BABEȘ-BOLYAI UNIVERSITY

FACULTY OF BIOLOGY AND GEOLOGY

DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

EVALUATING THE SPREAD OF ANTIBIOTICS, ANTIBIOTIC RESISTANCE, AND BACTERIAL CONTAMINANTS IN SELECTED WATER ENVIRONMENTS FROM ROMANIA

- Summary -

Scientific supervisor

PROF. DR. NICOLAIE DRAGOȘ

PhD Candidate

SZEKERES EDINA-KRISZTA

CLUJ-NAPOCA

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List of abbreviations

AMR - antimicrobial resistance
ARB - antibiotic-resistant bacteria
ARG - antibiotic resistance gene
ARNA - Antimicrobial resistance and the causes of non-prudent use of antibiotics project
DAD - diode array detector
DDD - defined daily doses
DNA - deoxyribonucleic acid
EEA - European Economic Area
EARS-Net - European antimicrobial resistance surveillance network
ECDC - European centre for disease prevention and control
EMEA - European medicines evaluation agency
ESAC-Net - European surveillance of antimicrobial consumption network
ESVAC - European surveillance of veterinary antimicrobial consumption
EU - European Union
EUCAST - European Committee on antimicrobial susceptibility testing
FCA - fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol
FD – field desorption
FEDESA - European Federation of animal health
HGT - horizontal gene transfer
LC - liquid chromatography
MGE - mobile genetic element
MIC - minimum inhibitory concentration
MLSB - macrolide-lincosamides-streptogramin B
MRSA - methicillin-resistant *Staphylococcus aureus*
MS - mass spectrometry
OTU - operational taxonomic unit
qPCR - quantitative PCR
SOS – the DNA-damage repairing mechanism typical to prokaryotes
SPE - solid-phase extraction
WHO - World Health Organization
WWTP - wastewater treatment plant

Key words: antibiotics, antibiotic resistance genes, bacterial community composition, hospital wastewater, groundwater.

Chapter I: General introduction

Since their introduction in clinical use, antimicrobial agents, such as antibiotics, have significantly impacted human and animal health and paved the way for modern infectious disease medicine. These compounds were originally natural products synthesized by environmental fungi and bacteria that possessed the ability to eradicate other microorganisms, thus constituting one of the evidence that antimicrobials may have existed for long time before their discovery by man.

The most common antibiotic classes and groups are: β-lactams (penicillins, cephalosporins, carbapenems) sulfonamides, aminoglycosides, glycopeptides, macrolides, tetracyclines, and quinolones. As noted by Davies et al. (2006) antibiotics have a concentration-dependent action, functioning as bacterial weapons for "territorial warfare" at high concentrations but also as signaling molecules at low concentrations. As signaling molecules, antibiotics control the homeostasis of communities by regulating gene expression involved in motility, stress response, pigmentation, production of metabolites, biofilm formation, virulence, and colonization. The way that antibiotic molecules are used in their fluctuating natural environments makes it easy to imagine how the resistance genes may also have protective roles in their antibiotic producing hosts (i.e. to regulate the response to these signaling molecules and protect against their overproducing).

Antibiotic resistance is the ability of microorganisms to tolerate the effects of antibiotics and to grow in the presence of these therapeutic agents. The extensive increase of antibiotic resistance in taxonomically divergent commensal and pathogenic bacteria is a recent event that followed the massive antibiotic use in medicine, agriculture, aquaculture, and horticulture. Horizontal gene transfer mediated by mobile genetic elements is the main mechanism underlying ARGs mobilization and transfer from the existing environmental genomic reservoir, often between taxonomically distant microorganisms. The adaptive response to antibiotic stress triggers an increase in both resistance-acquiring mechanisms, i.e., in mutation rate and in recombination and HGT events. The genetic determinants of resistance mechanisms are mainly located on MGEs, with less than 5% encoded chromosomally. The first is the acquired resistance obtained through HGT and the latter is the natural intrinsic resistance of bacteria which is transferred only through vertical pathways. Plasmids became

one of the major players in the HGT of antibiotic resistance because they can incorporate other MGEs. Thus resistance genes can be inserted into integrons that comprise part of a transposon which can be embedded and carried on a plasmid. Class 1 integrons are the most widespread integron types. They are found extensively in clinical context and were correlated to most of the resistance cassettes, with over 80 different gene cassettes described from this class. Their widespread distribution is due to the fact that they are usually embedded in a plasmid or transposon. Most of the modern class 1 integrons have a conserved 3' end consisting of *qac*EΔ1 (low-level of resistance to quaternary compounds) that is the functional deletion of *qac*E cassette, followed by *sul*I gene (sulfonamide resistance), and *orf*5 (an open reading frame with unknown function). Anthropogenic pressure has a major role in class 1 integron dissemination, thus they are being considered as "pollutants" in natural environments.

ARGs have been identified in various environments, like ancient permafrost, ground- and drinking water, agriculture and animal husbandry, wastewaters, and receiving surface water. An environmental factor that may disseminate MGEs among bacteria and promote their fixation is co-selection. Resistance determinants for an anthropogenic pollutant can mediate the co-selection of the others fixed on the same mobile element by simple linkage. So the more resistance determinants are present on an element, the more likely co-selection will provide a selective advantage given the high probability of exposure to at least one selective agent, especially in areas with growing human impact. Selection for resistance to disinfectants and heavy metals fixes ARGs linked to the same MGE. In recent years, concern keeps growing about aquatic environments acting as an appropriate milieu for complex interactions between selective agents, MGEs, and resistance determinants from commensal and natural bacteria, as well as a spreading route of antibiotic-resistant organisms to human and animal populations through drinking water.

There was an overall increase in antibiotic consumption in the EU during the last decade. In 2016, the European consumption of antibiotics in the community ranged between 10.4 and 36.3 defined daily doses (DDD) per 1000 inhabitants per day in Netherlands and Greece, respectively. Since higher level of antibiotic use in EU member states was associated with higher resistance levels, erroneous use of antibiotics is reflected in high levels of microbial resistance, for Romania, in most cases, exceeding the EU/EEA average. EARS-Net annual reports repeatedly place Romania in the top five problem countries with the highest percentages of community- and healthcare-associated resistant bacteria.

Chapter II: Aims of the thesis

The aim of this thesis was to explore the occurrence and abundance of antibiotic resistance genes and resistance determinants, such as antibiotic residues, mobile genetic elements, and microbial contaminants in the urban and peri-urban aquatic environments on Romanian territory.

More specifically:

- to assess general levels of antibiotics, antibiotic resistance genes, as well as the bacterial community composition in hospital wastewaters;
- to assess the removal potential of an activated sludge and chlorine disinfection process to investigate the impact that a conventional wastewater treatment may have on polluted effluents;
- to assess the relation between levels of antibiotic resistance markers and anthropogenic pollution in groundwater environments at various distances from urban areas;
- to identify marker ARGs to be further used as anthropic activity tracers that may be applied in evaluation of resistance-associated pollution.

