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Antibiotic and Disinfectant Resistance in Tap Water Strains
– Insight into the Resistance of Environmental Bacteria

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Abstract

Although antibiotic-resistant bacteria (ARB) have been isolated from tap water worldwide, the knowledge of their resistance patterns is still scarce. Both horizontal and vertical gene transfer has been suggested to contribute to the resistance spread among tap water bacteria. In this study, ARB were isolated from finished water collected at two independent water treatment plants (WTPs) and tap water collected at several point-of-use taps during summer and winter sampling campaigns. A total of 24 strains were identified to genus or species level and subjected to antibiotic and disinfectant susceptibility testing. The investigated tap water ARB belonged to phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. The majority of the isolates proved multidrug resistant and resistant to chemical disinfectant. Neither seasonal nor WTP-dependent variabilities in antibiotic or disinfectant resistance were found. Antibiotics most effective against the investigated isolates included imipenem, tetracyclines, erythromycin, and least effective – aztreonam, cefotaxime, amoxicillin, and ceftazidime. The most resistant strains originate from *Afipia* sp. and *Methylobacterium* sp. Comparing resistance patterns of isolated tap water ARB with literature reports concerning the same genera or species confirms intra-genus or even intra-specific variabilities of environmental bacteria. Neither species-specific nor acquired resistance can be excluded.

K e y w o r d s: susceptibility testing, antibiograms, MIC testing, drinking water bacteria, environmental strains

Introduction

Antibiotic resistance of environmental bacteria, including bacteria dwelling in aquatic ecosystems, is a thoroughly investigated phenomenon (Baquero et al. 2008; Martinez 2009). Due to increased antibiotic consumption (ESAC-Net 2020; Roberts and Zembower 2020), antibiotic resistance is considered emerging environmental contamination (Pruden et al. 2006). Natural waters can be reservoirs of autochthonous antibiotic-resistant bacteria (ARB) and could be additionally contaminated by antibiotics, ARB and antibiotic resistance genes (ARGs) due to human activities such as wastewater effluents discharge, aquaculture, or agriculture (Berglund 2015). Antibiotic resistance determinants may not be removed entirely in water treatment plants (WTPs) and can enter distribution systems. Although tap water is commonly considered drinking water in many countries, knowledge regarding

ARB biodiversity in drinking water distribution systems (DWDSs) is still scarce.

Bai et al. (2015) found antibiotic-resistant *Bacillus* sp., *Sinorhizobium* sp., *Bradyrhizobiaceae* sp., *Comamonadaceae* sp., *Enterobacter hormaechei*, *Sphingomonas* sp., *Enterobacter* sp., and *Ensifer* sp. in finished water at WTP in Shanghai, China. Antibiotic-resistant *Proteobacteria* were frequently isolated from tap water in Porto, Portugal (Vaz-Moreira et al. 2011; 2012; 2017; Figueira et al. 2012; Narciso-da-Rocha et al. 2013; 2014). Furthermore, antibiotic-resistant *Pseudomonas* spp. were found in tap water produced from a karstic springs system during turbid events in Le Havre, France (Flores-Ribeiro et al. 2014). Khan et al. (2016a; 2016b) isolated antibiotic and disinfectant resistant *Paenibacillus*, *Burkholderia*, *Escherichia*, *Sphingomonas*, and *Dermacoccus* representatives and other bacteria possessing ARGs from tap water in Glasgow, Scotland. Antibiotic-resistant *Methylobacterium* spp. were found

in a nation-wide study of hospital tap water in Japan (Furuhata et al. 2006). Various ARB were identified in tap water in Wrocław, Poland, in the previous study (Leginowicz et al. 2018). Moreover, Shi et al. (2013) found intestinal ARB in finished water and tap water in Nanjing, China. According to these reports, tap water bacteria's antibiotic resistance can be regarded as a global problem that requires further research. Even if most tap water bacteria remain unculturable, culture-dependent methods should not be neglected because they could broaden the current state of knowledge regarding environmental ARB.

Resistance dissemination could be facilitated by horizontal gene transfer (HGT) within water supply networks (Shi et al. 2013; Ma et al. 2017). Moreover, subinhibitory concentrations of disinfectants were revealed to enhance the intra-genus conjugation transfer of genes (Zhang et al. 2017), suggesting the potential spread of antibiotic resistance in suboptimally chlorinated drinking water. Other findings indicated the critical role of vertical gene transfer (VGT) in this phenomenon because some ARB's resistance patterns were revealed to be species-specific (Vaz-Moreira et al. 2011; 2012; Narciso-da-Rocha et al. 2013; 2014).

