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Study on the Total Coliforms Count and Coli Titter in the Waters of Kardzhali Reservoir, Bulgaria

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Abstract. The aim of this study was to examine TC (total coliforms) and coli titter from two sampling stations in the aquatory of Kardzhali Dam Lake and one station in River Arda, in August, 2011. The values of the TC index in the reservoir vary from 1900±674 cfu/100ml in station I, to 1293±194 cfu/100ml in station II, while the TC value of River Arda reaches 1698±134 cfu/100ml. In Reservoir Kardzhali, the smallest volume of water in which *Escherichia coli* cells were found, varies between 5 and 15 ml, while for the River Arda the value of coli titter is equal to 1. With highest percentage, regarding the presence of microbiological species in the reservoir waters, is the genus *Klebsiella* (70%), followed by *Citrobacter* (15%), *Enterobacter* (10%) and *Serratia* (5%), respectively represented by the species *Klebsiella oxytoca, Citrobacter freundii*, *Enterobacter cloacae* and *Serattia marcescens*. In the river Arda two genuses were found - *Serratia* (50%) and *Salmonella* (50%).

Key words: coli titter, total coliforms, Kardzhali Reservoir.

Introduction

The permanent control of the sanitary parameters, determining water quality, is used worldwide to monitor and control the quality and safety of various types of water reservoirs, and for prevention of illnesses caused by the polluted water.

These infections can arise from the bacteria naturally present in the aquatic environment or microorganisms present in the water as a result of contamination with human or animal faeces (ABRAHAM, 2011). According to a series of authors (ZMYSTLOWSKA *et al.*, 2000a,b; ZMYSTLOWSKA *et al.*, 2003; NIEWOLAK & TUCHOLSKI, 2000a,b), the presence of *Escherichia coli* in the water is evidence for the possible presence of variety of microorganisms, that can cause gastrointestinal diseases (DHSS, 1982; BLACKWOOD, 1978; FAPOHUNDA *et al.*, 1994).

Many potential pathogens could be associated with water, so it is thus impractical to screen samples for all possible disease causing agents. Instead various, indicator organisms have been used as surrogate markers of risk. (BARRELL *et al.*, 2000).

Most waterborne infectious diseases are related to faecal pollution of water sources therefore aquatic microbiology is mainly based on the need to identify indicators of faecal pollution such as coliforms and *E. coli*. (HUNTER, 1997).

The desirable qualities for a useful water quality indicator are (WHO, 1996):

- Presence in large numbers in human and warm blooded animal faeces.
- Easy to identify by simple methods.
- Does not grow as part from the natural microflora of various types of water reservoirs.
- Their presence and elimination after treatment of the water is similar to the pathogens.

The coliforms are part of the *Enterobacteriaceae* family, which includes Gram-negative, non-spore-forming, oxidize-negative, straight rods, facultative anaerobic bacteria that ferment lactose with the formation of acid and gas within 24-48h at 35- 37^{o}C. They appear as non-specific indicators of faecal pollution.

During the years different schemes are developed for the classification of coliforms. The earliest are the one of MCCONKY (1909) and BERGEY & DEAN (1908). Enough biochemical reactions for the identification of coliforms are established during the 20th years of the 20th century (HENDRIKS, 1978). Large numbers of those reactions are still in use today. The main disadvantage is their long duration.

In the modern microbiology the efforts are directed in developing reliable, fast methods for detection and determining presence of indicator microorganisms. This directs the efforts towards developing tests for fast identification of coliforms, including basically the genus *Escherichia, Klebsiella, Citrobacter, Enterobacter* and *Serratia.*

This became possible after the introduction of chromogenic substances, which may be added to the conventional nutrient media. The chromogenic components are modified by the bacterial enzymes or by specific bacterial metabolites; as a result the chromogenic substance changes its colour or fluorescent after the modification. This makes it possible to establish which colonies, possess this specific metabolic activity. It also makes it possible to determine the number of microorganisms from a specific indicator group in a mixed sample. The time required for the determination of different indicator bacteria can be cut down to 16-18h (ASHBOLT, 2001).