Chapter III: Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals

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1. Introduction

Considering that 20-30% of European patients receive antibiotics during their hospitalization, hospital effluents may be considered as hotspots for HGT, which can facilitate inter- and intraspecies transfer of antibiotic resistance determinants and virulence factors. Hospital effluents represent a major source of pollutants, such as antibiotics, heavy metals, and disinfectants, which are discharged into sewer systems, and/or receiving surface waters, often without any prior treatment. Only a few countries recommend pretreatment of these wastewaters before their release to water bodies, and no restrictions are foreseen in the European Directive 91/271/EEC for this special category of waste. Moreover, current EU legislation does not include specific regulations, neither the potential presence of ARB and ARGs in these waters nor their concentration thresholds.

The aim of this study was to determine the occurrence and abundance of antibiotics and ARGs, as well as bacterial community composition in wastewater effluents from different hospitals located in the Cluj County, Romania. We used culture-independent approaches to provide a better understanding of bacterial community composition and function. It should be noted that one of selected hospitals applies an activated sludge and chlorine disinfection process before releasing the effluent to the municipal collector. Raw and treated wastewater samples were thus collected to determine the removal potential of these pollutants.

2. Material and methods

- Wastewater effluent **samples were collected** in June 2015 in triplicate from three hospitals located in different cities from the Cluj County, Romania. Among selected hospitals: hospital 1 (H1) has an oncological profile and a number of 535 beds; hospital 2 (H2) is a small general hospital with 113 beds, providing service to around 30,000 inhabitants; hospital 3 (H3) has a general profile with 453 beds for 43,472 inhabitants. The effluent from H1 is treated using an activated sludge and chlorine disinfection process before being discharged into the municipal collector. Raw (H1) and treated (H1TWW) wastewater samples were collected for comparative purposes.
- The **quantification of the antibiotics** were carried out using a high-performance liquid chromatography (HPLC) system equipped with diode array and mass spectrometry (MS) detectors.
- The samples were filtered in triplicate through 0.2 µm-pore-size mixed cellulose ester membrane filters (Fioroni, France) (150 mL each) to collect microbial biomass. Each filter was cut into small pieces and used for total **DNA extraction** using the ZR Soil Microbe DNA kit (ZymoResearch, USA).
- Sixteen genes were **quantified** from the 36 genes tested in the **pre-screening** process, of which 14 genes confer resistance to different antibiotic classes such as β-lactams (*bla*$_{VIM}$, *bla*$_{SHV}$), aminoglycosides (*aac*C2), chloramphenicol (*cat*A1, *flo*R), MLSB (*erm*A, *mef*A), sulfonamides (*sul*I, *sul*II), and tetracyclines (*tet*A, *tet*B, *tet*C, *tet*O, and *tet*C), a gene from the 3' conserved region of class 1 integron conferring resistance to quaternary ammonium compounds (*qac*EΔ1) and a transposon-related element (*tnp*A). The 16S rRNA gene was also quantified to normalize the abundance of target genes and to assess the total bacterial population in hospital wastewater samples.

- Genomic DNA was extracted in triplicate and pooled, followed by an Illumina **sequencing** approach, targeting the V3-V4 region of the 16S rRNA gene, previously amplified using universal bacterial primers PRK341F/PRK806R.

3. Results and discussion

Several antibiotic compounds were detected in the wastewater collected from the three hospitals in Cluj County (Romania) at concentrations ranging from 3.67 to 53.05 µg/L (Figure 1). The most abundant antibiotic classes were β-lactams, glycopeptides, and trimethoprim. Ampicillin was detected in all wastewater samples, and the highest concentration was found in H1 (53.05 µg/L). Variations in the antibiotics administered in each facility, the number of beds, amount of water consumed or type of medical services provided may be reflected in antibiotic occurrence noted for hospital wastewater samples.

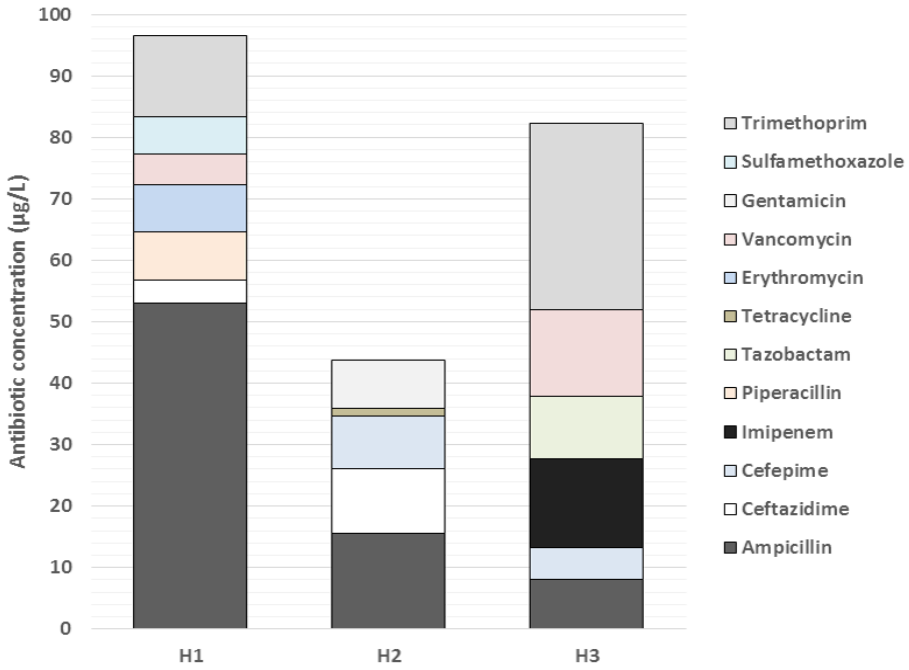

Figure 1. Concentrations of antibiotics detected in hospital wastewater samples. Hospital 1 (H1) has an oncological profile, while hospital 2 (H2) and hospital 3 (H3) have a general profile.