The influence of ARB from drinking water on consumer health is still unclear and requires further investigation (Vaz-Moreira et al. 2014; Sanganyado and Gwenzi 2019). Swallowed bacteria have been determined to exchange genes with human intestinal microflora (Salyers et al. 2004). Moreover, Khan et al. (2020) have recently confirmed the possibility of disseminating the *mcr-1* gene, known as the last resort ARGs, from drinking water to the healthy mouse gut.

Next to being resistant to antibiotics, bacteria dwelling in drinking water have been reported to be chlorine or monochloramine tolerant (Shrivastava et al. 2004; Furuhata et al. 2007; Chiao et al. 2014; Khan et al. 2016a). Like antibiotic resistance, resistance to disinfectants could be facilitated by HGT (Stokes and Gillings 2011). A risk of co-selection of ARB associated with drinking water chlorination has been suggested (Shi et al. 2013; Pruden 2014; Proctor and Hammes 2015), although this hypothesis requires verification (Lin et al. 2016). Antibiotic and disinfectant susceptibility testing of tap water bacteria could contribute to elucidating this issue.

This study's objective was to investigate ARB dwelling in bulk tap water supplied by two independent WTPs within one DWDS during the summer and winter seasons. Next to antibiotic resistance, the resistance to disinfectants was tested. The resistance patterns of isolated strains were also compared with literature reports to gain a preliminary insight into resistance prevalence in tap water bacteria and expand knowledge in this issue.

Experimental

Materials and Methods

DWDS, sample collection, and ARB cultivation. The DWDS in Wrocław, Poland, is primarily supplied by two independent WTPs: Na Grobli (NG) and Mokry Dwór (MD). Both WTPs (NG and MD) draw source water from the Oława and Nysa Kłodzka Rivers. In WTP NG, however, groundwater recharge is implemented as the first step of treatment, resulting in water adopting groundwater properties. WTP NG consists of the following treatment processes: groundwater recharge, aeration, filtration, ozonation, adsorption on activated carbon, pH correction, and disinfection. WTP MD consists of the following treatment processes: coagulation, filtration, ozonation, adsorption on activated carbon, pH correction, and disinfection. In both WTPs, chlorine and chlorine dioxide are used for disinfection purposes; residual chlorine is also provided in the distribution system (Siedlecka et al. 2020b).

Finished water samples from both WTPs (NG1 and MD1) and tap water samples from point-of-use taps, three in each WTP supply area (NG2, NG3, NG4 and MD2, MD3, MD4), were collected twice a season (in July and August 2018 for summer and in January and February 2019 for winter), as described previously (Siedlecka et al. 2020b). The Municipal Water and Sewerage Company kindly provided free and total residual chlorine concentrations of each water sample.

Before sample collection, each tap was disinfected and flushed until the water temperature stabilized to avoid plumbing influence. Samples were collected in sterile, plastic containers, supplemented with 0.1 g/l sodium thiosulfate (Chempur) to neutralize the disinfectants (Vaz-Moreira et al. 2017), and immediately transported to the laboratory. Then, 1 l of each sample was divided into four, and each of 250 ml aliquots was concentrated by filtration through a mixed cellulose membrane of 0.2 μm pore diameter (Whatman) with the application of a sterile filtration set (Nalgene). Next, each membrane was placed on a plate of R2A (BTL) supplemented with an antibiotic (Sigma-Aldrich), prepared following guidelines (EUCAST 2020) as presented in Table I.

These antibiotics represent the groups of high consumption rates in Poland (ESAC-Net 2020). Plates with membranes were incubated at 22°C for seven days. For quality control, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (BioMaxima) were inoculated on each prepared batch plates. Next, ARB representatives of different colony morphology (size, shape, color, opacity, surface, and texture) from various antibiotic supplemented plates, both WTPs and each sampling campaign, were selected.

Table I
R2A media supplementation for the cultivation of antibiotic-resistant
bacteria (ARB) (Siedlecka et al. 2020a; 2020b).

Antibiotic (abbreviation, final concentration)	ARB
Amoxicillin (AML, 8 mg/l)	Bacteria resistant to β-lactams
Ciprofloxacin (CIP, 2 mg/l)	Bacteria resistant to fluoroquinolones
Ceftazidime (CAZ, 8 mg/l)	Bacteria resistant to 3rd generation cephalosporins
Tetracycline (TE, 16 mg/l)	Bacteria resistant to tetracyclines

Molecular identification of strains. Each selected colony of ARB was subject to the streak-plate inoculation technique to isolate the pure strain. The bacteria were streaked on an R2A medium supplemented with the same antibiotic as previously incubated. The strains were Gram stained and observed under an optical microscope to confirm their purity, and apply the appropriate DNA extraction procedure. Next, genomic DNA was extracted with a Genomic Mini kit (A&A Biotechnology) under the manufacturer's instructions, depending on Gram-staining results. DNA concentration and purity were measured on NanoPhotometer N60 (Implen).

