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# SEASONAL VARIABILITY OF ANTIBIOTIC RESISTANCE AND BIODIVERSITY OF TAP WATER BACTERIA IN WROCŁAW, POLAND

Antibiotic resistance of bacteria has become a worldwide problem. Drinking water distribution systems (DWDSs) can be regarded as reservoirs of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). This study aims to provide a preliminary evaluation of seasonal changes in the occurrence of ARB and ARGs in Wrocław tap water samples. It also investigates the biodiversity of bacterial communities dwelling in Wrocław DWDS and compares them with worldwide literature reports. Third generation cephalosporins resistant bacteria were present in each season, with relative abundances reaching from 40.46% in spring to 99.86% in summer. β-lactams and tetracyclines resistant bacteria were present only in spring and autumn samples, with relative abundances reaching from 0.51% to 3.80%. Relative abundances of ARB fluctuated across the year, and no season-dependent trend was found. This suggests that other factors influenced the resistance phenomenon in Wrocław tap water. The investigated resistomes were represented only by several ARGs (*qnr*B, *tet*W, *erm*B, *qac*EΔ1). Class 1 integrons gene *int*I1 was also detected. Biodiversity of bacteria collected from large amounts of tap water was similar to that reported previously for Wrocław and worldwide DWDSs, with a prevalence of *Proteobacteria*, followed by *Actinobacteria, Cyanobacteria*, and *Firmicutes*.

## 1. INTRODUCTION

Antibiotic resistance of bacteria has become a global threat to human health and life [1, 2]. The resistance phenomenon can be investigated in the context of phenotypical resistant bacteria, able to grow at laboratory conditions, and genes encoding resistance mechanisms (so-called resistomes [3]). Antibiotic-resistant bacteria (ARB) are known to exist and proliferate in aquatic environments, and contribute to further dissemination of resistance [1, 4]. Nevertheless, the role of tap water bacteria in driving resistomes

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needs to be further investigated [4]. It is assumed that selective pressure exerted by disinfection processes in water treatment plants (WTPs) could contribute to proliferation of ARB in drinking water distribution systems (DWDSs), because disinfectant-tolerant bacteria, able to survive in disinfected tap water, are more likely to be antibiotic resistant [1, 2, 4, 5]. Moreover, free-DNA present in tap water samples as an effect of bacterial cell destruction during water treatment can potentially interact with biofilms in DWDSs (including opportunistic pathogens) and intestinal bacteria of drinking water consumers via horizontal gene transfer (HGT) [1]. Therefore, tap water can be regarded as a resistance reservoir [1, 2, 4, 5].

Because tap water is the primary source of drinking water for many people, it should be safe to drink and free of excessive microbiological contaminants, particularly opportunistic pathogens, and ARB. WTPs are obliged to provide drinking water suitable for consumption in terms of physicochemical and microbiological properties [6]. However, some microorganisms can survive all treatment steps, including disinfection, and may enter distribution networks. Despite the sufficient quality of drinking water produced in WTPs, its characteristics may also deteriorate through pipeline transfer due to the presence of pipe-origin contaminants. Accidental pipeline failures may contribute to the intrusion of external contaminants to distribution systems [7]. Furthermore, internal installations of individual buildings may adversely affect tap water quality. All of the above may contribute to the occurrence of undesired microorganisms in tap water.

Biodiversity of bacteria dwelling in DWDSs has been described in global literature [2, 7–12]. The majority of studies point to *Proteobacteria* as the most common phylum occurring in DWDSs. Next to *Proteobacteria*, among others *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes*, and *Planctomycetes* were frequently found in tap water and WTPs samples. Nevertheless, the vast majority of prokaryotic species present in DWDSs remain unknown or poorly understood [13].

Few studies have attempted to characterize spatiotemporal changes in bacterial community composition [7, 10, 12, 14–16], suggesting a major role of temperature, water source, and disinfection processes in shaping bacterial biodiversity in DWDSs. Some pathogens were found in tap water samples [8, 9, 16, 17], highlighting the need for improvement of the existing routine monitoring, so far primarily performed by culture-dependent methods.

Many studies to date have detected ARB and antibiotic resistance genes (ARGs) in tap water [1, 2, 4, 18, 19], although the seasonal variability of occurrence of ARB and ARGs in tap water has poorly been investigated. This study aims at filling this gap. To provide representative samples for each season and concentrate the planktonic bacterial biomass of tap water, the study employed a water purifier with a set of filters and a capillary membrane, like the studies of Shi et al. [2] and Ma et al. [4]. Because many environmental bacteria remain unculturable [2], both culture-dependent and culture-independent methods were applied. The study attempted the following: (i) preliminary evaluation of potential seasonal fluctuations of occurrence of ARB and ARGs in

Wrocław tap water, and a potentially observed pattern of such changes, (ii) investigation of the biodiversity of bacterial community dwelling in Wrocław tap water, including the occurrence of opportunistic pathogens; (iii) comparison of bacterial community composition with global literature reports. To the authors' best knowledge, it is the first study concerning antibiotic resistance and biodiversity of bacterial communities of Wrocław tap water collected through a long-term, capillary membrane filtration process.

