Environment Protection Engineering

Vol. 44 2018 No. 4

DOI: 10.37190/epe180406

MONIKA LEGINOWICZ[1], AGATA SIEDLECKA[1], KATARZYNA PIEKARSKA[1]

BIODIVERSITY AND ANTIBIOTIC RESISTANCE OF BACTERIA ISOLATED FROM TAP WATER IN WROCŁAW, POLAND

Microbial contamination and biodiversity were determined for the drinking water samples collected from selected points of the water supply system in Wrocław, Poland. All tested samples met the requirements of Polish law, i.e., the Regulation of the Minister of Health. However, the antibiotic resistant bacteria were found. The correlation between the distance of sampling points from water treatment plants and their microbial contamination was not established. Nevertheless, the Na Grobli treatment plant seemed to produce water of higher microbial quality than the Mokry Dwór treatment plant at the moment of sampling. The identification of representative isolates was performed with 16S rRNA gene sequencing and MALDI-TOF mass spectrometry and the results of these two methods were compared, indicating some discrepancies. Nevertheless, bacteria dwelling in drinking water in Wrocław belonged to the phyla *Actinobacteria*, *Proteobacteria (α-*, *β-*, *γ-Proteobacteria)* and *Firmicutes*. The determination of antibiotic resistance profiles showed that 12 from 17 tested isolates revealed resistance to at least one antibiotic and two strains were multi-drug-resistant.

1. INTRODUCTION

Water supply systems are intended to provide safe water, free of microbiological contamination to all consumers. As tap water is often considered to be drinking water, its high quality in terms of organoleptic, physicochemical and microbiological properties is demanded. From the epidemiological point of view, the microbiological quality, i.e., the presence and biodiversity of tap water microorganisms, is the most prominent issue. Intake of water contaminated with pathogens may lead to numerous diseases [1]. According to Polish law, the supplier is bound to produce and transfer drinking water free of indicator microorganisms, i.e., *Escherichia coli* and *enterococci*. To be more precise, there must not be any of the mentioned bacteria in 100 cm^3 of tested water [2].

[1]Wrocław University of Science and Technology, Faculty of Environmental Engineering, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland, corresponding author A. Siedlecka, e-mail address: agata.siedlecka@pwr.edu.pl

The novel Regulation of the Minister of Health does not specify the number of colony forming units (cfu) of psychrophilic microorganisms in 1 cm^3 of water, stating that this number should be *without abnormal changes*. This statement may lead to misunderstandings and misuses. However, it is suggested, that the number of cfu should not exceed 200 cfu in 1 cm^3 of consumer's tap water. Moreover, the former aspect of the mesophilic microorganisms in tap water is neglected [2].

The mandatory regulations are focused on indicators of pathogens and microorganisms associated with the phenomenon of faecal contamination [1]. The biodiversity of microorganisms dwelling in tap water is, indeed, a much more complex issue. The tap water microorganisms may form the biofilm structure or be present as planktonic cells [3]. Genetic exchange and the prevalence of antibiotic resistance often occur in dense, compact structures as biofilm [4]. However, from the consumers' point of view, the planktonic cells, which can reach a tap, are more important, as they could be a direct source of health problems.

Biodiversity of tap water microorganisms may be determined by culture-dependent and culture-independent methods [5]. Despite the development of many technologies, based on molecular and phenotypical features of bacteria and fungi, a reliable identification of microorganisms is still a challenge, especially in the case of infrequent isolates. In this study, two commonly applied methods were chosen and compared: 16S rRNA gene sequencing and MALDI-TOF. The 16S rRNA gene sequencing is the most common identification method, based on genetic variation. The other promising tool is the matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS), which allows the identification based on a unique protein profile of microorganisms. Undoubtedly, the advantage of the method is the rapidity of a test performance: the procedure from sample collection to test results could take only a few minutes [6]. Moreover, the API 20 E kits were applied in this study for the non-fastidious, gram-negative rods identification to compare and evaluate the results of biochemical and molecular identification approaches.

Because bacteria can acquire the resistance features, not only the pathogenicity but also antibiotic resistance of tap water microorganisms may be considered a threat to human health. Antibiotic resistance genes (ARGs) from microorganisms of the same and other species or directly from the environment may be absorbed in the transformation process. It is known that environmental strains may serve as the ARGs vectors [7]. In other words, tap water may be considered as a reservoir of resistance, even if tap water strains remain harmless. Unfortunately, the recognized and reputable committees dealing with the antibiotic resistance phenomenon such as The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) provide the resistance breakpoint values mostly for pathogenic bacteria, due to, among other, a lack of sufficient data on resistance profiles of environmental strains. It makes the evaluation of tap water strains resistance difficult to compare among laboratories and prone to under- or overestimation. Therefore, not only

resistance phenotypes but also ARGs should be considered in studies of environmental strains resistance [8].