Nearly full 16S rRNA gene was amplified with a primer set 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Siedlecka et al. 2020b). The PCR mixture consisted of: 4 µl of 5 × Silver Hot Start PCR Mix (Synegn), 0.4 µl of each 10 µM primer, 2 µl of DNA, and 13.2 µl of water (A&A Biotechnology). Touchdown PCR protocol was as follows: initial denaturation at 95°C for 15 min, followed by 25 cycles of denaturation at 95°C for 15 s, annealing at 55–50°C for 30 s, elongation at 72°C for 60 s, and final elongation at 72°C for 7 min. Negative control was applied to confirm the lack of contamination in the reaction. After PCR amplification, 5 µl of each sample was mixed with 1 µl of 6x loading buffer (A&A Biotechnology) and separated by electrophoresis in 1% agarose gel (Sigma-Aldrich) stained with Green DNA Gel Stain (Syngen). The products were electrophoresed at 120 V for 15 min, and at 80 V for 60 min in 1 × TBE buffer and visualized by UV (UVITEC). The amplicon size was compared with DNA Marker 3 (A&A Biotechnology). The remaining products were purified with Clean-up Concentrator (A&A Biotechnology) following the manufacturer's instructions. The DNA concentration of the purified PCR products was determined using NanoPhotometer N60 (Implen) and subjected to Sanger sequencing (Genomed).

Sequences 27F and 1492R were subject to quality control and aligned with MEGA-X. The consensus of each aligned pair of strands was created with BioEdit. The obtained consensus sequences were identified with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antibiotic and disinfectant susceptibility testing. Before susceptibility testing, each strain was transferred to Mueller-Hinton (BioMaxima) agar plate to ensure adaptation to the richer medium. Eighteen antibiotics and two disinfectants were tested through the Kirby-Bauer disk diffusion method (antibiogram). The antibiograms were prepared in accordance with guidelines (EUCAST 2020). Briefly, fresh bacterial culture was suspended in sterile saline (0.89% NaCl, Chempur) to achieve turbidity of 0.5 McFarland standard (BioMerieux). Then, the suspension was inoculated on Mueller-Hinton by swabbing three times, every time turning the plate by 60 degrees. Next, the disks containing the antibiotics (BioMaxima) were placed on plates with inoculated bacteria utilizing a dispenser (BioMaxima). The antibiotics selected for testing included (abbreviation, disk content in µg): ampicillin (AM, 10), aztreonam (ATM, 30), ertapenem (ETP, 10), imipenem (IMP, 10) meropenem (MEM, 10), ofloxacin (OFX, 5), cefotaxime (CTX, 5), cefepime (FEP, 30), doxycycline (DO, 30), oxytetracycline (T, 30), vancomycin (VA, 30), gentamycin (CN, 30), streptomycin (S, 300), sulphamethoxazole/trimethoprim (SXT, 23.75/1.25), erythromycin (E, 15), rifampicin (RA, 5), chloramphenicol (C, 30), and polymyxin B (PB, 300). Sterile disks were simultaneously soaked in 14.5% sodium hypochlorite (Chempur), commercial disinfectant Melsept (Braun) at the working solution, and sterile water as a control and placed on plates with inoculated bacteria. All disks were placed within 15 min after inoculation of the strains on the plates. The plates were incubated at 22°C for 7 d due to the psychrophilic properties and prolonged growth of environmental bacteria. For quality control, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (BioMaxima) were subject to antibiotic susceptibility testing in the same manner. Then, the diameter of the zone of inhibition was measured for each disk. Because the guidelines for inhibition zone data interpretation are provided only for clinically relevant species, the epidemiological cut-off values (ECOFFs) taken from the EUCAST database (https://mic.eucast.org/Eucast2/SearchController/) were adopted for the purpose of differentiation on susceptible (criteria as for wild type) and resistant (criteria

as for non-wild type) phenotypes. EUCAST provides different ECOFFs for various species. In this study, the lowest ECOFF among those proposed by EUCAST was adopted for each antibiotic to avoid resistant phenotypes' overestimation. If no ECOFFs was provided in the EUCAST database, bacteria presenting inhibition zone diameter ≥ 10 mm were considered susceptible. In the case of disinfectant susceptibility testing, the inhibition zone diameter ≤ 20 mm was adopted to consider bacteria to be resistant, as proposed by Khan et al. (2016a).

Minimal inhibitory concentration (MIC) testing. MIC testing was performed for four antibiotics representative of the most commonly consumed groups of antibiotics in Poland (ESAC-Net 2020), namely AML, CIP, CAZ, and TE, and two of such antibiotics with additives, i.e., AML with clavulanic acid and CAZ with avibactam. For antibiotic resistance screening, each strain was inoculated on four R2A plates supplemented with antibiotics: AML, CIP, CAZ, and TE, as described in section 2.1. Only strains able to grow on R2A in the presence of a given antibiotic were subject to further MIC testing. The strains that did not grow on R2A plates supplemented with a given antibiotic (prepared as presented in Table I) were considered sensitive, and they were not subject to MIC testing.

