Romanian Journal of Ecology & Environmental Chemistry, 4(2), 2024
https://doi.org/10.21698/rjeec.2024.208

Article

Proceedings of the 25th International Symposium "The Environment and the Industry" (E-SIMI 2022), 29 September 2022, online event

Natural and anthropic aquatic ecosystems – structural differences in bacterial populations

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Received:	*Accepted:*	*Published:*
01.09.2022	*19.12.2022*	*28.12.2022*

Abstract
The hydrosphere represents about 71% of the total surface of the planet of which only 2.8% is represented by freshwater ecosystems. The ecosystem maintains a state of balance between the component populations, throughout its self-control function, maintaining the numerical variations of these populations within certain limits of sustainability. The exceeding of these limits could causes the balance disturbance by changing the structure and functioning of the ecosystem. The complex human activities influence the structure and functioning of ecosystems, transforming the environment and adapting it to its purposes. Microorganisms are present in all types of ecosystems, being endogenous, exogenous or transient due to contamination with various sources of pollution. Water is an essential natural factor of ecological balance increasingly exposed to microbial pollution. Indicators of fecal pollution are used to assess the degree of water contamination and to locate its origin. The continuous and uncontrolled usage of antibacterial agents that contributed to bacterial resistance determined/ caused pollution of aquatic ecosystems with antibiotic resistant microorganisms. The emergence of antibiotic resistant bacteria is predictable in any environment given that the use of antibacterial substances is constantly growing worldwide. Thus, antibiotic resistance induced in the aquatic environment can have an effect both on bacterial populations in the ecosystem and on human health. Aquatic environments are recognized as one of the reservoirs for the transmission and dissemination of antibiotic resistance. The main goal of this paper is to highlight the differences of bacterial communities from anthropogenic and natural aquatic ecosystems and to assess the potential impact they have on environment and human health. The study area focused on two lakes in Bucharest, Lake Morii (anthropic) and Lake Snagov (natural). Microbiological and molecular biology methods were applied for a bacterial communities' characterization. The fecal indicators were quantified by Most Probable Number method. The identification and characterization of bacterial populations in both aquatic ecosystems (Morii Lake and Snagov Lake) were performed by Omnilog (Biolog, USA) and by iSeq100 (Illumina, USA) gene sequencing techniques from bacterial aquatic ecosystem. Antibiotic susceptibility was tested following CLSI recommendation.

Keywords: *bacteria, aquatic ecosystem, anthropic impact*

INTRODUCTION

The relationship between increasing population density and human activities is well known for the changing effects of the environment. With anthropogenic pressure there was also the modification of the bacterial populations from aquatic ecosystems.

Eutrophication is a biological response to a lakes' excess nutrient input that causes degradation of lentic aquatic systems [1]. Nowadays, this is an important challenging environmental problem because it is associated with the destruction of lake ecosystem around the world and decrease the value of its use in various purposes [2]. Rapid anthropogenic eutrophication of natural freshwater environments can lead to serious changes in the composition of the microbial community, affecting the functioning of the microbial loop and thus the entire aquatic food web [3, 4].

The main water stressors are human activities reflected by nutrients' inputs and climate change by increasing temperature and extreme weather phenomena. Human activities accelerate de degradation rate of water, the point sources may be wastewater effluents, infiltration from animal feedlots and human wastes [5].

The microbiota is known to be an important factor in the sustainability of the natural water ecosystem, but the microbial community also might include pathogens that results in very serious waterborne diseases in human and animals [6]. An increased abundance of antibiotic resistance genes (ARGs) in aquatic environments has been linked to environmental pollution.

The microbial community is not only a natural component of the aquatic ecosystems, but also one of the main indicators of their ecological situation. Water reservoirs can be affected by regular or accidental contamination which greatly influences their water quality [7]. According to EU Water Framework Directive, the surface water ecological status includes five categories: high, good, moderate, poor and bad [8]. In Romania it was transposed by the Water Law 107/1996 updated 2022 according to the National Management Plan of Hydrographic Basins [9].

In recent decades, various molecular approaches have been applied to characterize microbial community structure in aquatic ecosystems. The use of antibiotics was the most important way to control infectious diseases in the 20th century. Many infectious diseases were kept under control by the administration of antibiotics, but infections remain the main cause of mortality in the world. Moreover, infections that were previously controlled are becoming more common in immunosuppressed patients due to bacterial resistance to antibiotics. The ability of pathogens to grow in the presence of antibiotics by developing resistance has made victims as vulnerable as in the pre-antibiotic era. Resistance to all groups of antibiotics has emerged in parallel with the widespread use of antibiotics in the clinic and in animal husbandry, rendering many of them ineffective. Antibiotic-resistant bacteria can survive and even multiply in the presence of therapeutic antibiotic concentrations. The development of resistance is inevitable after the introduction of a new antibiotic in the clinic. The rate of emergence of bacterial strains resistant to new drugs is of the order of 1%.

