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COVID-19 lockdown shows how much natural mountain regions are affected by heavy tourism

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HIGHLIGHTS
• Heavy tourism-related anthropopressure on water revealed by multifactor analysis.
• COVID-19 lockdown allowed renaturation of water in Bialka river catchment.
• Content of antibiotic residuals increased during lockdown.

GRAPHICAL ABSTRACT

ABSTRACT

Mountain areas in Poland are among the most frequented tourist destinations and such intensive tourism negatively affects the natural environment. The COVID-19 pandemic and the resulting lockdown restricted travel for a few months in 2020, providing a unique opportunity to observe the studied mountain environment without the impact of typical tourist traffic. This study is based on the determination of antibiotic content, hydrochemical parameters, enumeration of culturable bacterial water quality indicators, antimicrobial susceptibility tests together with extended spectrum beta-lactamase (ESBL) gene detection in waterborne E. coli and NGS-based bacterial community composition at six sites along the Bialka river valley (one of the most popular touristic regions in Poland) in three periods: in summer and winter tourist seasons and during the COVID-19 lockdown. The results of individual measurements showed decreased numbers of bacterial indicators of water contamination (e.g. numbers of E. coli dropped from $99 \times 10^4$ CFU/100 ml to 12 CFU/100 ml at the most contaminated site) and the share of antimicrobial resistant E. coli (total resistance dropped from 21% in summer to 9% during lockdown, share of multidrug resistant strains from 100 to 44%, and ESBL from 20% in summer to none during lockdown). Antibiotic concentrations were the highest during lockdown. The use of multivariate analysis (principal component analysis – PCA and heatmaps) revealed a clear pattern of tourism-related anthropogenic pressure on the water environment and positive impact of COVID-19 lockdown on water quality. PCA distinguished three major factors determining water quality: F1 shows strong effect of anthropogenic pressure; F2 describes the lockdown-related quality restoration processes; F3 is semi-natural and describes the differences between the most pristine and most anthropogenically-impacted waters.
1. Introduction

Mountain areas occur on all continents and are among the most important and valuable regions for biological diversity. They play a key role in supporting the global environment and providing for the freshwater needs of nearly half of the world’s population (Korner and Ohsawa, 2005). In developed countries mountain regions are also among the most desired tourist destinations. At the same time, mountain environments are very sensitive to natural and anthropogenic changes. A representative example of highly frequented mountain destinations, where ski tourism plays a dominant role in the economic structure of the region, is the area located in Poland’s southernmost regions, Podhale. Within this region, the Biała river valley is particularly interesting due to its high popularity among tourists, having one of most highly developed skiing infrastructure in Poland, whilst also being included in the Natura 2000 program of the European Union (http://www.ineeko.org.pl/index_areas.php?rek=512; Krzesiwo, 2016). It is difficult to precisely assess the number of tourists visiting this area as very few studies dealing with this issue have been published. The most recent study of Krzesiwo (2016) estimated that in the winter season of 2014/2015, only one of the ski stations located in the Biała river valley was visited by about 318,000–342,000 people. However, the ski stations in the region are constantly developing meaning that the visitor numbers have most probably increased, whereas the number of permanent residents in most villages in the Biała river catchment oscillate around 2000. Such a significant tourism intensity negatively affects the natural environment in a number of ways, among which increased water consumption and excess production of sewage have proved to be the main issues in the study area and its vicinity (Lenart-Boroń et al., 2016a, 2016b; Bojarczuk et al., 2018; Lenart-Boroń et al., 2019, Lenart-Boroń et al., 2020), just as in other mountain regions of the world (Kangas et al., 2012; Pickering et al., 2003; Pop et al., 2011; Senetra et al., 2020; Sunlu, 2003). As a result of extensive research, both temporal and spatial variation of physicochemical and microbiological indicators of water quality have been previously documented. The most pristine region within the Biała river catchment is situated in the uppermost sector of the river, in the Tatra National Park, where the values of most physicochemical and microbiological parameters are the smallest (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2020). Water quality in this site is shaped by “natural” factors, such as surface runoff and snowmelt water (Lenart-Boroń et al., 2016a). Water contamination increases along the course of the river due to illegal discharge of sewage from households located upstream of the municipal sewage treatment plant (STP) (Lenart-Boroń et al., 2016a). The water quality upstream of the STP is shaped by a mixture of factors, i.e. by anthropogenic impacts (tourist traffic-related sewage inflow causing the presence of bacterial contaminants) and natural cycle (variable weather conditions, snowmelt, soil leaching and surface runoff) (Lenart-Boroń et al., 2016a; Bojarczuk et al., 2018). Further along the course of the Biała river, the river waters are severely contaminated by the discharge of sewage from the municipal sewage treatment plant in Czarna Góra (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2016c; Lenart-Boroń, 2017; Lenart-Boroń et al., 2017; Lenart-Boroń et al., 2019; Lenart-Boroń et al., 2020). The factors affecting the water quality at this site are of anthropogenic character and are strictly associated with fecal contamination (Lenart-Boroń et al., 2016a). Discharge of sewage from the STP along with other point sources of water pollution contribute to the deterioration in water quality of the Biała river downstream of the STP (Lenart-Boroń et al., 2020).

The temporal changes in water quality of the Biała river valley occur on different levels, i.e. variation throughout the year mostly coincides with changes in the tourist traffic intensity, which is the predominant factor causing severe water contamination in the winter ski season and only slightly lower in summer (Lenart-Boroń et al., 2016a). A less significant, but still observable, impact on the yearly changes in water quality is associated with environmental factors such as snowmelt, soil leaching or surface runoff which is characteristic of the mountain regions (Lenart-Boroń et al., 2016a, 2016b). The mentioned intensive ski tourism results in generation of vast amounts of sewage and thus a significant overload of the local sewage treatment plant (STP) (Lenart-Boroń et al., 2016a), whose capacity is insufficient even after its modernization in 2015 (Lenart-Boroń et al., 2019).

The ineffectively operating STP is coupled with poor water and wastewater management as well as poor environmental awareness in the municipalities surrounding the Biała river valley (Lenart-Boroń et al., 2019). Only half of the population (54.3%; Central Statistical Office, 2016) is connected to the sewerage system and there are numerous points of illegal sewage discharge from private guesthouses and households. All the above mentioned factors contribute to a significant deterioration of water quality along the Biała river, including the contamination of water with antimicrobial agents (Lenart-Boroń et al., 2020) and antibiotic-resistant bacteria (Lenart-Boroń et al. 2020; Lenart-Boroń et al., 2017; Lenart-Boroń, 2017; Chmiel and Lenart-Boroń, 2019).