The total bacterial load (16S rRNA gene copy numbers) in samples ranged from 5.46×10^7 to 1.15×10^8 copy numbers per mL of hospital wastewater sample. Among the detected ARGs, the *sul*I gene conferring resistance to sulfonamides had the highest relative abundance ranging from 5.33×10^{-2} in H2 to 1.94×10^{-1} copies/16S rRNA gene copies in H1. Previous studies have suggested that

highly contaminated sites often have a relative abundance of ARGs higher than 10^{-4} copies/16S rRNA gene copies. It is therefore reasonable to assume that our wastewater samples have relatively high pollution levels of ARGs. The abundances of ARG types (Figure 2) showed an apparently similar pattern as the corresponding classes of antibiotics, where β-lactams had the highest concentration in H1, aminoglycosides in H2, sulfonamides in H1, and tetracyclines in H2. A recent study has demonstrated that the *sul*I gene frequency is positively related to the level of urbanization and *qac*E1/*qac*EΔ1 gene shows an adaptive role to several habitats. In this study, *sul*I and *qac*EΔ1 genes had similar frequencies and high copy numbers in all hospital wastewater samples with values between 1.94×10^{-1} and 4.89×10^{-2} copies/16S rRNA gene copies, respectively, in H1. These genes have been usually associated to class 1 integron structures that may reside on MGEs, thus indicating a high frequency of HGT.

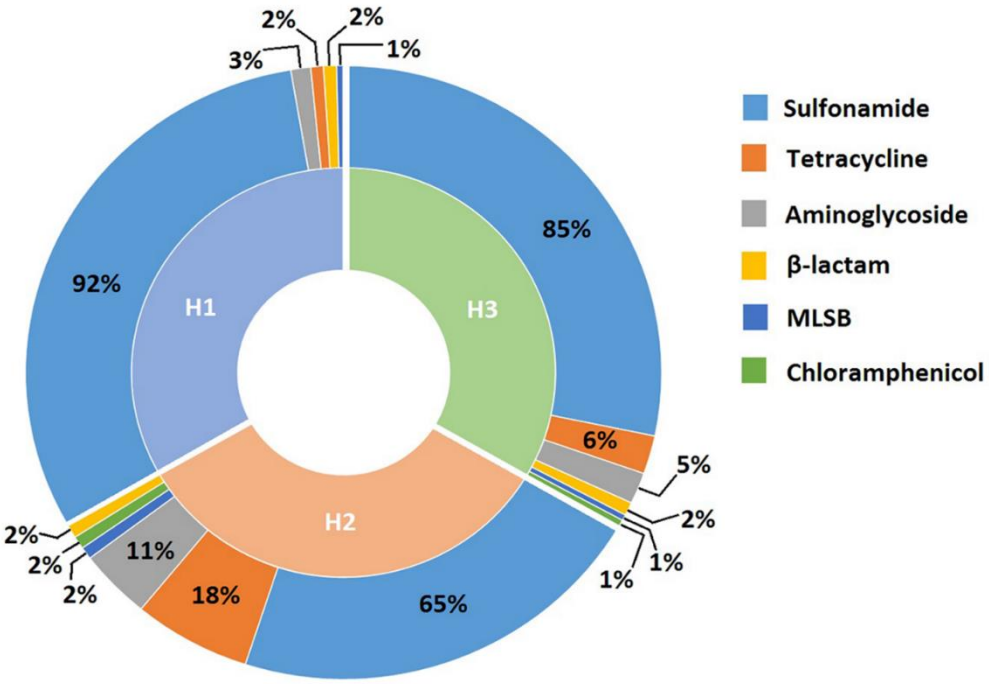

Figure 2. Percentage of the ARG types in the three hospital wastewater sample.

Phylogenetic classification of sequences, by using the default classifier in QIIME, assigned most of the sequences to 4 different phyla (Figure 3A). In H1, 97.27% of the sequences were assigned to Proteobacteria phylum. Members of the phyla Proteobacteria (44.7%), Firmicutes (33.68%), and to a lesser extent, Bacteroidetes (16.4%), and Actinobacteria (4.9%) were dominant in H2, while the community composition of H3 included three different phyla Proteobacteria (71.4%), Bacteroidetes (13.7%) and Firmicutes (13.1%). This taxonomic composition was found to be ubiquitous in hospital

wastewater effluents, non-hospital medical facilities, municipal WWTPs and untreated wastewaters from a wide range of geographic locations. Deltaproteobacteria, Epsilonproteobacteria, Clostridia, and Bacilli can be specifically associated with antibiotic-containing aquatic environments. As can be observed in Figure 3B, the investigated effluent microbiota was dominated by Epsilonproteobacteria (58.6% in H1, 6.9% in H2 and 23.9% in H3) and Gammaproteobacteria (26.9% in H1, 31.8% in H2 and 41.1% in H3). Deltaproteobacteria (1.2% in H1, 0.2% in H2 and 0.5% in H3) was found in relatively lower proportion. Clostridia (26.5% in H2 and 12% in H3) were found in high abundance in hospital wastewaters from the general profile hospitals. Other abundant classes were Bacteroidia (15.8%) as well as Bacilli (4.6%) and Actinobacteria (4.6%) in H2.