Resistance can be natural (intrinsic) or acquired through mutational resistance genes or through the acquisition of exogenous genes. The natural resistance of microorganisms to antibiotics means the resistance of all members of a bacterial species to one or more antibiotics present in maximum doses, which can inhibit the growth of other bacterial species. [10].

Among these, DNA fingerprinting techniques have one of the most frequently used and more recently, the advance of next-generation sequencing technologies has enabled a deeper and better resolution of taxonomic characterization [6].

The induced toxic effects start from the molecular level (highlighted by biomarkers) to the body level inducing phenotypic (structural) and physiological changes (growth and development), causing amplified imbalances in the food chain. The main scientific concern is to highlight any changes in bacterial DNA levels and to obtain molecular models based on which structural changes in the bacterial population can be predicted under the influence of anthropogenic factors.

This experimental study targets both microbiological and physical-chemical elements in order to highlight the level of fulfilment of the quality criteria specific to surface waters both in anthropogenic and natural conditions.

EXPERIMENTAL PART

The study area focused on two lakes in Bucharest, Lake Morii (anthropic) and Lake Snagov (natural). Microbiological and molecular biology methods were applied for a bacterial communities' characterization.

Sampling

Two sampling sites were selected in Bucharest-Ilfov, Romania, Morii Lake – anthropic lake (Fig. 1) and Snagov Lake – natural lake (Fig. 2). The collection/sampling points were selected based on motivational characteristics of an ecological nature - natural and anthropogenic ecosystems, respectively Snagov Lake and Morii Lake. These represent 2 lakes in Bucharest, an intensely populated and industrialized urban area that could have an impact on aquatic ecosystems. If Morii Lake - anthropogenic lake is located near the centre of the capital - socio-economically developed area, Snagov Lake - natural lake is located in a metropolitan area adjacent to the capital under development in the last 10 years.

The sampling was carried out in two sampling campaigns, in the winter and summer of 2020, from each control section collecting 2L of surface water sample. For microbiological analysis, samples were collected in sterile 1 L containers [11].

The surface water was collected during the winter and summer of 2020, according to SR EN ISO 19458:2007 then the samples were transported and preserved at 4-5°C and analysed in the same day to the laboratory in a maximum of 6 hours after sampling.

Physical and chemical analyses such as temperature, pH, conductivity and oxygen saturation were measured at the sampling sites using a multiparameter (ORION Star A329, Thermo Scientific).

Fig. 1. Morii Lake

Fig. 2. Snagov Lake

Microbiological analyses

The densities of coliform bacteria were quantified by Most Probable Number technique using Colilert-18 culture medium [12].

The densities of enterococci were quantified by membrane filtration method using Slanetz & Bartley Agar (Oxoid, UK) for incubation at 36±2°C for 48 hours and bile aesculine agar (Oxoid, UK) for the confirmation step at 44°C for 2 hours [13].

The identification of bacterial strains was based on their metabolic characteristics using an Omnilog automated system (Biolog, USA).

The susceptibility testing was performed in duplicate by disk diffusion method on Muller-Hinton agar (Oxoid, UK). The antibiograms were performed according to CLSI recommendations [14] with 6 beta-lactam antibiotics such as Amoxicillin clavulanate (AMC), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ampicillin (AMP) and also Imipenem (IMP).

PCR detection genes

The environmental DNA samples of Morii Lake and Snagov Lake were isolated according to the protocol of the PowerSoil kit (Qiagen, Germany). The extracted bacterial DNA was spectrophotometric quantified at 280 nm and 260 nm and subject to PCR with universal primer (UBP), primers for resistance genes (SHV, TEM, OXA, CTX-M) and efflux pumps primers (OprM, TolC, Acra). The PCR products were identified by 1% agarose gel electrophoresis.

The DNA was incubated for one hour in the presence of the restriction enzymes Hind III (DNA cutting site at A – AGCTT) and Tas I (DNA cutting site at TTCGA – A) – Fig. 3.

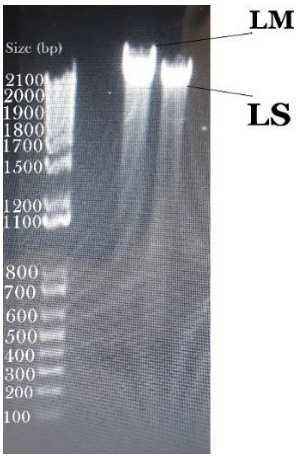


Fig. 3. Profile of restriction enzymes Hind III and Tas I for DNA samples isolated from surface water taken from Lake Snagov (LS) and Lake Morii (LM).