The coronavirus disease-2019 (COVID-19) pandemic struck in early 2020, and very soon became the most important public health emergency. In order to prevent its massive spread, many countries imposed mass quarantines, lockdown periods and restrictions on travelling across the country. Although the sudden lockdown had severe effects on the economy of many countries, the condition of the environment, particularly air and water, has improved due to decreased anthropogenic activity and the associated reduction in pollutants entering the ecosystem (Chakraborty et al., 2021; Pacaol, 2021). Restoration of water quality was observed and described in a number of previously polluted regions. Several studies were conducted in India, where the water quality improved during the lockdown period (e.g. in the river Ganga, in the Gangetic delta and in the river Damodar (Chakraborty et al., 2021) or Vembanad lake, one of the most severely polluted freshwater lakes in India (Yunus et al., 2020). In the Philippines, closing of restaurants, malls and hotels decreased wastewater discharge into water bodies (Pacaol, 2021); one of the case studies, conducted in Malaysia, showed that the suspension of non-essential activities had a positive impact on water quality (Najah et al., 2021). Dobson et al. (2021), using integrated modelling approach investigated a number of water quality parameters during the COVID-19 lockdown in London and confirmed the general (although not obvious) positive impact on in-river water quality. Finally, there are very few studies on the environmental effects of the COVID-19 lockdown in mountain areas. Bates (2020) reports that satellite images and Landsat data revealed less snow contamination in the Indian mountain ranges, whereas Bayar (2020) warns that Turkish mountain regions are more likely to become polluted as more people engage in outdoor activities and produce more garbage.

The impact of COVID-19 in Poland was similar to that in other European countries. On March 14th 2020, the Polish government declared an epidemic threat and citizens were instructed not to leave their houses unless necessary, entertainment facilities and restaurants were closed and the majority of companies closed their offices implementing home office working. From March 24th until April 20th 2020 travel across Poland was limited to necessary activities and associated only with performing work duties. From March 24th until May 18th 2020 restaurants, pubs and cafes were closed, but even after this time they could only function provided that the number of people per m² was maintained below a constrained number. This severely affected the Polish economy and, according to the Statistical Office in Rzeszów (Statistics Poland, 2020), tourism became one of the most significantly disturbed sectors by the pandemic-related crisis.

Thus, the COVID-19 lockdown conditions created a unique opportunity to investigate the properties of surface water in the Biała river valley during the winter season but without the associated tourist traffic. The use of statistical multivariate analysis techniques allowed to test the research hypotheses according to which: (a) the COVID-19 related
lockdown and the resulting decreased intensity of tourism substantially affected a number of parameters that determine the quality of surface water and (b) intensive tourism is a key factor shaping the water environment in tourismally developed mountain regions. In particular, the aim of the study was to determine how much the natural environment of the Biała river recovered after heavy anthropogenic pressure abruptly ceased.

2. Material and methods

2.1. Study area and sample collection

The selection of the sampling sites was based on 7 years of experiments conducted in the Biała river catchment. This experience allowed the most representative sites, in terms of the varying anthropogenic pressure, to be selected along the course of the river. Six sites were selected for the study (Fig. 1).

The water samples were collected at three dates: in the summer tourist season (August 2019), in the pre-lockdown late winter tourist season (early March 2020) and during the COVID-19 lockdown (May 2020). Water temperature (T), electrolytic conductivity (EC_TL) and pH were measured onsite during sampling with a handheld multimeter (YSI Pro 2030; USA). The samples were collected into four sets of bottles: for microbiological analysis 1000 ml water samples were collected into sterile polypropylene bottles; for analyses of hydrochemical parameters into 500 ml polyethylene bottles; for the assessment of antibiotic content and for DNA extraction for illumina sequencing - into 1000 ml sterile polyethylene bottles.

2.2. Solid phase extraction and analysis of antimicrobial agents

Antibiotics were selected on the basis of their wide applications in human and veterinary medicine on a global and national scale (European Centre for Disease Prevention and Control, 2019; European Medicines Agency, 2018) and these were: ampicillin, amoxicillin, cefoxitin, cefuroxime, clindamycin, doxycycline, erythromycin, gentamicin, netilmicin, ofloxacine, oxytetracycline, sulfamethoxazole, tetracycline, trimethoprim and vancomycin. Antibiotics from water samples were extracted using solid-phase extraction (SPE) cartridges (Oasis HLB 6 cc Vac Cartridge, 500 mg Sorbent per cartridge, 60 μm Particle Size, Waters, Milford, USA). Each cartridge was conditioned using 10 ml of methanol and three times with 5 ml of ultrapure water at a flow rate of 1–2 ml/min. One l of pre-filtered water sample (0.45 μm Sartorius filters) was then passed through the SPE cartridge at a flow rate of 10–20 ml/min, after which the SPE cartridges were dried under vacuum pressure for 30 min and the compounds eluted with 10 ml of methanol at a flow rate of 1–2 ml/min. Finally, the samples were completely dried and dissolved in 1 ml of methanol (Zhou et al., 2012).

The quantification of antibiotic content in water samples was carried out using an Ultra High Performance Liquid Chromatography (UHPLC) device equipped with an automatic autosampler (Agilent 1290 Infinity System) and mass spectrometer (MS) Agilent 6460 Triple Quad Detector (Santa Clara, USA). For the separation of compounds, an Agilent Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 μm) was used at 30 °C. The gradient of water with 0.1% formic acid and organic phase (acetonitrile with 0.1% formic acid); 0–5.50 min 5% organic phase, 5.51–8 min 100% organic phase, 8.01–9 min 95% organic phase was applied in order to separate the compounds. The volume of the injected sample was 5 μl and the flow rate was 0.4 ml/min. An MS Agilent 6460 Triple Quad tandem mass spectrometer with an Agilent Jet Stream Electro spray Ionization interface was used in both positive and negative ionization using Dynamic Multiple Reaction Monitoring (dMRM) mode. Nitrogen was used as the drying gas and for collision-activated dissociation (flow rate 10 l/min). The temperature of both the drying gas and sheath gas was 350 °C. The capillary and nozzle voltage were set to 3500 V and 500 V, respectively. System control, data acquisition and data processing for UHPLC-MS were done by MassHunter software (Version 10.0, Agilent, Santa Clara, CA, USA). SPE recovery ranged from 9.94% to c.a. 100%.

