Indian Journal of Geo Marine Sciences
Vol. 47 (07), July 2018, pp. 1495-1501

Distribution of enteric bacteria by means of GIS and detection of *Escherichia coli* with *uidA* gene in Kapıdağ Peninsula of Marmara Sea

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Received 01 November 2016 ; revised 02 March 2017

Present study consists the incidence of enteric bacteria in the Kapıdağ Peninsula. Nine sampling stations were selected and taken seasonally from 2011-2012. Presence of *Escherichia coli* was noted at all stations, except K1 and K6, throughout the sampling period irrespective of season. K1 and K6 stations (utilised as public beaches during the summer months) and depths below 30m displayed distinct genetic profile suggestive of diverse community of enteric bacteria other than *E. coli*. Analysis of *uidA* gene showed temporal and spatial genetic homogeneity in these *E. coli* populations. Ability to characterize *E. coli* communities might not only facilitate the mitigation of fecal pollution on Kapıdağ Peninsula, but also address the impact of environmental disturbances and seasonal and spatial variation on pathogen indicators.

[Keywords: Enteric bacteria, *E. coli*, *uidA*, Kapıdağ Peninsula, Marmara Sea, Turkey]

Introduction

Contamination of surface waters by faecal pollution constitutes a serious environmental and public health threat. Identifying and eliminating the source of contamination is not straightforward because assessment of faecal pollution generally relies on a limited number of surface water samples to measure faecal indicator organism densities[1]. Methods to determine sources of fecal pollution generally used include phenotypic and genetic characterization of faecal indicator bacteria[2].

The ability to distinguish between anthropogenic sources of fecal contamination is an important assessment tool, both for evaluating possible health risks and for developing effective control strategies[3]. Although there have been numerous attempts to discriminate between human and non-human sources of fecal contamination in water bodies, these attempts have in general, been unsuccessful[4]. Recently, however, advances in molecular based techniques have been used to describe and identify fecal bacteria[5].

Detection of *Escherichia coli* remains a key factor in determining the microbiological quality of surface sea water. Since, *E. coli* is present in high abundance in the gut of warm-blooded animals; it is widely used as an indicator of faecal pollution and to estimate the risk of exposure to other types of pathogenic organisms present in animal or human wastes[6]. Use of *uid*-targeted PCR for the direct detection of *E. coli* is useful and faster than many other methods. The *uidA* gene product is a frequently used enzymatic marker for *E. coli* identification in recently developed detection media[7,8,9].

The aim of this study was to isolate and analyse the prevalence of faecal coliform bacteria, with an emphasis on *E. coli*, and to determine the incidence of enteric bacteria in the coastal region of the Kapıdağ Peninsula by means of GIS analysis. In addition, the feasibility of using the polymerase chain reaction (PCR) to monitor *E. coli* was investigated, in intense stations on the Kapıdağ Peninsula. In addition, genetic heterogeneity was determined using *uidA* PCR/DGGE analysis of subsequent seawater samples.

Materials and Methods

Marmara Sea that is located between the European and Asian continents is an important aquatic ecosystem

for Turkey. Together with the Bosporus and the Dardanelles, it forms the Turkish Strait System that is an internationally important waterway. From an ecological point of view, Marmara Sea is under high stress because of the municipal, industrial and agricultural sources of pollution as well as the intensive maritime activities and is at risk of extensive environmental deterioration such as eutrophication.

The Marmara Sea, distinct from the Aegean Sea and Black Sea which it links, is an inland sea characterised by a two-layered water system. The waters of the upper layer are under the direct effect of the waters of Black Sea which has intense river inputs, whereas the lower, denser waters which do not mix with the upper layer are under the influence of the Mediterranean. The Kapıdağ Peninsula is located on the south coast of Marmara Sea forms the Gulf of Bandırma on its east and the Gulf of Erdek on its west. Its oceanography is represented by the two stratified water layers typical of the Marmara Sea, that consist of low salinity (23-29 ppt) Black Sea waters and high salinity (38.5 ppt) Mediterranean waters[10].

Surface samples from six coastal locations (K1-K6) from three open sea locations (St1-3 representing the west, north and east of the Peninsula respectively) at variable depths (surface, 5, 10, 20 and 30m) were taken from the coastal region of Kapıdağ Peninsula, seasonally during 2011 and 2012 (Fig. 1). Pre-noon water samples were collected with a bacteriologic sampler (500 ml) in sterilized dark glass bottles[11]. Samples were analysed under standard laboratory conditions and processed within 2 h of collection.