## 2. MATERIALS AND METHODS

*Sample collection*. Tap water samples were collected utilizing a set of water purification filters (FP3-HJ-K1, Aquafilter Europe), presented in Fig. 1. The set consisted of:

- sediment filter made of polypropylene nonwoven – mechanical pre-purification of water from sediments of particles larger than 5 μm, such as sand, mud, rust, etc.,
- softening and iron-removing filter – ion-exchange reducing water hardness,
- activated carbon filter with a bacteriostatic agent – reduction of heavy metals, hydrogen sulfide, phenol, benzene, pesticides, chlorine,
- capillary membrane – elimination of particles larger than 0.02 μm.

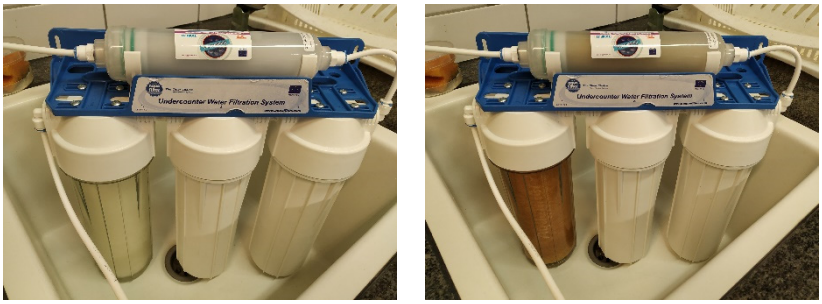


Fig. 1. Set of water purification filters: before (left) and after water filtration (right)

The set of water purification filters was installed at the Laboratory of Environmental Biotechnology of the Wrocław University of Science and Technology. Each sample was collected by filtering approximately 9000 $dm^3$ of tap water for 48 h. For assessment of seasonal variations, sampling was repeated in winter, spring, summer, and autumn of 2019. Before sampling, water was flushed for 5 min to avoid filtration of stagnated water [20]. The source water originates from the Oława and Nysa Kłodzka Rivers. After ground infiltration, it is treated by WTP Na Grobli. No flushing of the supply network or any failures were reported during any sampling campaign.

After water filtration, the capillary membrane was cut off in a sterile manner, submerged in 0.5 $dm^3$ of physiological solution (0.85% NaCl), and shaken for 1 h at 240 rpm at room temperature (Heidolph Unimax 1010). Next, the remaining bacterial

cells were detached using an ultrasound bath at 35 kHz (POLNED). To avoid cell destruction, ultrasounds were applied for only 30 s. The resulting suspension was divided:

- for inoculations (i.e., culture-dependent methods), the suspension was used immediately,
- for DNA extraction (i.e., culture-independent methods), the suspension was centrifuged for 10 min at 8000 rpm at 4 °C (Sigma 2K15), then the supernatants were decanted, and the pellets were frozen and stored at –20 °C.

*Relative abundances of ARB*. Relative abundances of bacteria resistant to β-lactams (β), fluorochinolones (FQ), 3rd generation cephalosporins (3GC), and tetracyclines (T) in heterotrophic plate count (HPC) were determined. These antibiotics were selected for the study because they belong to the most commonly consumed groups of antibiotics in Poland [21].

Table 1

Agar media supplementation for enumeration of HPC and ARB

| Agar medium | Drug and concentration | Purpose |
|---|---|---|
| R2A | – | psychrophilic HPC |
| R2A + β | Amoxicillin, 8 mg/dm$^3$ | % of bacteria resistant to β-lactams |
| R2A + FQ | Ciprofloxacin, 2 mg/dm$^3$ | % of bacteria resistant to fluoroquinolones |
| R2A + 3GC | Ceftazidime, 8 mg/dm$^3$ | % of bacteria resistant to 3rd generation cephalosporins |
| R2A + T | Tetracycline, 16 mg/dm$^3$ | % of bacteria resistant to tetracyclines |

A series of dilutions in the range of $10^0$–$10^{-5}$ was prepared from the initial suspension. Then, the dilutions were inoculated on R2A (BTL) plates and R2A (BTL) plates supplemented with antibiotics (Sigma-Aldrich) for HPC and ARB enumeration, respectively. The plates with antibiotics were prepared following Andrew et al. [22] and EUCAST [23] guidelines. The detailed information of media supplementation is presented in Table 1. For quality control, the *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains (BioMaxima) were inoculated on each batch of prepared plates. The plates were incubated for 7 days at 22 °C. Only plates with a CFU number in the range of 30–300 were subject to calculations.

*DNA extraction*. The pellets were subject to DNA extraction employing a DNeasy PowerBiofilm kit (QIAGEN) following the manufacturer's instructions. To provide the equability of DNA extraction for each sample, each pellet collected on a given sampling date was extracted thrice. Then, the three extracts obtained for each sample were pooled to represent seasons: winter, spring, summer, and autumn. DNA concentration and purity were measured using a NanoPhotometer N60 (IMPLEN).