In this study, the number and biodiversity of microorganisms isolated from water samples collected from the water supply system in Wrocław were investigated. The influence of the distance from water treatment plants (WTPs) on the microbial contamination of the sample was verified. The isolated strains were identified by 16S rRNA gene sequencing, MALDI-TOF MS and API 20 E kits methods. The antibiotic resistance phenotypes of selected strains were determined and the PCR for ARGs of *bla* group (β-lactamases genes) were applied.

To the authors best knowledge, this is the first study on antibiotic resistance phenomenon in tap water in Central Europe. The obtained results may be compared to scientific reports from other parts of the world. Furthermore, some routes of resistance spreading may be observed across Europe. Therefore, the aim of this study was to preliminary investigate the biodiversity and antibiotic resistance of strains dwelling in tap water in Wrocław (Poland).

2. MATERIALS AND METHODS

The sampling procedure and isolation of microorganisms. Samples of tap water were collected from 10 points located on the Wrocław's water supply system pipeline. The sampling points were selected based on their distance from the 2 WTPs, thereby providing an ability to compare different tap water sources. The 10 points included 2 points located in the area supplied only by Na Grobli WTP, 5 points – by Mokry Dwór and 3 points – in the area of mixed streams.

1 dm^3 of drinking water from every selected point was collected in sterile, glass bottles in July (one sampling campaign), as this month is considered to be the hottest during the year, resulting in the highest bacterial density. Afterwards, each sample was concentrated in a sterile manner by filtration through cellulose acetate membrane filter of 0.2 μm pore diameter (Whatman, Germany). Subsequently, the membrane filters were transferred on R2A (BTL, Poland) agar plates and incubated for 72 h at temperatures optimal for psychro- and mesophilic strains, i.e., 22 and 37 °C, in aerobic conditions. After the incubation, the cfu were counted and the morphologies of the obtained colonies were compared. A streak plate method was applied to isolate the pure strains. From all obtained colonies of each variant of temperature conditions, colonies of various pigmentation, size, height, shape, surface, texture or edge were assumed to be the representatives of various species and subjected to further investigations. Pure colonies were inoculated on R2A agar slants, and then subjected to further incubation for 24 h, at adequate temperatures (22 and 37 °C). Afterwards, genomic DNA was isolated with

the *Genomic mini* kit (A&A Biotechnology, Poland), according to the manufacturer's instructions.

Identification of microorganisms. Each strain incubated on a R2A agar slant was subjected to Gram staining for the preliminary classification. For further identification, three methods were applied: 16S rRNA gene sequencing (Genomed, Poland), MALDI--TOF MS (ALAB, Poland), and API 20 E kits (bioMerieux, France).

For the genetic identification, the PCR amplification was performed with 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTACCTTGTTACGACTT-3′) primers (Genomed, Poland). The PCR mixture consisted of: 10×PCR buffer, 10 mmol/dm^3 dNTPs, 10 μmol/dm^3 of each primer, 1 U RUN Polymerase (A&A Biotechnology, Poland), matrix DNA (2 μl). The mixture was filled to 20 μl with dd H_2O. The amplification steps were as follows: denaturation at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 2 min, and at the end final elongation at 72 °C for 10 min. The PCR products were purified with a Clean Up Kit (A&A Biotechnology, Poland) and subjected to sequencing (Genomed, Poland). The identification was performed with the BLAST tool and sequences were compared with the GenBank NCBI database. For the MALDI-TOF identification, the biomass of each pure strain was used. API 20 E kits were applied according to the manufacturer's instructions.

Table 1

Applied antibiotics with their classes, assays and symbols

Classes of antibiotics		Antibiotic	Assay [μg/disc]	Symbol
Aminoglycosides		amikacin	30	AK
		streptomycin	10	S
		gentamycin	10	CN
β-Lactam antibiotics	aminopenicillins	amoxicillin	25	AML
		ampicillin + sulbactam	10 + 10	SAM
	penicillin	piperacillin	30	PRL
	cephalosporins	cephalothin	30	KF
Tetracyclines		tetracycline	30	TE
		minocycline	30	MH
Synthetic drugs	sulphonamides	trimethoprim	5	W
	quinolones	ofloxacin	5	OFX
	nitrofurans	nitrofurantoin	300	F
Lincosamides		clindamycin	2	Da
Macrolides		erythromycin	15	E
Amphenicols		chloramphenicol	30	C