In order to carry out the analyzes at the DNA level, first, the quantification of the extracted DNA was carried out using the kit (Table 1).

Table 1. Concentration of bacterial DNA extracted from biological samples

Sample symbol	A260/A280	A260 (ng/µL)
LS	1.89	27.95
LM	1.87	28.91

The result of the quantification of the bacterial DNA concentration obtained after DNA extraction and isolation determined that the DNA extraction process fell within the optimal parameters and the contamination is low, the 260/280nm ratio being close to 1.8, and the two samples have a relatively similar concentration of DNA.

RESULTS AND DISCUSSION

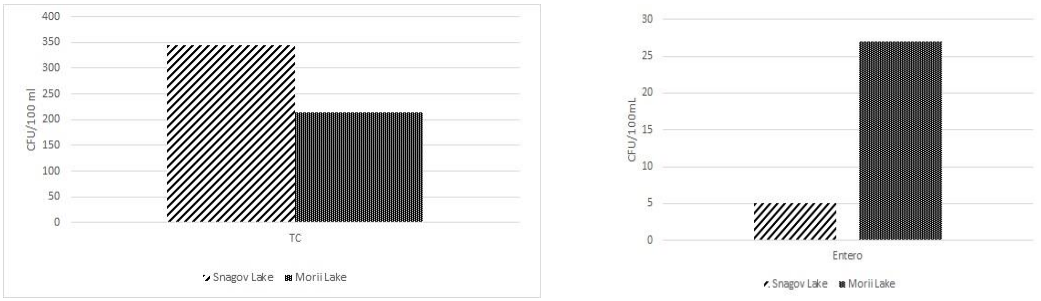
The physical-chemical parameters measured at the sampling sites for the natural or anthropic lakes showed no major variation between seasons excepting the temperature and conductivity parameters (Table 2).

Table 2. Physical-chemical characteristics of natural and anthropic lakes during winter and summer 2020

Physical-chemical indicator of water	Measure Units	Snagov Lake		Morii Lake	
		winter	summer	winter	summer
pH	pH units	7.8	7.6	8.2	8.0
Conductivity	µS/cm	320	360	350	380
Dissolved Oxygen	mgO2/L	7.2	7.0	6.9	6.6
Water Temperature	°C	10.5	20.5	12	21.4

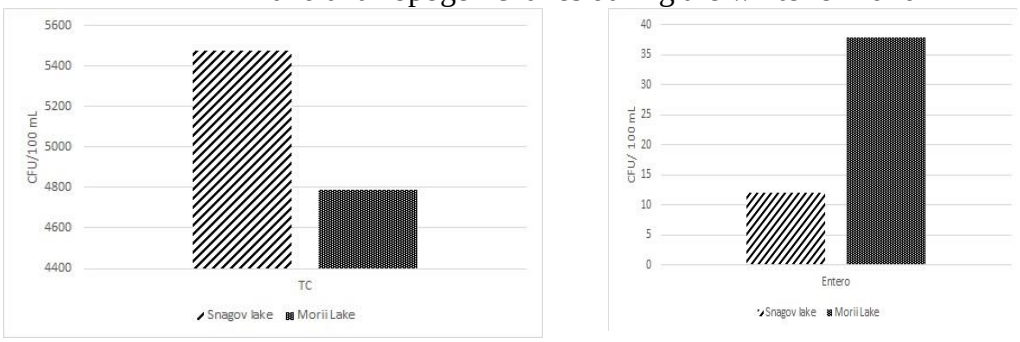
When the two lakes were compared, the higher values of the anthropic lake for pH and conductivity were higher than the natural lake. This observation could be explained by the higher pollution of the anthropic lake, especially in the summer time when the temperature value doubles compared to winter. In the warm period, the dissolved oxygen values decrease in both lakes, proof of the presence of the eutrophication process. The change in values that indicate both the eutrophication process and the pollution process is evident during the summer, especially for the anthropic lake.

Considering that this is an accumulation lake, the vegetation is not rich and the potential for the development of cyanobacteria is great.
The density of enterococci (Fig. 4A and 4B) highlights the anthropic character of Morii Lake and at the same time demonstrates the contribution of pollution from external sources for both lakes. The increase of the temperature seemed to induce an increase of the coliform bacteria populations (Fig. 5A and 5B) in both natural and anthropogenic lakes. Overall, the density of faecal coliform bacteria was higher in the anthropic lake during the winter.
The recorded values maintain this classification both in the cold season and in the warm season, despite the influence of the low temperature which causes a decrease in the degree of pollution in the cold season. In addition, a major influence in decreasing the bacterial density during the winter can be presented by the lower intensity of anthropic activities.



