

An Investigation on the Bacterial Contents of Natural Springs in a Rural Area of the Middle Blacksea Region in Turkey

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Abstract Bacterial contamination levels of representative 4 natural springs were detected seasonally through indicator bacterial contents in a rural area of Ordu, Türkiye. Total coliform bacteria, enterococci, *Escherichia coli* and *Clostridium perfringens* spores were used as the indicator organisms. According to the bacterial parameters, none of the detected springs comply with the standards implemented by the state health authorities. Contamination level of the springs increases with human and domestic animal activities in spring and summer. Using PCR and duplex PCR, samples collected from the most densely contaminated spring, Soğuksu, were analyzed for three pathogenic bacterial groups. After eight-hour enrichment of the samples, positive results were found only for *Salmonella* spp. In conclusion, this investigation revealed that the natural springs used for drinking water supply in the research area are exposed to bacterial contaminations and the level of contamination increased with settlement density.

Keywords: spring water, bacterial contamination, cultural tests, PCR analyses

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1. Introduction

Natural springs are the locations in which underground water reaches to the surface spontaneously through the fractures of earth strata [1,2]. Under natural environmental conditions, bacterial content of natural springs is extremely poor. Such habitats contain a few heterotrophic and chemolithotrophic bacteria because of poor availability of organic nutrients and lack of sunlight. This makes natural springs a convenient resource of drinking water for rural populations [3,4].

Bacterial communities present in groundwater are divided into two groups: permanent community and temporary community. Permanent community is represented by non spore-producing Gram negatives most of which are members of *Acromobacter* and *Flavibacterium*, and Gram positive bacilli including members of *Mirococcus*, *Nocardia* and *Cytophaga* in fewer numbers [5,6]. Using molecular techniques much more bacterial taxa have been found in subsurface habitats [7,8] Temporary community of groundwater, on the other hand, is not continuously growing and it is only temporarily present in groundwater. This community may be divided into two subgroups: i) soil bacteria and ii) the bacteria which are inhabitants of human and warm-blooded animals' body and mainly their gastrointestinal tract.

Even though most nonpathogenic soil bacteria drain through earth strata and rarely reach groundwater

reservoirs, they cannot survive there long because of nutrient limitation. Animal originated temporary community is mostly consisted of animal and more often human gastrointestinal community through the reach of digestive system waste into groundwater reservoirs because of dense settlement on the catchment area of groundwater reservoir, untreated deposition and/or incorrect storage of fecal waste and heavy rains help the waste to penetrate deep into the soil [9].

Health risk of temporary community is essentially the reach of human digestive system wastes into groundwater reservoirs by draining through the soil [2,10]. Natural intestinal community which is mostly nonpathogenic or rarely pathogenic is not able to survive in the environment long as they die in a relatively short time [11]. Nevertheless they may cause infections if contaminated water is used in this short survival period. In past and present days a number of epidemic incidences caused by waterborne microbes have been reported especially in developing countries [12].

A group of organisms are called index organisms that are used as an indicator of one or more target organisms such as pathogenic strains in a habitat. Index organisms used for monitoring treated water are called indicator organisms. The term indicator organisms are often used in place of both index and indicator organisms [2,12,13,14,15]. Amongst the others, a number of bacteria or bacterial groups such as coliform bacteria, *Escherichia coli*, enterococci and *Clostridium perfringens* spores have been used as indicator organisms for fecal contamination of

water reservoirs [16]. Each group possesses both advantages and disadvantages. In time, coliform bacteria included a number of genus according to the properties of acid and gas forming or only gas forming and β -galactosidase activity [15,17]. The origins of the genus included in coliforms may be only environmental or both environmental and fecal or only fecal [2]. Therefore, an existence of coliform bacteria is not always an indication of fecal contamination. On the other hand, *E. coli* becomes a better indicator for fecal contamination since it is a natural member of intestinal bacterial community of human and the other warm-blooded animals [15]. Enterococci are also intestinal origin and used as indicators for long term fecal contamination [18]. *Clostridium perfringens* spores are used as indicators not only for bacterial spores, but also for viruses and protozoan cysts detection in water [19].