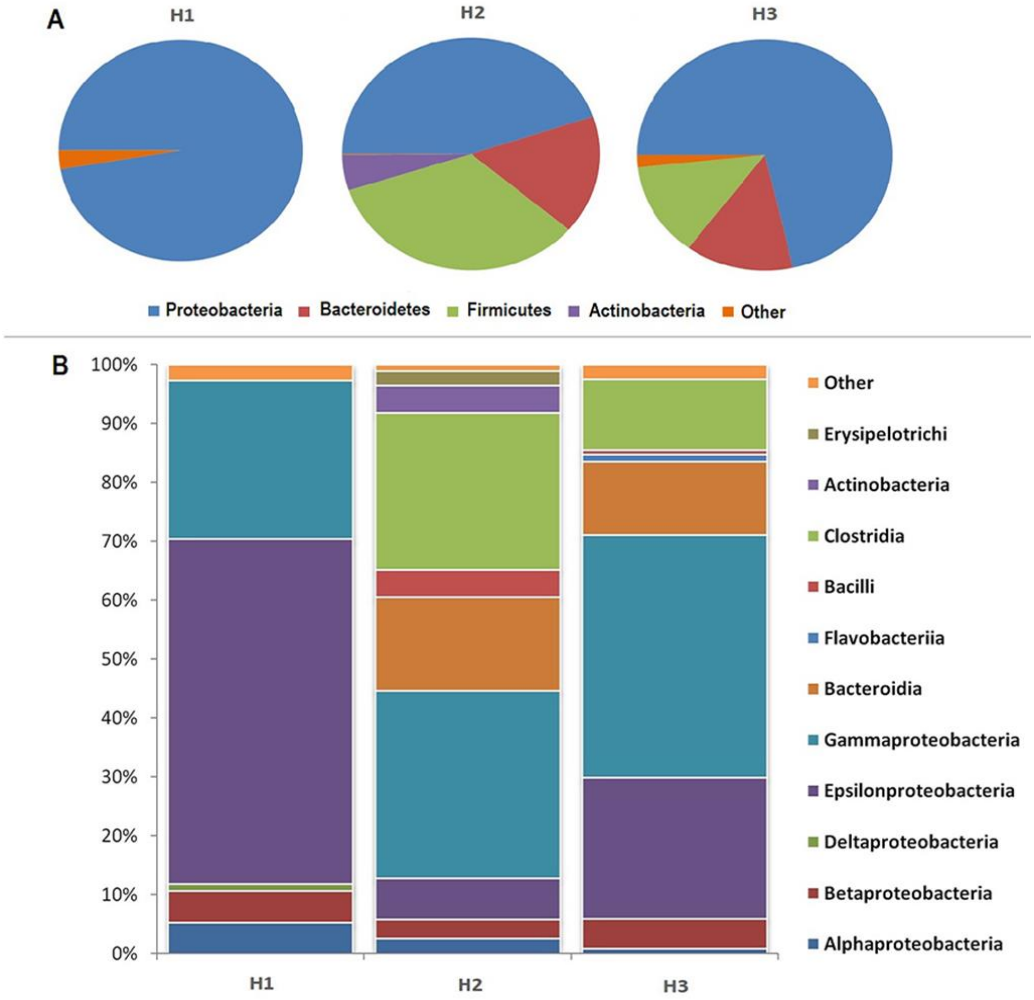

Figure 3. Bacterial community composition at phylum (A) and class (B) levels of hospital raw wastewater samples.

H1 was the only hospital which applies a wastewater treatment process before releasing the effluent into the municipal collector. In this study, antibiotic concentrations were reduced by 55-81% after the wastewater treatment process (Figure 4). Trimethoprim was reduced with 81%, followed by erythromycin with 62% and ampicillin with 55%. Piperacillin, vancomycin, sulfamethoxazole could not be detected in the treated wastewater (H1TWW). Interestingly, ceftazidime concentration increased about 74% in treated wastewater in comparison with hospital raw wastewater.

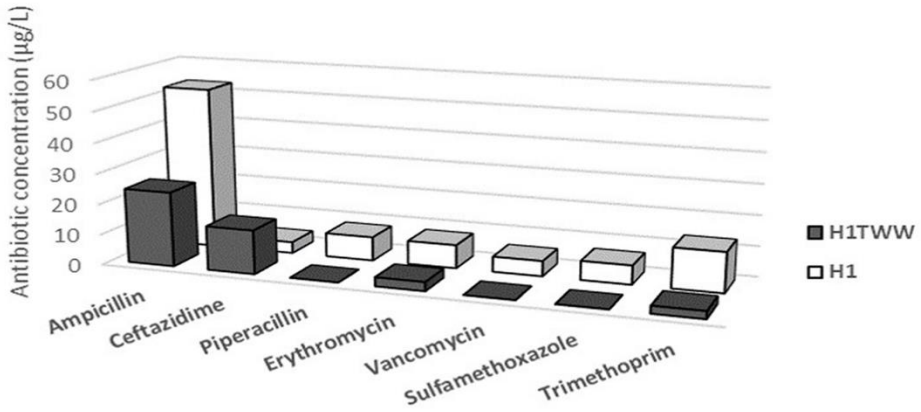

Figure 4. Concentration of antibiotics in raw (H1) and treated (H1TWW) hospital wastewaters.

Although the process of wastewater treatment is not specifically designed to decrease the levels of pollutants such as ARGs, their abundance was moderately reduced in the treated wastewater (Figure 5). The relative concentration of ARGs was found to be 1-3 order of magnitude lower (~10^{-7}) after the treatment, except for *tet*A, *bla*SHV, and *sul*I genes, which were slightly removed. Change in bacterial community abundance was observed, with a slight increase in Proteobacteria (from 97.5% to 98.7%) and Bacteroidetes (from 0.1 to 0.5%) phyla.

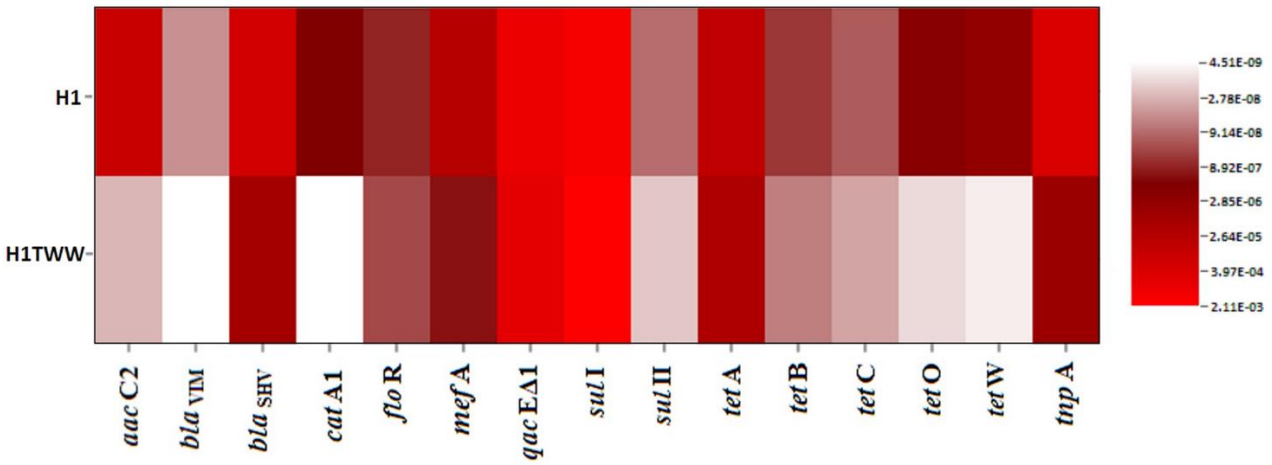

Figure 5. Relative concentration of antibiotic resistance genes in raw (H1) and treated hospital wastewater (H1TWW).

Chapter IV: Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas

[This chapter was published as: Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.-L., Rudi, K., Dragos, N., Coman, C. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas (2018) in Environmental Pollution: doi.org/10.1016/j.envpol.2018.01.107]

1. Introduction

In many countries, groundwater is the most important public water supply and also a drinking-water source. Thus, quality assessment and monitoring of these environments should receive particular attention. Due to the scale and impact of chemical and microbiological pollution, as well as the lack of clean-up technologies, the remediation of contaminated groundwater resources is difficult. Urban aquifers are especially vulnerable to anthropogenic pollution due to intense urban activities and industry. How direct and indirect anthropic activities may affect the ARGs profiles and related aspects of groundwater environments have been seldom investigated worldwide, partially because of difficulties and high costs involved. The selection of specific local environmental tracers could help in the comprehensive and consistent evaluation of these emerging pollutants that are currently needed to enable internal groundwater surveillance and protection policies for tackling human and environmental health risks posed by antibiotics and antibiotic resistance.

