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EVALUATION OF MICROBIAL AND CHEMICAL INDICATORS AS A MEASURE OF THE DEGREE OF POTABILITY OF WATER

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Abstract

The quality of drinking water is a challenge for consumers and scientists in our century. The bacterial diseases and deaths recorded in recent years, as a result of the consumption of fecal-contaminated water, are reasons for concern and microbiological evaluation of water in all populated areas on Earth. In this paper, microbiological and chemical analyses were performed for ten samples of water from wells drilled in households in the perimeter of Timis County, Romania. The analyses included the range of microbial groups and species considered indicators of water quality. Their isolation and identification were carried out on specific nutrient media and were completed with determinations of nitrates, phosphates and pH. According to the analysis reports, faecaligenous enterococci were not identified, instead clostridia were observed in most of the samples and a contamination with Escherichia coli. The microbial load is high in the majority of samples. The chemical analyses indicated the presence of phosphates and nitrates in most of the evaluated samples. The nitrates in the analyzed water samples did not exceed the value of 50 mgL^{-1}stipulated in the standard. The obtained results suggest the presence of some sources of faecal contamination and require taking measures to treat the waters in question.

Key words: *Clostridia, Escherichia coli, faecaligenous enterococci, fountain water, nitrates.*

INTRODUCTION

Water is one of the essential elements of life (Mitrănescu et al., 2007; Petcu, 2015). The United Nations supports the sustainable development of drinking water resources and wants to implement the project of access to sewage and drinking water for all nations with limited resources by 2030 (WHO, 2015). Chemical and biological monitoring is a concern for scientists in the 21st century due to the importance of water quality in human health. WHO (2022) points out that 1.2 billion people do not have access to drinking water services. Biological, chemical (WHO, 2017a), and physical pollutants can cause degradation in water quality. Among these pollutants, microorganisms (Figure 1) cause the most health problems worldwide (WHO, 2011). Microorganisms have a negative impact on children, the elderly, and immunodeficient individuals (Freeman et al., 2012).

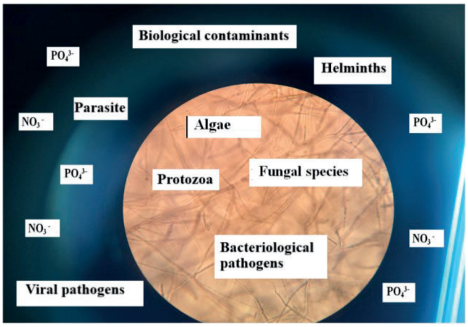

Figure 1. Table of biological and chemical water contaminants

Studies show that over 829,000 deaths are caused by pathogens present in water (Cabral, 2010; WHO, 2022). Diseases caused by microbiologically contaminated water are diarrhea (Baker, 2016; Ercumen et al., 2017), dysentery, cholera, poliomyelitis and typhoid. Among these diseases, diarrhea causes around 505.000 deaths annually (WHO, 2022). Among the most incriminated bacteria are coliforms (Bai et al., 2022), enterococci (Boehm & Sassoubre, 2014) and *E. coli,* also called "faecal indicators". Besides these, there are other new pathogens that are causing waterborne diseases.

These biological contaminants reach the environment (water, soil, food) through faeces, appear in the form of microbial biofilms in water systems because of their stagnation or the use of small amounts of disinfectant (Farhadkhani et al., 2014). Nitrates and phosphates are among the chemical pollutants that create major problems worldwide (Borozan et al., 2021). Nitrogen and phosphorus in moderate amounts support the growth of living organisms (Grzyb et al., 2021), but at high levels they cause eutrophication and deterioration of water quality, and have a negative impact on ecosystems and human health (Dodds & Smith, 2016; Ward et al., 2018). Animal, human, industrial, and agricultural waste are all potential sources of pollution. Sources of drinking water for consumers are represented by bottled water, automatic sources, tap water and water from dug or drilled wells (Hile Dama et al., 2023). Some studies show that water from individual wells is increasingly used, because access to groundwater is easy, well construction costs are reduced and it does not involve any additional fees. In developed regions, bottled water has a high percentage. In Romania there are several sources of drinking water, including drilled or dug wells. They are located in urban and rural areas. The water is intended to be consumed by humans, as well as for animals and irrigation. In every situation, ensuring water quality is essential, as it has the potential to affect the health of plants, animals, and humans. There is currently a lack of analysis concerning the quality of drinking water and available information regarding its potability in peri-urban and rural areas. Wells in these areas are often shallowly drilled or dug, raising the possibility of microbial contamination. Additionally, there is a growing percentage of septic tanks that could potentially contaminate water sources. According to the Ministry of Health, 30% of Romania's population uses water that is not monitored from a qualitative point of view, the water coming from traditional or drilled individual wells (https://www.asro.ro).

