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Total count and species composition of main microbial indicators of the water quality of Kardzhali reservoir, Bulgaria

ABSTRACT

The aim of this study was to determine the anthropogenic influence on the sanitary-bacteriological sate of the waters of Kardzhali reservoir. The seasonal dynamics of the total count of *Esherichia coli*, fecal streptococci (FS), and *Clostridium perfringens* were studied for the period April - March 2011/12 in the waters of the reservoir. Samples were taken from six stations located in the lake zone and the transitional zone of the dam-lake and one station at river Arda. With the exception of August and October the count of *E. coli* and FS did not exceed 30 cfu / 100 ml and 20 cfu / 100 ml respectively. In August was established an increase in the values of the two microbial indicators and this trend continued during November. Statistically significant differences were established for the *E. coli* and FS count between the stations located in the lake zone of the dam-lake and those at the net-cage farms and Glavatartsi village, subjected to strong anthropogenic influence. Presence of *Clostridium perfringens* in the studied samples was established only during August, mainly at the stations located near the net-cage farms and near the dam. Total of 220 strains fecal coliforms were isolated and identified, in order to establish the basic species composition. All of them fall into 5 genera of the *Enterobacteriaceae* family.

Key words: *Escherichia coli*, fecal streptococci, *Clostridium perfringens*, biochemical identification, *Enterobacteriaceae*, Kardzhali reservoir

Introduction

Due to the fact that there are a few large natural lakes in Bulgaria, there is a growing interest in artificially created reservoirs as water bodies with multi-purpose functionality. At the same time the human activities such as landscape alterations, urbanization, construction of net cage fish farms etc., can seriously affect the water quality and influence the normal microflora of the dam-lake.

Unlike farming in enclosed ponds, net cage farming has significant and immediate impact on the environment. Intensive fish farming has direct effect on the quality of the water, the qualitative and quantitative composition of the benthos, phytoplankton and bacterioplankton. Deploying a large number of net-cage farms has a significant effect on the rate of water flow and speed of sedimentation of substances. The sharp increase in sedimentation leads to a rapid accumulation of waste products in the area around the farms. That combined with the retention of water masses caused by resistance of the nets creates conditions for deterioration of the sanitary parameters of waters (FAO, 2008).

Kardzhali dam-lake is one of the reservoirs in the country towards which there is a growing investor interest for development of net cage aquaculture. At present 7 farms are operating in the dam-lake. All of them are situated near the shore line, and are concentrated in the zone in relative proximity to the dam wall.

In recent decades there has been increased urbanization and development of the coastal zone of the reservoir, which increases the risk of further contamination by untreated household sewage waters. These processes often lead to disruption of the ecological balance of the reservoirs. One major problem is the entry of enteric pathogens in the reservoir. They are the cause of a large percentage of water

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borne diseases (Karaboze, 2003). Often this leads to the occurrence of bacterial infection outbreaks (Karafistan et al., 2005).

According to number of authors (Zmystlowska et al. 2000a, 2000b, 2001; Niewolak & Tucholski, 2000a, 2000b) this secondary microflora entering the water bodies as a result of human activities have a strong impact on the microbiological status of fish and leads to a sharp increase in the number of microorganisms in the muscles, skin and digestive tract (Sugita et al., 1987).

During different periods of the operation of the Kardzhali dam-lake a number of researches have been carried out on the ecosystem and the water quality (Velcheva et al., 1997, 2000; Traykov et al., 2003; Traykov & Boyanovsky, 2003) mainly concerning the accumulation of heavy metals in the tissues of fish and the trophic status of the reservoir. On the backdrop of increased urbanization in the region of Glavatartsi village and the rapid development of net-cage aquaculture it is not taken into consideration the potential environmental threat that may arise or have already arisen in the reservoir.

The aim of this study was to trace the anthropogenic impact on the sanitary-bacteriological status of the reservoir, based on the seasonal changes in the number of *Esherichia coli*, faecal streptococci (FS), and *Clostridium perfringens* and species composition of fecal coliforms (FC) along the longitudinal axis of Kardzhali dam-lake.