The scope of the present work was to evaluate the distribution of antibiotic residues, ARGs, MGEs and the microbial contamination in groundwater environments at various distances from urban areas using a culture-independent approach and to determine specific aspects that could be related to the anthropic activity. We hypothesized that groundwater with close proximity to the urban areas is more vulnerable to contamination with antibiotics, sewage-derived microorganisms, and ARGs that could pose serious threats to human health. Furthermore, we investigated if specific genes could be used as reliable tracers for anthropic activity that could follow the pattern of environmental pollution in groundwater.

2. Materials and methods

- Site selection was focused on areas suspected to be susceptible to contamination from either animal or human wastewater and also to areas more isolated and *a priori* considered as pristine environments from Cluj-Napoca (Romania) and surrounding areas (Figure 6). The sampling sites of shallow groundwater in the experiment are dug wells (open wells with a direct access to the surface and stagnant water column; referred to as "well" samples) and driven wells (enclosed wells, referred to as "pump" samples) designed for varied uses like irrigation, domestic and stock use and occasionally for drinking water. Groundwater **samples were collected** at each site with clean and sterile amber polyethylene bottles in June-July 2015 and transported to the laboratory on ice for immediate processing.
- The samples were taken in triplicate, pooled and vacuum filtered through 0.2 mm-pore-size mixed cellulose ester membrane filters (Fioroni, France). Three filters were obtained for each sample, and from every individual filter, **DNA was extracted** using the ZR Soil Microbe DNA kit (ZymoResearch, USA).
- The **quantitative evaluation of antibiotics** in the samples was carried out on an HPLC-DAD/MS (Shimadzu, Japan), single quadrupole instrumentation.
- Absolute **quantification** was carried out for the 14 genes observed as present during the pre-screening step, which code for resistance to different antibiotic classes (aminoglycoside, β-lactam, chloramphenicol, MLSB, sulfonamide and tetracycline), antiseptic resistance, and MGE.
- To characterize the prokaryote microbial community structure and composition, a fragment containing the V3-V4 regions of the 16S rRNA gene was selected for amplification with the PRK341F: CCTACGGGRBGCASCAG and PRK806R: GGACTACYVGGGTATCTAAT universal primers and submitted for **sequencing** on Illumina MiSeq platform.

3. Results and discussions

Several antibiotic compounds were detected and quantitated in the examined groundwater resources at concentrations ranging from below detection limit to 917 ng/L in GW4 for cefepime (Figure 7). Overall, the most abundant antibiotic classes were trimethoprim, macrolide, sulfonamide, and β-lactams, followed by tetracyclines and fluoroquinolones. A threshold value of 100 ng/L was recommended as a limit between low and high antibiotic concentrations in groundwater. Thus, the elevated levels of these micropollutants, particularly in GW1 and GW3 (Figure 7), may pose an environmental risk associated with their persistence, bioaccumulation, and toxicity.

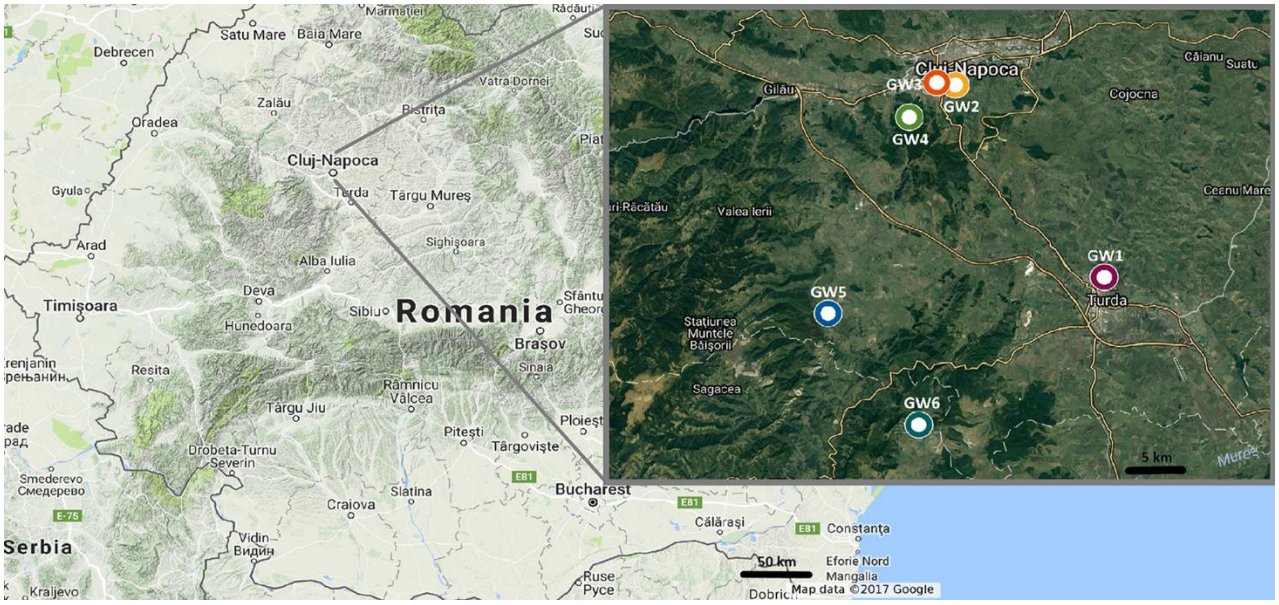

Figure 6. Location of wells and aquifers sampled in this study near the city of Cluj-Napoca and the surrounding areas (in the northwestern part of Romania).