This study aims to examine the impact of well depth (ranging from 16 to 62 meters) and household pollution sources on the microbiological and chemical properties of water from peri-urban drilled or dug wells. Its objectives include informing consumers about the chemical and biological risks they face, identifying pollution sources, highlighting relevant regulations, determining distances between water sources and critical pollution points, and tracing pollutants along trophic chains. Therefore, the study's objective is to promote sustainability and efficiency in managing underground water resources and constructing wells that minimize water degradation.

MATERIALS AND METHODS

Study area

The drinking water samples come from the northwestern part of Timis county (20°16′22°33′E, 45°11′46°11′N), from a locality (VA) located near the Mures river, characterized through a plain climate, where three types of climates intersect. The community consists of over 6000 inhabitants.

Sampling

The collection of water samples was carried out in accordance with the regulations in force. The amount of water collected was 1 L for each fountain. The water level in the bottle was about 1 cm below the edge. Each sampling bottle was accompanied by sample identification data (date, time, place). The samples were transported to the laboratory under optimal temperature conditions (4˚C) (Castilla et al., 2008). The water samples were processed in a maximum time of 6 hours.

Microbiological analyses

Microbiological analyses of drinking water were carried out by conventional methods. For the determination of mesophilic bacteria, faecal enterococci, clostridia and *E. coli*, the method of plate cultures was used, and for the evaluation of coliform bacteria, the method of serial dilutions was used. A number of 10 water samples were analyzed. Culture methods are preferred to qPCR, because they involve lower costs (Stelma, 2018). Water has legal regulations regarding the absence of bacteria or the numerical limitation of other microbial groups (Table 1).

Table 1. Microbiological parameters according to Law No.96/2024

Parameter	Maximum value allowed
Escherichia coli	0/100 mL
Intestinal enterococci	0/100 mL
Coliform bacteria	0/100 mL
Pseudomonas aeruginosa	0/250 mL
Clostridium perfringens	0/100 mL
Number of colonies at 22˚C	100/mL
Number of colonies at 37˚C	20/mL

For the isolation of mesophilic bacteria, 1 mL of undiluted water was inoculated in Petri plates with nutrient agar. The plates inoculated by the embedding technique were incubated at 37°C for 48 hours. The colonies developed on the surface of the medium were counted, and the results obtained were expressed as Colony Forming Units per milliliter ($CFU \cdot mL^{-1}$) (Zamorska et al*.*, 2023).

For the isolation of coliform bacteria, the presumptive test was applied (Rahayu et al., 2020). The inoculation of undiluted and diluted water samples (10^{-1} and 10^{-2}) was carried out on Tryptose-Lauryl-Sulphate medium, in three repetitions. In each medium test tube, a Durham tube was inserted to capture the gases resulting from the fermentation process. Incubation was carried out at a temperature of 37ºC for 24 hours. To confirm *E. coli* bacteria, positive test tubes from the presumptive test were confirmed on Levine medium (EMB agar). The growth of *E. coli* bacteria took place at a temperature of 37ºC, for 18 hours. *E. coli* produced metallic sheen colonies on EMB medium.

C. perfringens is a species that is anaerobic, sporogenous, bacillary, Gram-variable, motile, and can occur either solitarily or in pairs (UK Standards). The monitoring of this bacterium is mandatory in drinking water, especially in drilled wells, but it is not mandatory in surface waters subject to drinking water Law 96/2024. It is present in the faeces of animals and humans. It can contaminate water, soil, air and food (Petcu et al., 2007; WHO, 2011). To isolate the bacterium *C. perfringens,* undiluted water was inoculated on the basic medium, *Clostridium*. Incubation was carried out under anaerobic conditions, at a temperature of 37ºC, for 24 hours. Enterococci or group D streptococci, represent another group of bacteria that indicate faecal water pollution. These bacteria are common in nosocomial infections and present a high risk, because they can transfer resistance genes to other Gram-positive and Gram-negative pathogenic bacteria. *Enterococcus faecalis* is a group D bacterium, common in mineral, flat and spring water, which causes infections (Wei et al., 2017). For the isolation of enterococci in a single step, a solid medium, Azida-Bila-Esculina was used, a selective medium used for counting and confirming enterococci in water. The incubation temperature was 37°C for 24 hours.

Chemical analyses

To conduct an initial screening for areas of fecal contamination, the nitrate (NO_3^-) concentration was also assessed.

