**Escherichia coli** and **Serratia fonticola** ESBLs as a potential source of antibiotics resistance dissemination in the Tricity water reservoirs*

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Despite the fact that cephalosporins are rarely used in medical or veterinary treatment, the presence of Enterobacteriales strains resistant to this group of anti-bacterial drugs (ESBL) is an important issue that requires attention. Between 2019 and 2021, 14 retention reservoirs, 12 streams, 3 rivers and 1 lake situated in the Tricity area (in northern Poland) were sampled for the presence of ESBL strains. Out of 40 water samples, characteristic growth (**Escherichia coli** and the KESC group) on Chromagar ESBL plates was observed for 33 samples. The average number of ESBL **E. coli** was 42±132 CFU/100 ml, while the KESC group was 73±147 CFU/100 ml. Out of 33 positive samples, 57 ESBL Enterobacteriales strains were isolated, of which the most abundant species were **E. coli** (13 isolates) and **Serratia fonticola** (23 isolates). The **E. coli** ESBL isolates not only showed resistance to third generation cephalosporins but also to antibiotics from other groups, such as fluoroquinolones, aminoglycosides and sulfonamides. The **S. fonticola** ESBL isolates were also found to be mainly resistant to the third generation cephalosporins, with the exception of 5 imipenem and 2 ertapenem-resistant strains. These strains presented highly diverse fingerprinting profiles, as well as significant differences in phenotypic traits helpful for survival in the environment, such as biofilm formation and motility. Moreover, biofilm formation and the swimming ability were species and temperature dependent. We confirmed the presence of highly diverse ESBL strains with multiple drug resistance patterns in the Tricity water reservoirs. This could possibly pose a threat to human health and create a suitable ground for acquiring antibiotics resistance in the natural environment.

Keywords: Multi-drug resistant strains, ESBL-beta-lactamase, freshwater microbial contamination

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INTRODUCTION

Antibiotics have existed in the world since bacteria and fungi appeared on Earth, but people have noticed their effects relatively recently. The first potentially health-promoting use of tetracycline-containing beer was found in ancient Nubia around 350-550 BC (Kozińska et al., 2017). In 1897, Ernest Duchesne concluded that some moulds inhibit the growth of pathogenic bacteria (Duckett, 1999). The discovery of antibiotics became a turning point in the history of mankind. The “age of antibiotics” with antibiotics considered as “miracle drugs” that could fight almost any bacterial infection, began in 1928 after the discovery of penicillin by Alexander Fleming (Tan et al., 2015). Antibiotics have not only revolutionized medicine, but also had a huge impact on the socioeconomic well-being of people worldwide. Life expectancy has increased significantly, and mortality from many infectious diseases has decreased. People’s productivit y has increased by reducing absenteeism related to infectious diseases (Hutchings et al., 2019). The production of antibiotics and chemothapeutic agents is growing every year (Kümmerer et al., 2009a; Kümmerer et al., 2009b). The amount of antibiotics produced in the world is measured in millions of tons per year (ECDC, 2015). Antibiotics, when administered to humans or animals, are partially metabolized and then excreted via faeces and/or urine, both in metabolically active and inactivated forms. Furthermore, waste from the pharmaceutical industry and agri-food processing is of great importance with regards to the amount of metabolites and active forms of antibiotics released into the environment. They are released to municipal and industrial wastewater, and ultimately end up in surface waters. Waste and manure from animal husbandry is used as fertilizer on arable lands. This leads to the accumulation of antibiotics in the soil of farmlands and bottom sediments of water reservoirs, where antibiotics are being washed out from the soil, run off with rainwater and penetrate into the groundwater (Kümmerer et al., 2003). Fish farms likewise can be a source of antibiotics and their residues for other water reservoirs, such as rivers, lakes or ponds. However, municipal sewage, including sewage from hospitals, is considered to be the main source of contamination with antibacterial drugs. During biological waste water treatment, bioreactors contain various active and inactive forms of antimicrobial agents and a high content of microorganisms. The concentrations of drugs at this stage of treatment can vary, but even if low, they can affect microorganisms and promote horizontal gene exchange of resistance genes (Thanner et al., 2016). Next, the an-
tibiotic residues can be rinsed from the sewage sludge and move further, reaching rivers and the seas. While the majority of microorganisms resistant to antibiotics remains in the sludge of the sewage treatment plant, some of the drug-resistant bacteria may enter the natural environment (Galvin et al., 2010; Zhi et al., 2019). In fact, many antibiotics have been already found at detectable levels in the Polish water reservoirs and elsewhere (Koniuszewska et al., 2020; Rodríguez-Pérez & Bajorath, 2020; Szymańska et al., 2019; Rodríguez-Mozaz et al., 2015; Zhang et al., 2020).

The presence of antimicrobial contaminants in water, even at low concentrations, at first may not seem dangerous, but in the long run, a local accumulation of individual antimicrobial substances may occur, e.g. in the bottom sediments of water reservoirs, or in aquatic plants. At low concentrations, antibiotics are being detected both in the surface and drinking waters. Despite low levels, these drugs are constantly released into the environment, which additionally increases their hazardous potential. Furthermore, their constant presence in a given area affects many generations of microorganisms.

Effluents from municipal wastewater plants contain a mixture of various groups of drugs, including antibiotics. Such mixtures may have a synergistic effect on individual drugs, enhancing their impact on the environment. The presence of antibiotics in waters influences the composition of the microbiome of a given ecological niche: pond, lake or river (Koniuszewska et al., 2020). This may lead to selection of new drug resistance mechanisms in the environment or to enhanced spread of multi-drug resistant strains in water reservoirs. Antibiotics present in municipal wastewater pose a selective pressure, which not only promotes the growth of drug-resistant strains, but also causes a decrease in the number of drug-sensitive strains (Kümmerer et al., 2003).