GIS is basically defined as a set of a software and hardware which is able to link descriptive and spatial information for analysis. Using measured sample points in the study area, accurate predictions for unmeasured locations can be calculated using geostatistical analysis in the form of continuous surface[12]. The sample points may be measurements such as elevation, depth the water table, or levels of pollution. Using this method, created surfaces can be used to visualize, analyze, and understand spatial phenomena.

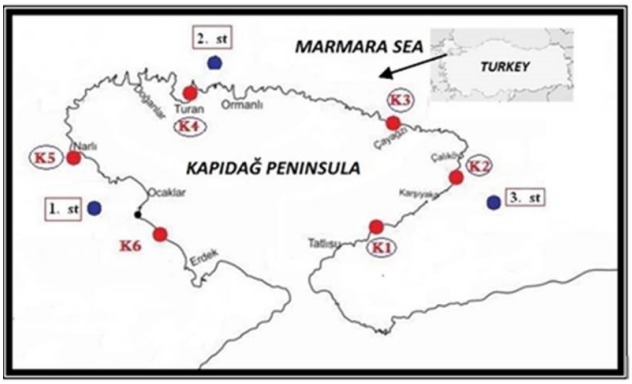

Fig. 1 — Sampling stations in the Kapıdağ Peninsula (adapted by[10])

In this study sea water temperature distribution of the vertical section of the study area was carried out using IDW method. In IDW method, it is assumed substantially that the rate of correlations and similarities between neighbors is proportional to the distance between them that can be defined it as a distance reverse function of every point from neighboring points[13].

In this study it is aimed that to show vertical variation of bacterial level in the Kapıdağ Peninsula with GIS base methods. The collected data were used as the input data to produce different maps which represent the spatial distribution of enteric bacteria in the Kapıdağ Peninsula. Spatial distribution map was prepared for a period of 4 seasons for displaying the differences taking place throughout the period in different months.

Multiple-Tube Fermentation Technique (five replicates) (9221)/Standard Total Coliform Fermentation Technique [Lauryl Sulfate Broth (Merck 1.10266)] was used to estimate the prevalence of faecal coliform bacteria and for the isolation of *E. coli* ([11], 9221B). Standard water and wastewater methods were applied for the analysis ([11], 9221). Lactose-fermenting colonies were further characterized by replica-plating on Eosin methylene blue agar (EMB, Merck 1.01347) and enteric chromagar (CHROMagar™ Orientation) followed by incubation at 37°C overnight[14]. Dehydrated powder was provided by the CHROMagar®. *E. coli*, pink colonies, were selected from the plate and inoculated into *E. coli* chromagar (HiMedia, MV1353) and incubated at 37°C overnight. For the identification of Enterobacteriaceae species, in particular *E. coli*, colonies were selected and confirmed by IMVIC [Indole (I), Methyl Red (M), Voges-Proskauer (V), and Citrate (C)] tests ([11], 9221F).

For each station that provided positive matches to *E. coli* by IMVIC test, a single pink *E. coli* colony was randomly chosen for molecular confirmation by DGGE and sequencing analysis. In absence of *E. coli* detection, a randomly selected enteric bacteria colony was selected. To determine the specificity of *uidA* for *E. coli* detection, DNA was extracted from exponential cultures using standard techniques[15].

A 166 bp fragment of the *uidA* gene was amplified from suspected *E. coli* isolates using primers UAL 1939 (5'-TATGGAATTTCGCCGATTTT-3') and UAR 2105 (5-TGTTTGCCTCCCTGCTGCGG-3')[16]. A GC clamp was attached to the 50 end of primer UAL-1939, leading to UAL-1939GC (208-bp)[17]. The PCR reaction mixture was prepared as follows: 1 × PCR buffer, 3 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (Promega Corporation, Wisconsin), 0.5 µM of each primer, 1 U of Taq DNA polymerase (Promega Corporation, Wisconsin) and DNA template, to a final volume of 50 µl. All amplification reactions were performed using a Rapid Cycler (Idaho Technology, Idaho). The PCR cycling conditions were a touchdown approach of 30 cycles as follows: genomic DNA was denatured at 94°C for 5 min, followed by 5 cycles of 92°C for 1 min, 65°C for 1 min, and 72°C for 1 min 30 sec, 5 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec, and 20 cycles of 92°C for 1 min, 50°C for 1 min, and 72°C for 1 min 30 sec with a final extension step of 72°C for 7 min. This was followed by a 2-min incubation at 72°C was added to the end of PCR program. A positive control of *E. coli* and nuclease-free water as a negative control was included in every procedure.

