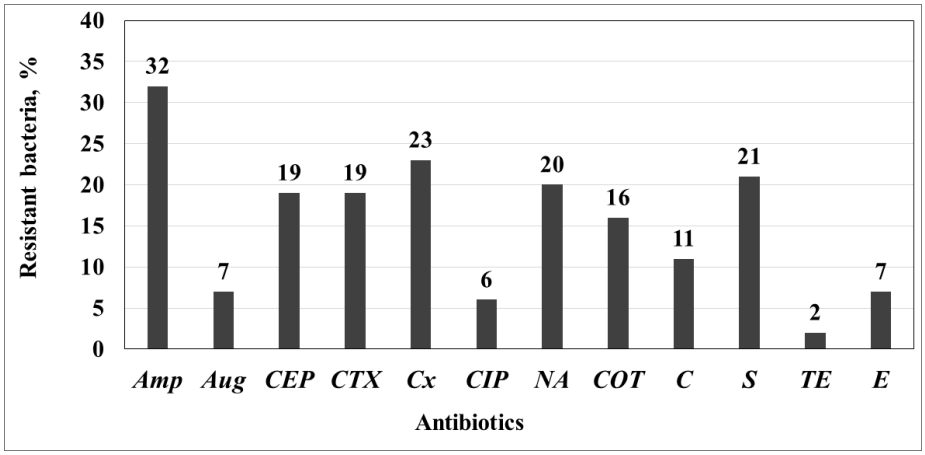
Table 1. Number of isolates from drinking water on its way from the source to the tap

Sampling point	**Denotation**	**Total number of isolates, *n***	**Relative proportion of**		**Number of tested isolates, *n***		
			Gram (-)	**Gram (+)**	**Total**	**Gram (-)**	**Gram (+)**
Entrance of DWPP, raw water	RW	48	81	19	35	31	4
Exit of DWPP, treated water	TDW	35	9	91	20	3	17
Tap water 1	TW-1	90	73	27	81	55	26
Tap water 2	TW-2	69	55	45	47	29	18
Tap water 3	TW-3	20	25	75	20	17	3
Tap water 4	TW-4	30	77	23	30	12	8
Total number of isolates, *n*		292	60	40	233	157	76

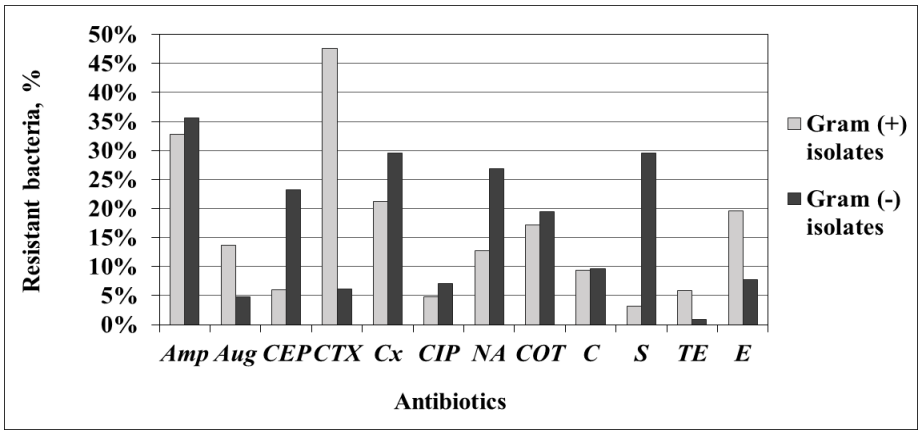
Due to the drinking water chlorination, 91% of the bacterial isolates from freshly chlorinated drinking water (TDW) were Gram-positive. A higher number of Gram-positive strains were also isolated from tap water sampling points TW-2 and TW-3, containing residual chlorine of 0.7 mg/l, compared to tap water TW-1 and TP-4 (with residual chlorine of 0.4 mg/l).