*Detection of ARGs in environmental DNA*. PCRs were conducted to detect ARGs and other genes related to HGT or resistance mechanisms: *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{OXA-48}}$, *amp*C, *mec*A, *qnr*A, *qnr*B, *qnr*S, *oqx*B, *tet*A, *tet*K,

*tet*L, *tet*W, *sul*I, *sul*II, *erm*A, *erm*B, *van*A, *mcr*-1, *mex*A, *flo*R, *qac*EΔ1, *qac*H, *tol*A, *int*I1, *tnp*A in environmental DNA samples, while 16S rRNA gene was amplified in terms of DNA quality control. Annealing temperatures ($T_a$), amplicon sizes and primer sequences with appropriate references have been reported elsewhere [24].

The PCR mixture consisted of 0.004 $cm^3$ of 5xGold Hot Start PCR Mix LOAD (SYNGEN), 0.0004 $cm^3$ of each 10 μM primer, 0.002 $cm^3$ of DNA, and 0.0132 $cm^3$ of water (A&A Biotechnology). The PCR protocols were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at $T_a$ for 30 s, elongation at 72 °C for 30 s, and final elongation at 72 °C for 7 min. In each reaction, negative and positive controls were applied. After PCR amplification, $0.005\times10^{-3}$ $cm^3$ of each sample was separated by electrophoresis in 2% or 3% (depending on the amplicon size) agarose gel (Sigma-Aldrich) stained with Green DNA Gel Stain (SYNGEN). The products were electrophoresed at 120 V for 15 min and 80 V for 60 min in 1×TBE buffer and visualized by UV (UVITEC, Cambridge). The amplicon size was compared with DNA Marker pUC/Msp I (A&A Biotechnology).

*Biodiversity of bacterial communities.* Environmental DNA samples representing the microbial consortia dwelling in tap water in four seasons were subjected to NGS for investigation of the bacterial biodiversity of each sample. The analyses were conducted by Genomed (Poland). The metagenomic analysis was based on hypervariable region V3-V4 of the 16S rRNA coding gene. Gene libraries were prepared with primers 341F and 785R, using Q5 HotStart High-Fidelity DNA Polymerase (NEBNext), following the manufacturer's instructions. Sequencing was conducted on MiSeq (Illumina) in paired-end technology (2×250 nt), using MiSeq Reagent kit v2 (Illumina) following the manufacturer's instructions. After sequencing, samples were demultiplexed and classified using MiSeq Reporter v2.6, 16S Metagenomics protocol. The applied taxonomy database was GreenGenes v13.8.

## 3. RESULTS

The results of the relative abundances of bacteria resistant to each antibiotic group are presented in Table 2. Calculated mean values were rounded up to the nearest unit.

Table 2

Calculated bacterial concentration in tap water samples and relative abundance of ARB in total HPC

| Season | R2A | β | FQ | 3GC | T |
|---|---|---|---|---|---|
| | CFU/$cm^3$ of tap water | % in total HPC | | | |
| Winter | 4 | 0.00 | 0.00 | 78.13 | 0.00 |
| Spring | 2 | 2.92 | 0.00 | 40.46 | 0.51 |
| Summer | 17 | 0.01 | 0.00 | 99.86 | 0.00 |
| Autumn | 28 | 3.80 | 0.02 | 42.45 | 1.16 |

All samples gave positive results in 16S rRNA PCR, confirming the reliability of conducted reactions. The results of PCRs are presented in Table 3.

Table 3

Results of PCRs

| Gene | Gene classification, resistane target or mechanisms | Winter | Spring | Summer | Autumn |
|---|---|---|---|---|---|
| *bla*$_{TEM}$ | β-lactamase | – | – | – | – |
| *bla*$_{SHV}$ | | – | – | – | – |
| *bla*$_{CTX-M}$ | | – | – | – | – |
| *bla*$_{KPC}$ | | – | – | – | – |
| *bla*$_{NDM}$ | | – | – | – | – |
| *bla*$_{OXA}$ | | – | – | – | – |
| *bla*$_{OXA-48}$ | | – | – | – | – |
| *amp*C | | – | – | – | – |
| *mec*A | methicillin | – | – | – | – |
| *qnr*A | (fluoro)quinolones | – | – | – | – |
| *qnr*B | | + | – | – | – |
| *qnr*S | | – | – | – | – |
| *oqx*B | | – | – | – | – |
| *tet*A | tetracyclines | – | – | – | – |
| *tet*K | | – | – | – | – |
| *tet*L | | – | – | – | – |
| *tet*W | | – | + | + | – |
| *sul*I | sulfonamide | – | – | – | – |
| *sul*II | | – | – | – | – |
| *erm*A | erythromycin | – | – | – | – |
| *erm*B | | – | + | + | – |
| *van*A | vancomycin | – | – | – | – |
| *mcr*-1 | colistin | – | – | – | – |
| *mex*A | efflux | – | – | – | – |
| *flo*R | florfenicol | – | – | – | – |
| *qac*EΔ1 | quaternary ammonium compounds | – | – | + | – |
| *qac*H | | – | – | – | – |
| *tol*A | transmembrane activity | – | – | – | – |
| *int*I1 | class 1 integron | + | + | + | – |
| *tnp*A | transposon | – | – | – | – |
| Total | | 2 | 3 | 4 | 0 |

Unfortunately, only the samples of environmental DNA collected in spring and summer passed all NGS steps and provided reliable results. In the spring sample, 85 547 total reads were obtained, including 74 158 that passed through quality filtering. Percent total reads classified at each taxonomic level were as follows: 99.96% for kingdom, 98.63% for phylum, 98.11% for class, 96.97% for order, 95.06% for family, 93.60% for

genus and 34.68% for species. In the summer sample, 100 454 total reads were obtained, including 87 847 that passed through quality filtering. Percent total reads classified at each taxonomic level were as follows: 99.96% for kingdom, 98.49% for phylum, 97.96% for class, 96.46% for order, 94.716% for family, 93.12% for genus and 51.07% for species.