PCR-amplified DNA was visualized using 1.5% agarose gel electrophoresis. The products of each PCR reaction for each environmental isolate were run in a 0.5 TAE buffer. The gels were stained in ethidium bromide solution (0.5 × TAE buffer with a final concentration 0.5 mg/l EtBr), PCR products were visualized with a UV transilluminator to confirm the presence of appropriately sized amplicons. DGGE was performed using an Ingeny PhorU-2 system 15 µL of PCR product was applied directly onto an 8% w/v polyacrylamide gel (acrylamide /N, N'-methylene bisacrylamide, 37:1, w/w) in 1 × TAE buffer (40 mmol/L Tris pH 7.4, 20 mmol/L Na Acetate, 1 mmol/L Na2EDTA). A 48 to 68% linear denaturing gradient was formed using 20% and 80% denaturants (100% denaturant being 7 mol/L urea and 40% v/v formamide). Electrophoresis was performed at a constant voltage of 100V and a temperature of 60°C for 16 h. Following electrophoresis, gels were stained for 1 h in Milli-Q water containing 1 µg/mL Ethidium Bromide then de-stained in Milli-Q water for 1 h, visualised on a UV transilluminator (Syngene Gene Genius) and photographed using the Syngene Gene Snap software. Bands of interest were excised and incubated in 30 µL of DNA water at 4°C overnight prior to sequencing. The sequencing reactions were carried out by LGC AGOWA (Germany). Sequence data were verified using Sequencher version 4.1, compared to the GenBank nucleotide database using BLAST searchers, and aligned in BioEdit to reference sequences downloaded from GenBank.

Results

Physico-chemical parameters showed typical seasonal variations (See Table 1). The mean sea surface temperature values varied between 8.5°C and 26.9°C (summer 2011) over the sampling period, the highest (29.0ºC) and lowest (5.7ºC) temperatures being recorded in summer 2011 and winter 2012, at the K5 and the 2nd station (St2) respectively. The effect of the temperature among the environmental variables on the microorganisms was characterized by slower rates of microbial inactivation for all enteric bacteria concentration, including *E. coli* and *E. fecalis* under temperature conditions within the range of 5-30°C (Fig. 2, 3, 4, and 5). Salinity fluctuated between 14.5–33.1ppt during the sampling period.

The set of primers, UAL-1939 and UAR-2105, located at the carboxyl coding region of the *uidA* gene produced amplified DNA bands of 166 bp for *E. coli*. But in this study, we used UAL-1939 with GC clamps and amplified DNA bands of 208 bp for *E. coli* (Fig. 6). PCR results indicated the presence of significant levels of *E. coli* strains at various stations where untreated sewage is discharged. *E. coli* was present at all stations, except K1 and K6, throughout the sampling period irrespective of season. K1 and K6 stations (utilised as public beaches during the summer months) and depths below 30 meters display distinct genetic fingerprints suggestive of a diverse community of enteric bacteria other than *E. coli*.

E. coli was detected in all stations, except K1 and K6, sampled during the monitoring period (Table 1, Fig.6). Other enteric species was dominant with the percentage of 40 to 100 at K1 and K6 stations during the study. Enteric bacteria was not found at the first and third stations 30 m depths in Summer, and at the second and third stations 30 m depths in Winter seasons. The most abundant enteric bacteria species was *E. fecalis* with the percentage of 50 to 100 in winter. In autumn, other enteric species was dominant with the percentage of 40 to 100, while *E. coli* and *E. fecalis* represented less than 50% except depths of 20 and 30 meters of station 2.