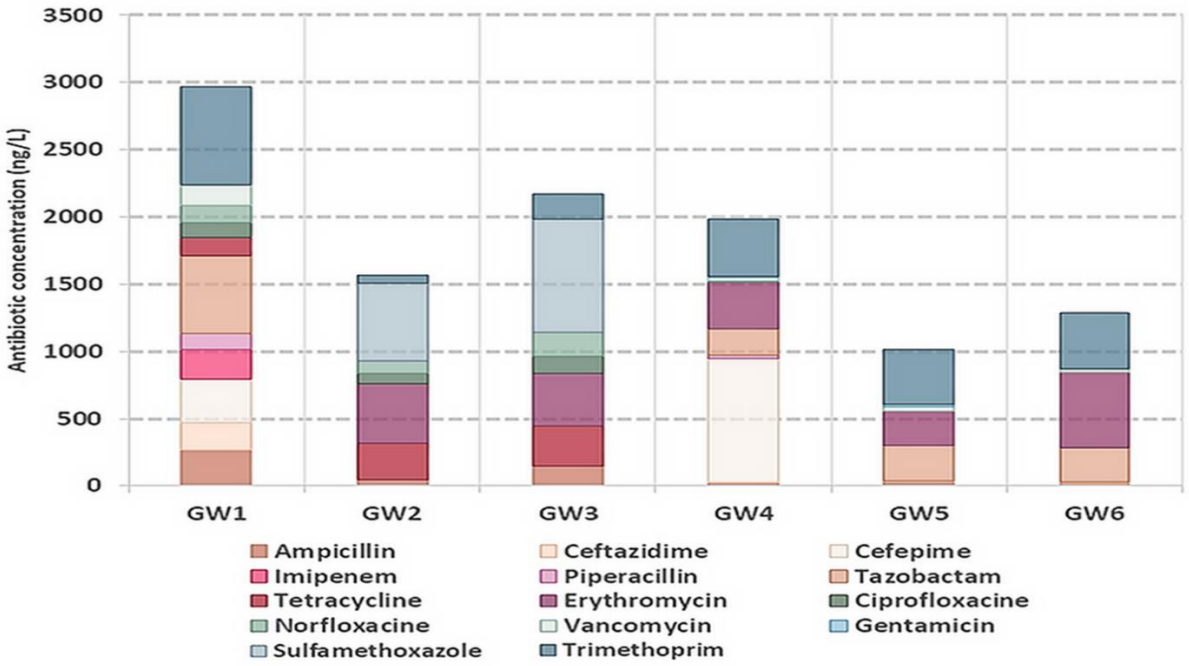

Figure 7. The concentration of antibiotics detected in groundwater samples.

From the total of 37 genes tested during the pre-screening step, a total of 11 ARGs were observed as present, coding for aminoglycoside (*aac*C2), β-lactam (*bla*SHV), chloramphenicol (*flo*R), MLSB (*erm*A, *mef*A), sulfonamide (*sul*I, *sul*II) and tetracycline (*tet*A, *tet*C, *tet*O, and *tet*W) resistance. Furthermore, one antiseptic resistance gene (*qac*EΔ1) and two ARG mobility-associated

genes (*tnp*A and *int*1) were identified (later referred to as other/associated). The presence of bacterial DNA in groundwater samples was verified by amplifying the 16 rRNA gene, which ranged from 2.53 × 10^3 to 5.01 × 10^6 gene copy number per mL of groundwater. The relative abundance of ARGs varied between 6.61 × 10^{-7} and 2.30 × 10^{-1} copies/16S rRNA gene copies in the investigated groundwater samples. In general, the order of ARG type frequencies in the groundwater samples was β-lactam > sulfonamide > tetracycline > other/associated > MLSB > chloramphenicol > aminoglycoside (Figure 8).

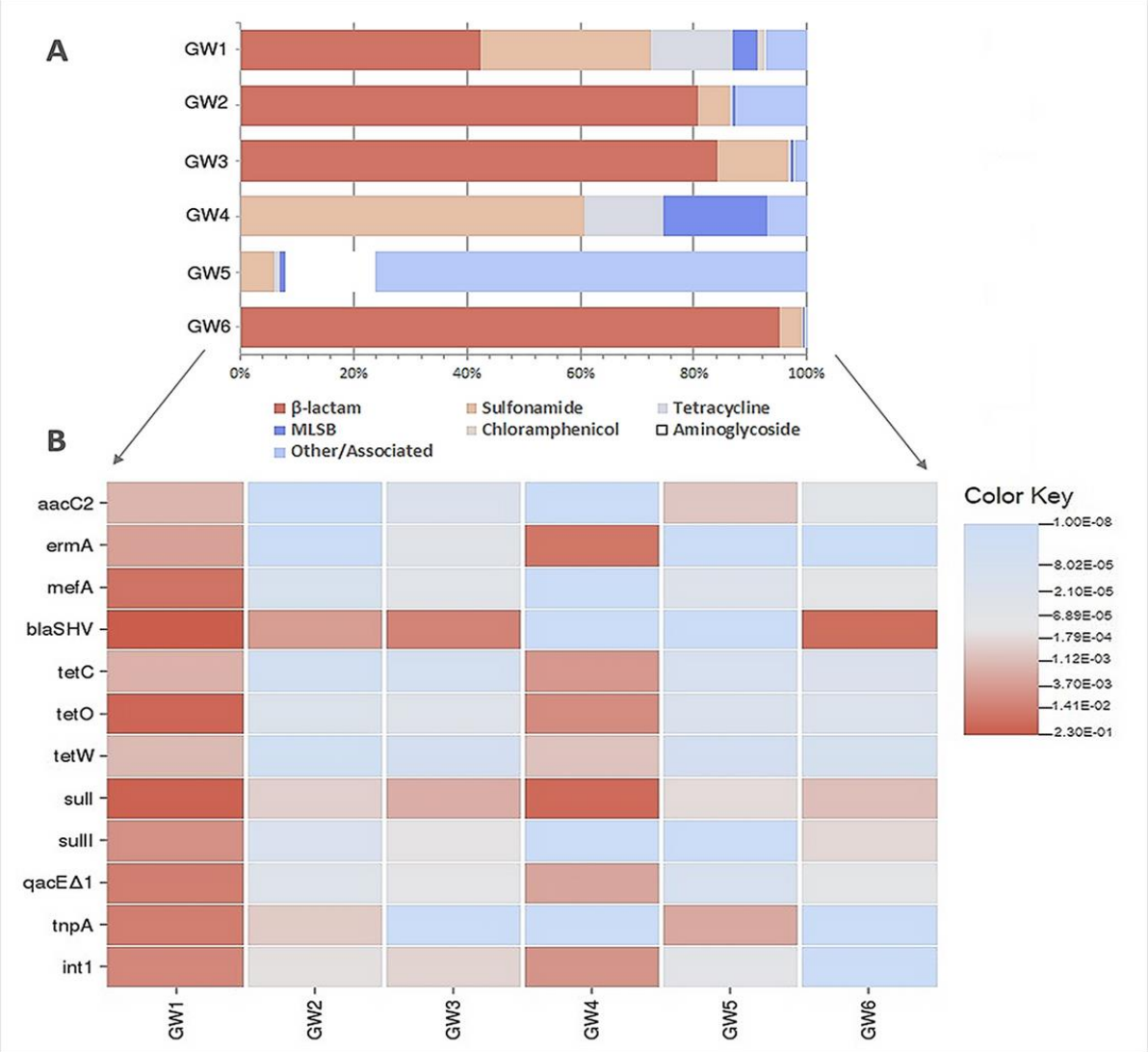

Figure 8. (A) Percentage of the ARG conferring resistance to the antibiotic families (x axis) in groundwater samples investigated (y axis). **(B)** Heat map of the relative concentration (target gene copies/16S rRNA gene copies) of ARGs measured in all six investigated groundwater samples.