2.3. Enumeration, isolation and identification of bacteria

In order to enumerate Escherichia coli, 100 ml of water was filtered through cellulose nitrate 0.45 μm membrane filters (Sartorius, Germany), placed onto Tryptone Bile X-glucuronide (TBX) agar and incubated at 44 °C for 48 h. After incubation, blue-green colonies were counted and expressed as colony forming units per 100 ml of water (CFU/100 ml). Then, blue-green colonies, preliminarily identified as E. coli, were subcultured by plate streaking and their species was confirmed using MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization - Time Of Flight) mass spectrometry. Staphylococcus spp. were enumerated using serial dilutions method (cultured on mannitol salt agar at 37 °C for 48 h). After incubation, the potential Staphylococcus colonies were verified by microscopic observations of Gram-stained smears. After being confirmed, the number of Staphylococcus spp. was expressed as CFU/ml.

2.4. Antimicrobial susceptibility tests

Forty-six E. coli strains were selected from the three examined dates, from different sites. Antimicrobial resistance was tested using the disk diffusion method following the recommendations of the Polish National Reference Centre for Antimicrobial Susceptibility (Gniadkowski et al., 2000). Antimicrobial disk cartridges were obtained from Oxoid (Great Britain). Bacterial isolates (0.5 MacFarland suspensions) were streaked onto Mueller-Hinton II agar (Biozaxima, Lublin, Poland) and antimicrobial disks were applied. The antimicrobial disks used in this study were: amikacin (AK 30 μg), amoxicillin/clavulanic acid (AMC 20/10 μg), ampicillin (AMP 10 μg), aztreonam (ATM 30 μg), cephalin (KF 30 μg), cefazolin (KZ 30 μg), cefamandole (MA 30 μg), cefoxitin (FOX 30 μg), cefotaxime (CTX 30 μg), ceftazidime (CAZ 30 μg), cefepime (FEP 30 μg), ciprofloxacin (CIP 5 μg), gentamycin (CN 10 μg), netilmicin (NET 30 μg), tobramycin (TOB 10 μg), piperacillin (PRL 100 μg), ticarcillin (TIC 75 μg), piperacillin/tazobactam (TIZP 100/10 μg), tetracycline (TE 30 μg) and trimethoprim/sulfamethoxazole (SXT 1.25/23.75 μg). The ESBL (extended-spectrum beta-lactamase)-positive strains were confirmed with the double disk synergy test (Drieu et al., 2008). After incubation for 18–24 h at 36 ± 1 °C, the growth inhibition zone diameters were measured and the results were compared with the breakpoint values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019).

2.5. Determination of the presence of ESBL genes in bacterial cultures and in total water DNA extracts

For the purpose of molecular analyses, bacterial DNA was extracted from all 46 E. coli strains and the control ATCC 25922 strain, using the Genomic Mini DNA extraction kit (A&A Biotechnology, Gdańsk, Poland), following the manufacturer’s protocol. PCR tests were conducted using specific primers: blaCTXM3 (Costa et al., 2006), blaCTXM9 (Simarro et al., 2000) and blOXA, blOXA and blOXA (Sænæs et al., 2004). The reactions were performed in a volume of 25 μl containing 50 ng of DNA template, 12.5pM of each primer, 2x (12.5 μl) of PCR Mix Plus Green (A&A Biotechnology, Poland) filled up with ultrapure water up to 25 μl. The following temperature profile was used for the reactions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, annealing for 45 s at temperatures corresponding to individual primers, then extension at 72 °C for 1 min with the final extension at 72 °C for 10 min. The reactions were performed in a T100 Thermal Cycler (Bio-Rad, USA). The PCR products were electrophoresed for 60 min in 1 x TBE SimplySafe (EurX, Poland)-stained 1% agarose gel and visualized in UV light.
2.6. Hydrochemical analyses

The chemical composition of water (concentrations of: Ca$^{2+}$, Mg$^{2+}$, Na$^+$, K$^+$, HCO$_3^-$, SO$_4^{2-}$, Cl$^-$, NH$_4^+$, NO$_3^-$, NO$_2^-$, PO$_4^{3-}$, Li$^+$, Br$^-$, F$^-$) was determined by ion chromatography using a DIONEX ICS-2000 chromatograph and an AS-4 autosampler (Dionex, Sunnyvale, USA). Water mineralization (total dissolved solids, TDS) was calculated as a sum of the determined ions.

Fig. 1. Study area and sampling sites along the Białka river. The 1st site is located at the border of the Tatra National Park (TNP) and is characterized by the cleanest waters. The 2nd site is situated c.a. 1 km upstream of the sewage treatment plant (USTP) of the Bukowina Tatrzańska municipality. The 3rd site is located by the effluent from the Bukowina Tatrzańska sewage treatment plant (STP). The 4th site is situated approximately 3 km downstream of the STP discharge, in the center of Białka Tatrzańska ski resort (DSTP1) and the 5th site - approximately 7 km downstream of the STP discharge in the Trybsz village (DSTP2). Groundwater collected in the Tatra National Park was treated as control, i.e. the most pristine water (GW).
2.7. Illumina sequencing of 16S rRNA amplicon

Water samples (1000 ml) were vacuum filtered through a 0.45 µm filter (Sartorius, Germany) and bacterial genomic DNA was extracted using Genomic Mini AX Bacteria + (A&A Biotechnology, Poland), followed by DNA purification using Anty-Inhibitor Kit (A&A Biotechnology, Poland).

The amplicon libraries of the hypervariable V3-V4 region of the 16S rRNA gene were prepared according to the 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B (Illumina), followed by a two-step PCR using Herculase II Fusion DNA Polymerase Nextera XT Index Kit v.2. The sample libraries were loaded on an Illumina MiSeq Platform and 2 × 300 bp reads were generated by Macrogen (South Korea).

2.8. 16S rRNA gene sequence analysis

The 16S rRNA V3-V4 regions from the Illumina sequencing were identified by comparing the sequence reads against the Greengenes v.13 database (97% similarity, minimum score 40). The resulting sequences were taxonomically assigned at the phylum level or lower ranks. CLC Genomic Workbench v. 12 (Qiagen, Hilden, Germany) and Microbial Genomics Module Plugin v. 4.1. (Qiagen, Hilden, Germany) were used to measure alpha diversity (Shannon diversity and Chao-1 index) within sample, as well as beta diversity among samples (UniFrac distances – weighted and unweighted, Bray-Curtis and Jaccard).