Antibiotic resistance phenotype of all bacterial isolates from the DWSS network

The data on the total (inherent and acquired) ABR of the tested heterotrophic plate count (HPC) isolates are presented encompassing for the overall drinking water network (Fig. 1) and separately for each sampling point along the way of the drinking water from the source to the consumers' tap (Fig. 3). In total, 233 strains of heterotrophic bacteria were tested, and 71% out of them were resistant to at least one AB.



a)



b)

Fig. 1 ABR phenotype of HPC bacteria isolated from water samples of the DWDS network: a) total HPC bacteria; b) Gram-negative and Gram-positive HPC bacteria. Tested antibiotics: *Amp*, *Aug*, *CEP*, *Cx*, *CTX*, *CIP*, *COT*, *C*, *S*, *TE*, *E*, and *NA*.

The pooled data for the DWSS (Fig. 1a) show that the largest fraction of isolates was resistant to beta-lactams ampicillin (32%) and cefoxitin (23%), followed by nalidixic acid (20%) and streptomycin (21%). The resistance to third-generation cephalosporin cefotaxime was detected among 19% of bacteria. The fraction of tetracycline-resistant isolates was the lowest, and all isolates were sensitive to gentamicin. If the phenotype ABR data for the examined DWSS are interpreted according to the criteria proposed by ECDC for clinically important pathogens, the incidence of ABR isolates in drinking water can be classified: high to

ampicillin (when 25% to 50% of isolates are resistant); medium (when 10% to 25% are resistant) towards 7 ABs from 5 classes (*CEP*, *CTX*, *Cx*, *COT*, *NA*, *C*, and *S*); low (5% to 10% are resistant) to 3 ABs (*CIP*, *Aug*, and *E*), and very low to *TE* (< 5%).

The comparative data (Fig. 1b) show that a higher proportion of Gram-negative bacteria were resistant to *Amp*, *CEP*, *NA*, *COT*, and *S* compared to Gram-positive bacteria, while among the latter the resistance to *Aug*, *CTX*, *E*, and *TE* was predominant.

The effect of water treatment and chlorination

The water treatment and chlorination in the DWPP provided full removal of the bacteria resistant to *CIP* and *TE*, as well as a change in the ABR pattern of the surviving bacteria (Fig. 2). In treated drinking water TDW, the number of isolates resistant to beta-lactams *Amp* and *CEP* decreased, in contrast to the increased resistance to *CTX*, *Cx*, and *Aug*. The water treatment and chlorination significantly decreased the incidence of the resistant phenotypes to the rest of the tested ABs.

Among the raw water isolates, the bacteria resistant to each tested beta-lactam exhibited high incidence level (> 25%), followed by the bacteria resistant to *COT*, *TE*, *S* or *NA* with a medium level (10-25%) and a low level for *CIP* and C (Fig. 2).

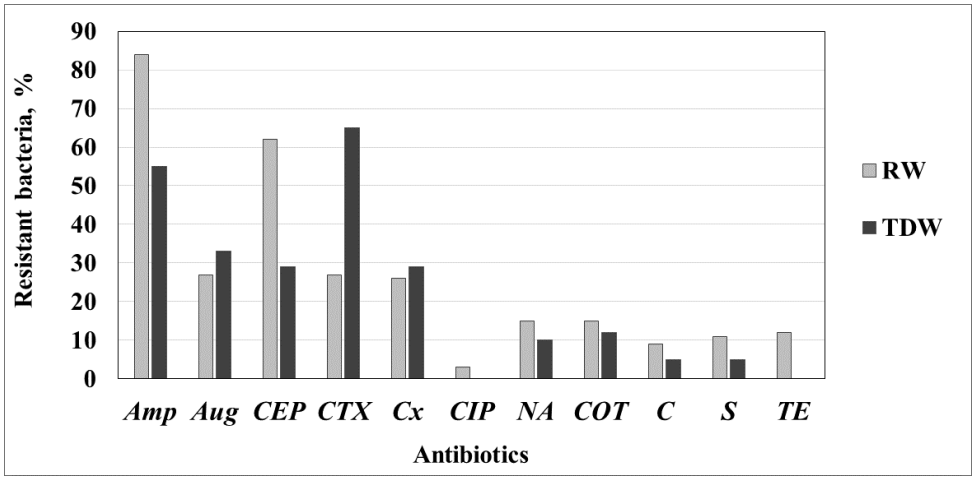


Fig. 2 ABR phenotype of isolated HPC bacteria depending on the treatment of drinking water

The effect of the drinking water distribution network

The data plotted on Fig. 3 reveal significant differences in the ABR pattern of the isolated bacteria depending on the drinking water sampling location.