Taxonomic classification of clustered OTU revealed the presence of 26 bacterial phyla. Abundant phyla (>1% of the population) included Proteobacteria, Bacteroidetes, Firmicutes, and

Actinobacteria (Figure 9). Several 16S rRNA gene sequences affiliated to fecal indicators such as *Escherichia coli* (less than 0.5%), *Enterococcus faecium* (less than 0.5% in GW1 and 0.7% in GW4), *Aeromonas hydrophyla* (2% in GW1 and 2.5% in GW2), *Aeromonas caviae* (0.7% in GW1 and 1.2% in GW4), and *Streptococcus* (less than 0.5%) were encountered with a relatively higher sequence number in GW1 and GW4, together with OTUs related to *Clostridium perfrigens* and the *Faecalibacterium* genus (Figure 10).

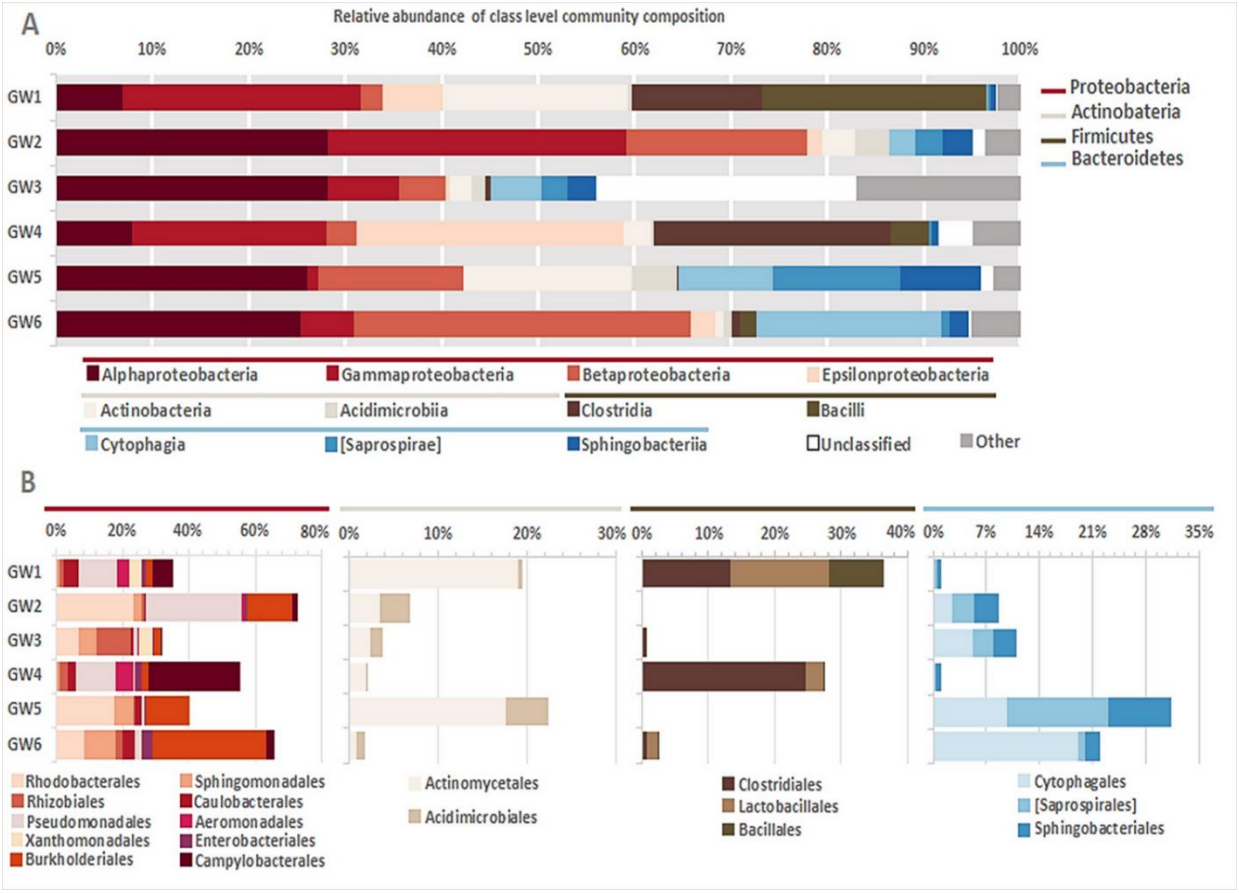

Figure 9. A taxonomic breakdown of bacterial communities by class **(A)** and order **(B)** in the groundwater samples. Classes and orders belonging to the same dominant phylum are color coded. Colors for each dominant phyla are shown in top right corner, red color palette for classes and orders belonging to Proteobacteria, tan for Actinobacteria, brown for Firmicutes, and blue for Bacteroidetes. Only taxonomic groups with more than 1% total abundance were included in the graphic.

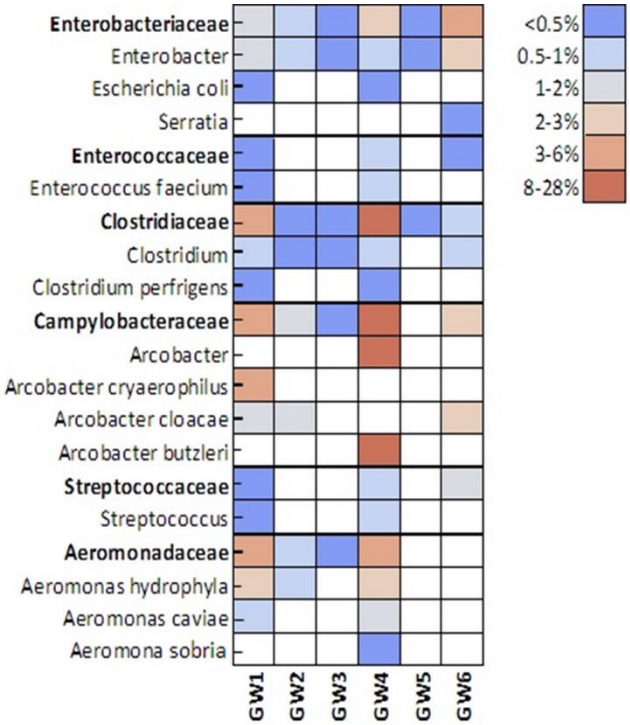

Figure 10. The relative abundance intervals of potential pathogens and fecal indicator bacteria in different samples. The family-level classification is accentuated in bold.

The co-selection of ARGs and MGE is suggestive for HGT in these aquatic environments and indicate a role of MGEs in the persistence and proliferation of the related resistance phenotypes. Correlation between the prevalence of ARGs, MGEs, and the microbial community structures at the phylum level indicated a connection with Proteobacteria, Bacteroidetes, and Actinobacteria (Figure 11). All three presented a weak correlation ($p < 0.05$) to ARGs, but a strong link ($p < 0.01$) to MGEs. It seems that Proteobacteria were the main integrase carriers, whereas Bacteroidetes and Actinobacteria showed a connection to transposases. The cooccurrence of class 1 integron-integrase gene (*int*1) with *qac*ED1, *sul*I, and *tet* (*tet*C, *tet*O, *tet*W) resistance genes in bacteria from groundwater samples was supported by previous findings documenting the coexistence patterns of these ARGs and integrons in different aquatic environments. Only the combined presence of these six genes showed trends towards the different degree of contaminant presence (antibiotics, ARGs, pathogens), suggesting the possibility of their use as tracer genes in the detection of antibiotic resistance related pollution.