2.9. Quality assurance and quality control

Quality assurance and quality control (QA/QC) was implemented to ensure the reliability of the obtained results. The sample collection was performed according to the guidelines on surface water sampling (Musselman, 2012), in three replicates. Each replicate was an individual grab sample. Moreover, each of the examined experiment was performed in two or three replications, in order to verify the repeatability of the obtained results. The results presented in this study are means of the replicates.

Quantitative analysis of antibiotic content was based on pure standards: oxytetracycline, vancomycin, gentamicin (VWR International LLC, Gdansk, Poland), erythromycin, trimethoprim, ofloxacín, sulfamethoxazole, clindamycin, cefoxitin, netilmicín, doxycycline, tetracycline, cefuroxime (POL-AURA, Zabrze, Poland), ampicillín, amoxicillin (Sigma-Aldrich, Saint Louis, USA). All solvents used in this experiment were high-performance liquid chromatography (HPLC) mass spectrometry (MS) grade (99.8%, VWR International LLC, Gdansk, Poland). The effectiveness of the method was verified by extracting a mix of all standards of antibiotics. Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on the standard deviation of the response (SD) and the slope of calibration curves (S), according to the formulas below:

\[
LOD = 3.3 \times \frac{SD}{S}
\]

\[
LOQ = 10 \times \frac{SD}{S}
\]

SD- the residual standard deviation of the regression line
S- the slope of the calibration curve of the standard analytes (Raponi et al., 2017).

For dMRM mode implemented in HPLC-MS measurements 4 product ions for most investigated compounds were recorded during analysis to ensure correct identification of compounds in real samples.

The quality control for the disk-diffusion based assessment of antibiotic resistance among bacteria was performed using the E. coli strain ATCC 25922. In the PCR tests, E. coli strain ATCC 25922 was used as a negative control, while the ESBL-positive strains detected in this study were used as positive controls. In hydrochemical analyses limits of quantification (LOQ) were determined by a standard curve. Quality control was performed by analyzing blanks and sample examination in duplicate runs. The calibration standards were standard solutions from Dionex. For the purpose of Illumina sequencing, DNA concentration was measured with a Qubit 4 Fluorimeter (Invitrogen, Waltham, USA). The presence of bacterial DNA in the examined samples was confirmed by qPCR run in a Mx3000P (Stratagene, USA) thermal cycler using SYBR Green as a fluorochrome and Universal 16S rRNA primers (Ferris et al., 1996). The obtained library quality was verified according to Illumina qPCR Quantification Protocol Guide.

2.10. Statistical analyses

Basic descriptive statistics were calculated and a Kruskal-Wallis analysis of variance (ANOVA) was applied to verify whether there were significant differences in the examined microbiological and hydrochemical parameters between the study periods. Spearman’s correlation coefficient was used to assess the correlation between E. coli, Staphylococcus spp., antibiotic concentrations and the Illumina reads identified to the genus level. Principal Component Analysis (PCA) was applied to explore the relationship between the following parameters: numbers of E. coli and Staphylococcus spp., hydrochemical parameters, antibiotic concentration and NGS data acquired for all samples. The number of principal components and the factors were selected according to the Kaiser criterion and the factors with eigenvalues > 1.00 were taken into consideration. The tests were conducted in Statistica v. 13 (StatSoft, USA). The statistical significance of differences in the prevalence of antibiotic resistant bacteria between the three dates of sampling was estimated with a chi-square test (https://www.socscistatistics.com/tests/chisquare2/default2.aspx). Heatmap construction, including clustering analyses, were conducted in R environment using heatmap package (Kolde, 2018).

3. Results and discussion

As stated in a number of articles (Bojarczuk et al., 2018; Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2016b; Lenart-Boroń et al., 2016c; Lenart-Boroń, 2017; Lenart-Boroń et al., 2017; Lenart-Boroń et al., 2019; Lenart-Boroń 2020), the dynamic development of tourism in the Bialka river valley affected the once pristine water environment in various ways. A significant share of human activities ceased as a result of the COVID-19 lockdown, providing a rare opportunity to observe the studied mountain environment without its normal tourist traffic, a unique situation in the region for well over a decade. This allowed an assessment of the impact of anthropogenic pressure on regions of the Bialka river catchment and to observe how the water environment reacts to this unexpected change.

3.1. Culturable bacteria in water

In terms of microbiological indicators of water quality, such as Escherichia coli and Staphylococcus spp., the differences between the three sampling dates are evident and clearly indicate the environmentally positive effect of the lockdown (Supplementary Table 1). For example, the numbers of E. coli and Staphylococcus spp. by the STP reached their maximums in August 2019 (99 × 10^4 CFU/100 ml and 40 CFU/ml respectively), whereas at the same site the number of E. coli was 12 CFU/100 ml and only 1 CFU/ml for Staphylococcus spp. in May 2020 (lockdown period). Moreover, during lockdown, E. coli and Staphylococcus spp. were absent in other sites, while in the previous dates these bacteria were absent only in groundwater (GW) (Supplementary Table 1). Such low numbers of bacteria have not been observed in the previous years during the winter and summer peak seasons. For
instance, the numbers of *E. coli* by the STP were similar to those observed during lockdown only during periods of low tourist traffic (i.e. early spring, late autumn) (Lenart-Boroń et al., 2016a). The absence or low numbers of *E. coli* at the three other sites (i.e. USTP, DSTP1 and DSTP2) was also noted during off-season sampling (October, November, early December) by Lenart-Boroń et al. (2016b), Bojarczuk et al. (2018) and Lenart-Boroń et al. (2019). The TNP site has been assessed as generally clean, with no *E. coli* most of the year (Bojarczuk et al., 2018). The most probable reason for such low number of bacteriological contaminants in water during the COVID-19 lockdown period is the significant decline in tourism not only in the studied region but in the entire country (Korinth and Ranasinghe, 2020). This hypothesis is also supported by the approximate amount of wastewater collected by the municipal STP, discharged in the examined months, which was the highest in August 2019 (c.a. 32,000 m³) and the smallest in May 2020 (less than half, i.e. 15,000 m³). Tourism has been demonstrated as the most significant factor of the fecal contamination of Bialka river water due to the STP overload and illegal discharge of sewage from private guesthouses (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2016c). On the other hand, as recommended by the government and sanitary authorities, people started using more alcoholic sanitizers, soaps, and disinfectants, which might have also contributed to the decrease in the number of microorganisms in wastewater (Aravinthasamy et al., 2021).