Table 1 — Seasonally variations of temperature (°C), salinity (‰) and enteric bacteria species (EC*: E. coli*, EF: *E. fecalis*, EO: other enteric species) percentages (%) at the sampling stations

Stations		*Spring 2011*					*Summer 2011*					*Autumn 2011*					*Winter 2012*				
		Temp	Sal.	Entericbact (%)			Temp	Sal.	Entericbact (%)			Temp	Sal.	Entericbact (%)			Temp	Sal.	Entericbact (%)		
		°C	‰	*EC*	*EF*	*EO*	°C	‰	*EC*	*EF*	*EO*	°C	‰	*EC*	*EF*	*EO*	°C	‰	*EC*	*EF*	*EO*
	S	9.5	24.8	50	50	-	26.0	20.1	10	60	30	17.0	18.8	30	30	40	8.0	24.4	25	50	25
	5	9.0	24.2	50	50	-	26.0	19.5	10	-	90	17.5	18.3	20	30	50	7.7	24.4	25	50	25
1	10	8.5	24.4	50	50	-	25.9	19.4	30	70	-	17.5	18.3	20	40	40	7.7	24.4	50	50	-
	20	8.0	25	-	50	50	15.9	24.0	-	20	80	17.5	18.5	20	40	40	8.0	24.4	-	100	-
	30	9.7	27.3	-	-	100	15.0	33.1	-	-	-	18.0	28.2	-	40	60	14.0	31.3	-	-	100
	S	10.0	22.1	50	50	-	26.0	21.2	50	50	-	17.0	18.5	30	30	40	7.5	23.8	-	100	-
	5	10.0	22.4	30	70	-	25.0	21.2	10	-	90	17.0	20.3	20	30	50	5.7	24.0	-	100	-
2	10	9.8	22.2	30	70	-	23.0	21.3	100	-	-	16.0	18.1	30	30	40	5.7	24.2	-	100	-
	20	8.5	23.8	-	-	100	13.0	24.5	-	10	90	18.0	26.8	-	50	50	8.0	24.5	-	-	100
	30	9.0	25.9	-	10	90	15.0	31.5	-	20	80	18.0	29.2	-	50	50	14.5	32.4	-	-	-
	S	9.9	22.9	30	40	30	26.0	20.5	20	80	-	16.5	24.7	-	-	100	7.5	23.9	-	100	-
	5	8.9	22.4	30	40	30	26.0	19.4	20	80	-	16.5	19.2	-	20	80	7.5	24.0	-	100	-
3	10	9.5	22.4	50	50	-	19.0	21.3	10	-	90	16.0	19.7	-	-	100	7.5	24.0	100	-	-
	20	8.0	24.6	30	40	30	12.0	27.6	-	25	75	17.0	28.8	-	-	100	9.9	26.5	-	-	-
	30	8.0	26.0	-	30	70	15.0	34.5	-	-	-	17.0	28.4	-	-	100	14.5	31.6	-	-	-
	K1	12.0	22.6	-	60	40	26.0	20.5	-	20	80	17.8	16.8	-	30	70	10.6	20.7	-	-	100
	K2	13.0	20.1	75	25	-	26.0	19.5	100	-	-	16.8	18.2	30	20	50	7.5	24.3	50	50	-
	K3	11.0	22.2	75	25	-	27.0	19.9	30	-	70	14.5	17.5	-	50	50	7.7	24.5	-	50	50
	K4	16.0	18.5	50	-	50	29.0	20.3	100	-	-	14.5	17.8	40	60	-	7.8	23.3	-	100	-
	K5	11.0	23.8	100	-	-	29.0	21.3	50	50	-	17.1	18.7	40	60	-	9.6	24.5	-	100	-
	K6	9.7	25.3	-	20	80	27.0	21.2	-	-	100	15.2	18.0	-	10	90	10.7	24.4	-	-	100

