Chemical, radiological and microbiological characterization of a drinking water source: a case study

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Significance and Impact of the Study: This paper presents the results of the environmental impact on the chemical, radiological, and microbiological quality and safety of raw water daily consumed by humans in local households located in a village in Central Serbia. The results indicated that the health risk related to the microbiological quality of raw water was much higher due to the absence of microbiological disinfection procedures, namely chlorination. The results presented in the manuscript pointed out the potential quality and safety risks regarding the consumption of untreated drinking water from environmental sources. Since there are many rural areas throughout the world where people consume untreated drinking water, these safety concerns are not related only to Serbia, but to all rural areas around the world.

Abstract

This study examined water samples from a local stream in Central Serbia, which was consumed as drinking water. The chemical parameters (chemical oxygen demand, pH, total concentration of dissolved substances and electrical conductivity), the concentration of major, trace, and radioactive elements in the water as well as the content of those from the environment, were examined. In addition, the microbiological quality of the water was inspected. The water samples were acidic (pH from 5.27 to 5.69) and chemical oxygen demand ranged in upper permissible limits (up to 6.25 mg O_2 l^{-1} (WR)). The concentrations of major, trace and radioactive elements, including radon, were below maximum contaminant levels. The water contained a higher number of total coliform bacteria than it was allowed (>10 colony forming units (CFU) in 100 ml of water) as well as enterococci and *Escherichia coli*. The characterization of the isolated bacteria indicated that two isolates demonstrated proteolytic activity, while full antibiotic resistance was not detected. The isolates showed moderate to strong ability to produce biofilm, while the isolates of *E. coli* were nonpathogenic. The results indicated that examined water samples were not microbiologically and chemically safe, therefore, the usage of analyzed water was not recommended as a water supply. Further research needs to include more frequent monitoring in order to propose measures for the improvement of the water quality and prevention of health risks for consumers.

Keywords: microbiological safety, chemical parameters, heavy metals, radioactivity, raw water quality

Introduction

Water is essential to life. However, many people in the world do not have access to clean and safe drinking water (Cabral 2010). Studies showed that population growth, increasing water scarcity, urbanization, and climate change are great challenges for drinking water supply systems. A clean and treated water supply to each household may be standard in Europe, however, in many other countries, access to clean water is not the case, and waterborne infections are common (WHO 2013).

The presence of natural organic matter (NOM) has a significant impact on the quality of drinking water sources (Ghernaout 2020). NOM is a complex matrix of organic substances produced in aquatic ecosystems via various biological, geological, and hydrological cycles (Sillanpää *et al*. 2018). Typically, NOM characteristic is dependent on the biodegradable dissolved organic carbon (BDOC) content in water sources (Ghernaout 2020).

Heavy metals are likewise important parameters of drinking water and represent a threat to human health. Population is exposed to heavy metals primarily through water consumption, and several heavy metals may bioaccumulate in the human body and may induce a disease (Chowdhury *et al.* 2016). Therefore, a determination of the level of heavy metals in various water sources is important for proper human health risk assessment (EPA 2012; WHO 2013). According to the Environmental Protection Agency (EPA) and Agency for Research on Cancer (IARC), exposure to toxic heavy metals is a major concern about drinking water, due to their carcinogenic and non-carcinogenic effects on human health.

Naturally occurring radioactive elements are present in almost all rocks and soils in the Earth's crust. Due to erosion and dissolution from rocks and minerals which form the aquifer, these radionuclides may as well be detected in drinking-water supplies. These include potassium ^{40}K, thorium, uranium, and their radioactive decay products such as radium ^{226}Ra, ^{228}Ra, and radon ^{222}Rn. The health risks associated with the presence of

radioactive elements in drinking water are generally very low. In fact, the radiation dose received due to the intake of natural radionuclides through the diet is typically about 0.3 mSv each year and only about 5% of that exposure comes from drinking water (WHO 2018). However, higher concentrations of radionuclides may be found in drinking water derived from groundwater sources and springs. Long-term exposure to relatively high levels of radionuclides in drinking water may be associated with serious health problems, such as cancer, anemia, osteoporosis, kidney disease, liver disease, and impaired immune system (Lesikar *et al*. 2006).

Radium isotopes are radiotoxic and dangerous, particularly due to the fact that ingested radium behaves similarly to calcium and may bioaccumulate in human bones (Martín Sánchez *et al*. 1999). Besides, ^{226}Ra has a half-life of about 1600 years and it decays to radioactive radon gas ^{222}Rn which is likely to be one of the most abundant radionuclides in drinking-water supplies. Radon in water occurs mostly by direct exhalation from radium-bearing aquifer rock structure rather than from the decay of dissolved radium itself. Water treatment, storage, and distribution generally reduce radon concentration in drinking water. However, untreated waters from natural springs, boreholes, or wells are more likely to cause increased exposure to radon (Nuccetelli *et al.* 2012). Radon exposure from ingesting water is typically small compared to that from inhalation. When water containing high levels of radon is used for domestic purposes (such as showering, washing dishes, cooking, etc.) radon gas escapes from the water and goes into the air, increasing indoor radon concentration. According to WHO (2009), inhalation of radon and its radioactive decay products have been identified as the second leading cause of lung cancer, after tobacco smoking.

In addition to the organic matter, heavy metals, and radioactive elements that can be found in naturally occurring drinking water, the members of waterborne pathogens and fecal coliform bacteria may be noticed as well. Contaminated water by waterborne pathogens may

be a source of infectious diseases, including cholera, dysentery, etc. Most waterborne pathogens are introduced to drinking water supplies from human or animal feces, and initiate infection in the gastrointestinal tract through the ingestion. The routes of transmission of these bacteria include inhalation and contact (bathing) as well (Gerba 2009). The ecology of waterborne pathogens should be assessed in relation to modern agricultural practices vis-à-vis anthropogenic activities. Genetic and phenotypic characterization of pathogenic bacteria is necessary to clarify zoonotic relationships with their animal hosts and factors influencing the transmission of human diseases through water (Rahman *et al.* 2020). Based on their large distribution and importance, it is vital to establish a bacterial presence in streams and small rivers that people use for water supply.

The area where the study was conducted is located in the municipality of Knić (Šumadija area). The climate of the examined locality could be characterized as temperate-continental with an annual average temperature from 9°C to 11°C and an average annual rainfall between 700 and 1000 mm depending on the position and altitude. Climate area is characterized by warm summers and moderately cold winters. The annual amount of precipitation is uneven over the year. The lowest amount of precipitation occurs in July and August, while the spring period is relatively rainy. The soil type is smonica, which is characterized by low content of humus and weakly acidic chemical reactions. The Kotljenjača stream on which the study was performed flows into the river Gruža, which is a tributary of the West Morava and belongs to the Black Sea basin (Paunović *et al*. 2018).

The aims of this research study were the evaluation of the presence and content of organic matter and the presence/absence of coliform bacteria in raw water samples used for drinking. In addition, the characterization of isolated bacteria was carried out to assess the risk of their presence to public health. Moreover, this study aimed to determine the presence of radon ^{222}Rn in water samples as well as naturally occurring radioactive elements and the

contents of major and trace elements in the raw water, soil, and sand samples. This investigation provided comprehensive data regarding the potential risk of using raw water from local streams as drinking water.