3.2. Antimicrobial resistance of *E. coli* strains

A trend similar to that seen in the bacteriological indicators of water quality could also be observed in the percentage of antibiotic resistant *E. coli* present within the total pool of strains (Table 1). The bacterial strains isolated in August 2019 showed the highest share of resistance to 5 antibiotics, and the highest share of ESBL-positive strains. Also, all strains isolated in this period were multidrug resistant (MDR). The strains isolated in March 2020 showed the maximum percentage of resistance to 9 antibiotics and the *E. coli* strains isolated in May 2020 – to 4 antibiotics. In terms of the total resistance (all strains isolated from each period showing resistance to any of the antimicrobial agents tested), the percentage share was the highest in August 2019 and the lowest in May 2020 (Table 1).

### Table 1

| Antimicrobial agent | Aug 2019 | Mar 2020 | May 2020 |
|---------------------|----------|----------|----------|
| Cefazidime (0/100)  | 9/91b    | 0/100a   |
| Ceftoxin (7/93a)    | 9/91a    | 0/100b   |
| Piperacillin / Tazobactam (0/100) | 0/100a | 0/100c |
| Ticarcillin (47/53a) | 27/73b  | 11/89c  |
| Cefepime (0/100)    | 9/91b    | 0/100b   |
| Cefotaxime (0/100)  | 5/95a    | 0/100b   |
| Netilmicin (27/73a) | 28/72a  | 11/89c  |
| Tetracycline (0/100) | 14/86a | 33/67a |
| Amikacin (7/93a)    | 9/91a    | 0/100b   |
| Aztreonam (0/100)   | 9/91b    | 0/100a   |
| Tobramycin (7/93a)  | 9/91a    | 0/100b   |
| Ciprofloxacin (0/100) | 5/95a   | 11/89c  |
| Trimeprprim / Sulfamethoxazole (0/100) | 5/95a | 22/78a |
| Cefalotin (87/13a)  | 32/68b   | 44/56c   |
| Cefazolin (33/67a)  | 14/86b   | 11/89c   |
| Cefamandole (0/100) | 9/91a    | 0/100b   |
| Ampicillin (100/0a) | 73/27a   | 22/78e   |
| Gentamycin (0/100)  | 9/91a    | 0/100b   |
| Amoxicillin / clavulanic acid (100/0a) | 18/82a  | 0/100a  |
| Total resistance    | 21/79a   | 14/86c   | 9/91b    |
| Other parameters of resistance | 2020 | 2019 |

| Presence [P/A] (%) | Aug 2019 | Mar 2020 | May 2020 |
|-------------------|----------|----------|----------|
| MDR (100/0a)      | 64/36b   | 44/56e   |
| ESBL (20/80a)     | 14/86c   | 0/100a   |
| blaTEM (33/67a)   | 9/91b    | 22/78e   |
| blaDOX (0/100)    | 9/91a    | 0/100b   |
| blacTX-M (0/100)  | 5/95a    | 100/0a   |
| blacTX-MP (0/100) | 5/95a    | 44/56e   |
| blacSHV (0/100)   | 5/95a    | 56/44c   |

Note: Values sharing the same letter (a, b, c) are not significantly different according to the Chi-square test, followed by the Fisher exact test (p < 0.05).

Bolded values are the highest among the examined dates.

- R – resistant or insusceptible; S – susceptible.
- P – type of resistance or resistance determining gene present; A – absent.

### 3.3. Concentration of antibiotics in the water samples

Interestingly, the opposite pattern was observed in terms of antibiotic residuals, as among 11 antimicrobials compounds detected in this study, 8 reached their highest concentrations during the lockdown period. Seven of these maximum values were observed by the STP and included vancomycin, erythromycin, trimethoprim, ofloxacin, ciprofloxacin, clindamycin and sulfamethoxazole. Tetracycline was detected only once and it was during the lockdown period at the DSTP1 site (Supplementary Table 2). One possible explanation of this phenomenon is the significant increase in the rate of antimicrobial agents prescribed and their “prophylactic” consumption which occurred in the first few weeks of the COVID-19 pandemics, as reported by several authors (Guisado-Gil et al., 2020; Armitage and Nellums, 2021; Rusic et al., 2021). Armitage and Nellums (2021) attribute the unexpectedly high rate of antibiotic prescriptions during the COVID-19 pandemics to their inappropriate and unjustified use as a result of telephone consultations. As suggested by Rusic et al. (2021), a significant proportion of people were taking antibiotics and demanding them from doctors in misguided attempts to prevent infections. The study by Galani et al. (2021) revealed a remarkable increase in the antibiotic content in wastewater in Greece during the first wave of COVID-19 infections. Most importantly, this increase cannot be strictly associated to the fact that some antibiotics (azithromycin and metronidazole) were considered possible therapeutic agents for patients with COVID-19 at the beginning of the pandemic, because the consumption of azithromycin increased by 36% in 2020 compared to 2019, while the consumption of other antibiotics excluding azithromycin increased by 61%. Rawson et al. (2020) report that even though only 8% of COVID-19 patients experienced bacterial or fungal co-infection during hospital admission, as many as 72% of patients received antimicrobial therapy, most often with broad spectrum antibiotics. According to Singer (2020), antibiotic consumption in England increased during the early pandemic, but during lockdown the use of at least a few antibiotics (e.g. doxycycline, co-amoxiclav, levofloxacin, clarithromycin and cefuroxime) was lower. Also, as stated by Reinstadler et al. (2021), who analyzed wastewater to monitor drug consumption in Innsbruck, the consumption of trimethoprim decreased during the COVID-19 lockdown. These two reports differ from the results obtained in this study, as the highest concentrations of most of the monitored antibiotics were observed during the lockdown (May 2020). As most of the consumed antimicrobials are excreted in an unchanged form, their content in the aquatic environment depends on the STP removal efficiency and – if it is low – the exposure of microorganisms to antibiotics will lead to increased antibiotic resistance post-COVID-19 (Lucien et al., 2021). Correspondingly, Kumar et al. (2021) found a substantial increased prevalence of antibiotic resistant *E. coli* in 2020 compared to 2018 in 6 locations of Ahmedabad city (India).