Materials and Methods

The research was carried out in the aquatory of Kardzhali reservoir for the period April 2011 - March 2012. Water samples were collected from six sampling stations located in the Lake Zone and the Transitional zone of the dam-lake and one at Arda River, in the area before its entry in the reservoir. Each station includes three sampling horizons, respectively at the epi-, meta- and hypolimnion water layer when there is a clear stratification of the reservoir. In November and March, during fall and spring turnover, samples were taken at 50 cm below the surface, at 1 m above the bottom and at the middle water layers. The exact location of the stations was determined by GPS receiver Garmin 76CSx (Figure 1).

The total count of the microbial indicators was determined after membrane filtration through filters (Membrane Solutions) with a pore size of 0.45 μm. For *Eschericha coli* and fecal coliforms filters were transferred for cultivation on SLS Coliform Agar (HiChrome) for 48 h at 44°C. The medium allows simultaneous counting of the number FC and *Escherichia coli* in the sample. Their number was established after direct counting of specifically colored colonies. The number of the fecal streptococci was determined by standardized test method ISO 7899-2, 2000. *Clostridium prefringens* count was established after incubation of the membrane filter on tryptose-sulphite agar with cycloserine under anaerobic conditions at 44°C ± 1°C for 24 h.

A total of 220 strains were isolated for biochemical characterization from the waters of Kardzhali reservoir and river Arda in August 2011. Identification was carried out by biochemical reactions, based on the rapid identification system Enterotube II (ENCISE).

Student`s T-test was applied for statistical evaluation of the results.

Figure 1. *Schematic representation of Kardzhali reservoir (a-general scheme, b - sampling stations in the studied area of the reservoir)*

Results

The seasonal dynamics of the total count of the studied microbial indicators in the aquatory of Kardzhali reservoir and river Arda for the period April 2011 - March 2012 are shown in Table 1.

There is no presence of fecal streptococci and *C. perfringens* in 100 ml of analyzed water samples during the spring (April, June) of 2011. Low numbers of *Escherichia coli* was detected in June only in stations near the village of Glavatarci and the net cage farms.

In early summer (July) was reported an increase in the values of the examined indicators. With the elevation of the water temperature in August a sharp increase was detected in

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the number of *Escherichia coli* in the thermocline zone and the bottom water layers around the net-cage farms. Their number in the hypolimnion varies from 238 cfu/ 100 ml at station III to 340 cfu/100 ml at station II. These values are over 10 times higher than those recorded at station V 15±8 cfu /100 ml and station VI 12±2 cfu /100 ml ($p<0.005$).

Similar correlation was reported for the FS. Their number varied from 4±4 cfu /100 ml at station V and VI to over 70 cfu / 100 ml in the hypolimnion at third and fourth stations. Presence of *Clostridium perfringens* in the studied samples was established only during August, mainly at the stations located near the net-cage farms and near the dam.

In November the number of *E. coli* was still significantly higher at the stations near Glavatartsi village and the dam wall. This high number along with the reported 40-60 cfu / 100 ml FS in the epilimnion at these stations, is evidence for a continuing influx of wastewater. The values differ from those reported at station VI 5±2 cfu / 100 ml for *E. coli*, where no presence of FS was established.

During the spring turnover in March 2012 the number of *E. coli* and FS along the longitudinal axis of the reservoir remained constant and it was within the range of 8±4 cfu/ 100 ml and 3±2 cfu/ 100 ml respectively. There was no established presence of *C. perfringens* in the samples. For the same period in the waters of river Arda was recorded a significant increase in the number of *E. coli* up to nearly 1300 cfu/ 100 ml.

In August 2011 the number fecal coliform bacteria (FC) was used as an additional indicator for assessment of the water quality. Changes in the values of this parameter for each station are shown in Table 2.