Each antibiotic-resistant strain was inoculated on a Mueller-Hinton plate, as described in section 2.3. Within 15 min, MIC strips (BioMaxima) were placed on the plates. The MIC strips included (abbreviation, concentration range in μg/ml): amoxicillin (AML, 0.016–256), amoxicillin with clavulanic acid (AMC, 0.016–256), ciprofloxacin (CIP, 0.002–32), ceftazidime (CAZ, 0.016–256), ceftazidime with avibactam (CZA, 0.016–256), and tetracycline (TE, 0.016–256). The plates were incubated at 22°C for 7 d due to the psychrophilic properties and prolonged growth of environmental bacteria. For quality control, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (BioMaxima) were subject to MIC testing in the same manner.

Statistical analysis. The normality of data was verified using a Shapiro-Wilk test. A Mann-Whitney *U*-test was conducted to evaluate the effect of the season (summer and winter) or WTP (NG and MD) on the total number of antibiotics to which each strain was resistant, as well as on inhibition zones of chlorine and commercial disinfectant disk diffusion testing.

Correlations between the total number of antibiotics to which each strain was resistant, and MICs results, as well as inhibition zones of chlorine and commercial disinfectant disk diffusion testing, were assessed using Spearman analysis. The significance level was set at $p < 0.05$ throughout the study. All statistical analyses were done in Microsoft Excel software (Microsoft Office 365 ProPlus).

Results and Discussion

Antibiotic and disinfectant susceptibility testing. As a result, 24 strains were isolated. The molecular identification of the strains, antibiotic and disinfectant susceptibility testing, and MIC testing are presented in Tables II, III, and IV, respectively. Free and total residual chlorine concentrations in collected tap water were in a range of 0.00 to 0.31 and 0.10 to 0.49 mg/l across the study, respectively. The data on the percentage of ARB in total bacteria, and other microbiological and physical-chemical analyses of the collected tap water samples, have been previously published (Siedlecka et al. 2020b).

The majority of bacteria belonged to the phylum *Proteobacteria* (15/24), including classes *Alphaproteobacteria* (13/24) and *Betaproteobacteria* (2/24). Representatives of *Bacteroidetes* (4/24), *Actinobacteria* (4/24), and *Firmicutes* (1/24) were also found. Unfortunately, *Caulobacter* sp. was excluded from further analyses because the strain did not grow evenly on Mueller-Hinton medium. A similar problem with tap water bacteria cultivation was reported by Khan et al. (2016a).

Some strains were identified to the same genera or species. These isolates generally presented similar resistance patterns despite their origin from different sampling points (*Achromobacter* sp., *Mycobacterium frederiksbergense*) or sampling campaigns (*Bosea massiliensis*). However, both *Sphingomonas* sp. isolates were obtained from the same sample, suggesting their affinity (the strains were primarily isolated on R2A medium supplemented with AML and CIP, respectively). Interestingly, all *Brevundimonas* isolates were isolated from WTP NG finished water. Two of them, identified as *Brevundimonas mediterranea*, from the same sample, also suggested their affinity (the strains were primarily isolated on R2A medium supplemented with AML and CAZ antibiotics, respectively).

The results of statistical analyses revealed that neither seasonal nor WTP dependent variabilities were found in the total number of antibiotics to which each strain was resistant, and inhibition zones of chlorine and commercial disinfectant disk diffusion testing. Weak but statistically significant correlations were found between the total number of ineffective drugs and AML ($rho = 0.45$) and AMC ($rho = 0.50$) MIC results for strains subject to MIC testing.

Among the antibiotics tested by the disk diffusion method, ATM and CTX, belonging to monobactams and 3rd generation cephalosporins groups, respectively (WHO ATC Index (https://www.whocc.no/atc_ddd_index/)), proved the least effective against the investigated bacteria, whereas all strains were susceptible to IMP. Apart from IMP, other carbapenems were not as effective against investigated strains. Tetracyclines and E also proved to be highly effective against tap water

Table II
Results of molecular identification of strains, % identity of sequence with the reference sequence
in the BLAST database, accession No. of the reference sequence.