Antibiotic's susceptibility testing. Resistance to antibiotics was tested using the modified Kirby–Bauer disc diffusion method. The turbidity of each bacterial suspension in sterile physiological solution was adjusted to 0.5 McFarland standard in order to provide even growth. Afterwards, the Mueller–Hinton agar plates were inoculated using the swabbing procedure. Antibiotic-impregnated discs were placed on the Mueller–Hinton agar plates within 15 min, according to the EUCAST guidelines. The antibiotics chosen in this study (belonging to several antibiotic classes) together with their assays and symbols are presented in Table 1. The plates were incubated for 24 h, at temperature conditions optimal for each strain, i.e., 22 or 37 °C. Zone diameters were interpreted according to EUCAST or CLSI guidelines. Due to the lack of data for many species, some breakpoints were adopted from literature (see Discussion for more details). If the zone diameters breakpoint values differed between the data sources, always the smaller value was chosen, in view of tightening the criteria. As the standards were adopted from a few independent guidelines and the literature, the strains were distinguished only as resistant or susceptible. Intermediate was consciously omitted.

PCR investigations of ARGs. To determine whether the isolated strains possessed ARGs responsible for resistance towards β-lactams, a PCR approach was applied. In this study, 3 ARGs responsible for the phenomenon of producing β-lactamases, i.e., *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX–M}}$ and *bla*$_{\text{SHV}}$ were selected [8]. The primer sequences, amplicon sizes and annealing temperatures of selected ARGs are presented in Table 2.

Table 2

Primer sequences, amplicon sizes and annealing temperature of PCRs [8]

Target gene	Primer sequence (5′–3′)	Amplicon size [bp]	Annealing temperature in standard PCR [°C]
bla$_{CTX–M}$	SCSATGTGCAGYACCAGTAA	544	55
	CCGCRATATGRTTGGTGGTG		
bla$_{\text{SHV}}$	GATGAACGCTTTCCCATGATG	214	61
	CGCTGTTATCGCTCATGGTAA		
bla$_{\text{TEM}}$	AGTGCTGCCATAACCATGAGTG	431	61
	CTGACTCCCCGTCGTGTAGATA		

Abbreviations: S – G or C, Y – C or T, R – A or G.

The PCR mixture consisted: 10×PCR buffer, 1 mmol/dm^3 dNTPs, 10 μmol/dm^3 of each primer, 1 U RUN polymerase (A&A Biotechnology, Poland), matrix DNA (2 μl). The mixture was filled to 20 μl with dd H_2O. The amplification steps were as follows: denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing

at given temperature for 1 min, elongation at 72 °C for 1 min, and at the end final elongation at 72 °C for 5 min [8].

3. RESULTS

3.1. MICROBIOLOGICAL CONTAMINATION OF DRINKING WATER SAMPLES

The sampling points together with the WTP serving as a tap water supplier and the numbers of obtained cfu per 1 dm^3 of sample are presented in Table 3.

Table 3

Number of cfu in samples of drinking water collected from selected sampling points

WTP	Sampling point	cfu per 1 dm^3	
Na Grobli	1	0	0
	2	0	0
Mokry Dwór	3	189	82
	4	129	140
	5	21	8
	6	297	206
	7	1080	808
Area of mixed streams	8	337	404
	9	129	168
	10	64	76

Based on morphologies differentiation, 23 strains were subjected to the streaking procedure and further investigations, as the representative ones. Strains of the same morphology, but incubated at other temperature conditions, were considered as different species.

3.2. IDENTIFICATION OF MICROORGANISMS

All species were identified by DNA sequencing and MALDI-TOF MS methods, simultaneously. As API 20 E kits are dedicated for *Enterobacteriaceae* and other non-fastidious, gram-negative rods, they were used only for the identification of the representatives of these bacteria types.

Table 4 presents the comparison of the results obtained by the three methods applied in order to identify the isolated strains. The overlapping identifications are highlighted in grey.