Table 1. *Average values of the monitored microbiological parameters (cfu/ 100 ml) in the aquatory of Kardzhali reservoir and river Arda for the period April 2011 – March 2012.*

Station	Horizon	April 2011			June 2011			July 2011			August 2011			October 2011			March 2012		
		E. coli	FS	*Clostridium perfringens*	*E. coli*	FS	*Clostridium perfringens*	*E. coli*	FS	*Clostridium perfringens*	*E. coli*	FS	*Clostridium perfringens*	*E. coli*	FS	*Clostridium perfringens*	*E. coli*	FS	*Clostridium perfringens*
I	Epilimnion	0	0	0	0	0	0	0	0	3	84	7	0	159	36	0	4	4	0
	Metalimnoin	0	0	0	0	0	0	10	10	4	112	6	18	107	5	0	3	5	0
	Hypolimnion	0	0	0	6	0	0	2	2	2	76	7	30	362	6	0	4	7	0
II	Epilimnion	0	0	0	0	0	0	7	0	4	168	30	0	377	68	0	3	6	0
	Metalimnoin	0	0	0	0	0	0	18	0	0	237	18	20	122	7	0	2	7	0
	Hypolimnion	0	0	0	0	0	0	30	10	2	342	20	57	59	7	0	3	7	0
III	Epilimnion	0	0	0	4	0	0	0	0	0	117	24	0	174	42	0	1	6	0
	Metalimnoin	0	0	0	0	0	0	8	3	2	204	20	14	37	6	0	4	6	0
	Hypolimnion	0	0	0	4	0	0	28	18	4	238	80	27	30	6	0	1	6	0
IV	Epilimnion	0	0	0	0	0	0	0	0	2	132	20	0	214	37	0	1	3	0
	Metalimnoin	0	0	0	7	0	0	10	0	3	186	30	21	25	6	0	1	5	0
	Hypolimnion	0	0	0	7	0	0	31	15	3	251	76	10	122	6	0	1	7	0
V	Epilimnion	0	0	0	0	0	0	0	0	0	8	2	0	78	0	0	5	7	0
	Metalimnoin	0	0	0	0	0	0	0	0	1	12	2	3	18	0	0	7	12	0
	Hypolimnion	0	0	0	4	0	0	7	0	0	24	8	0	11	0	0	5	16	0
VI	Epilimnion	0	0	0	0	0	0	0	0	0	10	3	0	1	0	0	7	20	0
	Metalimnoin	0	0	0	0	0	0	3	1	0	13	0	0	7	0	0	5	16	0
	Hypolimnion	0	0	0	0	0	0	0	0	0	12	0	8	7	0	0	4	10	0
River Arda		18	0	0	20	0	0	120	20	0	250	90	36	311	0	0	1288	52	0

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Table 2. *Total count of fecal coliforms (FC) in the aquatory of Kardzhali reservoir and river Arda recorded in August 2011.*

Horizon	Station						
	I	II	III	IV	V	VI	r. Arda
Epilimnion	840	892	1440	1630	400	1100	8600
Methalimnion	702	1320	1490	2100	540	1400	
Hypolimnion	240	1360	1160	1090	480	1410	
x	594	1191	1363	1607	473	1303	
SD	314	259	178	505	70	176	

At the stations located near the net-cage farms and Glavatartsi village again were reported significantly higher values ($p < 0.05$) compared to that at station V. In the transitional zone (station VI) was observed further increase in the number of FC up to 1300±176 cfu / 100 ml.

In order to evaluate the potential health risks caused by the high number of fecal coliforms the species composition of this group was analyzed. A detailed biochemical characteristic is presented in Table 3.