Traditional culture detection techniques for bacterial contamination are not effective in some cases such as longer testing time, inhibition of indicator growth by inhibitor of the other bacteria and presence of viable but uncultivable cells. [20,21,22]. In such cases molecular techniques promise more effective detection methods by increasing the limits of detection and shortening the test duration [21]. Commonly used molecular techniques are PCR-based ones such as monoplex PCR, multiplex PCR (m-PCR) and quantitative PCR (qPCR) [23,24,25,26,27]. Using specific primers, multiple loci of a target organism or more than one target organisms can be detected in a single PCR reaction. Quantitative PCR techniques allow detection of presence and quantity of target organism(s). To detect the presence and quantity of targeted organisms, mPCR and qPCR have been applied successfully to both the environmental and clinic samples [20,23,28,29]. A major drawback for molecular techniques for detection of target organisms is its sensitivity that has higher detection limits than that of traditional culture-based techniques [28], [30,31]. Nevertheless, various types of organisms can be detected and quantified by means of molecular techniques.

In rural areas of Blacksea Region, the groundwater which reaches to the surface as natural springs is almost the sole source of drinking water for the inhabitants. It is used as raw water without any treatment. There is no sewage system in this rural area. As a result, groundwater reservoir of the region is open to bacterial contamination with a high health risk which may originate from pathogenic bacterial contaminants. In this study, natural spring water was analyzed for presence of some indicator and pathogen bacteria using traditional culture-based and molecular techniques in Güvenyurt Village (Ulubey, Ordu) as a typical rural area for Middle Black Sea Region of Turkey.

2. Materials and Methods

2.1. Materials

Water samples, collected from a rural area of Ordu province in Middle Blacksea Region, were analyzed for certain selected indicators and pathogen bacteria. Indicators were total coliforms, *Escherichia coli*, enterococci and *Clostridium perfringens* spores while pathogens were *E. coli* O157:H7, *Salmonella* spp. and

Campilobacter jejuni. *E. coli* DH5 α and *Salmonella enterica* Typhimurium used as positive controls. Güvenyurt Village (Ulubey, Ordu, Turkey) was selected as the research area. 4 natural springs were selected amongst a total of about 35 springs which are used as drinking water supply by inhabitants in the area. Yarımcin and Harmancık springs are located in forested area away from populated area of the village. Yatakyeri spring is located on a lightly populated upper side of the village while Soğuksu spring is situated below the heavily populated area of the village. Locations of all the 35 natural springs of the research area are comparable with those four selected springs.

2.2. Methods

2.2.1. Collection, Transport and Storage of Water Samples

Water samples were collected to determine changes in bacterial content in four seasons of the year in November 2011, February, May and August 2012. Samples were collected in sterile 400 ml polypropylene vial by means of autoclave sterilized silicon pipe and stored in a cooler bag until the reach of the laboratory [32]. One part of each sample was used for traditional culture tests in the same day, while the other part was stored at 4°C until the following day for template DNA preparation.

2.2.2. Total Coliforms Test

A certain volume water sample was filtered through a sterile filter (Pall Corp.) with 0,45 μ m pore width using a filtration system (Sartorius). The filter then was transferred onto lactose TTC agar plates and incubated at 37°C for 24-48 h [32]. All the lactose positive colonies grown on the filter were counted as a total of coliform bacteria with *Pseudomonas* spp. [33]. The colonies were counted and the number of bacteria in 100 ml of original samples was estimated. To exclude *Pseudomonas* spp. from the total coliforms, randomly selected colonies were applied to oxidase test and oxidase positive colonies were excluded from the total number of colonies.

For oxidase test, 2 drops of freshly prepared oxidase reactive (1% Tetramethyl p-phenyldiamine HCl in water) were placed on a filter paper and a colony was spread on the filter with a non-chrome loop. The colonies which were coloured into dark blue- purple in 30 sec were taken as oxidase positive [32].

2.2.3. *E. coli* Test

Indole test was applied to find *E. coli* from the colonies grown on TTG agar plates. Yellowish colonies grown under the filter were inoculated into Tryptophan broth medium and inoculated at 44,5°C for 24 h in a test tube. Occurrence of a cherry-red ring on the surface of the medium was taken as *E. coli* positive [32].