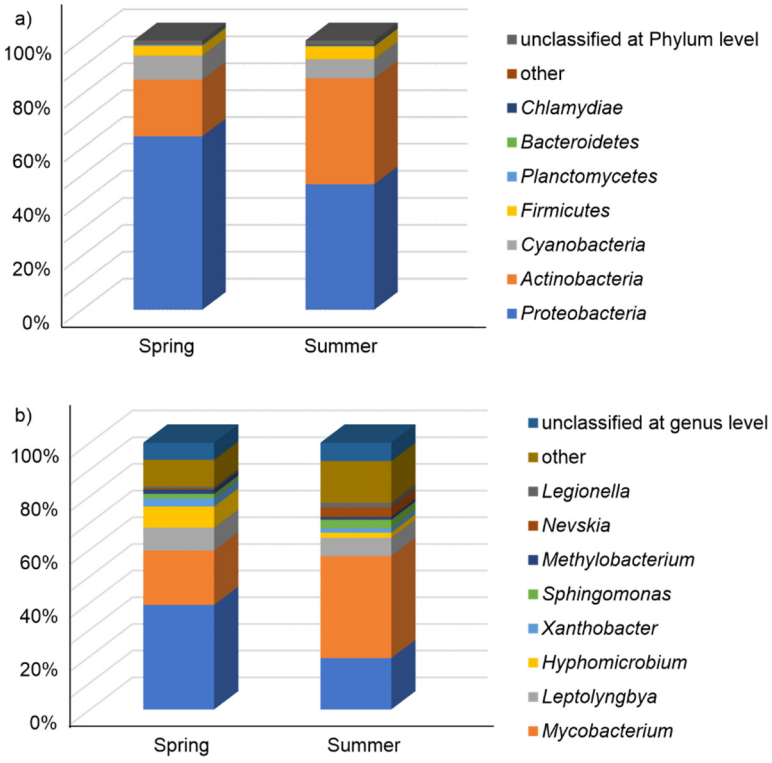


Figure 2. Relative abundances of bacteria at phylum level (a) and genus level (b) in spring and summer samples

Biodiversity of the spring and summer samples at the phylum and genus levels are presented in Fig. 2. The most abundant phyla in both samples were *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, and *Firmicutes*. *Planctomycetes*, *Bacteroidetes*, and *Chlamydiae* were also present in both samples. The dominant classes in phylum *Proteobacteria* in both samples were *Alpha-*, *Gamma-* and *Betaproteobacteria*. Among *Cyanobacteria*, the dominant class in both samples was *Synechococcophycideae*. Among *Firmicutes*, the dominant classes were *Clostridia* and *Bacilli*. *Actinobacteria* were primarily represented by order *Actinomycetales*.

In terms of biodiversity at the genus level, in the spring sample, phylum *Proteobacteria* was primarily represented by *Ancylobacter*, followed by *Hyphomicrobium*, *Xan-*

*thobacter*, *Sphingomonas*, and *Methylobacterium*, all belonging to *Alphaproteobacteria*, constituting 60.24% in classification at the class level. In the summer sample, phylum *Proteobacteria* was primarily represented by *Ancylobacter*, followed by *Nevskia*, *Sphingomonas*, *Hyphomicrobium*, and *Legionella*, including *Nevskia* and *Legionella* belonging to class *Gammaproteobacteria*. *Actinobacteria* were represented almost entirely by *Mycobacterium*, and the dominant genus of *Cyanobacteria* in both samples was *Leptolyngbya*. At the genus level, 387 and 421 taxonomic categories were identified in the spring and summer seasons, respectively.

None of the microbial indicators of drinking water quality mentioned in Polish Regulations [6], namely *E. coli*, *Enterococcus*, coliforms, or *Clostridium perfringers*, was detected in this study, although some pathogenic species from genus *Mycobacterium* were detected. More detailed information concerning representatives of genus *Mycobacterium* detected in the study is included in Table 4 (only species with relative abundance higher than 0.05% in at least one sample are listed).