Surface waters mostly contain drug-sensitive bacteria, however drug-resistant bacteria are also detected in growing amounts. Antibiotic-resistant bacteria have been detected virtually in all water bodies in the world (Manji et al., 2012; Mishra et al., 2018; Kurekci et al., 2017; El-Zanfaly, 2015; Azzam et al., 2017; Danner et al., 2019). People who use water bodies for recreational purposes constantly expose their skin and mucous membranes to bacteria which may be potentially antibiotic resistant. These bacteria can be either commensal, environmental or pathogenic strains which may cause infections, including Pseudomonas aeruginosa. On the other hand, drinking water contaminated with potentially pathogenic bacteria may be associated with food poisoning (Ling et al., 2018). The risk of contamination becomes even greater when the bacteria that cause the infections are not susceptible to antibiotics. Worldwide, 700,000 people a year die from bacterial diseases that would be largely curable if the bacteria had not acquired resistance genes (De Oliveira et al., 2020). The number of resistant bacteria is constantly increasing, and according to the World Health Organisation, by 2050 as many as 10 million people a year will have died from infectious diseases caused by drug-resistant microorganisms. The increasing number of people suffering from infectious diseases in areas where sewage treatment plants are unable to disinfect wastewater sufficiently causes in turn large-scale contamination of the environment with drug-resistant microorganisms (WHO Antimicrobial resistance: global report on surveillance 2014).

Despite the fact that cephalosporins are rarely used in hospital and veterinary treatment, the presence of strains resistant to this group of anti-bacterial drugs is an important fact that requires attention (Berendonk et al., 2015). Bacilli of the Enterobacteriales family, producing ESBL beta-lactamases (Extended-Spectrum Beta-Lactamases), have been and are still associated with nosocomial infections. Thus, during the 2019–2021 period we sampled 14 retention reservoirs, 12 streams, 1 lake and 1 pond situated in the Tricity area, Poland, looking for the presence of ESBL strains, which could possibly pose a threat to human health and create a suitable ground for acquiring antibiotics resistance in the natural environment. We have analysed not only their antibiotic resistance patterns, but also phenotypic traits important for bacterial survival in the environment, as well as for their pathogenesis.

**MATERIALS AND METHODS**

**Water sample collection**

40 water samples were collected in the Pomorskie voivodship (Poland) on 17.06.2019, 30.05.2019, 06.12.2019, 20.03.2020, 21.03.2020, 13.03.2021 and 17.03.2021, from different water reservoirs, such as the retention reservoirs, streams and rivers passing towns (Fig. 1, Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp). The water samples were collected into sterile 500-ml glass bottles, 1 meter away (if possible) from the shore of the water reservoirs, about 20 cm below the surface. Then, the samples were immediately transported to the laboratory in an isothermal box, kept at 4°C and processed within 24 h. There are no intensive animal husbandry, slaughterhouses, fish farms or pharmaceutical plants in the vicinity of the studied water reservoirs. However, there are several large hospital centres in the area, although they are not in close vicinity of any of the sampled water reservoirs. In Gdansk, there are 57 retention reservoirs localized at 7 streams. All of the streams flow into the Gulf of Gdansk. Samples were collected from 14 randomly selected retention reservoirs, 3 rivers, 1 lake and 12 streams localized in Gdansk, Gdynia, Sopot, Reda and Puck (Fig. 1, Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp).

**Isolation of ESBL strains**

Membrane filtration method (Baird et al., 2017) was used to examine the water samples. 50 mL samples were passed through sterile filters (0.22 µm pore diameter, Merck, Germany) with the use of a Merck Millipore membrane filtration set. Then, the filters were placed on the Chromagar ESBL medium (Grasso Biotech, Poland) and incubated for 17–24 h at 37°C. Positive colonies were then counted (pink colonies for the presence of E. coli, blue colonies for the presence of Enterobacteriaceae from the KESC group (Klebsiella, Enterobacter, Serratia, Citrobacter), and expressed as CFU/100 ml (Colony Forming Units) for each tested water reservoir. For positive plates, at least one colony was transferred onto a fresh medium and grown until pure culture was obtained. Isolated colonies were identified using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization with Time Of Flight Mass Spectrometry) (MALDI Biotyper, Bruker Daltonics, USA) according to the manufacturer’s procedure. Isolated colonies were also preserved by growing them in the LB medium (Grasso Biotech, Poland) at 37°C for 24 h (150 rpm) and transferring 1 ml of bacterial cultures into 1.5 ml Eppendorf tubes and freezing them at –60°C with 20% glycerol (Epoch, Poland).
Antimicrobial Susceptibility Testing

The double-disk synergy (DDS) assay was carried out to identify ESBL-producing strains (Jarlier et al., 1988). Subsequently, *E. coli* and KESC isolates were tested using a Kirby-Bauer disk diffusion assay for susceptibility to the following 16 antimicrobial agents according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) v.10.0 (2020) guidelines (European Committee on Antimicrobial Susceptibility Testing, Breakpoints tables for interpretation of MICs and zones diameters. Version 10.0, 2020; 2020): ampicillin (10 µg), amoxicillin/clavulanic acid (10/30 µg), piperacillin/tazobactam (30/6 µg), cefuroxime (30 µg), ceftazidime (10 µg), ceftazidime (10 µg), cefotaxime (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (10 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), ticarcillin (15 µg), sulfamethoxazole/tri-methoprim (25/25 µg), cefoxitin (30 µg) (Oxoid). *E. coli* ATCC 25922 (C49) was used as a reference strain (Supplementary Table 2 at https://ojs.ptbioch.edu.pl/index.php/abp).