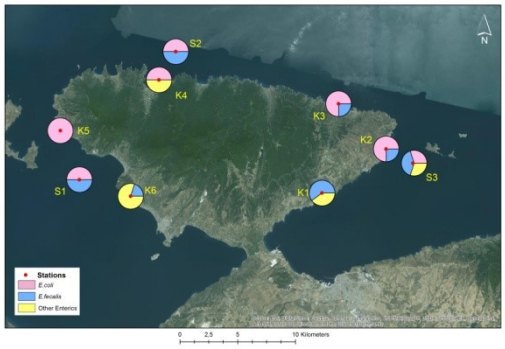

Fig. 2 — Distribution of bacterial levels at the surface in Spring 2011

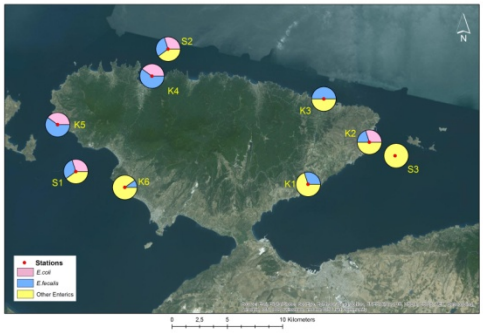

Fig. 3 — Distribution of bacterial levels at the surface in Summer 2011

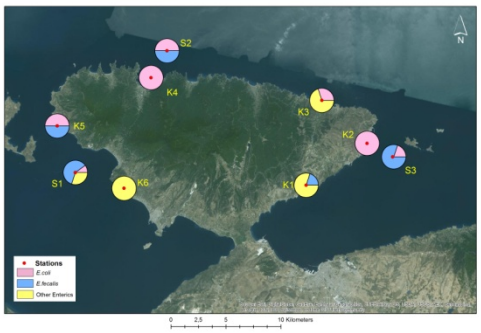

Fig. 4 — Distribution of bacterial levels at the surface in Autumn 2011

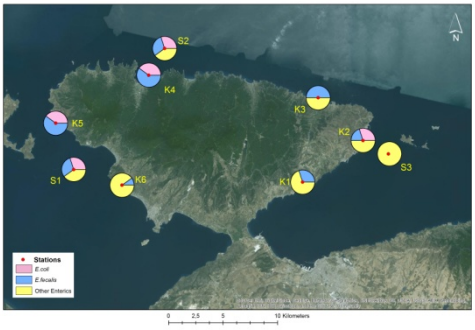

Fig. 5 — Distribution of bacterial levels at the surface in Winter 2012

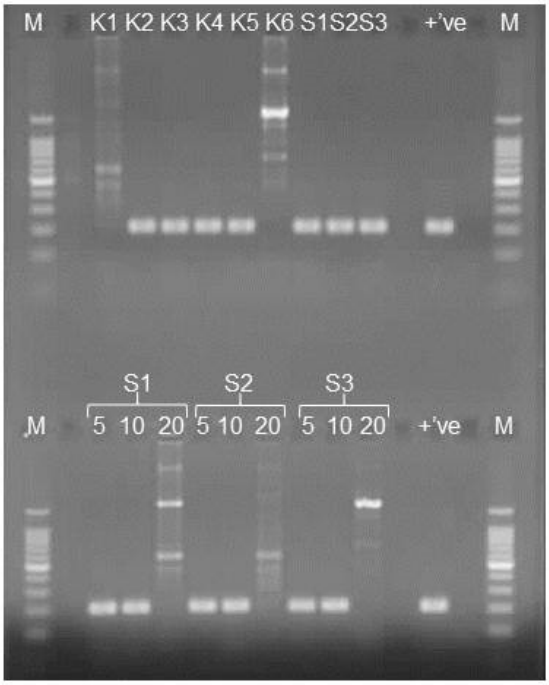

Fig. 6 — Amplification of β-D-glucuronidase gene fragment (uidA, 208 bp) from E. coli isolates using primer pair UAL-1939GC and UAL-2105 (M: 100-bp ladder (Promega) Lane +'ve : positive *E. coli* strain. Upper side denotes Stations K1 to K6and St 1,2,3 surface isolated in Summer 2011, bottom side, isolates depths (5,10 and 20 m) of St 1,2 and 3 isolated in Summer 2011. Station K1 and K6 in failed to generate any positive colonies for *E. coli* (see Table 1 and text for details), and a random, non-pink colony forming enteric isolate was analysed instead)

Discussion

Coastal waters are often used for multiple purposes such as receiving wastewaters, for recreation, and for aquaculture. Discharges of sewage effluents have a negative impact on the coastal environment and make it less attractive for recreation as well as for aquaculture, which is sensitive to the presence of pathogenic microorganisms and toxic compounds. Sources of coliform bacteria include runoff from feed lots and manure-amended agricultural land, inadequate septic systems, urban runoff and sewage discharges. The continuous seeding of these coastal waters from such sources, suggests that *E. coli* and other enteric bacteria can now be considered as established inhabitants of the local microbiological community, regardless of their transient presence as individuals cells. Occasional pollution by effluents containing human pathogens may result in prohibition of the sale of shellfish and economic loss[18].