Table 4

The results of identification by DNA sequencing, MALDI-TOF MS and API 20 E kits

No.[a]	DNA sequencing	[%][b]	MALDI TOF MS	API 20 E	[%][b]
			t = 22 °C		
1a/10	*Bacillus weihenstephanensis*	95	*Bacillus mycoides*		
3a/8	*Pseudomonas poae*	97	*Pseudomonas veroni*	*Ochrobactrum anthropi*	–
5a/8	*Bacillus megaterium*	94	*Bacillus megaterium*		
7a/8	*Pseudomonas poae*	95	*Pseudomonas veroni*	*Bordetella/Alcaligenes /Moraxella* sp.	61.1
9a/8	*Pseudomonas synxantha*	95	*Pseudomonas corrugata*	*Pseudomonas fluorescens /putida*	88.3
15a/3	*Microbacterium testaceum*	96	*Arthrobacter polychromogenes*		
22a/6	*Arthrobacter aurescens*	92	*Arthrobacter aurescens*		
23a/4	*Brevundimonas subvibrioides*	95	*Achromobacter insolitus*	*Myroides* sp./ *Chryseobacterium indologenes*	42.0
26a/5	*Brevundimonas subvibrioides*	92	*Brevundimonas diminuta*	*Myroides* sp./ *Chryseobacterium indologenes*	42.0
27a/9	*Bacillus cereus/thuringiensis /anthracis/toyonensis*	95	*Bacillus cereus*		
28a/7	*Lysinibacillus sphaericus*	95	*Lysinibacillus fusiformis*		
			t = 37 °C		
3b/8	*Micrococcus luteus*	98	*Micrococcus luteus*		
5b/8	*Bacillus cereus/toyonensis /antracis/thuringensin*	90	*Bacillus cereus*		
11b/6	*Bacillus toyonensis*	96	*Bacillus mycoides*		
14b/4	*Delftia acidovorans*	94	*Delftia acidovorans*	*Pseudomonas aeruginosa*	97.8
15b/3	*Bacillus pumilus*	97	*Bacillus pumilus*		
16b/10	*Bacillus pumilus*	94	*Bacillus pumilus*		
17b/10	*Bacillus pumilus*	95	*Bacillus pumilus*		
21b/9	*Micrococcus luteus*	96	*Micrococcus luteus*		
24b/5	*Bacillus toyonensis*	94	*Bacillus mycoides*		
25b/7	*Advenella kashmirensis*	92	*Alcaligenes faecalis*	*Pseudomonas fluorescens/putida*	94.1
28b/9	*Bacillus subtilis*	96	*Bacillus subtilis*		
30b/10	*Acinetobacter baumannie /oleivorans*	93	*Acinetobacter lwoffii*	*Pseudomonas luteola*	48.0

[a]Strain No./Sampling point.
[b][%] – degree of probability.

3.3. ANTIBIOTIC SUSCEPTIBILITY TESTING

After the incubation on the Mueller–Hinton agar plates with antibiotic discs, the diameters of inhibited bacterial growth were measured. Table 5 presents the antibiotic profiles of selected strains, grouped in terms of genus.

Table 5

The results of antibiotic susceptibility testing. Antibiotic resistance profiles of selected strains

Genus	*Acinetobacter* spp.	*Brevundimonas* spp.	*Pseudomonas* spp.			*Micrococcus luteus*		*Bacillus* spp.									
Strain No.	30b	26a	3a	7a	9a	3b	21b	1a	5a	27a	5b	11b	15b	16b	17b	24b	28b
Sampling point	10	5	8	8	8	8	9	10	8	9	8	6	3	10	10	5	9
AK	S	S	S	S	S	S	S	–	–	–	–	–	–	–	–	–	–
S	S	R	S	S	R	–	–	S	S	S	R	R	S	S	S	S	S
CN	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AML	S	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SAM	R	R	R	R	R	–	–	–	–	–	–	–	–	–	–	–	–
PRL	–	–	S	R	R	–	–	–	–	–	–	–	–	–	–	–	–
KF	–	–	R	R	R	–	–	R	S	R	R	R	S	S	S	R	S
TE	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
MH	S	R	S	S	R	S	S	–	–	–	–	–	–	–	–	–	–
W	–	–	–	–	–	R	R	–	–	–	–	–	–	–	–	–	–
OFX	–	–	–	–	–	S	S	–	–	–	–	–	–	–	–	–	–
F	–	–	–	–	–	R	R	–	–	–	–	–	–	–	–	–	–
DA	–	–	–	–	–	S	S	–	–	–	–	–	–	–	–	–	–
E	–	–	–	–	–	S	S	S	S	S	S	S	S	S	S	S	S
C	–	–	–	–	–	R	S	S	S	S	S	S	S	S	S	S	S

R means resistant, S sensitive, and the dash no guideline available to determine the susceptibility.

3.4. PCR INVESTIGATIONS OF ARGS

Most of the PCR results were negative. Only one strain, i.e., 15b, was revealed to possess bla_{TEM} sequence in its genomic DNA. This strain was identified as *Bacillus pumilus* using DNA sequencing and MALDI-TOF MS methods. As *Bacillus* spp. are not included in EUCAST and CLSI susceptibility testing guidelines, its resistance profile against β-lactams could not be established phenotypically using a disc diffusion method.