Results and discussion

In this paper, samples of water from a local stream in Central Serbia, which were used as drinking water, were examined for the first time. This study is unique since similar studies regarding the investigation of environmental impact on the chemical, radiological and microbiological quality and safety of raw water daily consumed by humans were not conducted in Serbia. Nevertheless, these parameters are crucial for public health, which is the major significance of this study. Investigations of this sort help suggest potential measures for a safe supply of drinking water in the researched area.

Chemical parameters of water samples

All water samples were tested for four chemical parameters: Chemical oxygen demand (COD), Quantitative measure of the acidity or basicity of drinking water (pH), total concentration of dissolved substances (TDS) and electrical conductivity (EC).

Chemical oxygen demand (COD) in analyzed water samples was within the permitted limits according to The Official Rules of RS (No. 42/98, 44/99, and 28/2019), ranging from 4.38 mg O_2 l^{-1} (WS) to 6.25 mg O_2 l^{-1} (WR). In Sample WT, COD was 5.55 mg O_2 l^{-1}. The results of quantitative measure of the acidity or basicity of drinking water showed that the analyzed samples of water were acidic. The lowest pH value (5.27) was measured in samples collected from the WS sample, while the highest pH value (5.69) was recorded in WR sample. The pH value of WT sample was 5.40. The water temperature was in the range from 12–16°C in a dry season. The total concentration of dissolved solids (TDS) was highest in the WR sample (40.50 mg l^{-1}). The lowest TDS was measured in WS sample (35.68 mg l^{-1}),

while in WT sample, TDS was 39.50 mg l^{-1}. Electrical conductivity ranged from 70 µs cm^{-1} (WS) to 81 µs cm^{-1} (WT). In the WR sample, electrical conductivity was 80 µs cm^{-1}.

According to the results of COD, the best quality of water was recorded at the water source (WS) and the worst at the water from the reservoir (WR), where low protection from falling debris was observed. Water collected from reservoir with COD above 6 mg O_2 l^{-1}, was not recommended for drinking according to the World Health Organization (2017b). When hard water is heated, deposits of calcium carbonate begin to precipitate. Depending on the interaction with other factors, such as pH and alkalinity, water with hardness above approximately 200 mg l^{-1} may cause scale deposition throughout the treatment in the distribution system, as well as in pipelines and tanks within buildings. The taste of drinking water may be affected by the presence of total dissolved solids. The palatability of drinking water is rated in relation to its TDS level as follows: excellent, less than 300 mg l^{-1}; good, 300 – 600 mg l^{-1}; fair, 600-900 mg l^{-1}; poor, 900-1200 mg l^{-1}; and unacceptable, > 1200 mg l^{-1} (Official Rules of RS, No. 42/98, 44/99, and 28/2019). Results from this study indicated that water samples possessed less than 300 mg l^{-1}, which was a water of good quality regarding TDS. Electrical conductivity depends on the concentration of the ions present in the water, their mobility, and the charge. The maximum allowed value of conductivity of drinking water is up to 1000 µS cm^{-1} (Official Rules of SRJ, No. 42/98, 44/99, and 28/2019; World Health Organization, 2017b). The minimum conductivity was measured in the WS sample (70 µS cm^{-1}), which was considered to be the highest quality of water considering its conductivity. pH value may be affected by substances that may alter the balance of the minerals and affect the activity of flora and fauna. Due to the influence of pH on the chemical and biological properties of water, the pH value of water is essential and needs to be tested. WHO recommends that the pH level of water sources should be at a pH measurement level between 6.5 and 8.5 (World Health Organization, 2017b). Water with a pH of less than 6.5 may

corrode metal pipes and is more likely to be contaminated with pollutants, which makes it unsafe for drinking (Saritpongteeraka and Chaiprapat 2008). The results demonstrated that the analyzed samples of water were acidic (pH values below 6) and thereby not recommended for usage.

The concentration of major and trace elements in water and their content in sediment samples

The results of the comparative analysis of the quantity of major and trace elements Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, S, Se, and Zn in the water, soil from the forest located around mountain spring coast (Soil 1) as well as in sand collected from spring of stream (Sand 1) are presented in Table 1. In Table S1, we presented guideline values for heavy metal concentrations in water.

Table 1 The concentration of major and trace elements in water and their content in sediment samples

Origin of sample	Water samples			Sediment samples	
	WS	WR	WT	Soil 1	Sand 1
Element/quantity	µg l^{-1}	µg l^{-1}	µg l^{-1}	µg g^{-1}	µg g^{-1}
Al	4965.00 ± 62.0	5488.00 ± 35.0	5724.00 ± 120.0	27680.00 ±551.90	20900.00 ± 320.40
As	n.d.	0.58 ± 0.22	n.d.	3.08 ± 0.16	1.66 ± 0.06
B	n.d.	n.d.	n.d.	1.09 ± 0.09	1.26 ± 0.06
Ca	7012.00 ± 5.0	8849.00 ± 16.0	8774.00 ± 26.0	4852.00 ± 73.30	5167.00 ± 55.70
Cd	0.27 ± 0.16	0.38 ±0.09	0.30 ± 0.24	0.32 ±0.02	0.19 ± 0.02
Co	0.51 ± 0.26	0.56 ± 0.15	0.51 ± 0.28	12.47 ± 0.01	11.85 ± 0.02
Cr	4.67 ± 0.07	5.24 ± 0.27	5.35 ± 0.27	80.13 ± 0.53	47.81 ± 0.05
Cu	0.59 ± 0.47	0.57 ± 0.20	2.09 ± 0.27	7.59 ± 0.08	6.36 ±0.03
Fe	2407.00 ± 18.0	2687.00 ± 12.0	2815.00 ± 65.0	20900.00 ± 275.4	20580.00 ± 52.50
K	1349.00 ± 82.0	1812.00 ± 624.00	1544.00 ± 176.00	2249.0 ± 136.60	952.40 ± 111.70
Li	2.70 ± 0.01	3.25 ± 0.01	3.33 ± 0.03	7.48 ± 0.06	8.11 ± 0.04
Mg	2243.00 ± 10.0	3418.00 ± 6.0	3419.00 ± 7.00	5333.00 ± 0.81	4293.00 ± 64.30
Mn	18.43 ± 0.10	17.99 ± 0.13	18.01 ± 0.25	375.70 ± 4.70	776.90 ± 6.20
Na	2926.00 ± 23.0	3295.00 ± 13.00	3243.00 ± 15.00	857.20 ± 6.20	532.10 ± 2.00
Ni	1.93 ± 0.42	2.16 ± 0.61	1.85 ± 0.16	20.88 ± 0.07	20.31 ± 0.05
P	39.77 ± 2.74	49.96 ± 1.34	45.23 ± 4.29	422.10 ± 0.40	495.80 ± 2.00
Pb	0.65 ± 0.27	2.45 ± 0.55	1.35 ± 0.36	46.33 ± 0.06	9.60 ± 0.05
S	5694.00 ± 16.0	5638.00 ± 15.00	5664.00 ± 12.00	179.60 ± 1.20	64.09 ± 0.18
Se	1.19 ± 1.21	1.50 ± 0.66	2.44 ± 0.28	n.d.	n.d.