These methods, although fairly easy, give information primary about the TC represented in the water, but they don't give a clear picture about species affiliation of microorganisms. Often, other species from the family *Enterobacteriaceae*, which are part of the natural micro flora of the water body, give positive reaction. Therefore further confirmative identification tests are needed.

The aim of this study was to determine the total coli forms (TC), coli titter and coli index, by the usage of chromogenic medium, in two sampling stations in the aquatory of Kardzhali dam-lake and in Arda River and to identify the isolated strains.

Materials and Methods

The research was carried out in August 2011, in the aquatory of Reservoir Kardzhali and Arda River, in the part where the river infuses in the reservoir. The water samples were collected from two sampling stations, located in the different parts of the reservoir: station I - located in close range of the settlement Glavatarci and station II- in the transitional zone of the reservoir (Halach Dere) and one station (III) on the Arda River, in the region where the river flows into the reservoir (Fig. 1). The precise location of the sampling stations is determined with the help of GPS receiver Garmin 76CSx.

Fig. 1. Sampling stations located on Kardzhali Dam Lake and Arda River.

The water samples were collected by direct immersion of sterile glass jars with a volume of 500 ml, 50 cm below the water surface and stored in cold conditions until their processing, not later than 6 h after sampling.

The coli titter is determined as the smallest volume of water in which the presence of *Escherichia coli* is established.

The coli index is determined as the number of *Escherichia coli* cells in 1l water sample, and it is calculated based on the coli titter value with the equation:

$$\text{COLI INDEX} = \frac{1000}{\text{COLI TITTER}}$$

Total coliforms (TC) and *E. coli* number were determined after filtration of 5 ml water sample through membrane filters (Membrane Solutions) with pore size 0.45 μm, and cultivation on chromogenic medium SLS Coliform Agar (HiChrome) for 48 h on 37°C.

Strains for identification were isolated based on their specific colouring during cultivation on SLS Coliform Agar. From every station 10 strains were isolated.

For verification of their purity, strike seeding on Levin medium was used. Biochemical reactions, based on the system for fast identification Enterotube II (ENCISE), were used for identification of the isolated strains.

T-test was applied for statistical evaluation of the results (MS Excel).

Results and discussion

The values for total coliforms, coli titter and coli index from the waters of Kardzhali Reservoir and Arda River are shown in Table 1.

Table1. Total coliforms, coli titter and coli index in the waters of Kardzhali Reservoir and Arda River for August, 2011.

Parameter	Station		
	I	II	III
TC (cfu/ 100 ml)	1900±674	1293±194	1698±134
coli titter (ml/ 1cfu)	5	15	1
coli index (cfu/ 1l)	200	67	1000

The values of TC, established in station II, which is located at the transitional zone of the reservoir between the lake like zone of the water

body and riverine zone, are within the range of 1293±194 cfu/100ml. They are significantly lower in comparison with the values from station III - 1698±134 cfu/100ml ($p<0.05$), and station I - 1900±674 cfu/100ml ($p<0.05$). At the same time, no reliable differences were found between the average values of total coliforms between station I and station III.

The long retention time of Kardzhali dam-lake, determines it as a lake like water reservoir, where a continuous process of self-purification takes place (TRAYKOV, 2003). The self-purification of the water is due to the action of physicochemical (continuous water exchange, settling of suspended particles along the longitudinal axis of the reservoir, where microorganisms are adsorbed; bactericidal effect of UV- rays, etc.) and biological (zooplankton; antagonism between the species, etc.) environmental factors. In this kind of water bodies the trophic diversity, and in consequence the quantity of microorganisms, reduces form the tail to the dam of the reservoir (STRASKRABA, 1998). The data deviates from this model. Increased values of the total coliforms in the region of station I, may be due to the influence of village Glavatarci, which is situated in close range of the station, and also from the fact that the study is conducted in a period of intensive tourist season. The settlement does not have two way sewage systems, and this gives the assumption that the sewage waters are filtrated in the reservoir. This assumption is supported by the high values of coli titter, which is an indicator of faecal contamination, from 5 ml water in this station and coli index 200 cfu *E. coli*/1l. Similar data is shown in the work of other authors, studying the processes of intensive urbanisation in the zones surrounding the large water reservoirs (MALLIN *et al.*, 2000).