DNA fingerprinting

The genomic DNA from all chosen strains was isolated with the use of Genomic Mini AX Bacteria Kit (A&A Biotechnology, Poland). The genomic DNA concentration was measured with the use of Epoch Microplate Spectrophotometer (BioTek Instruments) and the chosen strains were analysed using a repetitive-sequence-based rep-PCR with ERIC primers, as described by Versalovic and others (Versalovic et al., 1998). After PCR, 5 µl of the products were resolved in 0.8% agarose gels (0.5xTBE) at 50V for 2.5 h. After electrophoresis, the band patterns obtained for different strains were compared with the use of GelJ (Heras et al., 2015). Similarity trees were composed with the unweighted pair group method with arithmetic mean (UPGMA) and band differences were set to 1.0. As a reference, *E. coli* strains C38, C44, C47, C48, C49 and *S. marcescens* C19 were used for comparative purposes (Supplementary Table 2 at https://ojs.ptbioch.edu.pl/index.php/abp).

Enzyme production, motility and biofilm formation assays

For all enzyme production assays, as well as motility and biofilm formation, the bacterial cultures were freshly streaked on LA medium (Grasso Biotech, Poland) and grown at 37°C for 24 h. Then, bacterial suspensions were prepared from these fresh cultures in sterile 0.85% NaCl and adjusted to 0.5 MacFarland units (DensiChek plus, Biomeriux). For enzyme and motility assays, 10 µl of each bacterial suspension was placed on the medium surface. Protease production was measured on a medium containing skim milk (2 g/l) after incubation for 48 h at 37°C and at 20°C (Ji et al., 1987). The ability to produce DNases was assessed on DNase agar plates (Grasso Biotech, Poland) after 48 h of incubation at 37°C and flooding the plates with 1N HCl (Sigma Aldrich, Germany). For both tests, the diameter of clear halo around the colonies was measured. All experiments were performed twice, with 4 replicates. To determine the swimming and swimming motility, bacterial strains were inoculated onto LA surface (Thermo Scientific) plates and kept for 48h at 37°C without agitation. After the incubation period, OD₅₆₀ of bacterial cultures was measured and the bacterial cultures were removed from the wells. Then, 70 µl of 1% crystal violet solution was added into each well and left for 20 min without agitation. After incubation, the wells were washed 3 times with 900 µl of distilled water. Then, 900 µl of ethanol (96%, Sigma Aldrich, Germany) was added into each well and OD₅₆₀ of each well was measured. For calculations, the OD₅₆₀ value of the negative control was subtracted from the values obtained for the strains. Negative values obtained after subtraction were set to 0. The experiment was performed three times with two replicates.

As the reference for the above mentioned assays, *E. coli* ATCC25922 (C49) and *Serratia marcescens* KPD102-BA (C19) were used (Supplementary Table 2 at https://ojs.ptbioch.edu.pl/index.php/abp).

ESBLs are present in the Tricity waters

There are 57 retention reservoirs in Gdansk, and more are being designed and built. Due to the 3 geological elevation zones in the city, from depressed areas to the upland in the city, there is a high risk of flooding in case of heavy rainfall, when huge amounts of water flow from the upper terraces of the city towards the Gdansk Bay. Retention reservoirs in Gdansk have a total capacity of approx. 700,000 m³, and are located in cascades on 7 streams. It creates an exceptional system for draining rainwater into the sea, not implemented in other Polish cities (Gdanskie Wody, 2021). During the 2019-2021 period we sampled 14 retention reservoirs, 12 streams, 3 rivers and 1 lake situated in the Tricity area (Poland) searching for the presence of ESBL strains (Fig. 1). Of these, 19 samples were collected in Gdansk, 10 in Gdynia, 9 in Sopot and 1 each in Puck and Reda (the Płtnica and Reda rivers). Out of the 19 samples collected in Gdansk, 14 came from retention reservoirs, 1 from the Jasien Lake and 4 from streams. In Sopot and Gdynia, the samples were collected from 9 different
E. coli or the KESC group were isolated. The species of the isolated strains were identified with MALDI-TOF MS analysis (Table 1, Table 2). The most frequently isolated species were Serratia fonticola (41%) and Escherichia coli (21%). Moreover, 3 (5%) strains of Enterobacter cloacae were isolated, followed by 2 (4%) strains of Citrobacter braakii and Citrobacter freundii each. One strain belonging to Enterobacter xiangfangensis was identified, and as many as 11 (20%) strains from the Aeromonas genus were isolated that belonged to 5 different Aeromonas species (Table 1, Table 2).

**Table 1. Number of ESBL strains isolated from the Tricity waters (n=56) belonging to different species, as identified with MALDI-TOF MS.**

| Species                  | Number of isolates | % of isolates |
|--------------------------|--------------------|---------------|
| Aeromonas bestiarum      | 3                  | 5%            |
| Aeromonas eucrenophila   | 2                  | 4%            |
| Aeromonas hydrophilia    | 2                  | 4%            |
| Aeromonas salmonicida    | 1                  | 2%            |
| Aeromonas veronii        | 2                  | 4%            |
| Aeromonas spp.           | 1                  | 2%            |
| Citrobacter braakii      | 2                  | 4%            |
| Citrobacter freundii     | 2                  | 4%            |
| Enterobacter cloacae     | 3                  | 5%            |
| Enterobacter xiangfangensis | 1           | 2%            |
| Escherichia coli         | 12                 | 21%           |
| Rahnella aquatica        | 1                  | 2%            |
| Serratia fonticola       | 23                 | 41%           |
| Yersinia mollaretii      | 1                  | 2%            |