Zn	1.39 ± 0.04	1.99 ± 0.01	10.37 ± 0.09	36.38 ± 0.03	28.29 ± 0.04

WS – water sample from mountain spring; WR – water sample from village reservoir; WT – water sample from the tap in the household; Values are presented as mean ± standard deviation; n.d. – lover than 0.10 μg l^{-1} for water samples or <0.01 μg g^{-1} for soil and sand samples; / - not evaluated

The mean value of the concentration of the analyzed elements in water samples was ordered in the following way: Ca > S > Al > Mg >Na > Fe > K > P > Mn > Cr >Li > Zn > Se > Ni >Pb > Cu > Co > Cd. As was detected only in the WR sample while B was not detected in any water sample (the concentration was lower than 0.10 μg l^{-1}). The results of the Mann-Whitney test were applied in order to detect significantly different levels of concentrations of elements in water samples. The results demonstrated a significant difference in the concentrations of elements between WS/WR samples ($p < 0.05$) and WS/WT samples ($p < 0.05$) while between WR and WT samples, a significant difference was not exhibited.

Khan *et al*. (2021) indicated that human health risk is reflected through surface water contamination by heavy metals of the Gomti River Basin (India), which is classified as environmental pollution. According to He and Li (2020), the unique natural environment and the increase in human activities influenced the water quality in the Chinese Lesska plateau, which is confirmed by the results obtained in the presented study. He and Li (2020) indicated that contamination with hexavalent chromium (Cr^{6+}) is an issue that contributes to the pollution of the environment and water in that area. The occurrence and potential sources of Cr^{6+}suggest that residents in the field of research are facing high carcinogenic risks induced by Cr^{6+}.Apart from Cr, Hg is a common pollutant as well, as indicated Wang *et al*. (2022). According to Amiri *et al*. (2021), Hg sources were present in the coastal aquifer of Urmia in northwestern Iran which represented potential toxic sources of this metal. Liu *et al*. (2022) indicated the presence of manganese (Mn) in groundwater in Weining Plain, northwest China. The results exhibited that 50 groundwater samples (144 in total) were of very poor quality due to agricultural activities, industrial development, and local hydrogeological conditions. In our study, the presence of Cr, Hg, and Mn was found in investigated water

218 samples. However, their concentrations were well below those at which toxic effects may
219 occur (Table S1).

220 In order to evaluate the origin of some elements in water samples, their presence and
221 content in sediments were investigated. The mean value of the content of the analyzed
222 elements in sediment (Soil 1 and Sand1) samples was ordered in the following way: Al > Fe
223 > Mg > Ca > K > Na > Mn > P > S > Cr > Zn > Pb > Ni> Co > Li > Cu > As > B > Cd. Se
224 was not detected in any sediment sample (total content was lower than 0.10 μg g^{-1}).
225 Interestingly, the concentrations of Na, Se, and S were higher in water samples compared to
226 the content of the same elements in sediment samples (Table 1). Since the investigated water
227 was sampled from the spring of the stream, these elements presumably originated from its
228 mineral deposits or parent rocks. In the aquifer, groundwater came into contact with these
229 solid materials which dissolved them, releasing their constituents into the water.

230 The results from this investigation indicated that the concentrations of analyzed major
231 and trace elements in all water samples were well below those at which toxic effects may
232 occur. The exceptions were the concentration of Fe and Al. The concentration of Fe did not
233 pose health concern at concentrations normally observed in a drinking water, however, it
234 affected the acceptability of water since its concentration was ten times higher than the
235 concentration that did not affect the acceptability of water. The higher concentration of Al
236 was presumably the result of washing the rocks and minerals from the coast of the stream, in
237 which Al was present. Exposure to Al is generally not harmful, however, high concentrations
238 may cause serious health problems. This metal belongs to the group of potentially toxic ones.
239 When it comes to the aquatic environment, aluminum is, under certain conditions, toxic to
240 many organisms. According to the WHO (1998), the high concentrations of Al are present in
241 acidic water rich in organic matter, which is in accordance with results from our
242 investigation.

According to the Grujović *et al.* (2021), the concentrations of total P, PO_4, and P_2O_5 were in the optimum range for water, while concentrations of N, NO, NH_3 and NH_4 were under the range of detection in all water samples. In addition, the nitrogen and phosphorus contents were relatively low or below detecting limit, which made algae hardly grow. Therefore, there was a low risk of water pollution, such as algae growth and propagation (Liu *et al.* 2018).

Determination of radioactivity

The activity concentrations of radionuclides analysed in water samples (WS, WR, WT) as well as those in the soil obtained from the forest nearby mountain spring coast (Soil 1), soil around water reservoir (Soil 2), in the sediment (Sand 1and Stones 1) from the stream spring, are presented in Table 2. Applying Currie's method, minimum detectable activities (MDA) of ^{226}Ra, ^{228}Ra, ^{137}Cs and ^{40}K in water were estimated as 0.05 Bq kg^{-1}, 0.15 Bq kg^{-1}, 0.04 Bq kg^{-1}, and 0.61 Bq kg^{-1}, respectively. The levels of natural radionuclides ^{226}Ra, ^{232}Th(^{228}Ra) and ^{40}K (57 Bq kg^{-1}, 83 Bq kg^{-1}, and 613 Bq kg^{-1}, respectively) in the soil sampled around the water source were higher than the worldwide average values reported by UNSCEAR (32 Bq kg^{-1}, 45 Bq kg^{-1}, and 412 Bq kg^{-1}, respectively). However, activity concentrations of ^{226}Ra and ^{228}Ra in water samples were below minimum detectable activities and therefore presumably did not exceed the maximum contaminant level of 0.19 Bq l^{-1}(5 pCi l^{-1}) proposed by the U.S. Environmental Protection Agency (US EPA) for combined radium isotopes in drinking water. Ingested ^{40}K, on the other hand, commonly does not pose a significant health risk since it occurs naturally in a fixed ratio to stable potassium. Potassium is an essential element that does not accumulate in the human body and its constant level is maintained by physiological processes. Artificial radionuclide^{137}Cs measured in the soil was presumably the result of surface deposition which occurred after the Chernobyl nuclear accident in 1986. Due

to the relatively long half-life (30 years), ^{137}Cs still persisted in the environment, however, the analysed water was not significantly contaminated by this radioactive isotope.