4. DISCUSSION

As the information about the areas of water distribution of the WTPs was provided by the Municipal Water and Sewerage Company in Wrocław, the rough conclusion

about the effectiveness of each plant and the influence of water transport (i.e., the distance between the plant and the sampling point) was made. Surprisingly, there is an evident difference between numbers of cfu obtained from sampling points belonging to the area supplied by Na Grobli WTP which were equal 0 in two points tested in this study, and Mokry Dwór WTP or the area of mixed streams. This may suggest the advantageous performance of Na Grobli plant on the day of sampling. However, to confirm this statement, sampling campaigns should be repeated, and the analyses should include other factors, such as hydraulic and physicochemical properties of water (as for example the residue concentration of disinfectant) due to their crucial impact on microbial contamination. Nevertheless, from points supplied by Mokry Dwór, the sample point No. 5 had the lowest cfu numbers of psychro- and mesophilic bacteria. The sample point No. 7 had the highest bacterial contamination in both, psychro- and mesophilic strains cases. This may suggest an accidental contamination. The cfu values are evidently uncorrelated with the distances from WTPs (data not shown), as points No. 1 and 2 are distant from Na Grobli WTP and point No. 5 is much farther from Mokry Dwór WTP than point No. 7. Therefore, other factors than the distance from WTPs must have influenced the microbial contamination of sampling points. As mentioned above, these could be the hydraulic and physicochemical properties of water, such as water stagnation and the concentration of the residual disinfectant. An analogous situation may be observed in the area of mixed streams, but in this case there is no evidence which WTP is the main supplier, so the real distance, measured as the route of water distribution, could not be established precisely. Nevertheless, the microbial contamination of the tested water samples seems to be negligible. The cfu values do not exceed the standards suggested in the novel Regulation of the Minister of Health.

In the study of Grabińska-Łoniewska et al. [1], cfu values of microorganisms incubated at 26 °C ranged from 11 600 to 120 000 per dm^3 in samples collected between December 2000 and November 2002 in Warsaw. Moreover, the cfu concentrations depended on the distance from WTP (what cannot be proven in this study), but the other factors could have contributed to those results. The currently presented values differ distinctly from the results obtained by Grabińska-Łoniewska et al. [1]. This may be due to the constant improvements in water treatment introduced since then. The comparison between water supply systems of various cities may be misleading, as each network may be regarded as a distinct ecosystem. Furthermore, the physicochemical quality of tap water, as residue disinfectant concentration, may contribute to the biological stability [3] and strongly influence the results.

The results of identification, obtained using two molecular and one biochemical methods, display some differences. Only 3 of 11 strains of psychrophilic bacteria were identified accordingly using 16S rRNA gene sequencing and MALDI-TOF methods: 5a as *Bacillus megaterium*, 22a as *Arthrobacter aurescens*, and 27a as *Bacillus cereus*. As these strains are Gram-positive, API 20 E kits tests were omitted. Strains 15a and 23a were misidentified. In other cases, the results overlapped to the genus level. It is

worth to note that the misidentification even to the phylum level was noticed in the case of strain No. 23a. In the case of mesophilic bacteria, 8 of 12 strains were identified as the same species by two applied molecular methods. Among them, only one was Gram-negative. Strain 25b was misidentified. In other cases, the results overlapped to the genus level. API 20 E results never agreed with the results of sequencing and MALDI--TOF, often indicating bacteria from other phyla or families.

The 16S rRNA gene sequencing and MALDI-TOF identification accuracy and accordance were evaluated in other studies [9, 10]. Mellmann et al. [9] compared results of both methods, obtaining the proper identification of 57 from 80 known species using 16S rRNA gene sequencing and 67 from 78 known species using MALDI-TOF. Bizzini et al. [10] argue, that the results of identification obtained by MALDI-TOF and 16S rRNA gene sequencing overlapped for 62% of the isolated strains at the species level (what corresponds roughly with the results of mesophilic strains identification presented in this paper). Interestingly, 3 more strains from the study of Bizzini et al. [10] are revealed to be the same as identified by 16S rRNA gene sequencing after update of MALDI-TOF database, suggesting the need of further development of this method. According to some authors, MALDI-TOF is a promising tool for the rapid identification or characterisation of environmental strains [6, 11]. In the study of Christ et al. [11], the identification accuracy achieved by MALDI-ATOF and API 20 Strep for *Enterococcus* spp. identification showed that the results overlapped for 63% of the isolated strains at the species level. The inconsistently identified strains were subjected to 16S rRNA gene sequencing, which revealed that 74% were correctly identified by MALDI-TOF, whereas only 11% by API 20 Streps [11]. Nevertheless, in this paper the results of API 20 E kits are assumed to be unreliable.

Investigations of identities of drinking water microorganisms are neglected in routine tests, as they are time-consuming and laborious, and the identification of all isolates is almost impossible. In this study, the attempt was made to identify psychro- and mesophilic aerobic bacteria which were able to grow in laboratory conditions on R2A agar. As the selection of obtained colonies was made based on their cellular and colony morphologies, there is a probability, that some strains might be omitted, but results indicating the predominance of *Bacillus* spp. strains reduce this likelihood.