The concentration of phosphate ions (PO_4^{3-}) and the pH value were also established in several wells, chosen according to the depth.

NO_3^- was quantified using 4500-NO_3^- E. Cadmium Reduction Method (https://www.edgeanalytical.com/ wp-content/uploads/Waste_SM4500-NO3-.pdf) and PO_4^{3-} - was quantified using ammonium molybdate and antimony potassium tartrate (catalyst) under acidic conditions, in order to form to form a blue heteropoly compound (12-molybdophosphoric acid complex) and measured spectrophotometrically at wavelength 660 nm (https://assets.thermofisher.com/TFS-Assets/CMD/manuals/PI-D09244-DA-Phosphate-PID09244-EN.pdf).

RESULTS AND DISCUSSIONS

The mesophilic bacteria

The results obtained were reported to the regulations of Law No.96/2024. This law includes the requirements of European legislation regarding the quality of drinking water. This was made possible by Ordinance No. 7/2023 on the quality of water intended for human consumption, issued by the Government of Romania and published in the Official Gazette, Part I No. 63 of January 25, 2023, effective from January 28, 2023.

The results of the microbiological evaluation of the water from the ten wells demonstrate that in the analyzed water samples, most of the bacteriological indicators have high values and exceed the values provided by the standards in force. For example, mesophilic bacteria register a high number in almost all samples. The number of mesophilic bacteria is between 0-4 x 10^2/mL).

In the same samples, the number of *C. perfringens* bacteria (which falls between 0-5.85 x 10^2/mL) and coliform bacteria (whose number was between 36-110 germs/cm^3) also increased. According to Law 96/2024, the number of mesophilic bacteria, grown at a temperature of 37ºC, must be 20/mL. Our research shows that after 48 hours of incubation, mesophilic bacteria exceeded this limit in most samples, apart from sample 4, which comes from a 43 m deep well (Figure 2 and Figure 3). These results are supported by other studies showing that shallow groundwater systems are prone to fecal and chemical contamination (Islam et al., 2016; Lorentz et al., 2015; UNICEF, 2019).

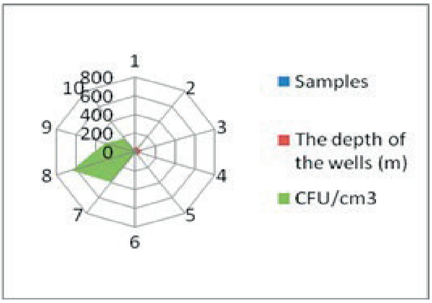

Figure 2. Estimation of mesophilic bacteria

Depending on the bacterial load (UFC/mL), the samples can be grouped in the following descending order: 6.5 x 10^2 (in sample 8) > 4.02 (in sample 7) 3.08 x 10^2 (in sample 9) > 2.61 (in sample 2) 1.77x10^2 (in sample 10) > 1.05 (in sample 5).

Wells analyzed microbiologically and chemically have different depths. The depths of the 10 wells are between 16-62 m. Maran et al. (2016) claim that the contamination with microorganisms is also correlated with the depth of the wells. The highest number was discovered in the well with a depth of 55 m. Samples 1 (the depth of the well is 60 m) and 6 (the depth of the well is 16 m) have a low concentration of bacteria compared to the other samples, but nevertheless exceed by 2.5-12.5% the value provided by the standard. The absence of pollution sources from households or the greater distance from the wells may be the reason for the lower number of bacteria in some wells. Some studies mention that the microbiological quality of water also depends on the season (Zamorska et al*.,* 2023). The studies on the water from the wells were carried out in the spring.

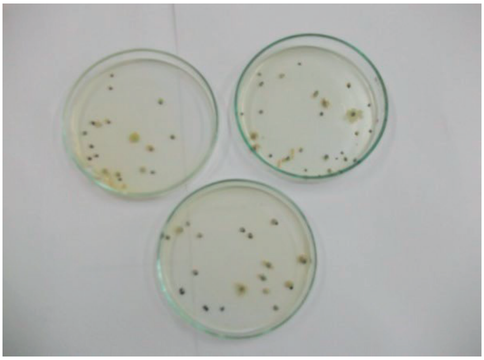

Figure 3. Mesophilic bacterial cultures

The load of Clostridium perfringens and faecal enterococci

C. perfringens spores are reliable indicators of polluted waters and are a stable ecological indicator (Stelma, 2018). They are widely distributed in sewage and constantly associated with faeces (Bisson, 1980). They are the parameters of contamination with human feces (Stelma, 2018). A genomic analysis claims that this bacterium is demanding from a nutritional point of view, because it prefers certain amino acids and is not present in the fecal of herbivores (Vierheilig et al., 2013) (Figure 4).