Table 4

Relative abundance at the species level for *Mycobacterium* spp.

| Species | Relative abundance at the species level [%] | | Pathogenicity | Reference |
|---|---|---|---|---|
| | Spring | Summer | | |
| *M. diernhoferi* | 0.03 | 0.06 | + | [25] |
| *M. frederiksbergense* | 13.68 | 22.65 | – | |
| *M. gordonae* | 0.01 | 0.12 | + | [17] |
| *M. hackensackense* | 0.52 | 1.04 | + | [25] |
| *M. hodleri* | 0.12 | 0.00 | – | |
| *M. isoniacini* | 0.31 | 0.01 | – | |
| *M. lentiflavum* | 0.11 | 0.00 | – | [26] |
| *M. neglectum* | 0.03 | 0.23 | – | |
| *M. pinnipedii*[a] | 0.42 | 2.14 | + | |
| *M. salmoniphilum* | 0.01 | 0.05 | + | [17] |
| *M. smegmatis* | 0.18 | 0.33 | + | [26] |
| *M. tusciae* | 0.01 | 0.21 | – | |
| *M. ulcerans* | 0.26 | 3.80 | + | [26] |
| *M. vanbaalenii* | 0.01 | 0.12 | – | |

[a]Data from https://my.absa.org/tiki-index.php?page=Riskgroups

Next to *Mycobacteria*, genera *Acinetobacter* and *Clostridium* are considered opportunistic pathogens and indicators of insufficient water treatment because the latter is able to produce spores under environmental stress [8, 9]. Relative abundances of *Acinetobacter* were 0.05% and 0.04% in spring and summer, respectively, whereas those of *Clostridium* were 0.37% and 0.70% in spring and summer, respectively.

# 4. DISCUSSION

## 4.1. SEASONAL VARIABILITY OF ANTIBIOTIC RESISTANCE IN WROCŁAW TAP WATER

*Relative abundances of ARB*. ARB were detected in each sample. 3GC resistant bacteria dominated across the year, with evidently lower relative abundances of β and T resistant bacteria (only in spring and autumn). No relevant abundances of FQ resistant bacteria were determined. It seems that ambient temperature itself was not responsible for the detected seasonal changes because no ambient temperature-dependent trend could be observed in any group of the investigated ARB. At tap water temperature, mean values during the sampling campaigns probably varied too slightly (data not shown) to affect bacteria. Another factor potentially contributing to the occurrence of ARB in tap water is selective pressure exerted by the presence of drugs in the environment [1, 19]. Unfortunately, no precise data regarding seasonal antibiotic consumption in Poland are available [21], and concentrations of antibiotics in collected tap water samples were not measured in this study. Because the concentration of doxycycline (belonging to tetracyclines group) was below the limit of quantification in river water [27], it might be impossible to measure the concentration of antibiotics in tap water samples. Notice, however, that doxycycline concentrations in wastewater samples were higher in winter than in autumn [27].

Data on the prevalence of ARB in tap water vary between studies. For example, no cultivable ARB were found in tap water in southeast Louisiana, USA [19]. In contrast, bacteria resistant to amoxicillin, ciprofloxacin, chloramphenicol, gentamicin, rifampin, sulfisoxazole, and tetracycline were present in each tap water sample collected from four cities in Michigan and Ohio, USA [5]. Relative abundances of these ARB were in the range of 3.02–15.22%, 0.18–13.14%, and 0.04–3.78% for amoxicillin, ciprofloxacin, and tetracycline, respectively, exceeding values obtained in this paper for the same antibiotics. Unfortunately, no ceftazidime resistant bacteria were investigated in the study [5]. Moreover, intestinal bacteria resistant to ampicillin, cephalothin, tetracycline, chloramphenicol, and trimethoprim were found in tap water in Nanjing, China [2], demonstrating that antibiotic resistance is a global problem.

CFU calculated per 1 $cm^3$ of a tap water sample were far below limits stipulated by the Polish Regulation of the Ministry of Health [6], pointing to sufficient microbial quality of Wrocław tap water. Nonetheless, the HPC values obtained in this study are slightly higher than those obtained by Perrin et al. [11] in Parisian tap water that were in a range of 2–4 CFU/$cm^3$ over one-year sampling campaign [11]. The present study provides no clear evidence of the influence of ambient temperature on seasonal variation of HPC. The highest value was observed in autumn and lowest in spring. In contrast to results presented in this paper, Asghari et al. [15] determined HPC on R2A in Maku, Iran, in ranges of 25–60 CFU/$cm^3$ and 30–360 CFU/$cm^3$ in cold and warm seasons, respectively, suggesting seasonal trends. Moreover, according to Nescerecka et al. [14],

bacterial growth in a distribution system is dependent on the season and water source. The results of a study [14] conducted in WTPs and DWDS in Riga, Latvia, showed higher seasonal variability of bacterial concentration in surface water than in groundwater because the latter is less prone to seasonal temperature changes and other external factors related to seasons. Because tap water collected in the present study originated from surface water subject to infiltration (data from Municipal Water and Sewerage Company in Wrocław), it could acquire properties of both surface and groundwater. The study [14] showed clear seasonal variability of both total and intact cell counts in tap water samples, with higher bacterial counts in warmer months. No such trend was observed in the present study. Moreover, according to Nescerecka et al. [14], dominant bacterial growth occurs in a distribution system due to disinfectant decay. This study involved no measurement of residual disinfectant because instantaneous $Cl_2$ measurements were assumed not representative of a sample due to large amounts of filtrated tap water. The type of source water and residual disinfectant could obscure seasonal changes in HPC in Wrocław tap water.