Strain No.	Origin*	Identification	% Identity	Accession
1	MD4VII(AML)	*Chryseobacterium* sp.	91.28%	MK095762.1
2	MD4VII(CIP)	*Bosea massiliensis*	100.00%	KM114964.1
3	NG1VIII(CAZ)	*Mycobacterium frederiksbergense*	99.55%	LN613126.1
4	NG1VIII(AML)	*Brevundimonas mediterranea*	99.84%	CP048751.1
5	NG2VIII(CIP)	*Sphingomonas sanxanigenens*	99.23%	KY078833.1
6	NG4VIII(CIP)	*Sphingomonas* sp.	99.69%	HM191725.1
7	NG4VIII(AML)	*Sphingomonas* sp.	99.77%	HM191725.1
8	MD2VIII(CIP)	*Dyadobacter* sp.	98.78%	MK271730.1
9	MD4VIII(CIP)	*Microbacterium* sp.	99.70%	MT542332.1
10	MD4VIII(TE)	*Afipia* sp.	99.54%	MK402948.2
11	MD4VIII(CIP)	*Bosea massiliensis*	99.69%	MF101018.1
12	NG1I(AML)	*Brevundimonas mediterranea*	99.69%	CP048751.1
13	NG1I(CAZ)	*Brevundimonas mediterranea*	99.84%	CP048751.1
14	NG2I(CAZ)	*Nocardia asteroides*	99.85%	MT355847.1
15	NG2I(CAZ)	*Sphingobium abikonense*	98.92%	MK696981.3
16	NG3I(AML)	*Achromobacter* sp.	99.39%	KT826375.1
17	NG4I(AML)	*Pedobacter* sp.	100%	EF660750.1
18	NG4I(CAZ)	*Flavobacterium* sp.	99.32%	JQ977667.1
19	MD1I(CAZ)	*Bacillus zhangzhouensis*	95.24%	MG651160.1
20	MD3I(AML)	*Achromobacter* sp.	94.86%	KT826375.1
21	MD4I(CIP)	*Caulobacter* sp.	97.92%	KM252977.1
22	NG1II(CIP)	*Brevundimonas* sp.	99.92%	CP045456.1
23	NG3II(CAZ)	*Mycobacterium frederiksbergense*	100%	LN613126.1
24	MD3II(CAZ)	*Methylobacterium* sp.	91.84%	HM327817.1

* – sample collection site and month, where: NG refers to WTP Na Grobli, MD refers to WTP Mokry Dwór,
Arabic numerals refer to consecutive sampling points (1 – finished water at each WTP, 2, 3, 4 – consecutive
sampling points in the distribution system within each WTP supply area), Roman numerals refer to the month
of sample collection, abbreviations in brackets refer to R2A media supplementation – the plate from which
the strain was initially isolated. Strains are ordered by the month of collection.

bacteria in this study. Among the antibiotics tested by means of MIC, the least effective was CAZ, followed by AML, both belonging to β-lactam antibiotics (WHO ATC Index (https://www.whocc.no/atc_ddd_index/)). Interestingly, AML and CAZ MICs exceeded 256 mg/l for 7 and 15 strains, respectively, suggesting strong resistance of tap water bacteria to these antibiotics. Moreover, the results of MIC testing suggest that all but one (strain No. 14) strains tested for AML exhibit β-lactamase activity. The addition of clavulanic acid, known as a competitive inhibitor of β-lactamases (Kim et al. 2009), decreased effective drug concentration. Among strains tested for CAZ, 8 (i.e., strains No. 1, 5, 6, 11, 12, 15, 16, and 20) proved to be sensitive to the avibactam additive, also suggesting β-lactamase activity (Wang et al. 2016). Nevertheless, the presence and activity of β-lactamases in the strains need to be confirmed by further studies. The majority of strains subject to AML or CAZ MIC testing (except for strains No. 15, 18, and 23) were resistant to at least one other antibiotic belonging to the β-lactam antibiotic class. All strains subject to CIP MIC testing were resistant to OFX, another representative of fluoroquinolones. Two strains resistant to TE (9 and 10) were also resistant to T and both tetracyclines used in the disk diffusion method, respectively. Contrary to the presented results, aminopenicillins and aminoglycoside resistance were reported to be common in tap water bacteria in Porto, Portugal (Vaz-Moreira et al. 2011; 2012; 2017; Narciso-da-Rocha et al. 2013). The differences in resistance of tap water bacteria in Wrocław and Porto could be region-dependent or taxon-dependent, because the other genera were investigated in these two DWDSs.

The majority of strains were resistant to 14.5% standard sodium hypochlorite, suggesting strong disinfectant resistance of tap water bacteria. However, no

Table III
Results of antibiotic and disinfectant susceptibility testing.