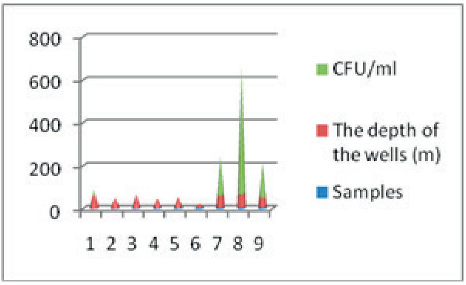

Figure 4. Estimation of bacteria *Clostridium perfringens*

According to the data presented in Figure 4, clostridia are found in large numbers in sample number 8 (5.85 x 10^2), followed in descending order by samples 10 (2.2 x 10^2), 7 (1.78 x 10^2), 9 (1.56 x 10^2) and 1 (0.25 x 10^2). *Clostridium* can be found in high numbers due to multiple factors. *C. perfringens* is an anaerobic bacterium, a condition that can be fulfilled by a drilled well. It is a sporogenous bacterium that easily survives through spores. It can also withstand lower temperatures.

Furthermore, the presence of animals and agricultural activities in rural areas contributes to the proliferation of clostridia and enterococci. Our studies are also supported by the research carried out by Hiroyuki and co-workers (2021). These authors demonstrated that *C. perfringens* is an effective indicator for water contamination with human faeces. The lowest number of *C. perfringens* was observed in samples 3 and 4. Clostridia were absent in samples 2, 4 and 6.

While enterococci are commonly present in fecal material, specific researchers suggest that the viability of *E. faecalis* might fluctuate based on the methodology of analysis utilized (Pourcher et al., 1991). Compared to clostridia, enterococci did not have such a high percentage. According to the Law 96/2024, enterococci and *C. perfringens* bacteria must be absent in drinking water.

Coliform bacteria and Escherichia coli

Coliform bacteria are facultatively anaerobic, Gram-negative bacilli, capable of fermenting glucose and producing acid and gases. They are present in the environment and in human and animal faeces (Martin et al., 2016). The regulations in force show that coliform bacteria and *E. coli* must be absent/100 mL drinking water.

Contrary to these norms, high levels of coliforms were observed in some water samples (Figure 5 & Figure 6). This refers to the same samples in which a high load of mesophilic bacteria and clostridia was detected. The highest number of coliforms was reported in sample no. 8 (1.10 x 10^2), followed by samples 9, 10 and 6. Inadequate sanitation systems and unhygienic human practices (Wright et al., 2004; Mahmud et al., 2019) contributes to a large extent of water sources the pollution. Mahmud and his team (2019), observed that in the water samples from tube wells the contamination with coliforms was 28%, and with *E. coli* 10.5%. Our research is also supported by other authors, who showed that vulnerability to contamination is also linked to the use of septic tanks placed at small distances from wells (Allevi et al., 2013; Back et al., 2018; Nayebare et al., 2020; Gumoteyo et al., 2021), improper management of household waste, improper maintenance of wells (Jimmy et al., 2013) presence of animals, unless they are kept in paddocks located a considerable distance from the water source (Navab-Daneshmand et al., 2018). Also, the mobility of faecal bacteria and chemical contaminants can be caused by anthropogenic activities in households, the depth and diameter of the well (Hynds, 2014), the type of well and the method of construction, buckets or motor pumps that come into contact with the water from the well (Maran et al., 2016), the properties of the soil and the underground water source.

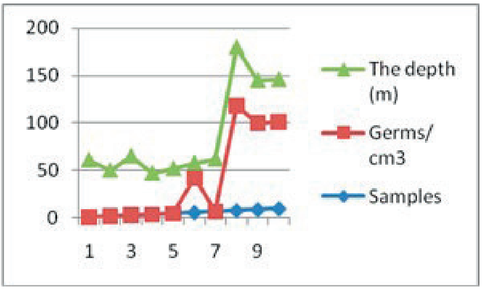

Figure 5. Estimation of coliform bacteria

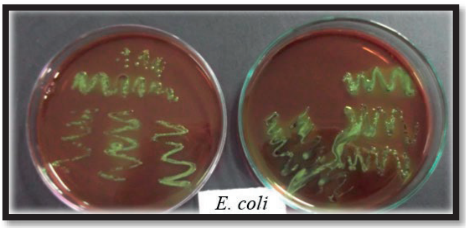

Figure 6. *E. coli* on EMB medium

Assessment of pH, nitrates and phosphates

pH plays a crucial role in affecting both the chemical and biological parameters that ultimately define water quality (Saalidong et al., 2022). Some researchers have proven that