Nevertheless, ARB relative abundances obtained in this study suggest a high prevalence of 3GC resistance among cultivable bacteria. Viable and cultivable resistant microflora is of particular importance from the consumer's point of view. Such bacteria can potentially infect drinking water consumers, and proliferate in their organisms. DWDS may also serve as an important reservoir for the dissemination of antibiotic resistance to recipient pathogens [1]. Therefore, not only phenotypical but also genetic resistance properties deserve attention.

*Detection of ARGs in environmental DNA.* Despite the filtration of large amounts of water for the preparation of each sample, only several genes were detected by PCRs. No β-lactamases encoding gene was detected in any of the samples, which could be surprising because high relative abundances of 3GC resistant bacteria were observed in all samples. Among genes conferring resistance to FQ, only *qnr*B was detected in the winter sample, although no relevant abundance of FQ resistant bacteria was observed. T resistance gene, namely *tet*W, was present in the spring and summer samples. Nevertheless, the occurrence of T resistant bacteria does not overlap with the presence of the *tet*W gene, because these bacteria were observed in relevant abundances in the spring and autumn samples. These findings suggest the importance of resistance mechanisms other than those encoded by the investigated ARGs in phenotypic resistance of bacteria dwelling in tap water. On the other hand, the presence of genes *qnr*B and *tet*W does not favor FQ resistance in the winter sample, or T resistance in the summer sample, respectively. Among other genes, *erm*B, encoding erythromycin resistance, was detected in the spring and summer samples, and *qac*EΔ1, encoding quaternary ammonium compounds resistance, was detected in the summer sample. Moreover, class 1 integronase gene *int*I1, one of mobile genetic elements [2, 4], was present in three of the tested samples.

The occurrence of ARGs in tap water samples varied between studies. For example, none of the six tested ARGs, namely *mec*A, *tet*A, *tet*W, *tet*X, *sul*I, and *erm*B, was detected in tap water in Louisiana, USA [19]. On the other hand, genes *cat*, *cmr*, $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, *sul*I, and *sul*II were found in tap water samples collected from four cities in the USA [5]. Moreover, Su et al. [18] found genes *qnr*S, *oqx*B, *qep*A, *tet*M, *tet*O, *tet*Q, *tet*W, *sul*1, *sul*2, *erm*B, *flo*R, *cfr*, *cml*A, *fex*A, and *fex*B in tap water samples in Guangzhou, China, and Shi et al. [2] found genes $bla_{\text{TEM-1}}$, *amp*C, *tet*A, *tet*G, *sul*I, *erm*A, and *erm*B by the PCR method in tap water in Nanjing, China. However, applying the metagenomics approach, 20 other ARGs were found in the study [2]. This demonstrates the limitations of the PCR method. Many ARGs relevant for the resistance of a given microbiota might be omitted with the PCR approach. It could partially explain the results of the present study.

Interestingly, Hao et al. [1] distinguished two groups of ARGs present in tap water, namely intracellular ARGs (iARGs) and extracellular ARGs (eARGs), the latter constituting free-living DNA fragments occurring in tap water. All 22 tested genes were present as iARGs in tap water samples collected over one year in Tianjin, China, including 15 present in each monthly sampling campaign. Moreover, 14 genes were present as eARGs. Noteworthy, Hao et al. [1] found a seasonal pattern in the occurrence of iARGs and eARGs in tap water samples – both most abundant in summer. Because only several ARGs were detected in the present study, no seasonal variation could be established.

*Relationship between ARB and NGS results*. The determined relative abundances of ARB show that almost all cultivable heterotrophic bacteria collected in the summer season were resistant to 3GC. It should be emphasized, however, that the ability of genera listed in NGS results to grow on R2A media in laboratory conditions is not evidenced. Therefore, it could not be excluded that phenotypical 3GC resistant bacteria constituted only a part of the tap water community collected in the summer season. In favorable laboratory conditions, these bacteria could proliferate and mask the non-cultivable tap water microflora. The determined relative abundances of ARB cannot be directly linked with the results of NGS, because these findings consider viable and cultivable resistant bacterial microflora and total bacteria present in a sample, respectively. Henne et al. [20] claimed that bacteria of high abundances do not have to be the most active ones in communities. According to Perrin et al. [11], only 1.3–1.8% of taxa detected via NGS were also observed using culture-dependent methods. Interestingly, Perrin et al. [11] found that cultivated microflora was dominated by *Mycobacteria* and *Sphingomonadaceae*, constituting a large part of NGS reads presented in this study. The discrepancies between culture-independent and culture-dependent methods were highlighted by Boers et al. [13], who claimed that the micelle PCR/NGS approach permitted detecting microbiological changes along WTP and DWDS, while HPC or ATP measurement proved to be insufficient for that purpose.

A large-scale study [4] on ARGs prevalence in tap water samples points to *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Methylobacterium*, *Methyloversatilis*, *Mycobacterium*, *Polaromonas*, and *Pseudomonas* as hosts of ARGs. Among these genera, *Methylobacterium* and *Mycobacterium* were found in relatively high abundances in the present study. Moreover, *Polaromonas* and *Pseudomonas* were detected in both spring and summer samples, but in relative abundances not exceeding 0.11% (data not shown). Similarly, Su et al. [18] suggested that *Pseudomonas* could be an ARGs carrier. Although some putative resistance vectors were detected in the present study, further research is needed to establish the relationships between phenotypical ARB and total biodiversity of bacteria dwelling in Wrocław tap water.