Table 3. *Biochemical characteristic of the strains isolated from Kardzhali reservoir and river Arda in August 2011.*

Test	Enterobacter		Citrobacter		Klebsiella			Serratia		Escherichia
					oxytoca	pneumoniae subsp.				
	aerogenes	cloacae	freundii	amalonaticus		pneumoniae	ozaenae	marcescens	liquifaciens	coli
Gram stain	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-
O/F	F	F	F	F	F	F	F	F	F	F
H_2S	-	-	+	+	-	-	-	-	-	-
Glucose, acid	+	+	+	+	+	+	+	+	+	+
Glucose , gas	+	+	+	+	+	+	+	-	-	+
Indol	-	-	-	+	+	-	-	-	-	+
MR	-	-	+	+	-	-	+	-	+	+
VP	+	+	-	-	+	+	-	+	+	-
Citrate	+	+	+	+	+	+	+	+	+	-
Lactose	+	+	+	+	+	+	+	-	-	+
L-Arabinose	+	+	+	+	+	+	+	-	+	+
D-Adonitol	+	-	-	-	+	+	+	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	v
D-Sorbitol	+	+	+	+	+	+	-	+	+	+
Lysine decarboxylase	+	-	-	-	+	+	-	+	+	+
Ornithine decarboxylase	+	+	-	+	-	-	-	+	+	v
PA	-	-	-	-	-	-	-	-	-	-
Urea	-	+	-	+	+	+	-	-	-	-

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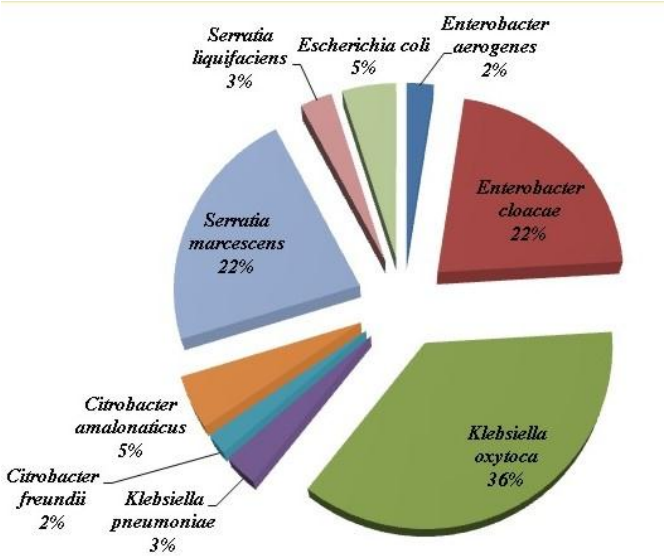

Figure 2. *Species composition of the family Enterobacteriaceae, in the aquatory of Kardzhali reservoir (August 2011).*

All 220 examined strains belong to only 5 genera (*Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter* and *Serratia*) of the family *Enterobacteriaceae.* The first four of them are used as an indicator of fecal contamination (WHO 1998) (Figure 2).

The most widespread is genus *Klebsiella* (39%). At present 4 species fall to this genus. Based on the obtained results, strains from the genus belong to two of these species - *Klebsiella oxytoca* and *Klebsiella pneumoniae*. A larger share (93%) takes *K. oxytoca*. The remaining 7% from this genus belong to the *K. pneumonia* species. Second in frequency of occurrence of FC is the genus *Enterobacter*, represented by *E. cloacae* and *E. aerogenes*. The strains that belong to this genus constitute 24% of all isolates identified. To *E. cloacae* fall 45 strains or 90% of representatives of the genus *Enterobacter* that we identified. To E. aerogenes genus fall a total of 5 (10%) of the isolated strains. To the genus *Citrobacter* fall 7% of the examined strains. The genus is represented by *C. amalonaticus* (10 strains) and *C. freundii* (5 strains), Over 25% of the examined strains belong to genus *Serratia*. Only 5 % of the isolated strains were identified as *Escherichia coli.*

During the study, the same 5 genera of the *Entrobacteriaceae* family were found in the waters of river Arda (Figure 3).

At stations I to IV, situated close to shore line, the ratio between fecal coliforms remained relatively constant with high percentage of genera *Klebsiella, Enterobacter* and *Citrobacter* (Figure 4). *Escherichia coli* accounts for about 10% of the total number. There were no significant differences in species composition between the stations.

The ratio of coliforms in station V significantly differs. They are represented mainly by species normally present in aquatic ecosystems. Widely found were species *Klebsiella oxytoca* (60%) and *Serratia mafcescens* (33%).The number of *Escherichia coli* formed less than 1% of the total number of isolated strains. In the transitional zone almost completely dominated the species *Klebsiella oxytoca*.