The biochemical characteristic of the strains isolated for identification and determining the coliform ratio in Kardzhali Reservoir, shows that all strains are Gram-negative, catalase–positive, oxidise–negative, facultative anaerobic, straight rod-shaped bacteria. When cultivated on Levin medium, they form colonies with purple colouring and metallic shine, which is typical for lactose–positive representatives of family *Enterobacteriaceae* (Bergey`s manual, 2005). Detailed results regarding the biochemical identification of the isolated strains are shown in Tables 2, 3 and 4.

The strains isolated from station I are characterized with the highest diversity of species and fall into four genera, based on their biochemical characteristics (Fig. 2a). The *Klebsiella* genus represents the main share. The strains assimilate glucose with release of acid and gas (CO_2), they produce acid from lactose, arabinose and sorbitol, utilize citrate, they have lysine and ornithine decarboxylase activity, tryptophan activity and decompose urea to ammonia. Based on the obtained results all strains of the genus were defined as *K. oxytoca* (strains 1, 6 and 8) and *K. ozaenae* (strain 4) (Bergey`s manual 2005, Enterotube II Code Book 2009).

Thirty percent (30%) of the studied strains fall into genus *Citrobacter*. Into this genus fall species that degrade glucose and other carbohydrate sources with the release of acid and gas. The genus is represented by *C. freundii* (strains 3, 9 and 10), which is normally present in the human microflora. Often it is isolated from clinical patients.

Table 2. Biochemical characteristic of the strains isolated from station I of Kardzhali Reservoir

Test	STRAIN №									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Gram	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Indol	+	-	+	+	-	+	+	+	-	-
MR	+	+	+	+	+	+	+	+	+	+
VP	-	+	-	-	+	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	+	-	-	-	-	-	+	+
Urea	+	+	+	-	+	+	+	+	-	-
PA	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	-	-	+	-	+	+	+	-	-
Ornithine decarboxylase	-	+	+	+	+	-	-	-	-	-
Glucose, acid	+	+	+	+	+	+	+	+	+	+
Glucose, gas	+	+	-	-	+	-	-	+	-	-
Lactose	+	+	-	-	+	+	-	+	-	-
L-Arabionse	-	-	+	+	-	-	-	-	+	+
D-Adonitol	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	-	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-
O/F	F	F	F	F	F	F	F	F	F	F
Enterotube II code	34723	32323	33173	26240	32323	24723	24223	34733	21061	21061

In the third place of occurrence is genus *Enterobacter*, represented by *E. cloacae* (strains 2 and 5). The species is widely spread in nature as part of the normal microflora of water and soil (Bergey`s manual 2005). They fall into the conditionally pathogenic bacteria in the colon of humans and animals. If they enter elsewhere they can cause urinary tract infections, wound, sepsis etc. (HAIDUSHKA *et al.,* 2011).

Strain №7 assimilates all tested carbohydrate sources, with the exception of dulcitol, with the release of acid, without forming gas. It decomposes citrate and decarboxylates amino acids lysine and ornithine in anaerobic conditions, doesn't hydrolyse urea and is indol negative. Based on its biochemical characteristic the strain falls into genus *Serratia*.

All isolated strains from station I fall into the so called faecal coliforms and are used as indicator for faecal pollution (WHO, 1999). Their presence in combination with the high values of coli titre, indicate recent contamination with sewage water of the studied region.

Table 3. Biochemical characteristic of the strains isolated from station II of Kardzhali Reservoir

Test	STRAIN №									
	1	2	3	4	5	6	7	8	9	10
Gram	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Indol	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-	-	-
Urea	+	+	+	+	+	+	+	+	+	+
PA	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+
Glucose, acid	+	+	+	+	+	+	+	+	+	+
Glucose, gas	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
L-Arabionse	+	+	+	+	+	+	+	+	+	+
D-Adonitol	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-
O/F	F	F	F	F	F	F	F	F	F	F
Enterotube II code	36763	36763	36763	36763	36763	36763	36763	36763	36763	36763

All isolated strains from station II fall into genus *Klebsiella* and are represented by the species *K.oxytoca* (Fig. 2b). Representatives of the species are found in human faeces, but more often are part of the normal microflora of water and soil.