Interestingly, most of the identified strains were Gram-positive and belonged mainly to *Bacillus* spp. genus. The identified psychro- and mesophilic bacteria belonged to the phyla: *Actinobacteria*, *Proteobacteria* (*α*-, *β*-, *γ*-*Proteobacteria*) and *Firmicutes*, what is in accordance with the other results concerning the biodiversity of tap water microorganisms [3, 5]. Among bacteria most often isolated from water supply systems are: *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, *Moraxella*, *Arthrobacter*, *Mycobacteria*, *Aeromonas*, *Bacillus* [1]. However, Grabińska-Łoniewska et al. [1] did not identified *Bacillus* spp. in Warsaw water supply system. According to Vaz-Moreira et al. [5], in tap water samples from Portugal, the most predominant phyla were *Proteobacteria* (*α*-, *β*-,

γ-, *δ*-, *ε*-*Proteobacteria*) and among other phyla, the *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, *Aquificae*, *Acidobacteria*, *Verrucomicrobia* and *Firmicutes* were identified. Similar observations were made by Holinger et al. [3] who established the phyla of microorganisms dwelling in tap water samples from 17 cities in USA as *Proteobacteria*, *Cyanobacteria*, *Actinobacteria* (the most predominant genus in 16 from 17 cities was *Mycobacterium* spp.), *Firmicutes* and *Bacteroidetes*. The most predominant pathogenic bacteria in tap water belongs to *Enterobacteriaceae*, for example *Salmonella* or *Shigella*. In this study, none of the selected strains was identified as a member of *Entrobacteriaceae*. However, some isolates may be regarded opportunistic pathogens [1].

As EUCAST and CLSI guidelines for antibiotic susceptibility testing by the disc diffusion method are incomplete for many strains, the breakpoint values established for other species of the same phylum or family are often used, if available. Nevertheless, such approach may lead to discrepancies, albeit is widely adopted in the literature [12].

Only strains identified as the same to the genus level were subjected to the antibiotic resistance testing. Among the identified bacteria, only *Pseudomonas* spp. and *Acinetobacter* spp. are included in the EUCAST and CLSI guidelines. Therefore, to expand the range of the study, the breakpoints established by other authors for other species against the antibiotics used in this study were adopted from the literature.

For *Acinetobacter* spp., the breakpoints from EUCAST, CLSI and the literature were used. Narciso-da-Rocha et al. [4] established epidemiological cut-offs (ECOFFs) values, i.e., the breakpoint for the differentiation between wild type and non-wild type. These criteria may be used to conclude about acquired resistance of *Acinetobacter* spp. strains. For *Brevundimonas* spp., the same criteria as for *Acinetobacter* were applied, as indicated in CLSI and by Corno et al. [13]. For *Pseudomonas* spp., the criteria from EUCAST, CLSI and the literature [14] were used. In the case of *Bacillus* spp., EUCAST does not provide any breakpoints, while the CLSI M45 document gives only MICs values, therefore the interpretation of zone diameters values for the disc diffusion method was adopted from the literature [15–17]. However, it is worth to note, that these zone diameters are dedicated to procedures deviated from EUCAST standards. For *Micrococcus luteus* the CLSI criteria of *Staphylococcus* spp. were adopted, as suggested by Corno et al. [13].

From the 17 tested strains, 12 were revealed to show resistance. From the antibiotics tested in this study, only clindamycin and erythromycin were efficient against all the strains, but this could be due to the lack of possibility to establish resistance profiles of the strains. Two strains, i.e., 26a and 9a, may be considered multi-drug-resistant (MDR), i.e., resistant to antibiotics from 3 or more groups. As the strains were incubated at temperatures assumed as optimal for their growth, i.e., 22 °C and 37 °C, the presented results may be considered only as preliminary.

In the case of strains No. 22a, 28a, and 14b, resistance profiles could not be established due to the lack of guidelines. Nevertheless, Falcone-Dias et al. [18] determined