The ABR phenotype of the isolates from the different tap water samples exhibited some specific traits:

- high level of resistance to *NA*, and medium level to *COT*, *Amp*, *Cx*, *E*, and *S* of the isolates from TW-1;
- high level of resistance to *Amp*, and medium to *CEP*, *CTX*, *COT*, and *C* among those from TW-2;
- in TW-3, high abundance of the isolates resistant to *CTX* and medium to *AMP*, *Cx* and *S*, but total susceptibility to five ABs (*NA*, *COT*, *C*, *TE*, and *E*);

- in TW-4, high resistance to *Amp*, *Cx*, *NA*, and *S*, but total susceptibility to five ABs (*Aug*, *CIP*, *COT*, *TE*, and *E)*.

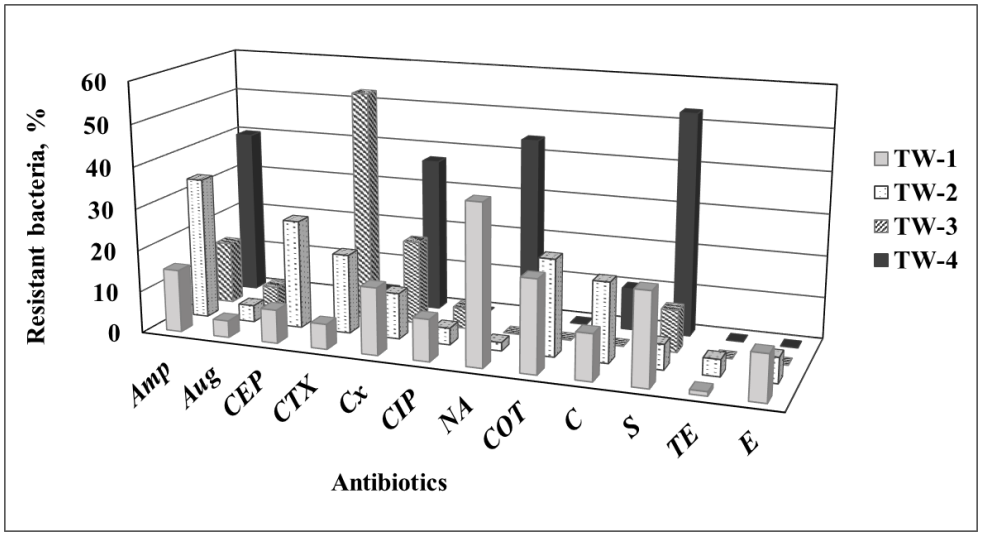


Fig. 3 ABR phenotype of the isolated HPC bacteria depending on drinking water sampling points of the municipal DWSS network

Among the isolates from TW-1 and TW-2, resistant patterns to all tested ABs were detected, while the isolates from TW-3 and TW-4 were fully susceptible to *COT*, *TE*, and *E*. The ABR phenotype of the isolates from TW-4 differed most significantly from the rest tap water sources.

When comparing the ABR patterns depending on the sampling locations, the following differences have been established: higher abundance of bacteria resistant to beta-lactams among the isolates from tap water TW-2 and TW-3, characterized by twice higher residual chlorine content compared to TW-1 and TW-4; Gram-positive bacteria resistant to *Amp* predominated among the isolates from TW-2, while the isolates resistant to *CTX* prevailed in TW-3; Gram-negative pigment-producing bacteria resistant to *S* and *NA* predominated among the isolates from TW-1 and TW-4. In addition, high level of resistance to *COT* was determined in TW-1, and high level of resistance to *Amp* and *Cx* in TW-4.