2.2.4. Detection of Intestinal Enterococci

To determine enterococci from the water samples, enterococci specific Slanetz-Bartley selective medium was used. After filtering of a certain volume of water sample, the filter was placed onto the selective agar medium and grown at 37°C for 24 h. To confirm the colonies as enterococci, the filter with colonies was transferred to a

preheated Bile Aesculin Azide Agar plate at 44°C for 2 h. The colonies, the colour of which was changed into tin to black, were counted as intestinal enterococci [34].

2.2.5. Detection of Anaerobic Bacterial (*Clostridium* sp.) spores

To find *Clostridium* sp. spores, solid SPS (iron sulphate agar) medium stored in a vial test tube was melted at 98°C. Water samples were heated at 75°C for 15 min to kill vegetative forms. Heated water samples were then inoculated into the bottom of the melted SPS medium in a ratio of 1:1 (v/v). This culture was incubated at 37°C for 24 h, and blackish coloured growth spots were counted as anaerobic spores ([35,36].

2.2.6. Detection of Indicator and Pathogen Bacteria by PCR

All the basic molecular biological techniques were carried out according to Sambrook and Russell [37]. PCR amplification was applied to detect pathogen strains of *Salmonella* sp., *E. coli* O157:H7 and *Campylobacter jejuni* in the collected water samples. *E. coli* DH5 α and *S. enterica* Typhimurium LT2 were used as positive controls. Genomic DNA isolation was carried out according to Park et al. [28]. Standard nutrient broth medium was used for bacterial growth. Pure genomic DNA, environmental DNA and cell-free extracts were used as template in PCR reactions. Cell free extracts were prepared according to Park et al. [28]. Specific primers used in this study were shown in Table 1. Monoplex and multiplex PCR amplifications were applied to the water samples collected from Soğuksu spring which is the most intensely polluted. 1 l of sample water was filtered through a filter with 0,45 μ m pore width and cells were resuspended in 1 ml nutrient broth. This suspension was used for either cell-free extract preparation or inoculums for nutrient broth enrichment culture.

Table 1. Sequences of the Primers Used in PCR Analyses

Names and sequences of the primer pairs (5'-3')	Organism
<i>lacA</i> -F2-A2: CCCGCGCCAATAACAGAATTATCC <i>lacA</i> -R2-A2: AGAAATGTTTGCCACGGTAGGGG	<i>E. coli</i> and <i>Shigella</i> [23]
<i>campsh</i> -F: CAAGTTGCTACAATCTCAGCCA <i>campsh</i> -R: GATAACACCATCTTTGCCCACT	<i>C. jejuni</i> [28]
<i>eae</i> 150-F: GGCGGATTAGACTTCGGGCTA <i>eae</i> 150-R: CGTTTTGGCACTATTTGCC	<i>E. coli</i> O157:H7 [38]
<i>invA</i> 3-F: AACGTGTTTCCGTGCGTAAT <i>invA</i> 3-R: TCCATCAAATTAGCGGAGGC	<i>Salmonella</i> spp. [39]

2.2.7. Statistical Analyses of the Data

Mann-Whitney U [40] (Ruxton, 2006) tests were applied for statistical analyses of the data using SPSS Statistics 17.0 package. Seasonal variations and relationship between contamination density and distance from the settlement area were analyzed separately.

3. Results

3.1. Natural Spring in Research Area

Initially, the total number of natural springs which are used for water supply by inhabitants was found. Total of 35 springs were found to be used by local population for drinking water supply. Almost all the springs are

transferred to the residences through pipes and used without any means of purification treatments. The locations of the springs are scattered through the area from lower to higher altitudes and from less or no populated areas to densely populated areas. Four of the springs were selected to represent all the springs of the area instead of testing all of them.

The spring Yarımcin is located in a non populated forest area at an altitude of 300 m. It is used as an on-site supply especially during hazelnut harvesting in August. Soğuksu spring is positioned below the most populated area of the village at an altitude of 530 m. The spring is used by quite a big part of the population through a pumping and a pipe system. As a representative of the less populated area, Yatakyeri spring has an 850 m altitude and supplies drinking water to a medium size of population through a pipe system. Lastly, Harmancık spring is located in a forested area at the altitude of 950 m which is the highest amongst the representing springs. There is no settlement near and above the spring and it is used as water supply to relatively small number of settlers through a pipe system.