ESBLs isolated from the Tricity waters present diverse resistance to antibiotics

For further studies, strains belonging to two of the most representative species, namely S. fonticola and E. coli, were selected (35 isolates, Table 2). All analysed strains were tested for the presence of ESBL beta-lactamases using the DDS phenotypic test. Among the 35 strains tested, 34 were ESBL positive. One negative DDS-test strain (155) showed resistance to all analysed third-generation cephalosporins, which may result from the AmpC

Figure 2. Average amount of ESBL strains detected with the ESBL Chromagar plates in different cities of the Tricity area (Gdansk, Gdynia, Puck, Reda, Sopot), expressed as CFU/100 ml of water sample +/- standard deviation.

Black bars represent ESBL E. coli strains, dark grey bars strains belonging to the KESC group, while light grey bars all other microorganisms able to grow on the medium. For details see Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp.
ESBLs are potential antibiotics resistance source in the Tricity waters

Table 2. The ESBL Enterobacterales strains isolated from the Tricity water reservoirs in 2019–2021, identified with MALDI-TOF MS analysis. The strains taken for phenotypic and genotypic analysis are marked in bold.

| Collection ID | Date of collection | City | Water reservoir | Species               |
|---------------|--------------------|------|----------------|-----------------------|
| 127           | 06.12.2019         | Gdansk | Zabornia retention reservoir | *Escherichia coli* |
| 128           | 06.12.2019         | Gdansk | Potokowa retention reservoir | *Escherichia coli* |
| 129           | 06.12.2019         | Gdansk | Potokowa retention reservoir | *Aeromonas hydrophila* |
| 130           | 06.12.2019         | Gdansk | Potokowa retention reservoir | *Aeromonas veronii* |
| 131           | 06.12.2019         | Gdansk | Potokowa retention reservoir | *Aeromonas hydrophila* |
| 150           | 12.03.2020         | Gdansk | Oliwa Park retention reservoir | *Escherichia coli* |
| 151           | 12.03.2020         | Gdansk | Grunwaldzka retention reservoir | *Escherichia coli* |
| 152           | 12.03.2020         | Gdansk | Grunwaldzka retention reservoir | *Serratia fonticola* |
| 153           | 12.03.2020         | Gdansk | Wilenska retention reservoir | *Serratia fonticola* |
| 154           | 12.03.2020         | Gdansk | Wilenska retention reservoir | *Serratia fonticola* |
| 155           | 12.03.2020         | Gdansk | Mysliwska retention reservoir | *Serratia fonticola* |
| 163           | 12.03.2020         | Gdansk | Osowa retention reservoir | *Enterobacter cloacae* |
| 164           | 12.03.2020         | Gdansk | Jasien lake | *Enterobacter cloacae* |
| 165           | 12.03.2020         | Gdansk | Jasien lake | *Enterobacter xiangfangensis* |
| 166           | 12.03.2020         | Gdansk | Swietokrzyska retention reservoir | *Serratia fonticola* |
| 167           | 12.03.2020         | Gdansk | Jabloniowa retention reservoir | *Aeromonas veronii* |
| 168           | 12.03.2020         | Gdansk | Jabloniowa retention reservoir | *Serratia fonticola* |
| 169           | 12.03.2020         | Gdansk | Cedrowa retention reservoir | *Escherichia coli* |
| 170           | 12.03.2020         | Gdansk | Labedzia retention reservoir | *Escherichia coli* |
| 172           | 12.03.2020         | Gdansk | Potokowa retention reservoir | *Serratia fonticola* |
| 173           | 12.03.2020         | Gdansk | Zabornia retention reservoir | *Escherichia coli* |
| 174           | 12.03.2020         | Gdansk | Potokowa retention reservoir | *Serratia fonticola* |
| 182           | 12.03.2020         | Gdansk | Oliwa Park retention reservoir | *Escherichia coli* |
| 876           | 18.03.2021         | Gdansk | Siedlicki stream | *Serratia fonticola* |
| 899           | 18.03.2021         | Gdansk | Nowicki stream | *Serratia fonticola* |
| 156           | 12.03.2020         | Gdansk | Cisowska stream | *Serratia fonticola* |
| 157           | 12.03.2020         | Gdansk | Cisowska stream | *Enterobacter cloacae* |
| 158           | 12.03.2020         | Gdansk | Cisowska stream | *Serratia fonticola* |
| 159           | 12.03.2020         | Gdansk | Cisowska stream | *Serratia fonticola* |
| 160           | 12.03.2020         | Gdansk | Chylotska stream | *Serratia fonticola* |
| 199           | 12.03.2020         | Gdansk | Chylotska stream | *Serratia fonticola* |
| 802           | 18.03.2021         | Gdansk | Cisowska stream | *Rahnella aquatica* |
| 810           | 18.03.2021         | Gdansk | Kacza river | *Serratia fonticola* |
| 845           | 18.03.2021         | Gdansk | Zrodlo Marii stream | *Serratia fonticola* |
| 874           | 18.03.2021         | Gdansk | Zrodlo Marii stream | *Citrobacter braakii* |
| 877           | 18.03.2021         | Gdansk | Chylotska stream | *Yersinia mollaretii* |
| 886           | 18.03.2021         | Gdansk | Chylotska stream | *Citrobacter braakii* |
| 88            | 30.05.2019         | Gdansk | Kolibkowski stream | *Aeromonas almonicida* |
| 89            | 30.05.2019         | Gdansk | Kolibkowski stream | *Aeromonas securinophila* |
| 90            | 30.05.2019         | Gdansk | Kolibkowski stream | *Aeromonas hydrophila* |
| 811           | 18.03.2021         | Gdansk | Kolibkowski stream | *Serratia fonticola* |
| 805           | 18.03.2021         | Gdansk | Plutnica river | *Serratia fonticola* |
| 809           | 18.03.2021         | Gdansk | Plutnica river | *Serratia fonticola* |
beta-lactam mechanism, however further confirmation is necessary.