The obtained data are in line with the trends reported by many researchers for alteration of the diversity and cultivability of aquatic bacteria, due to the water disinfection [3, 7, 20, 27]. Along the DWSS, the chlorination efficiency was decreasing and concentration gradients of chlorine exposure were developing. As a response, the vitality of aquatic bacteria may vary from death or complete inhibition at high chlorine concentrations to selective survival of resistant populations under sub-inhibitory concentrations. The bacteria surviving the disinfection may have innate high resistance to antimicrobials, as for example the case with the spore-forming bacteria. The obtained data for higher ABR levels to beta-lactams of the isolates from TW-2 and TW-3 are in accordance with the findings of Khan et al. [15] for greater survival of the ARB under the conditions of higher chlorine exposure compared to sensitive ones. Unlike our results, the researchers observed that chlorine-resistant bacteria in the drinking water they studied were more resistant to tetracycline, amoxicillin and sulfamethoxazole.

The differences in the ABR pattern of the bacterial isolates from different tap water samples of the studied DWSS could be related not only to the effect of the chlorination degree of the water, but also to the influence of the biofilms existing on the pipeline walls of the water supply network. The emission of bacteria detached from the biofilm communities existing in different sections of the DWSS may significantly affect the level and the ABR profile of the aquatic microbiota [30]. The impact of biofilms on the microbiological composition of drinking water is a thoroughly studied phenomenon [7, 12, 13, 23], and undoubtedly could had an impact on the abundance of ARB and on the profile of their ABR.

Multidrug resistance of bacterial isolates

The disc diffusion assay data on the susceptibility of the isolated bacteria to the tested substances from 7 antibiotic classes are summarized for the isolated Gram-negative and Gram-positive bacteria (Fig. 4), and for the overall DWSS (Fig. 5).

Among the Gram-negative isolates, the resistant bacteria to one class of AB were dominant. A higher incidence of isolates with multiple resistance was registered too. Among the Gram-positive bacteria, a higher incidence of antibiotic susceptibility was found.

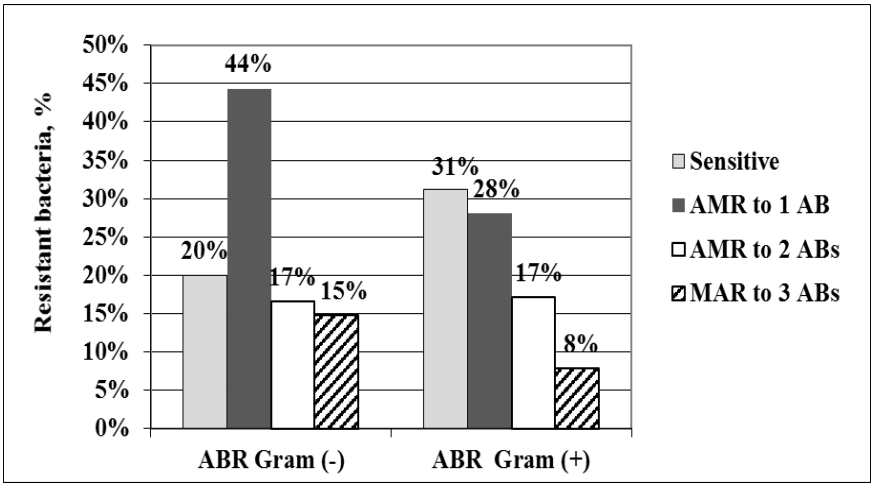


Fig. 4 ABR phenotype of the isolated Gram-negative and Gram-positive bacteria

For the overall DWSS (Fig. 5), up to 29% of all isolates were sensitive to the tested ABs, while 42% of them were resistant to one class of ABs, 18 % of the isolates manifested resistance to two classes of ABs, and 11% of them – to 3 classes.