Table 2 Activity concentrations of radionuclides measured in water, soil, and sediment

Origin of samples	Type of samples	Tested radionuclides/quantity				
		^{226}Ra Bq kg^{-1}	^{228}Ra Bq kg^{-1}	^{40}K Bq kg^{-1}	^{137}Cs Bq kg^{-1}	^{222}Rn Bq l^{-1}
Sediment samples	Soil 1	57 ± 3	83 ± 4	613 ± 15	28.1 ± 0.9	-
	Sand 1	37.2 ± 1.9	49 ± 3	727 ± 12	1.2 ± 0.2	-
	Stones 1	38.5 ± 1.9	48 ± 3	751 ± 13	1.3 ± 0.3	-
	Soil 2	26 ±3	38 ± 4	336 ± 13	4.2 ± 0.5	-
Water samples	WS	< MDA	< MDA	0.76 ± 0.15	< MDA	0.6 ± 0.2
	WR	< MDA	< MDA	3.0 ± 0.5	< MDA	2.3 ± 0.5
	WT	< MDA	< MDA	< MDA	< MDA	2.3 ± 0.4

WS – water sample from mountain spring; WR – water sample from village reservoir; WT – water sample from the tap in the household; Values are presented as mean ± standard deviation; MDA – minimum detectable activity

The results of measuring radon concentration in three water samples are likewise presented in Table 2. It is notable that considerably higher radon concentration was measured in the water reservoir and home tap (2.3 Bq l^{-1}), compared to the sample from the mountain spring of stream (0.6 Bq l^{-1}). This accumulation of radon was probably caused by the diffusion from deeper layers of soil or by radon exhalation from concrete walls of the water reservoir since the surrounding surface soil (sampled around the water reservoir) did not demonstrate high levels of radioactivity. Building materials are considered as one of the major sources of indoor radon due to the terrestrial origin of their components containing NORM (naturally occurring radioactive material) and addition of waste products such as phosphogypsum, coal fly ash etc. (IAEA 2015). Evidently, transportation and storage may significantly increase radon levels in household water. Nevertheless, radon concentrations measured in all three water samples did not exceed the maximum contaminant level of 11 Bq l^{-1}(300 pCi l^{-1}) set by US EPA.The results of the Mann-Whitney test were applied to detect

significantly different levels of radionuclides concentrations in water samples. The results showed a significant difference in the concentration of the radionuclides between WS/WR samples ($p < 0.05$) and WS/WT samples ($p < 0.05$) while between WR and WT samples, a significant difference was not demonstrated.

Using the methods described by UNSCEAR (2000), the annual exposure to radon in tap water was calculated to be about 0.5 μSv from water ingestion and 5.8 μSv from inhalation. These values were obtained assuming the average indoor occupancy time of 7000 h/y and measured direct tap water consumption of 60 l per year (proposed by UNSCER, since radon gas was readily removed from water by heating or boiling). The concentrations of radioactive elements in water, including radon, were in the optimum range which is essential since this water is used daily.

Microbiological analysis

The results of microbiological investigation of water samples are presented in Table 3. The total count of aerobic mesophilic bacteria was enumerated on the nutrient agar and ranged between 31 and 68 CFU ml^{-1} of the water sample, which was in accordance with the legislation in Serbia. According to the Rules on hygienic drinking water (Official Rules of RS No. 42/98, 44/99, and 28/2019), 300 CFU of aerobic mesophilic bacteria in 1 ml of drinking water are allowed.

Hi-chrome coliform agar indicated the presence of coliform bacteria in the range from 31-140 CFU in 100 ml of the water sample, which was higher than allowed. A total number of enterococci were enumerated in BEA plates and ranged between 4 and 12 CFU in 100 ml of the water sample. On TBX agar, the presence of ß-glucuronidase positive *E. coli* was detected which was not allowed in drinking water (Table 3).

Table 3 Enumeration of total aerobic mesophilic bacteria, total coliform bacteria, total enterococci, and *E. coli* on TBX agar

Type of substrate	Nutrient agar	Bile esculin agar	Hi-chrome coliform agar			TBX agar		
Colonies	Aerobic mesophilic bacteria	*Enterococcus* spp.	Blue colonies[1]	Pink colonies[2]	Total	Blue colonies[3]	White colonies[4]	Total
WS	31*	12	4	92	96	5	117	122
WR	68	9	8	132	140	8	144	144
WT	56	4	3	28	31	5	74	79

*The results are presented as CFU ml^{-1}; WS – water sample from mountain spring; WR – water sample from village reservoir; WT – water sample from the tap in the household; [1]*E. coli*; [2]*Klebsiella* spp.; [3]ß-glucuronidase positive *E. coli*; [4]ß-glucuronidase negative strains

After enumeration, the isolation of coliform bacteria was conducted. All the Gram-negative, catalase positive and oxidase negative isolates were subjected to Microgen GNA + B Oxidase Negative biochemical identification tests (Tables S2 and S3). The exceptions were isolates from genus *Aeromonas*, which were oxidase positive, thus subjected to Microgen GNA + B Oxidase Positive biochemical identification tests. The final identification was conducted by using a MALDI-TOF mass spectrophotometry, based on protein profile of every isolate (Fig. S1).

In Table S4, the distribution of bacterial genera isolated from each sample point is presented. In WR sample, genus *Enterobacter* was not isolated, while in WT sample genera *Aeromonas, Raoultella, Enterobacter* and *Acinetobacter* were not detected. Genera *Escherichia, Citrobacter* and *Enterococcus* were isolated in all tested samples. Therefore, it could be concluded that water was not microbiologically safe for drinking. Generally speaking, we noticed a higher number of bacteria in WR sample than in WT. It was due to the fact that in a water reservoir we could observe natural precipitation, and it was a place where water did not flow. The number of total aerobic mesophilic bacteria was in accordance with the results by Grujović *et al.* (2021), even though the season of sampling was not the same.

335 According to the Rules on hygienic drinking water in Serbia, 10 CFU of total coliform
336 bacteria in 100 ml of drinking water are allowed (when membrane filtration is used).
337 However, *E. coli* should not be detected in drinking water. If its presence is confirmed in
338 drinking water, that water is epidemiologically dangerous. *E. coli* was also detected by
339 Grujović *et al.* (2021).

340 Environmental waters do not represent a natural habitat for enterococci and their
341 presence in this milieu is considered to be the result of fecal pollution. The absence of
342 enterococci in 100 ml of water is the minimal requirement parameter for water quality
343 (Official Rules of RS No. 42/98, 44/99, and 28/2019; WHO 2017a). When the source of
344 enterococci to surface waters is not fecal, their presence may not indicate a health risk.
345 Epidemiological studies investigated the correlation between enterococci and swimmer
346 illness in recreational waters not impacted by wastewater, and the results are equivocal
347 (Boehm and Soller 2013). Another approach for the reduction of enterococcal numbers in
348 water is the cognition of their origin and the way of enterococcal transport to the water. Upon
349 entering surface water, enterococci concentrations vary due to dispersion and advection,
350 which are controlled by concentration gradients and fluid velocities, respectively. Enterococci
351 concentrations are further influenced by sedimentation/deposition, resuspension, particle
352 interactions, growth, predation, and light and dark inactivation due to the environmental
353 stresses, such as sunlight and oligotrophy, respectively (Boehm and Sassoubre 2013).