All tested E. coli and S. fonticola strains were resistant to ampicillin (Supplementary Table 3 at https://ojs.ptbioch.edu.pl/index.php/abp). The analyzed E. coli strains were more frequently resistant to more than one tested antibiotic than S. fonticola strains (Supplementary Table 3 at https://ojs.ptbioch.edu.pl/index.php/abp). However, one S. fonticola strain (155) was resistant to all tested antibiotics apart from tigecycline. This particular strain is of special interest since it was also negative in the DDS test for the presence of ESBL enzymes. Regarding groups of antibiotics, most strains (19/35) were resistant to at least one cephalosporin, while 10 strains were also resistant to fluoroquinolones. On the other hand, the most active antibiotic groups against bacteria were tetracycline and aminoglycosides (only 1 and 4 resistant strains, respectively). Similarly, only 5 strains were resistant to carbapenems and betalactams/beta-lactam inhibitors. Resistance to different third generation cephalosporins was different among the tested E. coli and S. fonticola strains. Noteworthy, all E. coli and S. fonticola strains were resistant to cefuroxime. Among the third generation cephalosporins, the most active against the tested strains was ceftriaxone, to which 2 (9.1%) S. fonticola strains, and as many as 7 (63.6%) E. coli strains were sensitive (Fig. 3). The number of E. coli strains sensitive to third generation cephalosporins was much higher than that of S. fonticola strains. All E. coli strains were resistant to at least one of third generation cephalosporins, while among S. fonticola strains only 8 out of 22 showed a similar resistance. Similarly, the number of E. coli strains resistant to ciprofloxacin and trimethoprim/sulfamethoxazole was much higher (7 strains, 63.6%) than the number of S. fonticola resistant strains (3 strains, 13.6% and 1 strain, 4.5%, respectively) (Fig. 3).

The E. coli and S. fonticola isolates were most sensitive to carbapenems and especially to meropenem, to which no resistant strains were found (Fig. 3, Supplementary Table 3 at https://ojs.ptbioch.edu.pl/index.php/abp). All E. coli strains were sensitive to imipenem and ertapenem, contrary to S. fonticola isolates, where 5 strains (160, 199, 155, 153, 166) were not sensitive to imipenem and 2 (155, 199) were resistant to ertapenem. In this study, the mechanism of lower sensitivity to carbapenems of S. fonticola strains was not examined.

Another group of tested antibiotics with high antimicrobial activity were aminoglycosides. Almost all tested strains were sensitive to gentamicin, except for 3 E. coli strains (127, 128, 151) isolated from two streams and one reservoir in Gdansk, and 1 S. fonticola strain (155) isolated from a reservoir in Gdansk (Supplementary Table 3 at https://ojs.ptbioch.edu.pl/index.php/abp). All E. coli strains were sensitive to imipenem and ertapenem, S. fonticola isolates, where 5 strains (160, 199, 155, 153, 166) were not sensitive to imipenem and 2 (155, 199) were resistant to ertapenem. In this study, the mechanism of lower sensitivity to carbapenems of S. fonticola strains was not examined.

Comparing isolates from both species, E. coli strains showed a higher incidence of multidrug resistance pattern, as 7 strains (104, 102, 127, 182, 150, 128, 151) were resistant to more than one antibiotic (Supplementary Table 3 at https://ojs.ptbioch.edu.pl/index.php/abp).

E. coli and S. fonticola ESBL strains show large diversity in phenotypic traits

Not only do the E. coli and S. fonticola strains show differences in antibiotics resistance, but they also present high diversity when looking at phenotypic traits important for pathogenesis and survival in harsh environments, such as motility, biofilm formation and production of extracellular enzymes. For 12 E. coli strains and 23 S. fonticola strains diverse phenotypic tests were performed at two different temperatures (20°C and 37°C) to
ESBLs are potential antibiotic resistance source in the Tricity waters

Swimming motility was expressed as an average colony diameter (mm) with standard deviation (n=4), while biofilm formation was expressed as an average OD_{560} value of crystal violet ethanol extractions after background subtraction, with standard deviation (n=2). Swimming motility was repeated twice, while biofilm formation three times. The results marked with different letters are statistically different as assayed with ANOVA and the Tukey post hoc test, p<0.05.

Interestingly, S. fonticola 155, which had low swimming ability at 20°C, showed high ability to form biofilm under the same conditions.

We also performed a combined principal component analysis (PCA) taking into account all phenotypic tests (swimming, swarming, biofilm formation) and antibiotic resistance for the tested E. coli and S. fonticola strains (Fig. 6). By combining all of these data, we could explain 40% and 27% of the variance among the strains with component PC1 and PC2, respectively. The E. coli strains seem to form clusters of strains which are more similar to each other, like for example strains 151, 150 and 182 and another cluster of E. coli 102 and 104 which were isolated either from the same retention reservoir or in close vicinity (Fig. 6, Table 2). However, there were also strains clustered with each other but isolated from different water reservoirs, such as E. coli 173 and 863, and E. coli 127 and 128. Contrary to E. coli isolates, S. fonticola isolates did not seem to form any clusters. Most of the strains were grouped together regardless of the place of isolation. Interestingly, there was one S. fonticola strain (155), which was highly different from other S. fonticola isolates and turned out to be more similar to E. coli isolates when looking at phenotypic traits (Fig. 6). Detailed analysis of phenotypic traits, together with antibiotic resistance, showed that biofilm formation at 37°C, swimming at both temperatures and resistance to cefuroxime are showing similar trends in the tested group of S. fonticola and E. coli strains (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp). Other traits, such as swimming, biofilm formation at 20°C and resistance to other antibiotics, form another cluster of features. In a global analysis based on Euclidean distance, some of the E. coli and S. fonticola isolates are clustered together. In this case, S. fonticola 155 again stands out from the group of strains, showing even more differences from other strains than S. marcescens C19 (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp).