Water and environmental temperatures of each spring were measured during sampling. The pH values were also determined immediately after reach of the laboratory. Temperature and pH data were shown in Table 2.

Table 2. water and environmental temperatures and pH values of the springs. Abbreviations: W stands for water E for environment, H for Harmancık, YY for Yatakyeri, S for Soğuksu and YC for Yarımcin

Sampling Time	Temp. & pH	Spring			
		H	YY	S	YC
Nov 2011	W (°C)	8	8	11	12
	E (°C)	4	10	6	3
	pH	8,29	8,30	8,31	8,33
Feb 2012	W (°C)	4	4	5	7
	E (°C)	2	2	0	2
	pH	8,00	8,20	7,97	8,20
May 2012	W (°C)	11	11	11	10
	E (°C)	13	15	19	15
	pH	8,25	8,22	8,00	8,30
Aug 2012	W (°C)	15	13	19	17
	E (°C)	20	22	25	23
	pH	8,30	8,00	8,20	8,30

3.2. Indicator Bacterial Contents of the Natural Springs

Selected indicator bacterial contents of the representative springs in the research area were determined by traditional culture technique in November 2011, February, May and August 2012. Parametric values of the parameters enterococci, total coliforms, *C. perfringens* spores and *E. coli* were shown in Table 3.

Harmancık spring was not contaminated with enterococci and *C. perfringens* spores through the year. *E. coli* was detected in autumn 2011 in only one of the three repeated tests but it was not detected in other three seasons. Coliform contamination was persistent through the year, more heavily in spring.

Yatakyeri spring did not contain any *C. perfringens* spores through year. Enterococci and *E. coli* were not detected in winter while they were represented by

relatively lower numbers in autumn compared with spring and summer. *E. coli* numbers were 5 to 10 times more than enterococci through year. Total coliforms were detected in all seasons and their numbers in spring and summer increased approximately 50 times to those of autumn and winter.

Table 3. Parametric Values of the Indicator Bacterial Groups Enterococci, Total Coliforms, *C. perfringens* Spores and *E. coli*. “cfu” stands for colony forming units, *C. perf* for *C. perfringens*, H for Harmancık, YY for Yatakyeri, S for Soğuksu and YC for Yarımcin

Spring	Sampling Time	Parameters and Parametric Values			
		Enterococci (cfu/100 ml)	Total Coliforms (cfu/100 ml)	<i>C. perf.</i> spores (cfu/50 ml)	<i>E. coli</i> (cfu/ 100 ml)
H	Nov. 11	0	53,33	0	1,33
	Feb. 12	0	63	0	0
	May 12	0	428,67	0	0
	Aug. 12	0	60,67	0	0
YY	Nov. 11	1,33	72,33	0	5,67
	Feb. 12	0	48,67	0	0
	May 12	61,33	3200	0	460
	Aug. 12	40,67	2266,67	0	423,33
S	Nov. 11	11	1233,33	35	47,33
	Feb. 12	174	656,67	0	11,33
	May 12	97,33	4500	1,67	453,33
	Aug. 12	48,67	1496,67	0	316,67
YC	Nov. 11	1,67	250	0	5,33
	Feb. 12	0	21,33	0	4
	May 12	64	1116,67	0	85
	Aug. 12	313,33	1763,33	0	355

Being located below the dense populated area, the most contaminated spring, by the indicator bacteria in the area through the year, is Soğuksu spring. It was contaminated heavily by total coliforms. In respect to contamination density total coliforms were followed by *E. coli* and enterococci respectively. This spring was the one among the representative springs that was contaminated with *C. perfringens* spore in autumn and spring. Winter and summer samples were free of the contamination.

Surprisingly Yarımcin spring was found to be densely contaminated with three of the indicators even though it is located away from the settlement area. It showed no *C. perfringens* contamination like Harmancık and Yatakyeri springs. Enterococci were present in three seasons except for in winter. Total coliforms contamination was found through the year with a lighter density than Soğuksu and Yatakyeri springs. Also *E. coli* contamination was found through the year (Table 2).