Strain No.	Aminopenicillins	Monobactams	Carbapenems			Fluoroquinolones	Cephalosporins		Tetracyclines		Glycopeptides	Aminoglycosides		Sulfonamides	Macrolides	Others			Total	Disinfectants	
	AM	ATM	ETP	IMP	MEM	OFX	CTX	FEP	DO	T	VA	CN	S	SXT	E	RA	C	PB		Cl₂	D
1	•	•	•		•													•	5	•	•
2		•									•								2	•	•
3		•																	1	•	•
4	•	•	•			•	•	•						•					7	•	•
5		•	•			•	•	•					•			•	•		8	•	•
6	•	•	•			•	•					•	•	•		•			9	•	•
7	•	•	•			•	•						•	•		•			8	•	•
8		•				•	•	•			•	•							6	•	•
9		•				•	•	•		•				•		•	•	•	9	•	•
10	•	•			•		•		•	•	•	•		•		•	•	•	12	•	•
11		•																	1	•	
12	•	•				•	•	•											5	•	•
13	•	•				•	•	•						•					6	•	•
14							•	•								•	•		4	•	•
15																•		•	2		
16	•						•					•				•			4	•	•
17	•	•				•	•				•	•						•	7	•	•
18																			0		
19							•												1	•	
20	•						•					•				•			4	•	•
21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22		•				•	•							•					4	•	•
23																			0	•	
24	•	•	•		•	•	•				•	•		•	•		•	•	12	•	
Total	11	16	6	0	3	11	16	7	1	2	5	7	3	8	1	9	5	6			

• – resistant; – – not included in the testing

Cl₂ – 14.5% sodium hypochlorite; D – commercial disinfectant

The abbreviations of antibiotics are explained in the Materials and Methods section.

Antibiotic groups are in accordance with ATC Classification System (WHO ATC Index).

correlations were found between inhibition zones against each disinfectant and the total number of ineffective antibiotics, nor antibiotic MICs, contrary to the results of Khan et al. (2016a), who found weak (but significant) correlations between chlorine-tolerance and MIC against TE, sulphamethoxazole, and AML. Four strains (11, 19, 23, 24) resistant to 14.5% standard sodium hypochlorite were revealed to be susceptible to working solution of Melspet, suggesting that other antimicrobial agents present in commercial composition disinfectant were more effective against tap water bacteria than chlorine. The disinfectant susceptibility testing of the investigated strains suggests that resistance to strong chemical disinfectants is frequent among tap water ARB. An approach alternative to tap water chlorination should be considered in the future. For example, in some European WTPs, treatment is based on biofiltration without final disinfection or residual disinfectant use (Proctor and Hammes 2015). It remains unknown, however, whether this approach is successful in limiting ARB prevalence in tap water.

Methylobacterium sp. and *Afipia* sp. proved resistant to the highest total number of antibiotics tested in this study using the disk diffusion method. The MIC testing results confirm the strong resistance of these two strains. On the other hand, *M. frederiksbergense* (strain No. 23) and *Flavobacterium* sp. were susceptible to all antibiotics tested with the disk diffusion method

Table IV
Results of MIC testing (µg/ml).

Strain No.	AML	AMC	CIP	CAZ	CZA	TE
1	>256	0.75	–	32	0.75	–
2	10	<0.016	–	32	32	–
3	10	0.25	–	>256	>256	–
4	>256	2	4	>256	>256	–
5	16	0.5	>32	>256	<0.016	–
6	96	1.5	12	10	<0.016	–
7	24	8	4	–	–	–
8	10	0.047	>32	>256	>256	–
9	–	–	6	>256	>256	24
10	64	4	–	>256	>256	>256
11	–	–	–	10	0.38	–
12	>256	2	–	96	64	–
13	>256	1.5	–	>256	>256	–
14	10	24	–	>256	>256	–
15	10	0.5	–	>256	64	–
16	>256	1.5	–	10	4	–
17	32	24	>32	>256	>256	–
18	10	0.5	–	>256	>256	–
19	–	–	–	>256	>256	–
20	>256	0.5	–	10	1	–
21	–	–	–	–	–	–
22	10	0.125	4	>256	>256	–
23	12	1	–	>256	>256	–
24	>256	24	>32	>256	>256	–

– – not included in the testing (strain susceptible to the antibiotic)
The abbreviations of antibiotics are explained in the Materials and Methods section.

and resistant only to AML and CAZ. The majority of strains proved multi-drug resistant (MDR), i.e., they were resistant to three or more antibiotic groups (Falagas and Karageorgopoulos 2008). The present study's results seem to confirm MDR among bacteria dwelling in tap water, reported previously (Vaz-Moreira et al. 2011; 2012; Narciso-da-Rocha et al. 2014; Leginowicz et al. 2018). Among genera identified in this study, MDR isolates of *Brevundimonas*, *Microbacterium*, *Pedobacter*, *Bosea*, and *Afipia* were also found in bottled mineral water (Falcone-Dias et al. 2012). Interestingly, the resistance of bacteria belonging to these genera might have been acquired because taxonomically related strains isolated from various mineral water brands presented different resistance profiles (Falcone-Dias et al. 2012).

Intra-genus and intra-specific variability in antibiotic resistance of environmental bacteria – comparison of the obtained results with literature reports. Lack of guidelines for antibiotic susceptibility testing for environmental species makes the comparison of scientific reports complicated (Leginowicz et al. 2018).