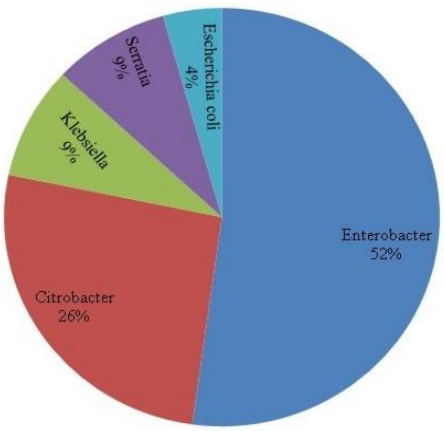

Figure 3. *Percentage ratio of the identified genera from family Enterobacteriaceace in the waters of river Arda.*

Discussion

The established seasonal variation of the studied indicators is influenced mainly by the operational characteristics of Kardzhali reservoir. On one hand, due to the low water temperature during the spring months (10-12^{o}C), limited amounts of food are fed to the fish in the net-cage farms, on the other hand during this period most of the commercial facilities along the shoreline of the dam-lake are not operational.

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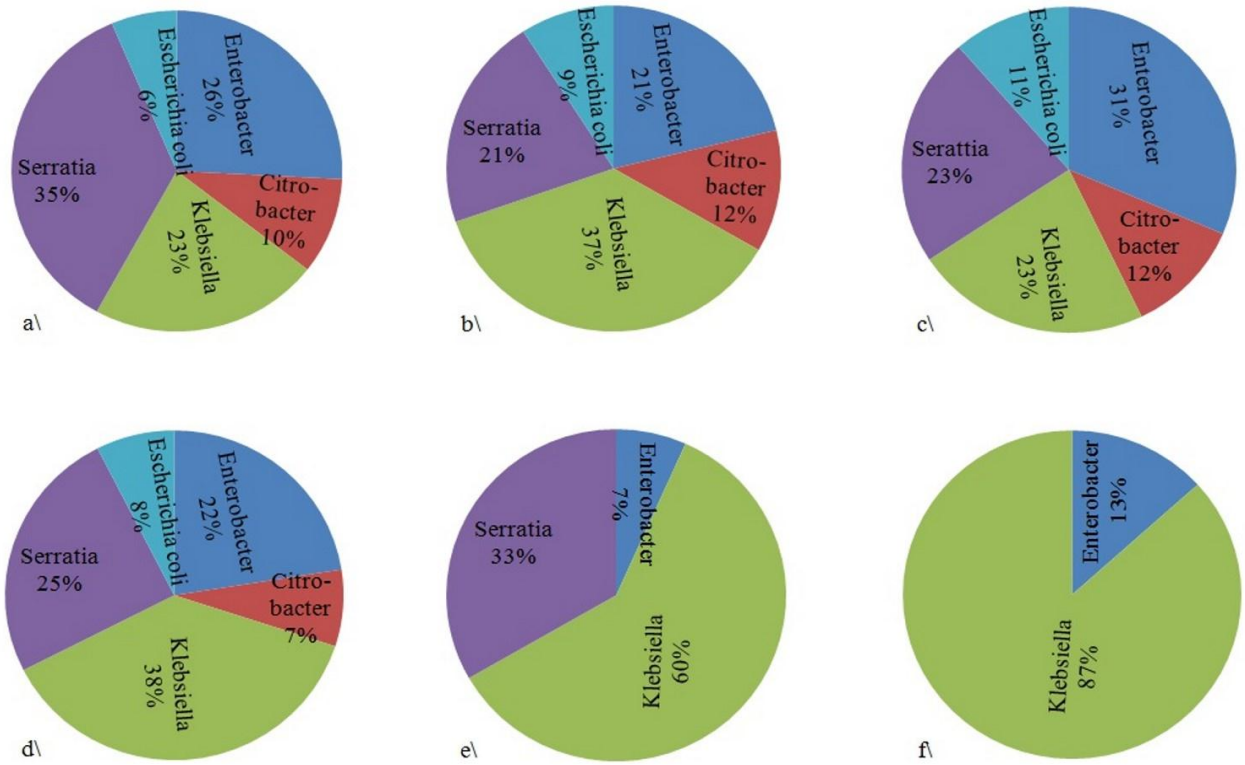