3.3. Comparison of Seasonal Contents of the Natural Spring

Enterococci were not detected in Harmancık spring through the year (Figure 1 a). The other three springs were contaminated with enterococci except for Yatakyeri and Yarımcin in winter. Contamination densities tend to increase in spring and summer. Contrary to Yatakyeri and Yarımcin, Soğuksu was densely contaminated in winter. Even though it is located away from the settlement area, Yarımcin spring was detected to exhibit the highest number of enterococci in summer (Figure 1 a).

Total coliforms were found in all the seasons in all the springs. The lowest numbers were found in autumn and winter in all the springs. Contamination densities increased both in spring and summer extensively. Soğuksu spring exceptionally exhibited a different pattern of a more dense contamination with a record number in spring at least three times more than those in the other seasons. Contamination densities were consistent with the location of the spring, closer settlement area results in higher contamination density (Figure 1 b).

C. perfringens spores were present only in Soğuksu spring. The spores were found in autumn and spring, tremendously higher in autumn. Locating below the densely settled area, the source of contamination was probably from human and/or livestock effect. The other springs were completely free from spore contamination (Figure 1 c).

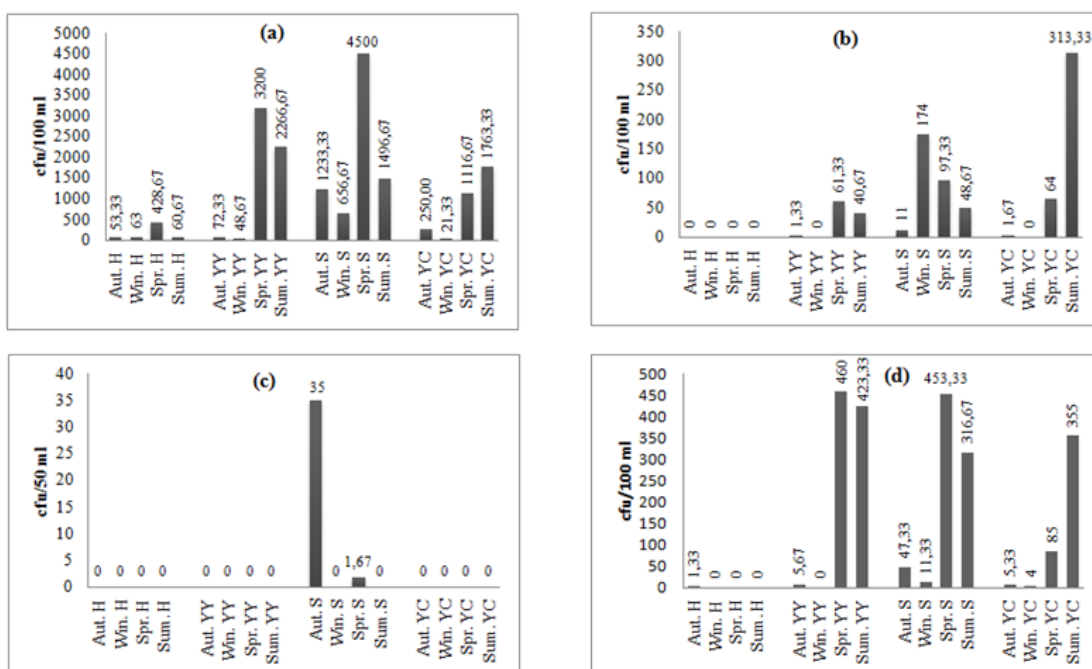


Figure 1. Seasonal variations in number of indicator bacterial parameters in representative natural springs. a) Total coliforms, b) enterococci, c) *C. perfringens* spores and d) *E. coli*. Parametric values for each indicator are given over the bar. Data is mean of three independent tests. “cfu” stands for colony forming units

E. coli was exhibited in all the representing springs. In Harmancık spring *E. coli* were found only in autumn, 5 cells were found in only one of the three repeat tests. During the other seasons of the year the spring was completely free of *E. coli*. The other three springs showed similar pattern of *E. coli* numbers. Contamination was relatively sparse in autumn and winter and increased substantially in spring and summer. Yarımcin spring was comparatively less affected while Yatakyeri and Soğuksu springs showed a similar higher contamination density (Figure 1 d).