354 The rationale for selecting *E. coli* and enterococci is their role in the evaluation of the
355 safety of drinking water concerning coliform pathogens (i.e., pathogenic microorganisms that
356 infect the intestinal tract). Such pathogens are spread via the excreta of humans or warm-
357 blooded animals. The contamination of drinking water with excreta containing these
358 pathogens may cause illness once the water is consumed. Contamination with excreta is still
359 the most significant and frequently occurring health risk through drinking-water exposure

(Cabral 2010; WHO 2017a). Since the water from our research originated from a mountain spring of stream, located in an uninhabited area, the microbiological contamination was most presumably related to dead animals or animal feces.

Aeromonas spp. is generally readily found in most fresh waters, and the species has been detected in many treated drinking-water supplies, mainly due to their growth in distribution systems. The factors that affect the occurrence of *Aeromonas* spp. in water distribution systems are not fully understood, however, organic content, temperature, the residence time of water in the distribution network influence this genus (Cabral 2010). *Acinetobacter* spp. may cause urinary tract infections, pneumonia, bacteraemia, secondary meningitis and wound infections. *Acinetobacter* has been reported as a persistent genus in tap water, as well as the presence of different species of this genus in drinking water (Carvalheira *et al*. 2021). The origin of *Acinetobacter* in surface water seems to be influenced by the wastewater treatment process which contributes to the selective increase of antibiotic resistant bacteria and the occurrence of multidrug-resistant bacteria in aquatic environments (Carvalheira *et al*. 2021).

Screening of bacterial isolates for virulence traits

Screening of bacterial isolates for virulence traits was evaluated by investigation of proteolytic and haemolytic activity, as well as the resistance to antibiotics and the ability to form biofilm.

The results of proteolytic activity indicated that *Aeromonas* and *Acinetobacter* isolates demonstrated proteolytic activity. All tested isolates showed α-haemolysis on blood agar plates. Alpha hemolysis was caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobin to green methemoglobin. Therefore, tested isolates exhibited a pathogenic potential.

According to the results obtained by disc diffusion method, none of tested isolates showed full resistance to the five tested antibiotics (Table S5). All tested isolates demonstrated sensitivity to tetracycline (inhibition zone in range from 18 to 30 mm), chloramphenicol (inhibition zone in range from 20 to 32 mm), and cefotaxime (inhibition zone in range from 18 to 26 mm). All tested isolates exhibited resistance to amoxicillin, while streptomycin produced selective effect. Bacteria from genus *Aeromonas*, as well as all isolates of *E. coli*, *C. brakki* and *R. ornithinolytica* were resistant to streptomycin. Other tested isolates were sensitive to streptomycin.

By using a biofilm formation assay, the ability of tested isolates to form biofilm was evaluated. As it can be noticed in Table S6, most *E. coli* isolates, as well as the members of genus *Enterobacter* and the isolates *R. ornithinolytica* V10 and *A. bestiarum* V11 demonstrated the ability to form biofilm. The rest of isolates showed no ability to form biofilm.

Biofilm formation poses a significant problem to the drinking water industry as a potential source of bacterial contamination, including pathogens, and, in many cases, furthermore, affecting the taste and odor of drinking water and promoting the corrosion of pipes (Liu *et al*. 2016). Bacteria isolated in our research were moderate to strong biofilm producers, therefore, the risk of corroding the pipes increased. Finally, the results indicated that purification, chlorination and more frequent microbiological monitoring were demanded.

Detection of *E. coli* virulence genes by PCR

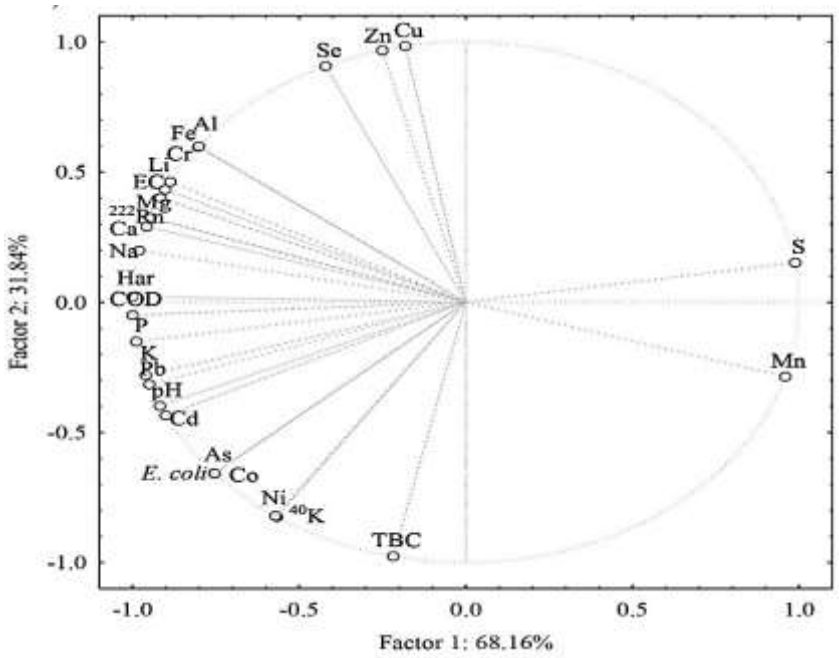
In order to detect the presence of Enteropathogenic *E. coli* isolate, they were subjected to polymerase chain reaction (PCR) for detection of *stx1*, *stx2*, *eaeA* and *hlyA* genes. The results indicated that none of the tested isolates showed the presence of *stx1*, *stx2*, *eaeA* and *hlyA* genes. Therefore, isolates of *E. coli* could not be designated as Enteropathogenic or Enterohaemorrhagic *Escherichia coli,* i.e. isolates were nonpathogenic.

Principal component analysis (PCA)

The results of the PCA of major and trace elements and radionuclides concentration in the water samples, as well as COD, pH, Hardness, EC values, and the total number of coliform bacteria (TCB) and *E. coli*, are presented in Fig.1. The first PCs were assumed with a total variation of 68.16%. Factor loadings for S (0.988) and Mn (0.958) in the water samples possessed positive values, while all other parameters had negative values. For PC1 the concentration of COD (-0.999), Har (-0.999), P (-0.988) had the major loadings in the water samples (Table S7).

The two PCs differentiate water samples from each other due to various concentrations of major and trace elements, radionuclides physical properties (COD, pH, Hardness, EC values), as well as the number of total coliform bacteria(TCB) and number of *E. coli*. Along with the Principal Component (1 and 2), axes showed that all water samples differed from each other.