Figure 4. Swimming motility (A; B) and biofilm formation (C; D) of E. coli ESBL strains isolated from the Tricity water reservoirs tested at 37°C (A; C) and 20°C (B; D).

Swimming motility was repeated twice, while biofilm formation three times. The results marked with different letters are statistically different as assayed with ANOVA and the Tukey post hoc test, p<0.05.

When looking at S. fonticola strains, they were compared to the S. marcescens C19 reference strain. S. fonticola environmental isolates presented higher swimming motility at 20°C than at 37°C. At 20°C, the swimming motility for all the S. fonticola strains (except for 882) was significantly lower than the one found for S. marcescens C19, while at 37°C only a few isolates were less motile than the reference strain (155, 172, 805, 876, 890) (Fig. 5AB). When looking at biofilm formation capability of S. fonticola, it was generally higher at 37°C than at 20°C, while swimming motility was generally higher at 20°C than at 37°C. However, at 37°C the strains with low biofilm formation capability also expressed low swimming motility (152, 166, 809, 810, 811, 845, 866, 876, 878) (Fig. 5AC).
ESBLs from *S. fonticola* and *E. coli* genera isolated from the Tricity waters present diverse fingerprinting profiles

Given a large phenotypic diversity among the isolated *E. coli* and *S. fonticola* ESBL strains, it was decided to verify their genotypic relationships. With the use of rep-PCR fingerprinting profiles obtained with ERIC primers, it was possible to assess the genetic variability among the isolated strains. Two strains (*E. coli* 103 and *S. fonticola* 845) were excluded from the analysis as their isolated genomic DNA was unstable, preventing appropriate execution of the experiments.

When taking into account profiles obtained for *E. coli* strains, 5 *E. coli* reference strains were used in the analysis that possessed similar or identical profiles forming a separate clade. Another clade was formed by *E. coli* environmental isolates, which in turn presented unique profiles for each strain (Fig. 7A).

For *S. fonticola* isolates, similar to *E. coli*, diversity among obtained rep-PCR profiles could be observed (Fig. 7B). Two *S. fonticola* strains (153 and 154) isolated from the Wilenska retention reservoir (Gdansk) showed almost the same band patterns, while other strains presented diverse profiles. Still, the strains could be divided into a few clades. However, these clades do not reflect geographical origin nor phenotypic analysis, apart from a few cases. For example, differently from all other strains in the phenotypic analysis, *S. fonticola* 155 formed a clade with several other strains (152, 153, 154, 156, 159, 805, 890) in the fingerprinting analysis. *E. coli* strains (150, 151, 182) that prove to be similar in the PCA analysis of the

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**Figure 5.** Swimming motility (A; B) and biofilm formation (C; D) of *S. fonticola* ESBL strains isolated from the Tricity water reservoirs tested at 37°C (A; C) and 22°C (B; D).

Swimming motility was expressed as an average colony diameter (mm) with standard deviation (n=4), while biofilm formation was expressed as an average OD<sub>540</sub> value of crystal violet ethanol extractions after background subtraction, with standard deviation (n=2). Swimming motility was repeated twice, while biofilm formation three times. The results marked with an asterisk are statistically different from the reference strain *S. marcescens* C19 as assayed with ANOVA and the Tuckey post hoc test, p<0.05.

**Figure 6.** Principal component analysis (PCA) of biofilm formation, swimming and swarming motility, and antibiotics resistance of *S. fonticola* and *E. coli* ESBL strains. *E. coli* C49 and *S. marcescens* C19 were used as a reference.

The biofilm formation and motility assays were performed at 37°C and 22°C. *E. coli* strains are marked with black dots, while *S. fonticola* with violet dots.
phenotypic traits, also fall in the same clade in the rep-PCR profile analysis. In contrast, *E. coli* 102 and 104 which are similar in PCA, tend to have very different rep-PCR profiles (Fig. 7A, Fig. 6).

**DISCUSSION**

Faecal microorganisms can enter water bodies in diverse ways, including runoff, sewage discharge, and direct faecal deposition (Korajkic et al., 2015). Many investigations have shown the presence of multi-drug resistant coliforms in water (Manji et al., 2012; Mishra et al., 2018; Kurecki et al., 2017; El-Zanfaly, 2015; Azzam et al., 2017). In water reservoirs, such as rivers, streams, lakes and retention reservoirs, both pathogenic and commensal Enterobacteriales may be present (Muraledharan et al., 2019). Pathogenic bacteria present in the water reservoirs may in turn cause infections in humans, which often have to be treated with antibiotics (Manji et al., 2012). The antibiotic therapy is effective when the causative agent is drug-sensitive, otherwise the treatment is much more challenging. Thus, there is a growing concern regarding the occurrence of multi-drug resistant coliforms which can render antibiotic therapy ineffective (Mishra et al., 2018). Presently, antimicrobial pharmaceuticals are widely used in patient treatment, as well as in treatment of animals. Poland is one of the European Union countries with the highest usage of antibi-

**Figure 7. Rep-PCR profiles similarity of *E. coli* (A) and *S. fonticola* (B) ESBL strains isolated in the Tricity water reservoirs in 2019–2021.**

The profiles were obtained with ERIC rep-PCR primers and analysed with UPGMA and band similarity index set to 1.0. *S. marcescens* C19 and *E. coli* C36, C44, C47, C48, C49, were used as a reference. Gd, Gdansk; Gdy, Gdynia; Sp, Sopot; ‘S’, stream; ‘R’, retention reservoir.