The highest level of ABR was detected among the isolates from untreated raw water RW, and only 6% of them were fully sensitive to the tested ABs. Multidrug resistance to 4 classes of ABs was detected in 6% of isolates from RW, unlike to TW. The bacterial community of the treated drinking water TDW underwent significant changes as a result of chlorination and no bacterial strain with MAR to 3 classes of ABs was isolated, unlike the tap water from the rest of the DWSS sampling points.

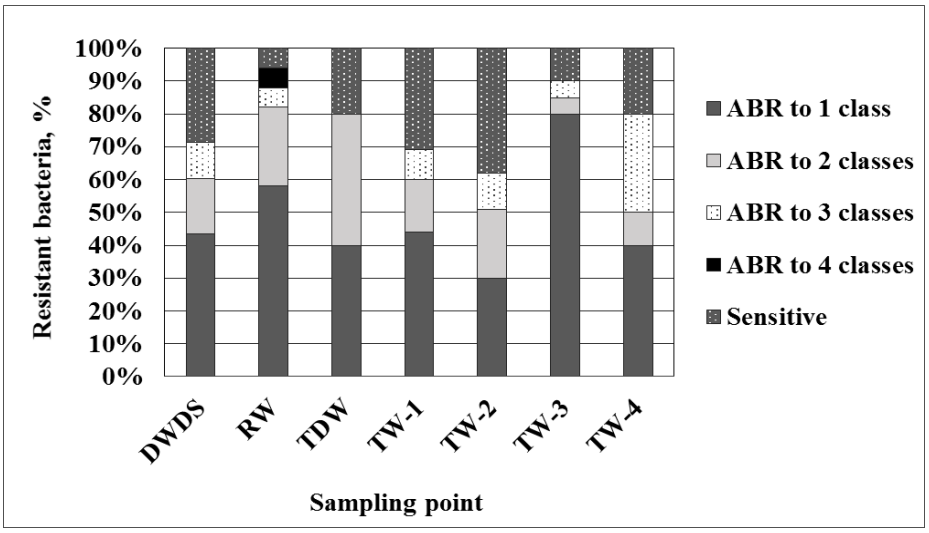


Fig. 5 ABR phenotype of bacterial isolates
depending on the sampling points at the DWDS

Comparative data on the ABR of the bacterial isolates depending on the sampling point showed prevalence of the isolates resistant to one antibiotic class in all drinking water (TW-1 – TW-4), followed by isolates resistant to two classes of ABs, except for TW-4. The highest share of MAR isolates was detected among the isolates from TW-4, while among the ones from TW-3, the bacteria resistant to one class of ABs prevailed. Among the isolates from TW-1 and TW-2, a higher proportion of susceptible bacteria was found compared to TW-3 and TW-4.

According to the guidelines for antibiotic susceptibility assessment of clinically relevant pathogens, in interpretation of the results the breakpoints for a particular species should be used [1, 2]. However, the aquatic bacterial species as the harmless ones are not covered by these guidelines. Appling the breakpoint values of *Staphylococcus spp*. or *Enterobacteriaceae* as criteria for interpretation of the results in our study supposes an approximation of ABR breakpoints of the aquatic bacteria. Despite of that, the resulting phenotypic data give important information of the total (intrinsic and acquired) ABR of the culturable heterotrophic bacteria in drinking water. The data reveal a medium or low incidence of ABR of the aquatic bacteria to the selected 13 substances of 7 classes of predominantly broad-spectrum ABs, with exception of erythromycin, which is effective mainly against Gram-positive bacteria, but many Gram-negative bacteria also were sensitive. However in order to determine the risk of some of these bacteria being carriers or vectors of ABR from the aquatic environment to humans, it is necessary the species to be identify and their ability to colonize the human body to be clarified [28].