Figure 4. *Percentage ratio of the identified genera from family Enterobacteriaceace in the waters of Kardzhali reservoir (a\station I; b\ station II; c\station III d\ station IV; e\ station V; f\ station VI).*

The increase in the number of *E. coli*, FS and *C. perfringens* in July and August at stations near the net cages coincides with the intensive tourist season in the adjacent village Glavatartsi. The lack of sewage system and WWTP in the village may lead to the filtration of polluted water from the septic facilities of the households and hotels into the waters of the Kardzhali reservoir. These results correlate with similar studies from other authors. Hong et al. (2010) and Maipa et al. (2001) found that in the waters near densely populated areas around the dam-lakes and coastal shorelines are recorded very high levels of fecal coliforms and *E. coli*, compared to more remote areas.

The reservoir is characterized by a long retention time which determines it`s lake like nature (Traykov et al., 2003). During the period from April to September was observed a clearly defined temperature stratification of the waters in the lake zone and transitional zone of the dam-lake, which results in a differences in the number of the monitored microbiological indicators in the water column. Larger number of *E. coli* and FC in the bottom layers usually is due to their association with the colloid sized small particles (Youn-Joo et al., 2002). Another reason is water temperature. Flint, 1987 has established that survival of FC is significantly higher at low water temperatures. High temperature and high amount of dissolved oxygen, typical for the euphotic water layer have a negative effect on the number of *Escherichia coli* (Pearson et al., 1987).

The increase in bacterial numbers by several degrees in early spring (March, 2012) in the waters of river Arda and at the stations in relative proximity to the river is a natural process. The spring period is characterized by a significant amount of rain falls, which results in the influx of large quantities of contaminated surface waters, which can contain fecal and pathogenic microorganisms. The increased drainage to the rivers leads to a significant increase in microbial titer (George et al., 2004; Djuikom et al., 2006).

The species composition of the coliforms shows significant differences in water quality between stations along the shoreline and those situated in the open water area of the

reservoir. The high percentage of FC in the samples from station I to IV is indicative for the deterioration of the sanitary-bacteriological parameters of the water. The total count of coliforms at station V and VI is formed mainly be species which are part of the natural microflora of the water bodies such as K. *oxytoca* and S. *marcescens.*

The monitored microbiological parameters showed a pronounced seasonal dynamic in Kardzhali reservoir and river Arda for the period of this study. Peak values of the total count of all indices were recorded in August 2011.

The significantly higher count FC ($p<0.005$) and the high ratio of over 4:1 between *E. coli* and fecal streptococci at the stations near the net cage farms and Glavatarci village compared to those at station V are indicative for local contamination of the waters of the reservoir during the summer period of 2011. These results and the established species composition of the coliform group suggest that the microbiological pollution is mainly due to an increased environmental pressure caused by human activities in the area. On the other side the low percentage of the FC and *E. coli* in the area of stations V and VI is indicative of the high self-purification capability of Kardzhali reservoir.

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References

Beveridge M, 1984. Cage and Pen fish farming. Carrying capacity models and environmental impact. FAO Fish. Tech. Pap., 255: 1-131.

Djuikom E, Njine T, Nola M, Sikati V, Jugnia L. 2006. Microbiological water quality of the Mfoundi River watershed at Yaound´e, Cameroon, as inferred from indicator bacteria of fecal contamination. Environmental Monitoring and Assessment, 122(1-3): 171–83.

Flint P. 1987. The long-term survival of Escherichia coli in river water. J. Appl. Bact., 63: 261-270.