ESBLs are potential antibiotics resistance source in the Tricity waters reaching as much as 25.87 DID (Defined Daily Doses per 1000 inhabitants per day) in 2015 (Olczak-Pietkowska et al., 2017). When regional usage is considered, the Pomorskie Voivodeship was the fourth largest antibiotic user in Poland (26.57 DID). Pharmaceutical residues excreted by treated humans and animals tend to end up in the water reservoirs where they can be a potential selection factor for multi-drug resistance arising in pathogenic, commensal and environmental microorganisms. In this phenomenon, horizontal and lateral gene transfers could be potent mechanisms (Emanuelpour et al., 2020).

The inland water catchment area of the Gulf of Gdansk, together with the Vistula River mouth, are important sources of bacterial contamination, including multi-drug resistant strains feeding into the Baltic Sea. In this study, we verified the presence of multiple ESBL species carrying resistance to different groups of antibiotics. Already in 2014, Bartoszewicz and others (Bartoszewicz et al., 2014) reported the presence of antibiotics resistant *E. coli* strains in the Sopot streams, while Łuczkiwicz and others (Łuczkiwicz et al., 2010) reported only one *E. coli* ESBL out of 155 strains isolated from effluent of the water treatment plant in Gdansk. Here, growth of ESBLs strains could be observed in 80% of samples, with greater load of strains observed in larger water reservoirs, such as the retention reservoirs (Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp). In fact, this is a larger number than the one observed in other rivers, streams and retention reservoirs in the territory of Poland. For example, Lenart-Boroń et al. (Lenart-Boroń et al., 2017) reported that 14% of the isolated *E. coli* strains were ESBL *E. coli* in the Białka and Zakopianka rivers (Southern Poland), while at a later date this percentage rose to 20.6% of ESBL *E. coli* strains in the Białka river (Lenart-Boroń et al., 2020a). On the other hand, no ESBL strains were detected in the Szreniawa river (Lenart-Boroń et al., 2020b). Contrary, as many as 37.1% of *E. coli* isolates from effluents from a hospital situated in Olsztyn (Poland) belonged to ESBL, while from the city wastewater treatment plant effluents there was only 17.7% of such strains present (Korzeniewska et al., 2013). In rivers, the load of ESBL bacteria was usually lower than in streams, but the water flow rate of the river is usually less turbulent, which enables deposition of the microorganisms in the sediments with the speed of 0.066 m/h (Garcia-Armisen et al., 2009). In contrast, streams with more ESBL *E. coli* than in the rivers, usually have a high flow rate, which can prevent microorganisms from depositing themselves on the way. It is not surprising that in the retention reservoirs much more ESBL strains were found, as these reservoirs are fed by both, the streams and in case of heavy rain also with rain waters flowing from the urban areas. Moreover, the retention reservoirs in Gdansk are rich with birds which can also deposit their faeces contaminated with ESBL strains (Rybak, data not published). In fact, already in 2009, faeces of birds caught in the Tricity area were in 27% contaminated with ESBL *E. coli* (Litterak et al., 2010). We managed to isolate 56 ESBLs strains out of all of the water reservoirs, the majority of which belonged to *E. coli* (12) and *S. fonticola* (23) species.

Within Enterobacteriales, strains of the *Serratia* genus are frequently encountered in the human nosocomial infections. Apart from *Serratia marcescens* and *Serratia liquefaciens* complex (*S. liquefaciens, Serratia proteamaculans, Serratia grisea*), which are regarded as a major agent causing human *Serratia* infections, there is little information...
about the remaining species of *Serratia* (‘unusual *ser-ri-tiae*), including their susceptibility patterns to antimicrobial agents or underlying mechanisms of resistance (Grimont et al., 1992). Strains of *S. fonticola* are widespread in the environment (drinking water, sewage and soil), with birds being reported as possible natural hosts (Stock et al., 2003). As a human pathogen, *S. fonticola* has been associated with multiple diseases, such as diarrhea, septic arthritis, wound, respiratory, urinary tract, bloodstream, or skin and soft tissue infections (Stock et al., 2003). Resistance to beta-lactams in *S. fonticola* has been mediated by chromosomal class A extended-spectrum β-lactamases ESBLs belonging to the FONA family (Philippon et al., 2016; Fuentes-Castillo et al., 2020).