### 4.2. BIODIVERSITY OF BACTERIAL COMMUNITIES DWELLING IN WROCŁAW TAP WATER

The study results correspond with the findings presented in the literature [2, 7–12]. At the phylum level, bacterial communities dwelling in Wrocław tap water were dominated by *Proteobacteria* in the spring and summer samples. The predominance of *Proteobacteria* over *Actinobacteria*, however, is more evident in the spring sample than in the summer sample, where relative abundances of these two phyla are more approximate. This suggests that *Actinobacteria* could take prevalence over *Proteobacteria* in the summer season. In the case of less abundant phyla, differences in their relative abundances are negligible. The study results are following previous findings concerning the bacterial biodiversity of Wrocław consumer tap water [8]. Nevertheless, the study [8] showed no *Cyanobacteria* and relative abundances of *Firmicutes* higher than in the present study in the majority of samples.

Considering the results of seasonal biodiversity changes at the genus level, the prevalence of *Mycobacterium* over *Ancylobacter* was observed in the summer sample in comparison to the spring sample. Moreover, changes in *Alphaproteobacteria* representatives were observed because *Hyphomicrobium*, *Xanthobacter*, and *Methylobacterium* relative abundances decreased, while *Sphingomonas* slightly increased from spring to the summer season. Relative abundances of *Gammaproteobacteria* representatives, namely *Nevskia* and *Legionella*, increased in the summer sample, suggesting that these genera could benefit from higher ambient temperatures. Similar discrepancies were found in bacterial community changes in samples collected from DWDS in Ann Arbor, USA [10]. At the class level, *Alpha-* and *Betaproteobacteria* constituted the majority in winter and summer months, respectively. At the genus level, however, this trend was not so obvious, because the most dominant OTUs belonging to *Betaproteobacteria*, namely *Hydrogenophaga*, reached higher abundances in winter seasons [10].

In this study, richness, i.e., the observed taxonomic categories at the genus level, was higher in the summer sample than in the spring sample which is consistent with the results obtained by Pinto et al. [10]. Contrary, richness was found to be higher in winter

than in summer months in DWDS in South Africa [12]. This issue highlights the complexity of bacterial community compositions in DWDSs.

*Occurrence of opportunistic pathogens in Wrocław tap water.* Many *Mycobacterium* species were detected in this study, including opportunistic pathogens and non-tuberculosis mycobacteria (NTM) [17, 25, 26]. Potentially identical 16S rRNA gene sequences of different NTM species limit the applicability of the sequencing method for investigating NTM populations [17]. Therefore, the species identification presented in this paper should be approached with caution. Nevertheless, the presence of *Mycobacteria* in relatively high abundances, particularly in the summer sample, draws attention.

Other examples of genera detected in this study but particularly undesired in tap water include *Acinetobacteria* and *Clostridia* [8, 9]. The latter may indicate the insufficiency of disinfection processes at WTP or former contamination of tap water, due to its ability to produce spores [8]. Bacteria could enter DWDS in the events of failures and renovations, and remain in the distribution network, both in planktonic and biofilm forms [7, 8]. These results demonstrate that despite overall sufficient microbial quality of tap water, the occurrence of opportunistic pathogens in consumers' point-of-use taps is inevitable.

### 4.3. COMPARISON OF THE COMPOSITION OF THE BACTERIAL COMMUNITY OF WROCŁAW TAP WATER WITH GLOBAL LITERATURE REPORTS

An insight into the composition of the bacterial community of Wrocław tap water could contribute to broadening knowledge concerning this issue. Many parallels are observed between DWDSs worldwide. For example, the predominance of *Alpha-* and *Betaproteobacteria* in DWDS was found in Pittsburg, USA [7]. Next to these dominant classes, *Gammaproteobacteria* were present in relatively low abundances in autumn, winter, and spring samples, and *Actinobacteria* and *Firmicutes* were present only in the winter sample [7]. Moreover, among the genera detected in the present study, *Hyphomicrobium*, *Sphingomonas*, *Methylobacterium*, and *Nevskia* (among most dominant, as presented in Fig. 2), as well as *Sphingopyxis*, *Delftia*, *Gallionella*, *Acinetobacter*, and *Pseudomonas* (not exceeding 1% of relative abundance, data not shown) were also found in Pittsburg DWDS [7], emphasizing similarities between these studies. Furthermore, *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, and *Actinobacteria* dominated in DWDS in Cali, Colombia [9], which is consistent with the results of the present study despite the different climate conditions and geographical distance between Cali and Wrocław. The fact that Cali is supplied by surface water and chlorine is maintained in the distribution network supports the hypothesis that the most important factors shaping bacterial communities in DWDSs are the type of source water and disinfection regime [12–14]. Among dominant genera in Cali, *Mycobateria*, *Sphingomonas*, *Methylobacterium*, *Bacillus*, and *Phyllobacteria* were also detected in Wrocław (the two last ones in relative abundances <1.0%, data not shown), whereas neither *Cyanothece* nor *Brucella* were found in the present study.