Incorporating indicator bacteria characteristics into source tracking studies is attractive since source identification would be intricately tied to the same biological indicator of the faecal contamination. A high amount of diversity within the *E. coli* population is necessary to reflect what might be expected in surface water contaminated with faecal pollution. In our study, only one gene was studied, the gene coding for β-D-glucuronidase. Analysis of *uidA* gene using DGGE analysis and BLAST results showed temporal and spatial genetic homogeneity in *E. coli* marine populations in this area for the first time.

As to make a general evaluation, the most intensively apparent species was *E. coli*. The second most abundant was *Enterococcus fecalis*. The species of *Enterococcus* were apparent as well, when *E. coli* was not present. This was expected since they are more resistant against environmental conditions than *E. coli*. Many researchers also determined the same findings and reached the same results in other areas[19,20]. The survival of *E. coli* in different types of water as well as in environments associated with aquatic milieus has been studied. When exposed to aquatic environments, the bacteria face a number of hostile factors, e.g. biotic (competition, predation) and abiotic (pH, light, salinity, oxidative stress, temperature, osmotic pressure and nutrients deficiency) and in order to survive induce a number of stress responses[21,22]. For the enteric bacteria in the seawater, the optimal temperatures for survival tend to be lower than those for active growth, and this has been demonstrated in a number of seawater experiments. In this study, within the range of 5-30°C, slower rates of inactivation have been demonstrated in the figures for all enteric bacteria concentration, including *E. coli* and *E. fecalis* (Fig. 2, 3, 4, and 5). Several studies point out that the survival of enteric bacteria in water is enhanced by lower temperatures[23,24]. Contrary, directly visible and UV light greatly affect the survival of enteric bacteria in water in a negative fashion. In the present study, *E. coli* was not observed in *winter,* presumably due to its location and negative effects of seasonal physical parameters, such as temperature.

The presence of a negative relationship between salinity concentration and the number of enteric bacteria in sea has been previously reported for the Marmara region[25]. However, enteric bacteria of sewage origin undergo a sudden osmotic shock when they enter seawater and may adapt their metabolism to the new medium by means of their osmoregulation systems. This ability of enteric bacteria aids them in gaining resistance to salt in sea environments and increases their probability of survival[26]. The less

saline waters of the Black Sea (22–26 psu) reach the Mediterranean via upper currents while the concentrated saline waters of the Mediterranean (38.5–38.6 psu) reach the Black Sea via the under currents of the Çanakkale and İstanbul Straits[27,28].

Meanwhile, some species have never been considered in evaluations, in spite of their apparent presence in some periods. Some of these species were non-fermentating bacteria; such as Gram negative (which live unrestrictedly in nature) and sporeless bacilli. The most well-known and apparent species are *Acinetobacter* spp., and *Pseudomonas* spp.[29,19]. The detailed analysis of these species were not conducted in this study, however they were evaluated as a group. It is important to note that Gram negative bacteria had different environmental bacteria however fecal coliform bacteria were not detected in this test.

Results of the present study show that the PCR is a potentially powerful technique for the rapid detection of enteric bacteria in routine water quality monitoring. In addition, this study indicated that PCR provides a powerful supplement to conventional methods for more accurate risk assessment and monitoring of pathogenic bacteria in the marine environment.

Conclusion

The ability to rapidly monitor for various types of microbial pathogens would be extremely useful not only for routine assessment of water quality to protect public health, but also allow effective assessments of water treatment processes to be made by permitting pre- and post-treatment waters to be truly analyses. At the last, because of the indigenous nature of *E. coli* considerations must be made about the importance of this bacterium as an indicator of recent fecal contamination and the necessity of knowing the source of contamination if public health is to be protected.

Acknowledgement

The authors would like to thank the Research Fund of Istanbul University for supporting the research project UDP-17861 and FYO-2016-3735. This manuscript was presented at the 13th International Conference on Protection and Restoration of the Environment, Mykonos Island, Greece, 3rd to 8th of July, 2016.

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