Among the tested *E. coli* and *S. fonticola* strains, large differences in resistance to groups of antibiotics could be observed. Strains that belonged to the *S. fonticola* (except 155) species were rarely resistant to other antibiotic groups except for third generation cephalosporins, contrary to *E. coli* isolates. Stock and others (Stock et al., 2003) have also pointed out the natural susceptibility of several *Serratia* species (*S. ficaria, S. fonticola, S. odorifera, S. pluminthicaeand S. rubidaea*) to a wide range of antimicrobial agents (Stock et al., 2003). *S. fonticola* strains are naturally sensitive to tetracycline and naturally resistant to ticarcillin and amoxicillin. At the same time, this species is sensitive or intermediately sensitive to aminopenicillins in the presence of β-lactamase inhibitors. Stock et al. (2003) have shown that their *Serratia* spp. isolates were able to express their own naturally occurring AmpC β-lactamase, which might be inducible (*S. ficaria, S. fonticola, S. odorifera*) or not inducible (*S. rubidaea*). Interestingly, *S. fonticola* 155 isolated from the Mysliwska retention reservoir (Gdansk) has shown a multi-drug resistant pattern for third generation cephalosporins and at the same time was negative in the DDS-test. Taking into consideration Stock and others (Stock et al., 2003), it seems possible that *S. fonticola* 155 may be facilitated with inducible AmpC β-lactamase. The unique β-lactam susceptibility pattern of *S. fonticola* 155, showing resistance to amoxicillin and several cephalosporins, may be due to expression of a chromosomally encoded class A β-lactamase with an enhanced cephalosporinase activity, which in case of *S. fonticola* 155 needs further investigation (Stock et al., 2003). Contrary to *S. fonticola*, *E. coli* ESBL isolates were generally more resistant to the tested antibiotics. However, a few *S. fonticola* strains were not sensitive to imipenem, a representative of carbapenems. Carbapenems are considered as antibiotics of the ‘last resort’, used for treatment of patients infected with drug-resistant pathogens. *S. fonticola* can harbour its drug resistance to imipenem on a plasmid, which in turn can be disseminated to other microorganisms present in the environment, such as the isolated ESBL *E. coli* strains. Similarly to *S. fonticola* 155, it was found that environmental *S. fonticola* UTAD54 was resistant to multiple antibiotics and possessed genes coding for multiple enzymes (A β-lactamase with carbapenemase activity, carbapenemases SFC-1 and SfH-1), which are not common among *S. fonticola* strains (Henriques et al., 2004; Saavedra et al., 2003).

Apart from the antibiotics resistance of the tested *E. coli* and *S. fonticola* strains, closer consideration was taken for other phenotypic features of these bacteria which may facilitate their survival in the water reservoirs, as well as be potential factors enabling efficient pathogenesis. Enzyme production, biofilm formation and motility were investigated at two different temperatures, reflecting both, the host environment (37°C) and the water environment (20°C). In general, we noticed differences in biofilm formation ability and swimming motility, which were both species and temperature dependent. Higher swimming motility and lower ability to form biofilm was observed for *S. fonticola* grown at 20°C. The *E. coli* isolates were more motile and produced less biofilm at 37°C, which may suggest that they are more suited to invade the host than to persist in the environment. Interestingly, 7 *E. coli* isolates, which were more motile at 37°C, simultaneously produced more biofilm (102, 104, 127, 169, 170, 173, 863), while 2 *E. coli* strains (103 and 128) were not efficient in biofilm production. This finding is in agreement with Wood and others (Wood et al., 2006) who showed that in some cases *E. coli* requires motility for biofilm formation in its early stage. Wood and others (Wood et al., 2006) compared motility and biofilm formation of multiple *E. coli* strains which proved to be correlated. The strains capable of producing the largest biofilms under tested conditions were also the most motile (Wood et al., 2006). In the case of *S. fonticola* species, 4 *S. fonticola* strains (153, 155, 156 and 882) also showed a similar pattern of being more motile and a better biofilm producer at 37°C. This ability is particularly important because production of biofilms often complicates chronic and difficult-to-treat infections by protecting bacteria from the immune system, decreasing antibiotic efficacy, and dispersing planktonic cells to distant body sites (Jackson et al., 2002).

Due to large diversity among the tested strains regarding their phenotypic traits, such as antibiotics resistance, motility and biofilm formation, their genotypic variability with the use of rep-PCR fingerprinting profiles was analysed. The tested *E. coli* and *S. fonticola* ESBL strains presented large diversity of profiles, which are dissimilar from the reference strains used in this study. High variability of rep-PCR profiles of bacteria isolated from water reservoirs is widely documented (Moreira et al., 2012; Potrykus et al., 2016; Khare et al., 2020; Chandrasekar et al., 2015; Kodarska et al., 2015; Lenart-Boroń et al., 2020a). Similar results for aquatic *E. coli* were observed in the Yamuna River (India) (Khare et al., 2020). The isolates were highly diverse at all sampling sites of the river except for the entry site (Delhi at Palla). However, in this site the influence of anthropogenic activities and the pollution was the lowest when compared to other sampling sites. Similar situation was observed in Ontario (Canada), where fingerprinting profiles of periphytic *E. coli* were variable (Moreira et al., 2012). Moreover, unlike Kon et al. (2007), we have observed diverse rep-PCR profiles even for the isolates coming from the same water reservoir, except for *S. fonticola* 153 and 154. In the case of *S. fonticola* no fingerprinting analysis in the literature had been found for comparison purposes. However, the profiles are as diverse as the *E. coli* profiles obtained in this study (Chandrasekar et al., 2015).

To sum up, the water reservoirs situated in the urban Tricity area are a habitat for ESBL Enterobacterales strains and thus can be a source of growing antibiotics resistance of the microorganisms in this area. The antimicrobial resistance (AMR), supported by a vertical and/or horizontal transfer of antibiotic resistance genes, is a serious public health challenge globally. AMR has been widely associated with pathogens in clinical settings, and it is becoming increasingly recognized that nonclinical environments and nonhuman hosts may also be reservoirs of AMR genes. As a result, constant monitoring of these bacteria (present not only in the water reservoirs but also in drinking water and at bathing areas localized by the Baltic Sea) should be taken under consideration,
since all of the freshwater reservoirs feed into the Baltic Sea with their waters containing ESBL strains.

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