Nevertheless, the comparison of data presented in this paper with resistance patterns of bacteria belonging to the same genera or species reported previously in literature could shed new light on antibiotic resistance spread in environmental bacteria. Unless stated otherwise, this review concerns only drugs tested in this study.

Some data regarding the antibiotic resistance of representatives of genera *Achromobacter*, *Chryseobacterium*, *Pedobacter*, and *Microbacterium* are available. Carbapenems and SXT have been claimed to be the most effective antibiotics against *Achromobacter* spp., opportunistic human pathogens (Almuzara et al. 2010). It is in accordance with the results presented in this paper. On the other hand, a literature review concerning genera *Chryseobacterium*, *Pedobacter*, and *Microbacterium* reveals some discrepancies. *Chryseobacterium* spp. were reported to be inherently resistant to E, C, linezolid, polymyxins, aminoglycosides, tetracyclines, and many β-lactams, and intermediately sensitive to VA and clindamycin (Kirby et al. 2004; Loch and Faisal 2015), but susceptible to RA, CIP and SXT (Kirby et al. 2004; Chen et al. 2013b). Another study revealed that out of

Chryseobacterium spp. isolates obtained from aquatic habitats, 97% were resistant to AM, 89% to PB, 62% to E, 54% to T, 21.5% to florfenicol, and 69% were sensitive to SXT (Michel et al. 2005; Loch and Faisal 2015). *Pedobacter* spp. were considered environmental superbugs, probably intrinsically resistant to many antibiotics and having β-lactamases. They have been reported to be resistant to AMC, AM, ATM, FEP, CAZ, C, CIP, CN, S, TE, and VA, but susceptible to IMP and SXT (Viana et al. 2018). *Microbacterium* spp. isolates have been reported to be resistant to CTX, CIP, DO, E, CN, RA, and VA (Gneiding et al. 2008). Findings regarding *Chryseobacterium* sp., *Pedobacter* sp., and *Microbacterium* sp. presented in this study are, therefore, partially in contradiction with literature reports.

Some discrepancies are also found in resistance patterns of representatives of genera *Dyadobacter* and *Flavobacterium*. For example, *Dyadobacter alkalitolerans* first isolated from desert sand in China was susceptible to TE and RA, but resistant to AM, E, VA, and AML (Tang et al. 2009), and *Dyadobacter arcticus* first isolated from Arctic soil in Svalbard was susceptible to PB, TE, VA, CIP, OFX, S, and SXT, but resistant to AM, CN, and CAZ (Chen et al. 2013a). *Flavobacterium psychrophilum* isolates obtained from trout in Turkey demonstrated reduced susceptibility to T, but susceptibility to AML (Saticioglu et al. 2019). In contrast, *Flavobacterium columnare* isolates obtained from pound cultures in Nigeria proved resistant to S, T, C, OFX, CN, and AML, but susceptible to CIP and SXT (Ogbonne et al. 2019). Moreover, clinical isolates of *Flavobacterium* spp. were resistant to E (Aber et al. 1978). The resistance patterns of *Dyadobacter* sp. and *Flavobacterium* sp. isolates investigated in this study differ from those mentioned above.

Afipia sp. and *Methylobacterium* sp. were resistant to most of the antibiotics tested 0 in this study. *Afipia* spp. has been formerly isolated from tap water samples (Zhang et al. 2009). Within this genus, *Afipia felis*, a putative cat-scratch disease agent, has been suggested to be resistant to most antibiotics, remaining susceptible to aminoglycosides, IMP, RA, amikacin, and tobramycin (Maurin et al. 1993). *Methylobacterium* spp. isolates formerly proved resistant to AML, E, and C, but the majority of them were susceptible to TE and CN (Hiraishi et al. 1995). On the other hand, *Methylobacterium* spp. isolates obtained from hospital tap water in Japan were resistant to AM, CN, E, VA, C, and OFX but susceptible to IMP and TE (Furuhata et al. 2006). Therefore, resistance patterns of *Afipia* sp. and *Methylobacterium* sp. investigated in this study, and those reported previously in the literature show certain differences.

Sphingomonadaceae are common inhabitants of tap water. This family has been suggested to be intrinsically resistant to colistin. Resistance to fluoroquinolones, cephalosporins, and sulphonamides is possibly acquired in these bacteria (Vaz-Moreira et al. 2011; Narciso-da-Rocha et al. 2014). The most antibiotic-resistant genera of the family *Sphingomonadaceae* proved to be *Sphingomonas* and *Sphingobium* (Vaz-Moreira et al. 2011), both investigated in the present paper. *Sphingomonas* representatives have been reported to be resistant to IMP, MEM, FEP, CAZ, CIP, CN, and SXT, and *Sphingobium* representatives to MEM, FEP, CAZ, CIP, CN, and SXT, respectively (moreover, resistance to β-lactams has been suggested to be intrinsic in genus *Sphingobium*) (Vaz-Moreira et al. 2011; Narciso-da-Rocha et al. 2014). The comparison of these resistance patterns with results presented in this paper, therefore, reveals some intra-genus differences.