A)



b)

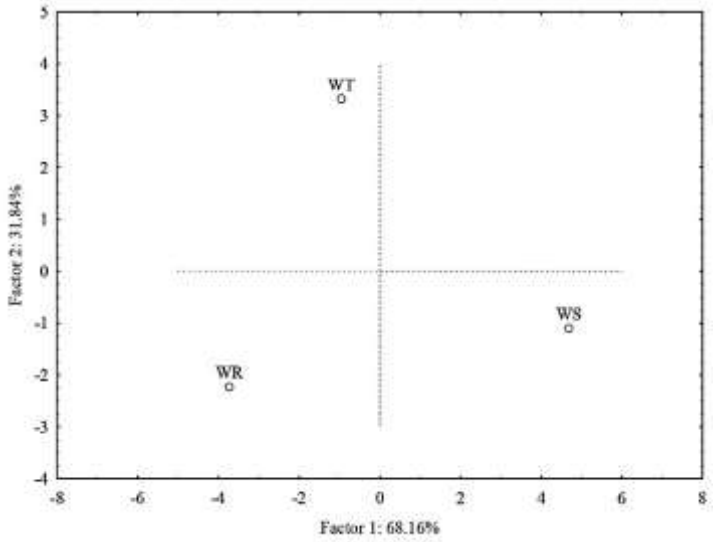


Figure 1 PC loadings (a) and Screen plot (b) of the water samples relative to the concentration of elements, radionuclides, COD, pH, Hardness, EC values and total number of coliform bacteria (TCB) and *E. coli*

The dependence between water samples, related to the concentration of elements, radionuclides physical properties (COD, pH, Hardness, EC values) and the number of total coliform bacteria (TCB), as well as the number of *E. coli* is presented in Fig. 1a. The water sample from the source (WS) was distinguished by the highest values of S and Mn. The water from the reservoir (WR) was characterized by a higher concentration of trace elements like Pb, Cd, As, Ni, as well as a higher number of TCB and *E. coli* (Fig. 1b). The water from the tap (WT) was differentiated by high values for physical properties (COD, Har, EC) and elements like Na, Ca, Cu, and Zn.

All in all, the significance of our results through all the chemical, radiological, and biological aspects of analyzed water samples was demonstrated. Based on the analysis of water samples from three significant points before human consumption, it could be concluded that some risks related to human health were exhibited. Some of the health risks of samples include acidic pH (all three samples) and high COD (WR and WT samples) as well as the presence of a high number of total coliform bacteria (TCB) and the presence of indicators of fecal contamination (enterococci and *E. coli*). The organic matter was present in water due to the washing of forest land while in the water reservoir, no purification process was noticed.

Regarding the major and trace elements, the concentration of Al and Fe was high (Table S1), however, these metals did not pose a health risk. Investigated water samples exhibited some good properties, like TDS and EC. The investigated radioactivity of water samples indicated that activity concentrations of ^{226}Ra and ^{228}Ra were below minimum detectable activities and the water samples were not significantly contaminated by this radioactive isotope ^{137}Cs. Radon concentrations measured in all three water samples did not exceed the maximum contaminant level.

In the investigated water samples, the presence of total coliform bacteria in high number, as well as the presence of *E. coli* and enterococci, were detected. The results obtained from safety evaluation of tested isolates indicated that isolates from genera *Aeromonas* and *Acinetobacter* demonstrated proteolytic activity. All tested isolates showed α-haemolysis on blood agar plates. Resistance to more than two of the five antibiotics tested was not observed among the isolates. However, isolates present a risk to public health by its very presence and abundance. Moreover, it is possible that, through the time, isolates develop either phenotypic or genotypic resistance. Thus, further monitoring of water is necessary.

Based on the comprehensive results presented in this study, it could be concluded that the chemical quality of raw water depended on the quality and type of stone and soil in the environment as well as on the radioactivity of the environment. However, the health risk related to the microbiological quality of raw water was much higher due to the absence of microbiological purification or chlorination. The results presented in the manuscript pointed out the potential safety problems regarding the consumption of untreated drinking water. Since there are many rural areas around the world where people consume untreated drinking water, these safety concerns are not related only to Serbia, but to all rural areas throughout the world. Therefore, there is a need for the implementation of certain measures, such as chlorination and constant monitoring in order to improve water quality and to prevent health

risks and epidemiological waterborne diseases. These kinds of manuscripts would complete the research about the environmental impact on the quality and safety of water in many rural areas throughout the world and encourage the authors to conduct further studies in this field. Further investigation needs to include the characterization of isolated coliform bacteria, especially *E. coli* isolates, in order to detect certain serotypes as well as to label resistance genes if isolates demonstrate resistance to the tested antibiotics.

Materials and methods

Sampling of water

Investigated raw water samples were collected in the three target points of stream in the village Pajsijevic (Sumadija area, Central Serbia) throughout the spring of 2021.The first target point was the mountain spring of Kotljenjača stream (Kotlenik Mountain) (WS sample -43°51′27″N; 20°42′48″E; alt. 402 m a.s.l.), where the water passed through layers of small and large rocks (natural filtration) and went into the pipes which transmitted water into the reservoir in the village, which represented the second target point (WR sample – 43°51′39″N; 20°43′54″E; alt. 336 m a.s.l.). The water reservoir was about 2.5 m deep, 1 m in diameter, buried in the ground with concrete walls. There was no water purification or chlorination of water in the reservoir. The only way of purification was the natural precipitation of water content into the bottom of the reservoir. At 50 cm from the bottom of the reservoir, a pipe was positioned through which the water was transmitted to households by natural fall, which is the third target point (the tap in the household; WT sample – 43°52′07″N; 20°44′39″E; alt. 312 m a.s.l.). Therefore, the tested samples presented raw water which people used for drinking. The first two target points were selected since they were the only places where tested water was outside the village tubing, while tap water was tested due to the fact it was used for human consumption. Apart from human consumption, the water was used for agricultural and industrial purposes.

Water samples were collected aseptically in sterile 500ml glass bottles by directly dipping the bottles into the surface of the water. The water samples from the tap were collected directly into the sterile bottles, after letting the tap run for a minute. All water samples were collected in triplicate based on standard water sampling procedures. At every sampling point, the pH and temperature of the water were measured. The samples were labeled properly and transported on ice to the laboratory of Microbiology, Faculty of Science in Kragujevac, for analysis. Aliquots of the samples were used for enumeration of total mesophilic bacteria and total coliforms as well as the detection of indicators of fecal contamination (enterococci and *E. coli*). Moreover, the concentration of major and trace elements, chemical parameters, and radioactivity were evaluated in all water samples.

Chemical parameters of water samples

The total content of organic matter (COD) was determined by titration with potassium permanganate in an acid solution using the Kubel-Tiemann method (Trajković *et al.* 1983).

The pH of each water sample was measured using pH meter CT-6020 (Shenzhen Ke Dida Electronics Co., Ltd., Baoan District City, Fuyong, Shenzhen Street Peace community, and King Industrial Zone). Electrical conductivity (EC) and total concentration of dissolved solids (TDS) were measured using a Combo pH/Conductivity/TDS Tester HI98129 (Hanna Instruments Ltd., Woonsocket, Rhode Island, USA).

The concentration of major and trace elements in water and their content in sediment samples

The water samples (three replicates at every locality) were collected in bottles of 500 ml and conserved by adding 1.5 ml of concentrated HNO_3 (US EPA 1994). The samples of water were not further treated; the presence and the concentration of the following elements: Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, S, Se, and Zn, were analyzed by direct aspiration of the sample.