### 3.4. Hydrochemical parameters of water

Among the hydrochemical parameters, sum of ions, EC<sub>25°C</sub>, NH<sub>4</sub>+, NO<sub>3</sub>− and PO<sub>4</sub>− are mainly associated with anthropogenic impact (i.e. wastewater contamination) on the water quality in the examined area (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2016b; Bojarczuk et al., 2018; Lenart-Boroń et al., 2019). The fluctuations in the values of these parameters between the three dates are particularly visible in the case of the STP, DSTP1 and DSTP2 sites (Supplementary Table 1). By the STP the sum of ions was highest in August, while in March and
May it had lower values, whereas at the sites DSTP1 and DSTP2 the lowest sum of ions was observed during lockdown, EC$_{25^\circ C}$ was the lowest in March (STP) and May (TNP, USTP, DSTP1 and DSTP2), while NH$_4^+$ was observed only in the STP in August. According to previous reports (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2016b; Bojarczuk et al., 2018; Lenart-Boroń et al., 2019), the fluctuations in the mentioned parameters co-occur with fluctuations in tourist numbers. Furthermore, similar values of these parameters, e.g. sum of ions or NO$_3^-$ to those observed during the COVID-19 lockdown were only observed in previous years in the DSTP1 or DSTP2 during the off-season (i.e. October, November) (Lenart-Boroń et al., 2016a, 2016b; Bojarczuk et al., 2018) and only very rarely by the STP (Lenart-Boroń et al., 2019).

### 3.5. NGS-based bacterial community composition assessment

Illumina sequencing provided a substantial number of reads for bacterial communities in all examined sites, however there were clear differences in the number of reads between the samples collected in various sites. The number of reads for 18 water samples collected from three dates ranged from 71,161 for groundwater (GW) in the pre-lockdown winter season (March 2020) to 22,741 in the DSTP2 site in August 2019. Generally, the highest numbers of reads were characteristic of the GW sample, whereas the samples located downstream of the sewage treatment plant (DSTP1 and DSTP2) were characterized by the smallest number of reads. On the other hand, the number of operational taxonomic units (OTUs) ranged from 993 in the STP in August 2019 to 87 in GW in May 2020. Contrary to the number of reads, the groundwater samples were characterized by the smallest number of OTUs and unique OTUs (Supplementary Table 3), suggesting that the microorganisms present there are more probably of different origin than those sampled at the remaining sites. Their dissimilarity from the ones present at the other sites may also be due to the limited supply of contaminants from surface water. For groundwater-borne bacteria to thrive, they need to be able to withstand the oligotrophic conditions of the groundwater environment, resulting in the reduced biodiversity of this type of water. As shown in Fig. 2, the most diverse water in terms of number of unique OTUs was collected by the STP. This is hardly surprising, as biological treatment plants deal with already microbiologically very active material (i.e. sewage) and the conditions within treatment plants themselves are very different from those prevailing in surface waters. This means that the treatment plant’s effluent is expected to harbor a lot of unique bacteria not easily detected in the uncontaminated surface waters. Such an effect, i.e. the higher number of OTUs, is still visible in the DSTP1 and DSTP2 sites.

The alpha diversity measures differed in various sites, depending on the anthropogenic pressure the sites were under, while the differences between the study periods were rather more subtle (Fig. 2). The Simpson and Shannon diversity indices showed that the more anthropogenically impacted sites were characterized by higher diversity (i.e. the highest diversity was recorded for the STP, followed by DSTP1, DSTP2, USTP, TNP, and GW). This was related to both higher OTU richness and evenness recorded for the more polluted sites. Thus, the OTU richness was the smallest in groundwater, the highest by the STP and then it decreased downstream with the distance from the sewage treatment plant. Seasonally, the OTU richness was the highest during the lockdown period and the smallest in March 2020. The Kruskal-Wallis test did not, however, confirm that the observed differences in the alpha diversity indices were statistically significant ($H = 3.24$, $p = 0.20$; $H = 3.03$, $p = 0.22$ and $H = 2.47$, $p = 0.29$ for No. of reads, No. of OTUs and No. of unique OTUs, respectively). Such results, i.e. the lack of significant differences in the diversity indices for different water types, were observed in the studies of e.g. Van Assche et al. (2019). Shannon diversity correlated positively with OTU richness (Supplementary Table 4), No. of unique OTUs, species evenness, No. of $E$. coli and Staphylococcus spp., concentration of the antibiotic cefoxitin, EC$_{25^\circ C}$, Na$^+$, K$^+$, NH$_4^+$, SO$_4^{2-}$, Cl$^-$ and PO$_4^{3-}$, OTU richness correlated positively with the numbers of $E$. coli and Staphylococcus spp., the concentration of cefoxitin, EC$_{25^\circ C}$, sum of ions, Na$^+$, K$^+$, NH$_4^+$, SO$_4^{2-}$, Cl$^-$ and PO$_4^{3-}$, indicating that, contrary to the results obtained by e.g. Paruch et al. (2019), higher richness is associated with greater water contamination. However, some studies indicate that the results obtained in our study are not unprecedented, as for example Simonin et al. (2019) demonstrated that shifts in bacterial communities and diversity are a response to the urbanization-driven pollution of the watershed, while de Oliveira and Margis (2015) found higher alpha diversity in river water samples collected from the most human activity-impact regions compared to samples from less impacted regions.

### 3.6. Multivariate statistical analysis of datasets – Principal Component Analysis (PCA)

Principal Component Analysis (PCA) is a useful tool when there is a need to simultaneously consider several (or more) random environmental parameters and has been successfully applied in a number of studies dealing with the variability of water quality (Mandal et al., 2020; Nishiyama et al., 2018; Lira et al., 2020). Therefore, this method was employed to comprehensively examine the results obtained from the conducted experiments, which produced a large amount of data for each sampling date and study site. The results showed that the first three factors accounted for 58.03% of the total variation in the chemical composition of water, bacterial contamination, antibiotic content and Illumina sequencing results. The three independent factors separated the water samples into groups and demonstrated a few interesting regularities (Fig. 3).

The first factor (F1) explains 31.18% of variance and demonstrates severe anthropogenic pressure. It demonstrates a strong positive relation between higher Na$^+$, Cl$^-$ and PO$_4^{3-}$ concentrations and higher numbers of Staphylococcus spp. and $E$. coli, higher concentrations of cefoxitin and the high number of Illumina sequencing-based reads, including microorganisms identified to the genus level, such as Bifidobacterium, Propionimonas, Allochromatium, Caldilinea, Nannocystis, Terracoccus, Dokdonella, Nitrospira, Streptococcus, Gordonia, Blautia, Propionibivirio, Leptospira and Desulfovibrio (Supplementary Table 5). Some of these identified genera have been recommended as potential indicators of human fecal pollution of surface waters, such as: Bifidobacterium (Lamendella et al., 2008) or Blautia (Koskey et al., 2014). Some other genera, such as Nannocystis, Dokdonella, Nitrosira, Streptococcus, Gordonia, Propionibivirio, Leptospira and Desulfovibrio, even though not suggested as bacterial indicators of poor water quality, have been found or isolated from human or animal feces, in wastewater or in STP effluents (Chen et al., 2019; De et al., 2020). Generally, the identified F1 is an atypical factor from the hydrochemical point of view. Sodium and chloride ions have primary importance within this factor and they are also positively correlated with phosphates, indicating the strong impact of water and wastewater management. In the natural environment, without strong anthropogenic pressure, hydrogen carbonates are the most important anions and calcium is among the most important cations. The importance of the remaining ions results from the geological conditions of the area. Thus, the primary factor (F1) that drives the above atypical relationships should be considered anthropogenic.