Interestingly, a high relative abundance of *Cyanobacteria* was reported in DWDS in Cali [9], which is consistent with the results presented in this paper. Nevertheless, because *Cyanobacteria* are known to be photosynthetic microorganisms, their ability to survive in a dark environment of DWDS requires explanation [9]. Moreover, it is worth mentioning that the detected richness and diversity of bacteria dwelling in biofilms was higher than in bulk water samples of DWDS in Cali [9].

In a large-scale study [11], 368 samples collected over one year from WTPs and DWDSs in Paris, France, were subject to NGS. The results revealed that relative abundances of genera *Phreatobacter*, *Maricaulis*, and *Mycobacterium* were higher in warm seasons, whereas relative abundances of genera *Hyphomicrobium* and *Pseudomonas* were higher in cold seasons. These findings are consistent with the results of the present study for *Mycobacterium*, *Hyphomicrobium*, and *Pseudomonas* (the last one was present in relative abundances <1.0% in both samples in this study), although neither *Phreatobacter* nor *Maricaulis* were detected in tap water samples investigated in this paper. However, *Sphingomonas* relative abundance was higher in the cold period in Parisian tap water [11], and lower in the spring sample than in the summer sample in Wrocław tap water. In contrast, out of seven most dominant OTUs detected in the study [13], only families *Gallionellaceae* and *Comamonadaceae* were present in samples investigated in this paper, with relative abundances <1.0% at the family level (data not shown). The observed difference in bacterial community composition may result from different source water and disinfection regime. The water sources included surface water and surface water after infiltration in the Netherlands [13] and Wrocław, respectively. Moreover, $ClO_2$ was added only before water storage in a reservoir and was not maintained in the DWDS in the Netherlands [13], whereas disinfectants, namely $Cl_2$ and $ClO_2$, are maintained in the Wrocław distribution system.

### 4.4. IMPLICATIONS

This study aimed at a preliminary investigation of the antibiotic resistance and biodiversity of bacteria dwelling in Wrocław tap water collected in one point-of-use in terms of seasonal variability. Both phenotypical (ARB) and genetic (ARGs) aspects of bacterial antibiotic resistance were investigated. The results show seasonal changes in ARB relative abundances in Wrocław tap water, although no ambient temperature-dependent trend was observed. Therefore, other factors seem to have influenced ARB prevalence in the tested samples. Because only several genes were detected in this study, namely *qnr*B, *tet*W, *erm*B, *qac*EΔ1, and *int*I1, the role of tap water as a reservoir of ARGs [1, 2, 4, 5] is questionable. Nevertheless, it seems that other ARGs, not included in this study, may confer resistance to ARB detected by the culture-dependent method. Moreover, bacterial biodiversity in two samples, collected in spring and summer, was investigated, demonstrating the differences in the community composition in these sea-

sons. Opportunistic pathogens were unfortunately detected in Wrocław tap water. Noteworthy, taxonomic categories detected in this study and their relative abundances are consistent with literature reports concerning bacterial biodiversity of other DWDSs worldwide, contributing to current knowledge regarding this issue.

Due to large amounts of tap water (up to 9000 $dm^3$) and a long collection time (48 h) provided in this study for sample collection, incidental phenomena such as biofilm detachments should be mitigated, and samples should be representative of the sampling season. Attempts to discover trends in bacterial structure, however, require further research. In future studies, NGS analyses should cover all four seasons. Moreover, the discrepancies in the obtained results encourage conducting a large-scale and long-temporal study for broadening the insight into the spatiotemporal variability of antibiotic resistance and biodiversity of bacteria dwelling in Wrocław tap water.

## 5. CONCLUSIONS

- The occurrence of ARB was detected in every sample collected over one year. 3GC resistant bacteria were present in each sample, with relative abundances reaching from 40.46% in spring to 99.86% in summer, whereas FQ resistant bacteria were absent in each sample. β and T resistant bacteria were present only in the spring and autumn samples, with relative abundances reaching from 0.51% to 3.80%.
- Only several genes, namely *qnr*B, *tet*W, *erm*B, *qac*EΔ1, and *int*I1, were detected. The majority of investigated ARGs were not detected in any sample. This suggests the contribution of other factors or other ARGs, not included in this study, to the phenotypical resistance phenomenon of bacteria dwelling in tap water. The resistance mechanisms of cultivated 3GC resistant bacteria need to be further elucidated.
- Wrocław tap water meets the legislation requirements, but the high relative abundance of ARB draws attention and indicates the need for further research. Nevertheless, the issue of antibiotic resistance in drinking water is not covered by the law. Moreover, some opportunistic pathogens of genera *Mycobacterium*, *Acinetobacter*, and *Clostridium* were detected in this study.
- The biodiversity of bacteria differed between spring and summer seasons. The NGS results of this study are consistent with scientific reports concerning the biodiversity of tap water bacteria worldwide.
- This study can contribute to the current knowledge regarding the dissemination of resistance in DWDS environments, and potential factors influencing this phenomenon as well as the biodiversity of tap water bacteria.

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