George I, Anzil A, Servais P, 2004. Quantification of fecal coliform inputs to aquatic systems through soil leaching. Water Research, 38: 611–618.

Hong H, Qiu J, Liang Y. 2010. Environmental factors influencing the distribution of total and fecal coliform bacteria in six water storage reservoirs in the Pearl River Delta Region, China. J. Environ. Sci. 22(5): 663-668.

Karaboze I, Ucar F, Eltem R, Ozdmir G, Ates M. 2003. Determination of existence and count of pathogenic microorganisms in Izmir Bay. JES, 26: 1-18.

Karafistan A, Colakoglu A. 2005. Phisical, chemical and microbiological water quality of the Manyas Lake, Turkey. Mitig. Adapt. Strat. Global Change 10: 127-143.

Maipa V, Alamanos Y, Bezirtzoglou E. 2001. Seasonal fluctuation of bacterial indicators in coastal waters. Microbial Ecology and Health Disease, 13: 143-146.

Niewolak S, Tucholski S. 2000a. Ability of bacteriological self-purification of common carp, tench and crucian carp fingerlings reared in pond receiving the discharge from sewage treatment plant and then kept in running river water of different quality. Arch. Ryb. Pol., 8(1): 49-61.

Niewolak S, Tucholski S. 2000b. Sanitary and bacteriological evaluation of common carp, tench and crucian carp reared in a pond supplied with biologically treated sewage. Arch. Ryb. Pol., 8(1): 35-48.

Pearson H, Mara D, Mills S, Smallman D. 1987. Physico-chemical parameters influencing faecal bacterial survival in waste stabilization ponds. Wat. Sci. Tech., 19: 145-152.

Sugita H, Oshma K, Fushino T, Deguchi Y. 1987. Substrate specificity of heterotrophic bacteria in the water and sediment of a carp culture pond. Aquaculture 64: 39-46.

Traykov I, Boyanovsky B. 2003. Changes in the trophic status of the Kardjali reservoir due to water level fluctuations. Sofia University Annual Report, 95(4): 227-230.

Traykov I, Boyanovsky B, Zivkov M, Mirinchev G. 2003. Spatial heterogeneity of physical and chemical parameters in Kardjali reservoir – Effect on trophic state. - In: Preprints of Proceedings. "Second International Conference Ecological Protectiton of the Planet Earth". June 2003. Sofia, p. 280-287.

Velcheva I. 1997. Research on the content of cadmium (Cd) in different organs of perch (*Perca fluviatilis* L.) from the artificial lakes "Kardjali'and "Studen Kladenetz" on the river Arda. Acta zool. Bulg., 50: 43-48.

Velcheva, I, Kostadinova P, Popov V. 2000. Lead, Zink and Cadmium Content in Carp (*Cyprinus caprio* L.) Muscle Tissue from Kardjali Dam Lake. Proceedings of the Scientific Conference "Ecology and Health, 2000", House of Science and Technology, Agricultural University, Plovdiv, p. 319-323.

WHO. 1998 Guidelines for Drinking Water Quality. Health criteria and other supporting information. World Health Organization, Geneva. Second Edition, Vol. 2

Youn-Joo A, Kampbell D, Breidenbach G. 2002. Escherchia coli and total coliforms in water and sediment at Lake Marinas. J.Envir. Poll., 120(3): 771-778.

Zmyslowska I, Guziur J, Wozniak M, Harnisz M, Mientki C. 2000b. Effects of culture of the sheatfish (*Silurus glanis* L.) on the hygienic state of fish and of water environment. Bulletin Vurh Vodnany, 3: 90-98.

Zmyslowska I, Harnisz M, Lewandowska D. 2001. Sanitary and bacteriological evalution of water quality during cage culture of wells (*Silurus glanis* L.) in cooling water. Pol. Arch. Ryb., 9(2): 191-199.

Zmyslowska I, Lewandowska D, Pimpicka E. 2000a. Microbial evaluation of water and digestive tract contents of tench (*Tinca tinca* L.). Arch. Ryb. Pol., 8(1): 95-105.