pH influences microorganisms and the availability of substances in water (Pal, 2017).
According to Saalidong et al. (2022), "the relationship between water pH and other water quality parameters are different in different water systems and can be influenced by the presence of other parameters".
The pH values influence the microbiological quality of the water (WHO, 2024). In the acidic pH range, the growth of *E. coli* bacteria can be inhibited (WHO, 1996).
According to The Directive (EU) 2020/2184 of the European Parliament and of The Council, of 16 December 2020on the quality of water intended for human consumption and Law 96/2024 shows that the pH value must be 6.5-9.5 pH units or ≥ 6.5 and ≤ 9.5 pH units (Senaldi et al., 2021).
In our research, the pH values in the four wells are close and are in the range of 6.25-6.65 (Figure 7).

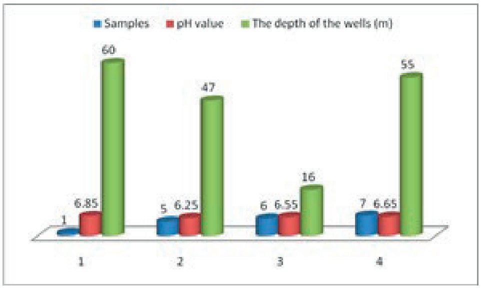

Figure 7. pH in wells drilled at different depths

Nitrogen and phosphorus are two essential elements for the growth of living organisms, but the excessive amount turns them into chemical contaminants, which depreciate the water quality and affect health (Chen et al., 1999; Davidson et al., 2012; Ward et al., 2018). Application of nitrogen and phosphorus fertilizers can result in groundwater pollution, potentially contaminating drinking water sources (Akiyama et al., 2006; Wang et al., 2019). The level of nitrates can increase in agricultural areas by applying manure in large amounts.
Nitrates (0÷2 mg·L^{-1}) and phosphates (0 ÷0.01 mg·L^{-1}) were noticed in most of the water samples (Figure 8). In his studies, Fones et al. (2020), discovered that phosphate (0.62 mg·L^{-1}) exceeded the average values allowed.

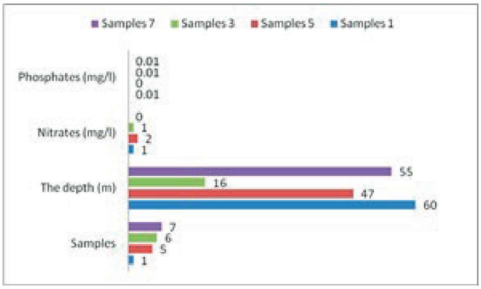

Figure 8. Estimation of nitrates and phosphates from wells drilled at different depths

According to the European drinking water regulations, nitrate maximum allowable value is 50 mg·L^{-1} while there is no specific limit for phosphate. The maximum allowed concentration of nitrates in drinking water in Romania is set at 50 mg·L^{-1}, this limit is aligned with the European Union standard. This reference value is also mentioned in the World Health Organization guidelines (WHO, 2016; 2017), with the aim of protecting the health of more vulnerable population groups. For example, infants are protected against methemoglobinemia. Many authors have observed that exceeding the allowed concentration causes thyroid hypertrophy (Van Maanen et al., 1996), macular degeneration (Klein et al., 2013), bladder and ovarian cancers (Ward et al., 2018). In our studies, the concentration of nitrates is low, below the limit imposed by the standards in force, which is why we consider that nitrates do not endanger the health of consumers. In addition, some authors mention that the reduced intake of nitrates has as potential benefits the lowering of blood pressure and immunoregulatory effects (Ahluwalia et al., 2016).

CONCLUSIONS

As a result of the fact that the water comes from the countryside and there are animals, septic tanks and inorganic fertilizers and chemical treatments for weeds, diseases and pests are used there, it is necessary to periodically evaluate the physico-chemical and microbiological parameters.
Overall quantitative water analysis has higher accuracy compared to qualitative analysis, but the latter confirms us the identity of the

contaminants. Most waters have a high microbial load of mesophilic bacteria, *C. perfringens,* respectively coliform bacteria and *E. coli* which makes the water unfit for consumption. All these microorganisms cause health problems. Nitrates were below standard limits (0÷2 mg·L^{-1}).

Each consumer was informed about the microbiological and chemical characteristics of the water. To avoid health risks, preventive and treatment measures were recommended for correcting water properties, boiling water, cleaning wells, limiting the access of animals to wells, connecting to a centralized distribution system (where applicable) and observing the optimal distance between septic tanks and wells. Also, septic tanks must meet standard construction requirements.

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