resistance phenotypes of *Brevundimonas*, *Arthrobacter*, *Microbacterium*, *Bacillus*, and *Lysinibacillus* genera representatives for tetracycline, cephalothin and streptomycin (among other) using the disc diffusion method, quoting CLSI databases (dated 2007), but, unfortunately, the authors did not present the assumed zone diameters. Bzdil et al. [19] also determined the susceptibility of, i.a., *Bacillus* spp. and *Lysinibacillus* spp. isolated from horse lesions using the disc diffusion method with reference to CLSI, but the applied zone diameters were not presented, as well. Hleba et al. [20] determined antibiotics resistance of, i.a., *Pseudomonas* spp., *Acinetobacter* spp., *Micrococcus luteus*, *Bacillus cereus*, *Lysinibacillus sphaericus* using the disc diffusion method quoting CLSI, but also did not presented the zone diameters values. Due to the lack of data concerning the interpretation of the disc diffusion method′s results, Corno et al. [13] implemented zone diameters established for other species, assigning them to *Brevundimonas intermedia* and *Micrococcus luteus*. Rahman et al. [21] considered all obtained isolated as resistant, when zone diameter was ≤16 mm. Similarly, Ankolekar et al. [22] used interpretive standards not specified in the research paper. Moreover, the authors applied the modified procedure of Mueller-Hinton inoculation. Some authors follow the commercial kits outlines [15–17, 22] and until these criteria are accepted by international committees, the results may be considered valid. On the other hand, many research papers do not provide sufficient data to evaluate the reliability of their findings. Without established guidelines, even if obtained zone diameters are equal 0 mm, the interpretation should be limited, as the various assays of drug in disc may influence the results. For example, too low assay may contribute to the false-positive outcomes. Moreover, the intrinsic resistance of the species must be taken into account. Similar observations were made by Chaves et al. [23], who investigated *Bacillus cereus* in terms of antibiotic resistance to vancomycin, gentamicin, tetracycline and clindamycin according to CLSI guidelines by the MIC method. The authors admitted that they need to question the results obtained by other researchers, as the resistance phenotypes of *B. cereus* determined based on the disc diffusion method should not be considered indisputable without international committees' acceptance.

The need to create disc diffusion guidelines for environmental strains is indirectly indicated by Zhang et al. [24] due to the emergence of resistant *Brevundimonas* spp.; the authors admitted, that CLSI outlines are sometimes neglected in the literature. Strains as *Brevundimonas vesicularis* might contribute to fatal infections [25, 26], so the disc diffusion method outlines need to be broaden.

The PCR results obtained in this study do not provide an evidence for widespread prevalence of ARGs in tap water strains. Although β-lactamase genes are often found in *Enterobacteriaceae* [8], the horizontal gene transfer allows for genetic exchange of ARGs by bacteria from distant genera [7]. However, the occurrence of *bla* genes in the selected strains was very low, as only one strain, identified as *Bacillus pumilus*, possessed the bla_{TEM} sequence.

5. CONCLUSIONS

- The microbial water quality indicates sufficient water treatment, but also the possibility of accidental contaminations. However, all the results were in accordance with the regulations of Polish law.
- Despite the application of two independent molecular methods and one biochemical method, the unquestionable identification of all selected strains was impossible, indicating the specificity of tap water isolates.
- Microorganisms dwelling in the water distribution system in Wrocław belong mainly to the phyla: *Actinobacteria*, *Proteobacteria* (*α*-, *β*-, *γ*-*Proteobacteria*) and *Firmicutes*.
- 12 of 17 tested strains were resistant to at least one antibiotic chosen for this study; 2 strains considered MDR were identified as *Brevundimonas* spp. and *Pseudomonas* spp.
- The antibiotic resistance profiles described in the literature should be considered cautiously, as international committees do not provide guidelines for many environmental strains.

ACKNOWLEDGEMENTS

The authors thank the Municipal Water and Sewerage Company in Wrocław for the provided information and substantive assistance. The work was co-realized within the allocation No. 0402/0138/17 awarded for the Faculty of Environmental Engineering, Wrocław University of Science and Technology by the Ministry of Science and Higher Education in years 2017–2018.

REFERENCES

[1] GRABIŃSKA-ŁONIEWSKA A., WARDZYŃSKA G., PAJOR E., KORSAK D., BORYŃ K., *Transmission of specific groups of bacteria through water distribution system*, Pol. J. Microbiol., 2007, 56 (2), 129.
[2] Regulation of the Minister of Health from December 7, 2017 on the quality of water intended for human consumption (in Polish), Dz. U. 2017, poz. 2294.
[3] HOLINGER E.P., ROSS K.A., ROBERTSON C.E., STEVENS M.J., HARRIS J.K., PACE N.R., *Molecular analysis of point-of-use municipal drinking water microbiology*, Water Res., 2014, 49, 225.
[4] NARCISO-DA-ROCHA C., VAZ-MOREIRA I., SVENSSON-STADLER L., MOORE E.R., MANAIA C.M., *Diversity and antibiotic resistance of Acinetobacter* spp. *in water from the source to the tap*, Appl. Microbiol. Biotechnol., 2013, 97 (1), 329.
[5] VAZ-MOREIRA I., EGAS C., NUNES O.C., MANAIA C.M., *Bacterial diversity from the source to the tap. A comparative study based on 16S rRNA gene-DGGE and culture-dependent methods*, FEMS Microbiol. Ecol., 2013, 83 (2), 361.
[6] ANGELETTI S., *Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology*, J. Microbiol. Methods, 2017, 138, 20.
[7] MARTINEZ J.L., *The role of natural environments in the evolution of resistance traits in pathogenic bacteria*, Proc. R. Soc. B, 2009, 276, 2521.
[8] KORZENIEWSKA E., KORZENIEWSKA A., HARNISZ M., *Antibiotic resistant Escherichia coli in hospital and municipal sewage and their emission to the environment*, Ecotoxicol. Environ. Saf., 2013, 91, 96.