521 The soil and sand were sampled from the mountain spring of stream for the
522 comparative analysis of the content and the determination of origin of the following
523 elements: Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, S, Se, and Zn.
524 Soil samples (3 replicates) were collected from the forest which was located around mountain
525 spring coast. A composite mixture of soil samples was air-dried at room temperature for 1
526 month, crushed, and pulverized in order to pass through a 2-mm sieve. The sampling and
527 laboratory preparation of the soil samples were carried out in accordance with the described
528 procedure for soil sampling (Kastori *et al*. 2006). Sand samples (3 replicates) were taken
529 directly from the mountain spring. A composite mixture of sand samples was air-dried at
530 room temperature for 48 h. The rest of the sand sample preparation was described in Alshahri
531 (2016). Soil and sand samples were dusted in an agate mortar prior to microwave digestion.

532 **Contents of major and trace elements**

533 The digestion of samples (soil and sand) was performed on Advanced Microwave
534 Digestion System (ETHOS 1, Milestone, Italy) using the HPR-1000/10S high pressure
535 segmented rotor. About 0.5 g of samples were precisely weighed with accuracy ± 0.1 mg and
536 mixed with 10 ml HNO_3 (70 wt. %, ACS reagent, Sigma Aldrich,) and 1 ml H_2O_2 (30 wt. %,
537 ACS reagent, Sigma Aldrich), and then heated using microwave energy for 30 min. The
538 temperature was gradually raised to 200ºC in the first 10 min, remained at 200ºC in the next
539 20 min, and then decreased rapidly to room temperature. After cooling, the solution was
540 diluted to a fixed volume into a volumetric flask of 50 ml with ultrapure water. Ultrapure
541 water with a resistivity of 18.2 MΩ cm^{-1} (equal to 0.05 μS cm^{-1}) was prepared using a
542 Barnstead™ GenPure™ Pro (Thermo Scientific, Germany).

543 The contents of major and trace elements were determined by inductively coupled
544 Plasma optical emission spectrometry (ICP-OES). ICP-OES measurement was performed
545 using Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, United

546 Kingdom) with parameters presented in Table S8. Three multi-elemental plasma standard
547 solutions were used to prepare calibration solutions for ICP-OES measurement: Multi-
548 Element Plasma Standard Solution 4, Specpure®, 1000 μgml^{-1} (Alfa Aesar GmbH & Co KG,
549 Germany), ILM 05.2 ICS Stock 1 and SS-Low Level Elements ICV Stock (VHG Labs, Inc-
550 Part of LGC Standards, Manchester, NH 03103 USA). For each digested sample, the ICP-
551 OES measurement was carried out in triplicate (n=3). The analytical process quality control
552 performed using EPA Method 200.7 LPC Solution certified reference material (CRM) for 30
553 analyte(s) at various concentrations (ULTRA Scientific, USA) indicated that the obtained
554 concentrations were within 97-103%.

555 **Determination of radioactivity**

556 **Gamma spectrometry.** Soil and sediments (sand and stone), prepared in the same way as the
557 content of major and trace elements, sampled in the immediate vicinity of the water spring
558 and water reservoir, were transported to the laboratory, dried until constant weight, and then
559 grounded in a glass mortar. Water samples were examined as raw, from all three sampling
560 points. All samples (water and sediments) were sealed in 450 ml Marinelli beakers and stored
561 for four weeks to ensure radioactive equilibrium between ^{226}Ra and its decay products.
562 Specific activities of radionuclides were estimated using coaxial HPGe detector (GEM30-70
563 ORTEC, 30% relative efficiency and 1.65 keV FWHM for ^{60}Co at 1.33 MeV) equipped with
564 a 10-cm-thick lead shield to reduce the background. Gamma spectrometric measurements of
565 soil samples were performed for 3 h, while water samples were measured for 48 h due to the
566 low count rates. The specific activity of ^{226}Ra was obtained by observing the gamma lines of
567 ^{214}Pb (351.9 keV) and ^{214}Bi (609.3 keV). ^{228}Ra (^{232}Th) was estimated using the photopeaks of
568 ^{228}Ac at 911.1 keV and 338.3 keV. Gamma lines at 661.6 keV and 1460.7 keV were used for
569 evaluating the activities of ^{137}Cs and ^{40}K, respectively.

570 Radon in water was measured using RAD7 (DURRIDGE Company, Inc. USA) active
571 measuring device supplied with RAD H_2O accessory. Water was sampled in standard 250 ml
572 sampling vials, following the procedure recommended by the manufacturer. Samples were
573 transported to the laboratory and radon measurements were performed within the next 24
574 hours to avoid radon decay. WAT250 protocol applied for measurement included aeration
575 process followed by four 5min counting cycles. Radon concentrations were obtained by
576 averaging these 5min measuring results and the values were finally rectified to the sampling
577 time.

578 **Microbiological analysis**

579 **Enumeration of total aerobic mesophilic bacteria and enterococci.** The enumeration of
580 total aerobic mesophilic bacteria was conducted by inoculation of nutrient agar plates with 1
581 ml of water sample in two repetitions. The plates were incubated at 37°C for 48 h. After the
582 incubation, the bacterial colony forming units (CFU) were enumerated.

583 Enterococci in drinking water are considered as indicators of environmental fecal
584 contamination. The enumeration of members from genus *Enterococcus* was conducted by
585 inoculation of bile esculin agar (BEA) plates with 1 ml of water sample in two repetitions.
586 After solidification, BEA agars were covered with a thin layer of the same medium to
587 establish microaerophilic conditions. The plates were incubated at 37°C for 24 h. After
588 incubation, the black bacterial colony forming units (CFU) were enumerated.

589 **Membrane filtration method.** The detection and counting of total coliform bacteria (TCB)
590 and *Escherichia coli* by membrane filtration method were performed according to the
591 standard SRPS EN ISO 9308-1:2017. The method was based on membrane filtration of a
592 certain volume of the water sample, incubation of the concentrate after membrane filtration
593 on chromogenic medium. After filtering the sample (100 ml of water sample), the membrane
594 filter was transferred to the HiCrome Coliform Agar. *Klebsiella* spp. formed pink colonies,

595 *Salmonella* spp. and *Shigella* spp. formed colorless colonies, while *E. coli* formed dark blue
596 colonies. Incubation was performed at 37°C for 24 h. In the case the colonies were single and
597 pure on a chromogenic medium (if their number was not higher in the sample), the colonies
598 could be numbered.

599 *E. coli* was detected by following: 100 ml of sample was filtered through 0.45 μm
600 nitrocellulose membrane; then the membrane was placed on a plate on TBX agar (Oxoid) and
601 incubated at 37°C for 24 h; finally, the number of positive (blue-green dark) colonies on the
602 plate was counted as ß-glucuronidase positive *E. coli* (ISO 16649-3:2015). The specificity of
603 ß-glucuronidase for *E. coli* bacteria generated considerable use of methods that identified the
604 β-D-glucuronidase activity as a definite indication of the presence of *E. coli*, without any
605 further confirmation (Vergine *et al.* 2017).

606 After the enumeration of specific groups of bacteria, the isolation of bacteria was
607 conducted. The isolated colonies were purified by double subculturing using the streaking
608 plate method. For confirmation, all strains were subjected to Gram staining, oxidase, and
609 catalase test. All strains that were Gram-negative, oxidase negative and catalase positive were
610 used for further examination. Well-known biochemical tests, Microgen GNA+B-ID Oxidase
611 Negative tests were used for preliminary identification (Microgen, Germany). The final
612 identification was conducted by MALDI-TOF protein analysis as described in Grujović *et al.*
613 (2019).