The second factor (F2) explains 16.77% of variance and shows the negative relation: the higher number of NGS reads of e.g. Acidithiobacillales, Solibacteres, Alcaligenaceae, Cyclobacteriaceae, Leadbetterella, the lower numbers of Staphylococcus spp., $E$. coli, and lower water pH (Supplementary Table 5). This is coupled with lower concentrations of the antibotic ciprofloxacin along with some of the sequencing-based identified genera, such as: Clavibacter, Hallicsonenobacter, Entmicellia, Leadbetterella, Fluvicolita, Pirellula, Phenyllobacterium, Anaeroспорa, Blastomonas, Limnohabitans, Cellvibrio, Chthoniobacter, Lutheolibacter or Prosthecobacter. This indicates that along with the presence of ciprofloxacin, and at lower water pH, the numbers of $E$. coli and Staphylococcus spp. decrease. One of the interpretations of this relationship might be that the presence of ciprofloxacin in the STP sample caused
a reduction in the numbers of culturable *E. coli* and *Staphylococcus* spp. Ciprofloxacine is one of the agents commonly used in treatment of urinary tract infections and respiratory diseases, hence its presence in the STP effluent. According to the ECDC Annual Epidemiological Report on Antimicrobial resistance in the EU (*European Centre for Disease Prevention and Control, 2020*), 33% of invasive *E. coli* isolates in Poland were assessed as resistant to fluoroquinolone antibiotics, including ciprofloxacin (i.e. 67% of invasive *E. coli* strains were susceptible to...
Bishop and Okwori (2017) detected 75% of ciprofloxacin-susceptible E. coli and 92.3% of ciprofloxacin-susceptible S. aureus strains isolated from carrots, which was attributed to the use of contaminated irrigation water. This suggests that the presence of ciprofloxacin in the water environment negatively affected the prevalence of indicator bacteria. On the other hand, the presence of ciprofloxacin co-occurs with higher numbers of reads of the other bacterial genera mentioned above. This is also not unusual, as according to e.g. Li et al. (2017) and Fu et al. (2021), fluoroquinolones, such as enrofloxacin or ciprofloxacin may cause microbial shifts as a result of selective antibiotic pressure. This factor describes the lower anthropogenic pressure put on water quality, which can be associated with the COVID-19 lockdown. Therefore, according to this interpretation the F2 factor can be...
considered a restoration factor, associated with economic and political (lockdown-related) decisions. The third factor (F3) explains 10.08% of variance. It also shows the following negative relation: the higher the concentrations of Mg$^{2+}$ and HCO$_3^-$ ions; the fewer the numbers of NGS reads of Rhodoforax, Burkholderiales, Cytophagaceae, Stramenopiles, Flavobacteriaceae, Sphingomonadales, Flectobacillus (Extended Data Table 4). On the other hand, the number of NGS reads of Cytophagaceae, Flavobacteriaceae, Stramenopiles and Sphingomonadales was the lowest in the GW sample and also present in very small numbers by the STP. This similarity between the most pristine and most polluted water may be associated with the fact that the third factor distinguishes the river waters and the two samples that do not belong to this group (groundwater and STP effluent). With all the above, this factor can be called a semi-natural one.

The projection of water samples onto the F1/F2; F1/F3 and F2/F3 planes demonstrates that the anthropogenic pressure most significantly determines the segregation of waters within the examined periods. The samples of groundwater flowing out of the Tatra National Park (GW) cluster in the right part of the graph, whereas strongly transformed water samples from the STP are scattered, but are generally located in the left part of the diagram (Fig. 3). Between those two extremes, there are samples of water characterized by various degrees of anthropogenic transformation due to inflow of contaminants from the areas with unregulated water and wastewater management or downstream of the STP discharge (DSTP1 and DSTP2). A very strong effect of preventive measures against COVID-19 spread, in the form of travel restrictions and socio-economic disruption, can be observed during the lockdown period (May 2020) on the chemical composition of water and microbiological parameters (both culture-based and Illumina sequencing reads). The Białka river catchment, especially the Białka Tatrzanska and Bukowina Tatrzanska regions, is characterized by very well developed ski infrastructure along with accommodation facilities (Heldak, 2016; Heldak and Szczepański, 2011). The resulting tourist “invasion” of the local ski resorts generates huge amounts of wastewater that cannot be efficiently processed by the treatment plant (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2016c). Therefore, the STP effluents in certain periods are strongly enriched with nutrients, and NH$_4^+$, Na$^+$ and Cl$^-$ play a major role in their chemical composition as well as in the chemical composition of water downstream of the STP. During the COVID-19 lockdown period, there was a fundamental change in the position of the STP sample against the F1-F2 factors (Fig. 3c). The STP sample became similar to waters associated with the lithological structure and c, F$_2$ showed the highest sensitivity to the lockdown-associated changes (Fig. 3a and c, F$_2$ showed the highest sensitivity to the lockdown-associated changes). The STP sample against the F1-F2 factors (Fig. 3c). The STP sample was the lowest in the GW sample. This indicates their more common occurrence, possibly due to more favorable conditions. Some of these above listed families of bacteria are typically present or have been isolated from clean, pristine or mineral water samples For example, the only strains from the family Gaiellaceae were isolated from a deep aquifer supplying a mineral water bottling plant in Portugal (Albuquerque and da Costa, 2014), Laperriere et al. (2020) in their study on bacterial distribution in relation to watershed land use and water quality suggest that Chthoniobacter, Verrucomicrobia, Pedosphaeraceae and Proteobacteria can be used as indicative of highly forested or agricultural watersheds, which are characterized by better water quality parameters than urban watersheds.