[9] MELLMANN A., CLOUD J., MAIER T., KECKEVOET U., RAMMINGER I., IWEN P., DUNN J., HALL G., WILSON D., LASALA P., KOSTRZEWA M., HARMSEN D., *Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria*, J. Clin. Microbiol., 2008, 46 (6), 1946.
[10] BIZZINI A., JATON K., ROMO D., BILLE J., PROD'HOM G., GREUB G., *Matrix-assisted laser desorption ionization–time of flight mass spectrometry as an alternative to 16s RRNA gene sequencing for identification of difficult-to-identify bacterial strains*, J. Clin. Microbiol., 2011, 49 (2), 693.
[11] CHRIST A.P.G., RAMOS S.R., CAYÔ R., GALES A.C., HACHICH E.M., SATO M.I.Z., *Characterization of Enterococcus species isolated from marine recreational waters by MALDI-TOF MS and Rapid ID API® 20 Strep system*, Mar. Pollut. Bull., 2017, 118 (1, 2), 376.
[12] ŁEBKOWSKA M., *The occurrence of antibiotic-resistant bacteria in water intended for human consumption*, Ochr. Środ., 2009, 31 (2), 11 (in Polish).
[13] CORNO G., COCI M., GIARDINA M., PLECHUK S., CAMPANILE F., STEFANI S., *Antibiotics promote aggregation within aquatic bacterial communities*, Front. Microbiol., 2014, 5 (297), 1.
[14] VAZ-MOREIRA I., NUNES O.C., MANAIA C.M., *Diversity and antibiotic resistance in Pseudomonas spp. from drinking water*, Sci. Total Environ., 2012, 426, 366.
[15] BANERJEE M., SARKAR P.K., *Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic microorganisms from spices*, Food Microbiol., 2004, 21, 335.
[16] ROY A., MOKTAN B., SARKAR P.K., *Characteristics of Bacillus cereus isolates from legume-based Indian fermented foods*, Food Control, 2007, 18, 1555.
[17] KUMARI S., SARKAR P.K., *Prevalence and characterization of Bacillus cereus group from various marketed dairy products in India*, Dairy Sci. Technol., 2014, 94, 483.
[18] FALCONE-DIAS M.F., VAZ-MOREIRA I., MANAIA C.M., *Bottled mineral water as a potential source of antibiotic resistant bacteria*, Water Res., 2012, 46, 3612.
[19] BZDIL1 J., HOLY O., CHMELAR D., *Gram-positive aerobic and microaerophilic microorganisms isolated from pathological processes and lesions of horses*, Vet. Med., 2017, 62 (1), 1.
[20] HLEBA L., PETROVÁ J., ČUBOŇ J., KAČÁNIOVÁ M., *Antibiotic resistance of microbial contaminations isolated from husbandry animals and foodstuffs*, Anim. Sci. Biotech., 2014, 47 (1), 104.
[21] AB RAHMAN N., CHOWDHURY A.J.K., ABIDIN Z.A.Z., *Antibiotic resistant bacteria from sediment of coastal water of Pahang, Malaysia*, J. Technol., 2015, 77 (24), 65.
[22] ANKOLEKAR C., RAHMATI T., LABBÉ R.G., *Detection of toxigenic Bacillus cereus and Bacillus thuringiensis spores in U.S. rice*, Int. J. Food Microbiol., 2009, 128, 460.
[23] CHAVES J.Q., PIRES E.S., VIVONI A.M., *Genetic diversity, antimicrobial resistance and toxigenic profiles of Bacillus cereus isolated from food in Brazil over three decades*, Int. J. Food Microbiol., 2011, 147 (1), 12.
[24] ZHANG C.C., HSU H.J., LI C.M., *Brevundimonas vesicularis bacteremia resistant to trimethoprim-sulfamethoxazole and ceftazidime in a tertiary hospital in southern Taiwan*, J. Microbiol. Immunol. Infect., 2012, 45 (6), 448.
[25] SHANG S.T., CHIU S.K., CHAN M.C., WANG N.C., YANG Y.S., LIN J.C., CHANG F.Y., *Invasive Brevundimonas vesicularis bacteremia: two case reports and review of the literature*, 2012, 45 (6), 468.
[26] PAPAEFSTATHIOU C., CHRISTOPOULOS C., ZOUMBERI M., DIAKAKIS A., GEORGALAS P., KOUPPARI G., *Fatal community-acquired Brevundimonas vesicularis bacteremia in an elderly patient*, Clin. Microbiol. Newsl., 2005, 27 (7), 57.