3.4. PCR Detection of Bacterial Strains in the Samples

Especially rivers, irrigation and recreation waters have been tested for certain bacterial strains by means of PCR and qPCR analyses. The pathogens *Campylobacter jejuni*, *E. coli* O157:H7 and *Salmonella enterica* Typhimurium LT2 strains were detected by multiplex PCR and qPCR with a reasonable sensitivity [28]. To find sensitivity of such techniques in natural spring water, we also tried to detect *E. coli* O157:H7, *Salmonella* spp. and *C. jejuni* by monoplex and duplex PCR amplification.

Firstly, PCR parameters were optimized by using *E. coli* DH5 α genomic DNA as template and specific primer for *E. coli* and *Shigella* spp. Minimally amplifiable genome numbers were found to be 3520 per reaction using pure genomic DNA. Then, PCR amplification reactions were performed using cell free extracts of *E. coli* DH5 α and *S. enterica* Typhimurium LT2 as template in PCR reactions, yielding expected PCR products.

Eventually Soğuksu spring which is the most densely contaminated in the area was analyzed for existence of the bacterial groups above by PCR. Exhibiting an intermediate contamination level among the other seasons which was 47,33 cfu/100 ml, autumn samples were used for PCR analyses. 1 l of the sample containing 473 *E. coli* cells were filtered and resuspended in 500 ml TE tampon. This concentrated sample was freeze-crashed and used as template DNA. No PCR products were amplified even with increased amount of templates.

To supply more template DNAs, 1 l of original spring water sample was filtered and inoculated in nutrient broth to enrich bacterial cell numbers. During enrichment 1 ml samples were removed from the culture after 1, 2, 4 and 8 hours after incubation and used for cell free extract preparation. Each sample was tested for PCR amplification using *E. coli* specific primers first. An expected 360 bp PCR product was produced by template from 8 hour incubated culture but no product from earlier samples. Same result was found with *Salmonella* spp. specific primers. No product was seen in samples from all incubation times with primers for *E. coli* O157:H7 and *C. jejuni*. Afterwards monoplex and duplex PCR amplification were applied using *E. coli* and *Salmonella* spp. specific two sets of primers using cell free extracts as template from 8 hour enriched culture. 360 bp *E. coli* and 260 bp *Salmonella* sp. products were seen as single bands in monoplex PCR, 360 bp and 260 bp two fragments in duplex PCR reactions (Figure 2). This result showed that *Salmonella* spp. can be detected in Soğuksu spring contaminated with *E. coli* in a density of 47,33 cfu/100 ml

if only 1 l filtered sample was enriched in nutrient broth at least 8 hours.

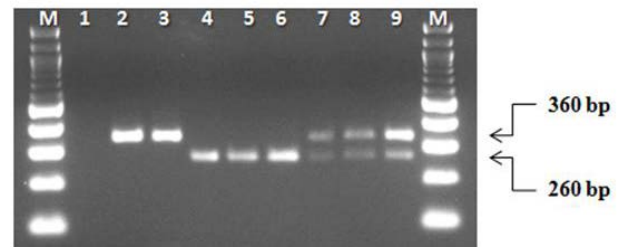


Figure 2. Monoplex and duplex PCR products amplified with *E. coli* specific *lacA* and *Salmonella* spp. specific *invA* primers. Line 2 and 3 shows *lacA* monoplex, lane 4-6 *invA* monoplex and lane 7-9 *lacA-invA* duplex PCR products. Lanes M are molecular markers (Biolabs 100 bp DNA ladder)