3.7. Multivariate statistical analysis of datasets – cluster analysis and heatmap

The bacterial community composition was examined with a cluster analysis and a heatmap (Fig. 4a and b). Pseudomonas, Pseudomonadaceae and Flavobacteriales were the most frequent in all samples. One of the hypotheses explaining the dominance of these three bacterial groups may be the one put forward by Chen et al. (2021), who class these bacteria as metabolically flexible habitat generalists, which become dominant in highly dynamic and disturbed ecosystems. This characteristics gives the habitat generalists an advantage over habitat specialists in environments with high spatial and temporal heterogeneity. It also favors the group of generalists after events of natural and anthropogenic habitat disturbance, which appears to be the case in the samples examined in this study. Some of the bacterial groups were present only in the STP and downstream of the STP sites, suggesting that the STP may be the source of these bacteria. These groups include Thiothrix and Candidatus Microthrix, which are bacteria associated with activated sludge (Nierchilo et al., 2020), Carnobacteriaceae (lactic acid bacteria), Campylobacteraceae, Clostridiales and Ruminoococcus (common representatives of gut microbiota; Rajilje-Stojanović and de Vo, 2014). Conversely, other groups were found only in groundwater samples, i.e., Nocardia, Sphingobium or Sphingopyxis. Also, there were a few genera and phyla that were absent only in the groundwater samples: Flavobacteriales, Cytophagaceae, Stramenopiles, Verrucomicrobiaceae, Xanthomonadaceae, Saprospiraceae, Flavobacteriales, Cytophagaceae, Stramenopiles, Verrucomicrobiaceae, Xanthomonadaceae, Saprospiraceae, Clavibacter, Moraxellaceae. Cluster analysis, combined with the heat map (Fig. 4a and b), supports the results obtained in the PCA, as some of the genera and groups characteristics of each of the distinguished factors (F1, F2 and F3), form common clusters. This is particularly visible in Fig. 4b, which shows bacterial abundance in the six examined samples in the three periods of study combined. The first cluster combines three taxa which are abundant in all samples and are not associated with any of the three designated factors. Another two clusters group taxa which are mostly associated with F3 (a semi-natural factor). The third cluster most evidently groups taxa that are abundant only in the GW sample. Interestingly, the sixth cluster combines two taxa (Flectobacillus and Sphingomonadaceae), which are associated with F3 and are absent or less abundant in GW and STP samples, but were present in TNP, USTP, DSTP1 and DSTP2. The bottom, seventh cluster presents very interesting regularity, as it groups taxa associated with F1 (anthropogenic), and it is clearly noticeable that they are absent in pristine and less anthropogenically changed samples (GW, TNP and USTP), while being detected in the STP and the two samples located downstream (DSTP1 and DSTP2). However, the most readily noticeable feature of the cluster analysis for the sampling sites (sampling dates combined – Fig. 4b) is the clear hierarchical grouping of the sites along the pristine – anthropogenically disturbed gradient. In this analysis, the GW sample is distinct from the rest of the sites, which were split into pristine upstream waters (TNP and USTP) and anthropogenically affected STP, DSTP1 and DSTP2 samples.

3.8. Concluding remarks

It had been made clear that anthropogenic pressure strongly affects the relations between the hydrochemical parameters of water, antibiotic residues and bacterial populations along the longitudinal profile of the Białka river valley. The regions along the Białka river valley located nearest to the STP are most severely affected by anthropogenic
pressure, especially due to wastewater contamination (Lenart-Boroń et al., 2016a; Lenart-Boroń et al., 2019). The use of multivariate statistical analysis (i.e. principal component analysis and a heatmap along with cluster analysis) demonstrated that the water quality variation in the pre-lockdown and lockdown period is shaped by three major factors. The first and most important one is strictly related to poor water and wastewater management. This is a nationwide problem as the environmental impact of tourism (particularly as a result of wastewater-related contamination of water) has been reported by many authors, among others by Pickering et al. (2003) in the Australian Alps, by Sunlu (2003) in the Mediterranean region, by Baoying and Yuanqing (2007) in the Chinese Lijiang Ancient Town, by Nicholson et al. (2018) in the Mt. Everest Region or by Sun and Liu (2020) in the West Lake Basin in China.

However, as a result of the COVID-19 pandemic lockdown, the sudden closure of various branches of industry (including tourism) allowed the water environment in the studied region to return to the quality observed only during the off-season in recent years (Lenart-Boroń et al., 2016a; Bojarczuk et al., 2018; Lenart-Boroń et al., 2019). This short respite period was sufficient for the natural chemical composition of the Białka river water to recover and its bacterial population to re-appear, demonstrating that the anthropogenic changes are not irreversible. Also important, in the context of this study, is that the lockdown effect on the changes in various water parameters was not obvious while interpreting individual groups of data, its positive impact on the quality of surface water become evident only with the use of multivariate statistics. The presented results are consistent with other reports of COVID-19 impact on the quality of water resources (Braga et al., 2020; Hallema et al., 2020; Dobson et al., 2021). However, this study is one of the very few conducted in mountain areas. For example, Duttagupta et al. (2021) reported a general improvement in the quality of water in the Ganges in the close vicinity of the Himalayas. Other reports, on the other hand, focus on the improvement in air quality. Diémoz et al. (2021) describes the reduction in air pollution in the Italian Northwestern Alps while Bates (2020) reports less air pollution in a network of mountain ranges and rivers in India, China and Pakistan. It may be assumed that the data obtained for the samples collected during the lockdown period could be considered as a baseline mainly in order to estimate the scope of anthropogenic impact on aquatic environment in terms of water degradation, particularly due to wastewater-related contaminants. Therefore, strategies should be considered in order to sustain this effect at least to some extent, as it is evident that the most important problem in the study region is improperly functioning sewage management. Rume and Didar-Ul Islam (2020) proposed a number of strategies to maintain the positive environmental effects of the COVID-19 lockdown. Among these was addressing the need for proper treatment of wastewater before discharge, as well as ecological restoration and promotion of ecotourism. In terms of safe waste, wastewater and water management there is an evident need for extensive campaigns aiming to increase awareness of environmental damage caused by incorrect disposal of household sewage.

Fig. 4. Abundance heatmaps constructed based on log10 + 1 transformed NGS data for 63 OTUs whose total number of reads across all the samples reached 1000 for sampling sites, three dates combined (a) and all individual samples (b). Please note clear hierarchical clustering of sites along the gradient of anthropogenic pressure (a) and clustering of OTUs partially corresponding to the most important parameters identified in the PCA (a and b).
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CRediT authorship contribution statement

Anna Lenart-Boroń: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, review & editing, Project administration, Funding acquisition.
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