



Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas[☆]

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ABSTRACT

Groundwater is an essential public and drinking water supply and its protection is a goal for global policies. Here, we investigated the presence and prevalence of antibiotic residues, antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), and microbial contamination in groundwater environments at various distances from urban areas. Antibiotic concentrations ranged from below detection limit to 917 ng/L, being trimethoprim, macrolide, and sulfonamide the most abundant antibiotic classes. A total of eleven ARGs (aminoglycoside, β -lactam, chloramphenicol, Macrolide-Lincosamide-Streptogramin B - MLSB, sulfonamide, and tetracycline), one antiseptic resistance gene, and two MGEs were detected by qPCR with relative abundances ranging from 6.61×10^{-7} to 2.30×10^{-1} copies/16S rRNA gene copies. ARGs and MGEs were widespread in the investigated groundwater environments, with increased abundances not only in urban, but also in remote areas. Distinct bacterial community profiles were observed, with a higher prevalence of Betaproteobacteria and Bacteroidetes in the less-impacted areas, and that of Firmicutes in the contaminated groundwater. The combined characteristics of increased species diversity, distinct phylogenetic composition, and the possible presence of fecal and/or pathogenic bacteria could indicate different types of contamination. Significant correlations between ARGs, MGEs and specific taxa within the groundwater bacterial community were identified, revealing the potential hosts of resistance types. Although no universal marker gene could be determined, a co-selection of *int1*, *qacE Δ 1* and *sull* genes, a proxy group for anthropogenic pollution, with the *tetC*, *tetO*, *tetW* resistance genes was identified. As the *tet* group was observed to follow the pattern of environmental contamination for the groundwater samples investigated in this study, our results strongly support the proposal of this group of genes as an environmental tracer of human impact. Overall, the present study investigated several emerging contaminants in groundwater habitats that may be included in monitoring programs to enable further regulatory and protection measures.

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1. Introduction

Antibiotics, the miracle drugs of the 20th century, have become environmental contaminants of emerging concern in only a few

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decades since their discovery, together with the genes associated with antibiotic resistance (Di Cesare et al., 2015; Fares, 2016). Research has shown that these contaminants are persistent and mobile enough to be transported from wastewater, farm, and agriculture effluents to natural waters, and their potential for long-term risk to environment, human and animal health is increasingly recognized as one of the most serious global threats of our times (World Health Organization (WHO), 2014; Sui et al., 2015; Chen et al., 2017; Yan et al., 2017). 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 (World Health Organization (WHO), 2006; Balzer et al., 2016). 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 (Sui et al., 2015). Urban aquifers are especially vulnerable to anthropogenic pollution due to intense urban activities and industry (López-Serna et al., 2013).

Microbiological analysis of ground and drinking water is usually focused only on the occurrence of fecal indicators and pathogenic bacteria in an effort to prevent outbreaks and infectious diseases that in countries with limited health surveillance often go undetected (World Health Organization (WHO), 2006). While a high bacterial count itself poses a health risk, their simultaneous presence with trace levels of antibiotics and antimicrobial resistant microorganisms makes this risk even more serious (Xi et al., 2009; Chen et al., 2017) by constituting a direct and primary selection pressure that accelerates the spread of antibiotic resistance genes (ARGs) in the environment (Ghosh and LaPara, 2007). One of the underlying mechanisms of ARG acquisition and dissemination is the transfer of these genes via mobile genetic elements (MGE) like plasmids, transposons or integrons. Class 1 integron, incorporating the quaternary ammonium compound (*qacEA1*) and sulfonamide (*sulI*) resistance gene, is common among multidrug resistance bacteria and their increased abundance was linked to urban activities (Stalder et al., 2014). Anthropogenic input, for example via contaminated water, stimulates horizontal gene transfer and creates an opportunity for bidirectional gene exchange between environmental and clinical bacteria (Di Cesare et al., 2015; Gillings, 2017).

The European Groundwater Directive (2006/118/EC) sets groundwater quality and measurement standards based on local characteristics and allows improvements supported by new monitoring and scientific results. Emerging pollutants in groundwater were not previously included in water quality legislation for several reasons, including lack of knowledge on contaminant sources and pathways, their effect and detection techniques (Lamastra et al., 2016). Most of the reviews exploring pharmaceutical and anthropogenic pollution in water matrices have focused mainly on surface and wastewater systems. Thus, ground- and drinking water monitoring results of these contaminants show significant discrepancy between different countries (López-Serna et al., 2013; Sui et al., 2015; Fares, 2016; Xu et al., 2016; Chen et al., 2017). Despite the fact that Europe provides the largest number of reports on compounds detected in groundwater, among which antibiotics are one of the most abundant groups, only three antibiotics from the macrolide group (erythromycin, clarithromycin, azithromycin) were included on the 1st Watch List (Decision 2015/495) of priority pollutants developed by EU WFD (Water Framework Directive, 2000/60/EC) (JRC, 2015; Fares, 2016). The urgency to reduce the environmental spread of ARGs has generated significant scientific and policy-planning interest, involving their consideration as biological contaminants of emerging concern (Berendonk et al., 2015; Igrejas et al., 2017). This is necessary for the identification of critical control sites where prevention and remediation measures can be implemented. Their inclusion as priority contaminants would be justified based on the fact that they are transferrable biological pollutants with higher abundance in aquatic systems affected by human activities, which can travel long distances and persist or increase over time (Berendonk et al., 2015; Martínez, 2017). Considering the diversity of ARGs and MGEs it is hard to assess risks and rank their importance in clinical and environmental settings (Martínez et al., 2015). So far, no feasible priority list has been developed for resistance

determinants that would support policy measures and pinpoint research gaps. The European Council, addressing the problem of pollutant emission from industry, agriculture, or urban wastewater sources, recommends that member states define their own internal monitoring parameters and additional protective measures until unified legislation can be submitted for implementation (Water Framework Directive, 2000/60/EC).

Even though about 30% of the water used in Romanian cities originates from groundwater, and most of the rural population supply their own drinking water via domestic wells, there is an increasing problem of pollution due to wastewater from large industrial and agricultural areas that lead to unusable water supplies (Tellam et al., 2007). A comprehensive plan for monitoring antibiotics and ARGs in environmental settings from Romania is lacking, even though there is an increased number of cases of antibiotic-resistant infections (Popescu et al., 2016). How direct and indirect anthropic activities may affect the ARGs profiles and related aspects of groundwater environments have been seldom investigated worldwide (Vaz-Moreira et al., 2014; Li et al., 2015; Chen et al., 2017), 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

2.1. Sampling sites, sample collection, and DNA extraction

The working hypothesis was that wells with close proximity to urban areas with high anthropic activity are more vulnerable to antibiotic and microbial contamination, as well as to antimicrobial resistance. Thus 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 (Supplementary Fig. 1). 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 (Table 1). Shallow subsurface water in areas around Cluj-Napoca is impacted to a greater extent by seepage from the municipal solid waste landfill, urban and hospital wastewaters and the fertilizers and pesticides used in agriculture (Rotaru and Răileanu, 2008; Iordăchescu et al., 2011). The GW1 sampling site is near a swine confinement facility, where no special waste handling system is employed and manure is periodically applied to crop fields. GW2 is a private well in the residential area (outskirt) of the city and GW3 is located in the city center, in the impact range of two hospitals.

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