4. Discussion

In terms of the quality of natural springs, the research area represents the rural area of not only Ordu Province but, perhaps also all the middle and eastern Blacksea Region of Turkey. The area exhibits a sparse settlement and natural springs are almost the sole drinking water source, without any treatment in most cases. Not an effective surveillance has been applied in the area, where selected indicator bacterial parameters have been tested in only one station for each village twice in a year [40]. The results of this study showed no potable spring water available in the research area according to the regulation of the health authority [41]. According to these regulations, total coliforms are an indicator for bacterial detection in drinking water. However, some sceptical ideas have been become widespread for these organisms as an indicator because many numbers of coliforms are known to be purely environmental origin [15]. Taking this into consideration, Harmancık spring may be accepted to accomplish the requirements for a drinking water supply provided that a very few contamination with *E. coli* in summer is omitted (1,3 cfu/100 ml). This result implies that underground water reservoirs located under non-settled areas are not completely open to bacterial contamination. However, Yarımcin spring which is positioned well away from settlement area exhibited some degree of contamination with enterococci and *E. coli* as well as total coliforms. Positioning away from settlement area like Harmancık spring, difference in the contamination levels is possibly because of the difference in their altitudes, that the altitude of Yarımcin is 300 m and Harmancık 950 m. Yarımcin area is probably affected by human activities during hazelnut harvesting in summer and by wild life in winter which is more moderate than Harmancık. Other two springs, Yatakyeri and Soğuksu, are badly open to bacterial contamination in 4 seasons of the year. Soğuksu spring is located below the most densely populated area and therefore is exposed to the worst contamination among the natural springs tested accordingly. Moreover, it exhibited *C. perfringens* spore contamination in autumn and spring. The results indicate that more human and livestock activities cause heavier bacterial contamination in natural springs of the area.

When only very low level of total coliforms and rare *E. coli* are represented, a seasonal effect comparison cannot

be meaningful for Harmancık spring. Remaining three springs exhibited a tendency to increasing bacterial contents in spring and summer. When the total of autumn and winter values were compared with those of spring and summer, total coliforms increased considerably in Yatakyeri, Soğuksu and Yarımcin springs. The higher coliform values were seen in spring in Yatakyeri and Soğuksu but in summer in Yarımcin. Enterococci were also showed similar pattern with total coliforms in Yatakyeri and Yarımcin but winter was the season that exhibited higher value in Soğuksu. *C. perfringens* spores were detected in only Soğuksu spring in autumn and spring indicating that it may be originated from human and especially livestock activities. Being the best indicator parameter for faecal contamination, number of *E. coli* exhibited a strong seasonal variation in Yatakyeri, Soğuksu and Yarımcin springs. Difference in the numbers of *E. coli* in autumn and winter was clearly visible when compared with the number in spring and summer. Increased number of *E. coli* in spring must be because of the increase in rainfall and snow melting in this region causing more bacteria to pass through the soil into underground water reservoirs. Two reasons may be considered for higher contamination levels in summer, one of which may be increased human population size and another possibility is increased survival time of enteric bacteria in the environment. In contrast, relatively low level of contamination in autumn and winter may be a result of decrease in human population size resulting less faecal contamination and lower temperatures which limits the survival time of enteric bacteria in environment.

Distance of the springs from the settlement areas seems to be correlated with the degree of contamination. Keeping in mind the recent idea that total coliforms cannot be used as a reliable indicator for intestinal bacteria, only Harmancık spring, which is located at higher altitude than the settlement area, may be taken as not contaminated with the indicator bacteria. Contrarily Yarımcin spring was contaminated through the year even though it is located away from the settlement area but at lower altitude. The other two springs are situated in settlement areas and were densely contaminated accordingly. Contamination levels of those springs increase in accordance with the density of human and livestock populations. Indeed, Soğuksu spring exhibited heavier contamination than Yatakyeri spring especially in summer and autumn. Also enterococci and especially *C. perfringens* spore contamination was strikingly dense in Soğuksu spring located in the most populated area.

The results of this investigation show that all the natural springs were contaminated by bacterial indicators tested except for those which were located in higher latitude than the settlement areas. Most of the springs of the area are positioned more or less in populated areas, this means contamination with intestinally originated bacteria through the year with various densities. There is no sewage system in the area and not likely to be in near future because of sparse settlement tradition in the Blacksea Region. There is neither a water supply network nor an effective monitoring program. Although rare individual treatment systems are present, most of the population use raw natural springs for their drinking water supply and accordingly open to the exposure of possible pathogens which originate from gastrointestinal tract of human and

warm-blooded animals. Certainly, the most urgent measure for the region is to build a clean drinking water supply. Even when a clean water network is constructed by local authorities, underground water reservoirs must be protected from microbiological, and of course also from chemical contaminations which are out of focus here. This is necessary for not only for protection of natural environment but also for the safety of natural springs in the area as an emergency water source.

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