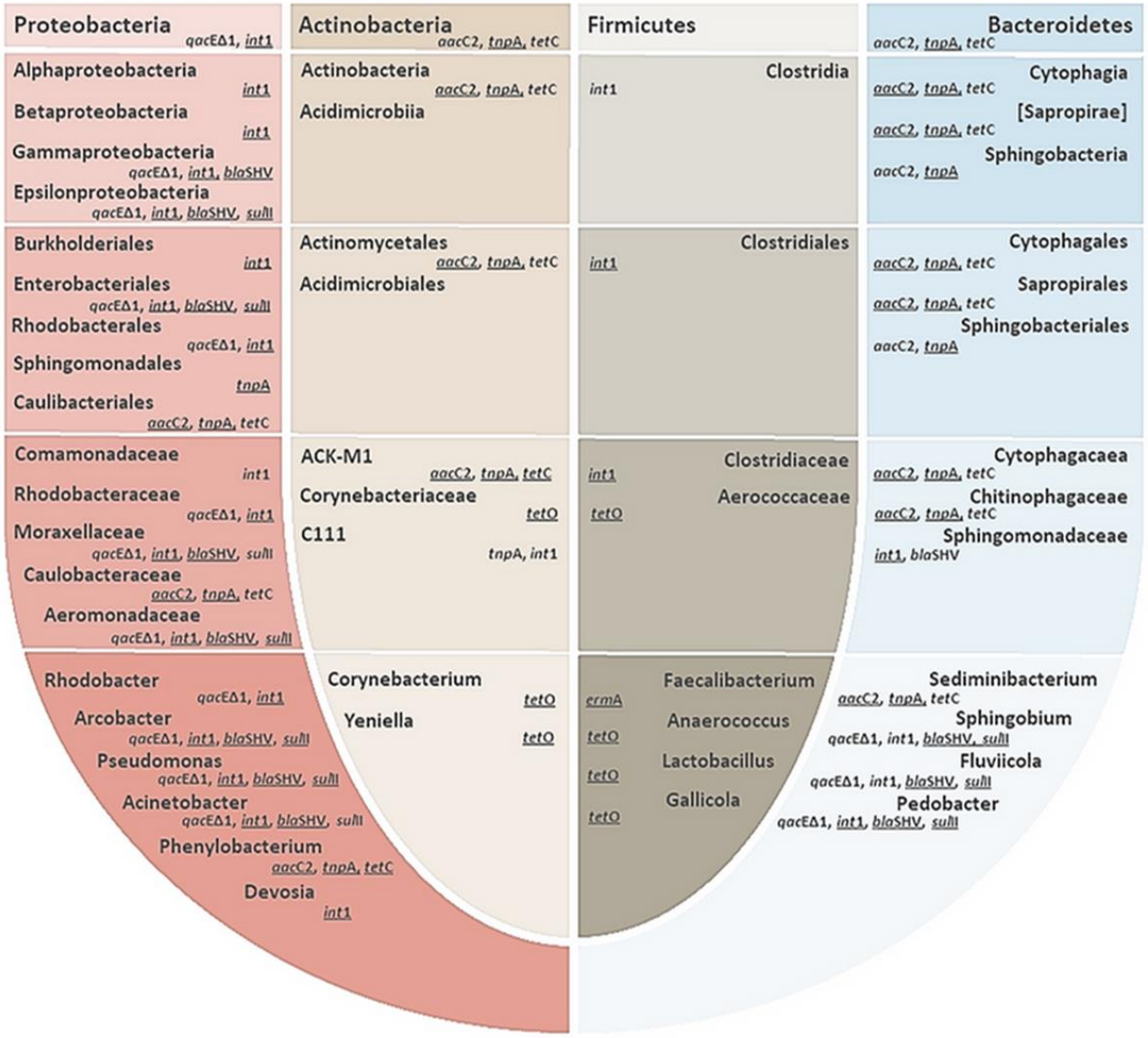

Figure 11. Significant correlation of the microbial community with ARGs. $p<0.01$ were considered as strong- (signaled with underline) and $p<0.05$ as weak correlations. The color palette of bacterial phyla corresponds to Figure 9: classes and orders belonging to the same dominant phylum are color coded, red color palette for classes and orders belonging to Proteobacteria, tan for Actinobacteria, brown for Firmicutes, and blue for Bacteroidetes.

Chapter V: Conclusions

- The most prevalent antibiotic classes detected in hospital effluents were β-lactams, glycopeptides, and trimethoprim with high, µg/L concentrations, and with reduction rates ranging from 55-81% after wastewater treatment. Trimethoprim, macrolide, and sulfonamide were the most abundant antibiotic classes, with ng/L concentrations, in tested groundwaters.
- Generally, ARGs showed a high relative abundance, with *sul*I and *qac*EΔ1 resistance genes as most prevalent in all the investigated hospital wastewater samples. Since these genes are part of the 3' conserved segment of clinical class 1 integrons, and considering that wastewater treatment did not affect their concentrations, it can be assumed that class 1 integrons are continously released into environmental waters. Moreover, the combined presence of class 1 integron associated genes (*int*1, *qac*EΔ1, and *sul*I) with *tet*C, *tet*O, *tet*W resistance genes can be used as reliable tracer for the measure of anthropogenic pollution in groundwater ecosystems.
- High abundance of Epsilonproteoacteria, Clostridia, and Bacilli (previously associated with antibiotic pollution) in addition to elevated levels of some opportunistic pathogen and fecal indicator groups were observed in the hospital effluents. Wastewater treatment increased the abundance of the *Enterobacteriaceae* family, a bacterial group well-known for its antibiotic resistance ability. Increased species diversity was observed in all contaminated groundwater samples. Additionally, a distinct phylogenetic composition consisting in OTUs affiliated to opportunistic pathogens and fecal indicator bacteria were observed.
- Conventional wastewater treatment that was applied before releasing the hospital effluents to the municipal wastewater network, showed only moderate removal efficiency of the studied emerging pollutants.
- Overall, general levels of antibiotics, ARGs, and MGEs are widespread in groundwater ecosystems not only near human settlements, but also at various distances from Cluj-Napoca city. This finding indicated that, most likely, local anthropic activities are affecting resistance-related contaminants in groundwater, rather than distance from the city. Potential ARG hosts, as well as possible mobilization patterns in groundwater environments were identified by correlations between ARGs, MGEs, and taxonomical groups.

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