Some intra-specific variability in antibiotic resistance was also observed based on the examples of the *Sphingobium abikonense*, *Nocardia asteroides*, and *B. massiliensis*. *S. abikonense* was first isolated in India. Its resistance pattern differed from the results presented in this study in terms of AM, RA, and PB (Kumari et al. 2009). The antibiotic resistance of *Nocardia* spp. has been suggested to be species-specific. Nevertheless, linezolid and SXT appear to be effective against this genus, although emerging resistance to SXT has also been reported (Schlaberg et al. 2014; Hashemi-Shahraki et al. 2015; McTaggart et al. 2015; Zhao et al. 2017). *N. asteroides* isolates have formerly proved susceptible to SXT, MEM, CTX, and CN, but resistant or moderate-resistant to AMC, CIP, FEP, AM, VA, and RA; whereas resistance to IMP differed among the studies (Schlaberg et al. 2014; Hashemi-Shahraki et al. 2015; Zhao et al. 2017). In the present study, the resistance pattern of *N. asteroides* differed in terms of AM, CTX, VA, and CIP, suggesting intra-specific diversity of antibiotic resistance. Moreover, *B. massiliensis* first isolated from hospital tap water in France has been reported to be susceptible only to DO (La Scola et al. 2003) in contrary to results presented in this paper. Both *B. massiliensis* isolates investigated in this study were susceptible to most drugs (including DO), also suggesting intra-specific diversity.

Finally, some data regarding *M. frederiksbergense*, *Brevundimonas*, and *Bacillus zhangzhouensis* are also available. *M. frederiksbergense* was first isolated from soil in Denmark (Willumsen et al. 2001). This species has been confirmed to potentially lead to infection while being sensitive to drugs commonly used to treat non-tuberculous mycobacteria (Senozan et al. 2015). *Brevundimonas* spp. isolates were most frequently resistant to AM, ATM, FEP, AMC, CIP, and CAZ, although other resistance patterns were also reported (Ryan and Pembroke 2018). Results presented in this paper generally appear to be in line with these reports. *B. zhangzhouensis* was first isolated from a shrimp farm in China. Unfortunately, no resistance pattern was described for this strain (Liu et al. 2016). The isolate investigated in

this study was only resistant to CTX and CAZ, both belonging to the 3rd generation cephalosporins group.

To sum up, the comparison of results presented in this paper with previous literature reports points to intra-genus differences in resistance patterns of tap water strains identified to the genus level in this study (*Chryseobacterium* sp., *Pedobacter* sp., *Microbacterium* sp., *Dyadobacter* sp., *Flavobacterium* sp., *Afipia* sp., *Methylobacterium* sp., and *Sphingomonas* sp.). This observation, however, does not exclude species-specific resistance. Nevertheless, some strains identified to the species level in this study (*S. abikonense*, *N. asteroides*, and *B. massiliensis*) also presented resistance patterns different from those reported in the literature, suggesting intra-specific diversity and acquired resistance. Similarly, according to Narciso-da-Rocha et al. (2013), *Acinetobacter* spp. isolates (obtained from WTP and tap water) of the same sequence types presented wild type or non-wild type against some antibiotics. On the other hand, some strains identified in this study as belonging to the same genus or species presented very similar (*M. frederiksbergense*, *B. massiliensis*, *B. mediterranea*) or identical (*Achromobacter* sp.) resistance patterns among each other, despite being isolated from different sampling points or campaigns, which reduces the likelihood of their affinity. Therefore, both HGT and VGT appear to play a role in the resistance spread among tap water ARB.

Conclusions

Tap water bacteria could be MDR and disinfectant-resistant. Neither seasonal nor WTP-dependent variabilities were found in terms of bacterial resistance to antibiotics and disinfectants. IMP proved the most effective, and CAZ the least effective drug against tap water isolates. The comparison of resistance patterns of the strains investigated in this study with previous literature reports indicated the existence of intra-genus and intra-specific variabilities, suggesting acquired resistance of tap water bacteria. Nevertheless, some isolates' species-specific resistance could not be excluded because most strains were only identified to the genus level. Moreover, some strains identified in this study as belonging to the same genus or species presented very similar (*M. frederiksbergense*, *B. massiliensis*, *B. mediterranea*) or identical (*Achromobacter* sp.) resistance patterns among each other. It appears that both horizontal and vertical gene transfer could shape resistance phenotypes of tap water bacteria.

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