614 The collection of identified bacterial species was kept in a 20% (v/v) glycerol/medium
615 mixture at -80°C at the Faculty of Science, University of Kragujevac.

616 **Procedures to screen bacteria for virulence traits**

617 The safety aspect of isolated bacteria included the investigation of proteolytic activity, the
618 ability to synthesize extracellular proteins, named hemolysins, the resistance to antibiotics
619 and the ability to form biofilm.

620 The substrate for evaluation of proteolytic activity of bacteria was formed by mixing
621 nutrient agar medium and milk (1.6% fat) in proportion 1:1. The inoculated media were
622 incubated at 37°C/24 h. The appearance of a clear zone around the colonies of bacteria
623 confirmed their proteolytic activity. As a positive control *Bacillus subtilis* ATCC 6633, and
624 as a negative control, *E. coli* ATCC 25922, were used.

625 Haemolysis, on blood agar (Oxoid, Hampshire, United Kingdom) supplemented with
626 5% (v/v) sheep blood, was determined after the incubation of the plates at 37°C for 24 hours.

627 The resistance to antibiotics was determined by using disc diffusion method. Bacterial
628 suspensions were prepared by the direct colony method. Initial bacterial suspensions
629 contained about 10^8 colony-forming units (CFU) ml^{-1}. Briefly, a standardized inoculum of
630 bacteria was swabbed onto the surface of Mueller-Hinton agar. Filter paper disks,
631 impregnated with a standardized concentration of an antimicrobial agent, were placed on the
632 surface, and the size of the inhibition zone around the disk was measured after overnight
633 incubation. Five antibiotics, with various mode of action, were selected: Streptomycin (10
634 µg); Tetracycline (30 µg); Cefotaxime (30 µg); Amoxicillin (10 µg) (Biolab, Budapest,
635 Hungary) and Chloramphenicol (30 µg) (Torlak, Belgrade, Serbia). The interpretation of
636 zones of inhibition (in mm) was conducted according to the EUCAST (2022).

637 The ability of isolated bacteria to form biofilms was assayed as described by Grujović
638 *et al.* (2019). Optical densities (OD) of stained adherent bacteria were determined with an
639 enzyme-linked immunosorbent assay plate reader (RT-2100C, Rayto, Shenzhen, China) at
640 630 nm wavelength.

641 **Detection of *E. coli* virulence genes by PCR**

642 *E. coli* isolates were subjected to polymerase chain reaction (PCR) of *stx1*, *stx2*, *hlyA* and *eae*
643 genes which was performed as described by Paton and Paton (1998) with slight modifications
644 in DNA isolation.

One bacterial colony was selected from the culture media and suspended in 1 ml of PCR-grade water (Invitrogen USA), vortexed and centrifuged at 13000 *g* for 5 min. Supernatant was discarded, 200 µl was added and the pellet suspension was vortexed, heated in thermoblock (99ºC) for 5 min, immediately followed by cooling on ice for 15 min and centrifugation at 13000 *g* for 5 min. The supernatant was transferred to nuclease free tubes (Eppendorf, Germany) and was used as template for PCR reaction in volume of 3 µl.

Multiplex PCR of *stx1, stx2, eae* and *hly*A genes was performed using Multiplex PCR Master Mix (EURx, Poland) in final reaction volume of 25 µl. Primers used in the reaction are presented in Table 4. PCR was performed on Mastercycler Personal (Eppendorf, Germany) and consisted of initial denaturation at 94ºC for 2 min, followed by 35 cycles, each consisting of denaturation at 94ºC for 10 s, annealing at 56ºC for 10 s and extension at 72ºC for 5 s, followed by final extension at 72ºC for 5 min.

Table 4 List of oligonucleotide primers used for detection of *stx1*, *stx2*, *eaeA*, and *hlyA* gene

Primer	Sequence (5′ –3′)	Target gene	Amplicon size
*stx*1-F	ATAAATCGCCATTCGTTGACTAC	*stx*1	180 bp
*stx*1-R	AGAACGCCCACTGAGATCATC		
*stx*2-F	GCACTGTCTGAAACTGCTCC	*stx*2	255 bp
*stx*2-R	TCGCCAGTTATCTGACATTCTG		
eae-F	GACCCGGCACAAGCATAAGC	*eae*	384 bp
eae-R	CCACCTGCAGCAACAAGAGG		
hlyA-F	GCATCATCAAGCGTACGTTCC	*hlyA*	534 bp
hlyA-R	AATGAGCCAAGCTGGTTAAGCT		

F: Forward primer R: Reverse primer

The potential PCR-amplified products were analyzed using 2% agarose (MBG, USA) gel electrophoresis and stained with ethidium bromide. Images were documented by a BioDocAnalyze system (Biometra, Germany).

664 **Statistical analysis**

665 Data were presented as mean ± standard deviation using Microsoft Excel where appropriate.
666 Mann-Whitney test was used for detection of significantly different levels of
667 concentrations/contents of major and trace elements and radionuclides among water samples
668 (WS, WR, WT) using SPSS 20 package (SPSS, Chicago,Delaware, USA).Principal
669 component analysis (PCA) was used to reveal the associations of the different elements,
670 radionuclides, chemical parameters as well as the total number of coliform bacteria and the
671 number of *E. coli* in water samples using Statistica 13.0 package (TIBCO Software, Palo
672 Alto, California, USA).

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678 MALDI-TOF.

679 **Conflict of interests**

680 All other authors declare no competing interests.

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Authors contribution statement

M. Ž. Grujović and K.G. Mladenović were the creators of the idea, and they were performed microbiological analysis of water and interpretation of results related to the hygiene of the tested water samples. S.M Marković and N.N. Đukić performed chemical testing of water samples and interpretation of the presence and amount of organic matter in water. J.M. Stajić examined the radioactivity of water and sand and soil samples as well as data processing. A.M. Ostojić and N.M. Zlatić determined the presence of major and trace elements in water samples and performed statistics analysis. All authors participated in collecting literature and preparation of the manuscript.

Supporting information

Additional Supporting Information may be found in the supplementary files.

Table S1. Guideline values for maximum concentration (μg l^{-1}) of metals in water

Table S2. Preliminary identification of *E. coli* according to biochemical tests and Microgen GNA+B-ID tests

Table S3. Preliminary identification of other coliforms according to biochemical tests and Microgen GNA+B-ID tests

Table S4. The distribution of coliform bacteria genera through water samples

Table S5. Sensitivity to antibiotics of tested isolates

Table S6. The ability to form biofilm

Table S7. Factor coordinates of the variables, based on correlation

Table S8. Instrumental operating conditions for ICP-OES

Supplementary Figure S1. Mass spectra of **A** – *A. calcoaceticus*; **B** – *A. bestiarum*; **C**– *E. cloacae*; **D** – *R. ornithinolytica*; **E** – *E. coli*; **F** – *C. braakii*