Ninety one bacterial isolates of mainly non-pathogenic environmental bacteria, but also several opportunistic pathogens, have been biochemically identified (The data are presented in detail as a part of a comprehensive ABR prevalence study in the same DWSS [24]). Most isolates were recognized as a source of ABR in drinking water despite that some exhibit intrinsic ABR probably being evolved in the long-term coexistence in a common habitat of competing species, such as producers of antibiotics (as secondary metabolites) and bacterial species that were evolved resistance to the antibiotics they have been exposed.

The Gram-negative yellow pigment-producing flavobacteria originating from the genera *Sphingomonas* and *Embedobacter*, as well members of genus *Methylobacterium*, often were found among the isolates from TW-1 and TW-4, while among the ones from TW-2 and TW-3, chlorine-resistant representatives of genus *Bacillus* and other Gram-positive bacilli that were resistant to ampicillin and cephalosporins were registered. The family *Sphingomonadaceae* isolates were resistant to streptomycin, ciprofloxacin or co-trimoxazole, and some of the isolated pseudomonads were resistant up to 4 AB classes. Coagulase-negative *Staphylococcus* isolates resistant to ampicillin, ciprofloxacin and fusidic acid also were detected [24]. Most of the identified bacterial species were intrinsically resistant to some of the tested ABs:

- *Pseudomonas* strains – toward some beta-lactams, tetracycline and chloramphenicol;
- *Sphingomonadaceae* – toward beta-lactams and fosfomycin;
- Gram-positive bacteria – to ceftazidime, fosfomycin, nalidixic acid [2].

Hence, the innate ABR probably greatly contributed to the established total ABR of the bacterial isolates from drinking water.

Given the small number of HPCs in drinking water from the studied DWSS, which is 0.1-16 CFU/ml, a negligible health hazard for ABR transfer to humans can be assumed. Despite the low incidence of multidrug resistant bacteria, in case of opportunistic pathogens care should be exercised especially when immunocompromised people were exposed. As a rule, it is accepted that a particular environment component can present risk for transfer toward people only if a particular vector bacterium is present in quantities enough to colonize human body [18]. Depending of the particular vector, it can represent risk even if it is present in a few number. Because of that, identification to species level establishing the ability to colonize and infect the human body is needed for characterization of vector bacteria of ABR from water environment to human. Since clinical breakpoints are based on the parameters relevant for therapeutic success their use for assessment of the antimicrobial susceptibility of the environmental bacteria has its limitations. As a more reliable alternative for interpretation of the ARB of environmental bacteria, implementation of the epidemiological cut-off (ECOFF) value, developed by EUCAST was considered, despite that the information for non-pathogenic environmental species that can be carriers of ABR is scarce [5]. Establishing a standardized methodology for assessment of ABR in environmental bacteria will allow a comprehensive environmental monitoring and meet the challenge of increasing antibiotic resistance and possible ABR transfer from the environment to human commensal or pathogenic bacteria.

Conclusion

As far as we know, the conducted study of the ABR phenotype among the heterotrophic bacteria in the Bulgarian drinking water is the first of its kind. The ABR phenotype of 233 strains of heterotrophic bacteria toward 13 antibiotics of seven classes was determined and the prevalence of ABR in the drinking water on its way from the water source to the end-users was assessed. The effect of the drinking water purification and chlorination and its transportation in the water supply network was assessed.

The data on the total ABR of the isolated bacteria obtained in this study could be summarized as follows:

- The ABR levels predominantly were medium or low; the tetracycline resistance was very low, and no gentamicin resistance was detected.

- 29% of all tested strains were sensitive to all tested ABs, and the ABR to one class ABs of 42% was prevalent; 11% of the isolates were MAR to 3 classes of ABs.
- The water treatment and chlorination were a leading factor for the restructuring of the aquatic community and the changes of the prevalent ABR phenotypes.
- The percentage of the bacteria resistant to the individual AB varied significantly in drinking water depending on the sampling point demonstrating the local influence of the drinking water distribution network.

Although there is no law regulation for the ABR of drinking water, the data on the ABR phenotype of HPC bacteria demonstrate prevalence of different types of antibiotic resistance, and a need of comprehensive monitoring and a greater concern the microbiological quality of the water.

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