Isolated species from station III fall into genus *Serratia* (strains 1, 2, 3 and 6) and genus *Salmonella*, respectively represented with the strains 4, 5, 7 and 8 (Fig. 2c). The bacteria of the genus Salmonella are among the main causes of food borne infectious diseases in humans (MOLBAK, 2006). They retain viability up to 4 months in still waters and 5 to 6 days in flowing waters (HAIDUSHKA, 2011). Their high titter in the waters of Arda River is an evidence of contamination with sewage water. This along with the high values of coli titter (1ml) and coli index (200 cfu *E. coli*/ 1l), creates the risk for occurrence of infectious outbreaks.

Conclusions

Highest levels of contamination with sewage water, based on the study of coli titter (1ml) and coli index (200 cfu *E. coli*/ 1l) in the three studied stations, are established in the waters of the Arda River.

Table 4. Biochemical characteristic of the strains isolated from station III of Kardzhali Reservoir

Test	STRAIN №								
	1	2	3	4	5	6	7	8	9
Gram	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Indol	-	-	-	-	-	-	-	-	+
MR	+	-	-	+	-	-	-	+	+
VP	+	+	+	-	+	+	+	-	-
Citrate	+	+	+	+	+	+	+	+	-
H_2S	-	-	-	+	+	-	-	+	-
Urea	-	+	-	+	+	-	-	+	-
PA	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+
Glucose, acid	+	+	+	+	+	+	+	+	+
Glucose, gas	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+
L-Arabionose	+	-	-	+	+	+	-	+	+
D-Adonitol	-	-	+	+	-	-	-	-	-
D-Sorbitol	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-
O/F	F	F	F	F	F	F	F	F	F
Enterotube II code	36161	36123	36321	37161	37161	36063	35161	37161	36560

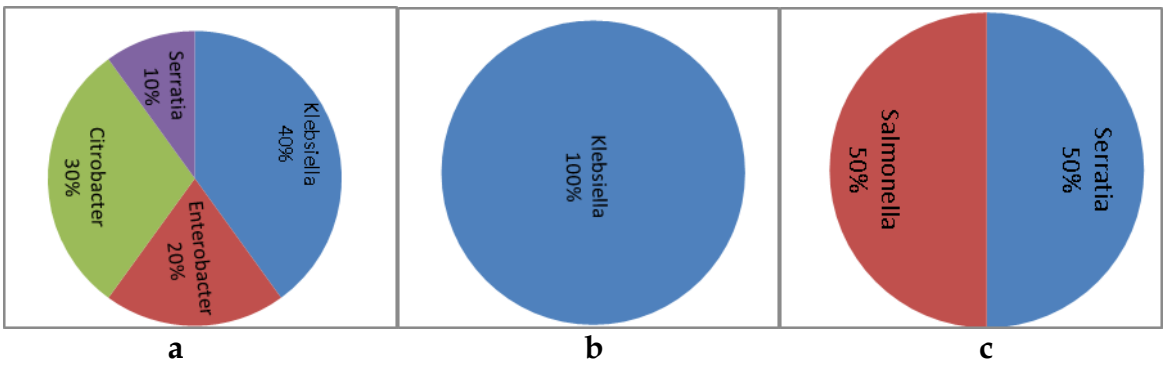

Fig 2. Percentage ratio of identified genus form family *Enterobacteriaceace* by stations (a\station I; b\ station II; c\station III)

The high number of coliforms and the species composition is indicative for contamination and loading of Arda River with sewage waters in the section

before it flows in the Kardzhali Dam-lake.

The absence of pathogenic species, combined with a decrease in the number of TC, coli titter and coli index in station II is evidence of the self-purifying capability of the studied reservoir.

The new rise in the number of TC in station I in proximity to Glavatarci village, as well as the presence of faecal coliforms, indicates secondary site of contamination with sewage waters in the region.

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