A. **B.**

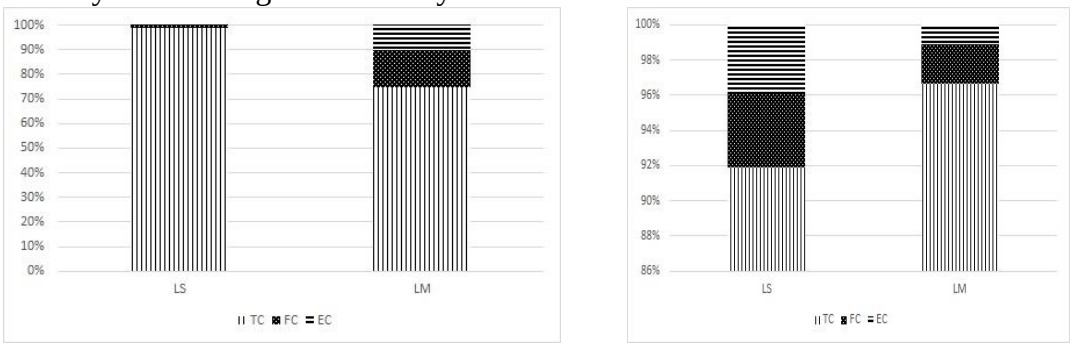
Fig. 4. The densities of **A**. total coliform bacteria (TC) and **B**. Enterococi in both natural and anthropogenic lakes during the winter of 2020



A. **B.**

Fig. 5. The densities of **A**. total coliform bacteria (TC) and **B.** Enterococi in both natural and anthropogenic lakes during the summer of 2020

Comparing the distribution of coliform populations (Fig. 6), the difference between the two types of slow ecosystems during the two analysed seasons was found.



A. **B.**

Fig. 6. The structure of the coliform bacteria community in A. winter and B. summer.

The structure of coliform bacteria populations showed a higher density of faecal coliform bacteria and *E. coli* in Morii Lake, with a higher intake in summer. The anthropogenic pressure is clearly

observed on the results from summer (Fig. 5) in natural lake where the density of fecal coliform bacteria increases with increasing temperature and the manifestation of the recreational season on Snagov area.

Although the density of total coliform bacteria increases to the same degree in both lakes during the summer in Snagov Lake, the proportion of fecal coliform bacteria increases significantly to 5% compared to 1% in winter. In the warm season, *E. coli* is also present in a proportion of 4% of the population of coliform bacteria.

In accordance with the national quality standards imposed for surface water used for drinking purposes [15], it can be observed that the coliform bacteria density values (Figure 4A, Figure 5A) fall into A2 category in winter and A3 in summer for both aquatic ecosystems. Taking into account the densities of enterococci, the surface water from Snagov Lake falls into A1category, and Morii Lake into A2. These reports are maintained throughout 2020, regardless of the winter-summer season.

According to national quality parameters for bathing water [16], the enterococci densities indicate an excellence quality of both lakes for the period of 2020.

The results of PCR (Fig. 7) showed that the genetic material extracted and analysed was mostly from bacterial strains because a majority amplification product of around 700bp was obtained. An interesting fact can be observed in the case of amplification of the genetic material with specific primers P2 and P3 (specific for the blaSHV and blaTEM resistance genes). These genes are present in the genetic material from bacterial strains isolated from Morii Lake, but are not present in bacterial strains isolated from Snagov Lake. These genes can fall into the category of molecular markers because they differentiate between anthropogenic and natural aquatic environment. In the anthropogenic environment (Morii Lake), these genes are effective components of the mechanisms of adaptation to anthropogenic pressure. In the case of Snagov Lake, natural lake, the anthropogenic pressure exists but it is insignificant and there is no need for specific adaptation mechanisms. The gene recognized by P4 (Oxa) primer is not present in the genetic material extracted from bacterial strains isolated from Morii and Snagov Lakes, which indicates that it does not belong to the mechanisms of adaptation to the present environmental conditions. The same can be seen for the presence of the P5 amplified resistance gene, the amplification product is nonspecific (100 and 250 bp) for the resistance gene which would have had an amplification product around 500 bp.

In the case of genes involved in the production of effluent pump units, a robust presence can be observed for bacteria isolated from both Morii and Snagov locations. Outflow pumps are components of a nonspecific defence / adaptation mechanism that is present in all bacteria isolated from LM and LS.

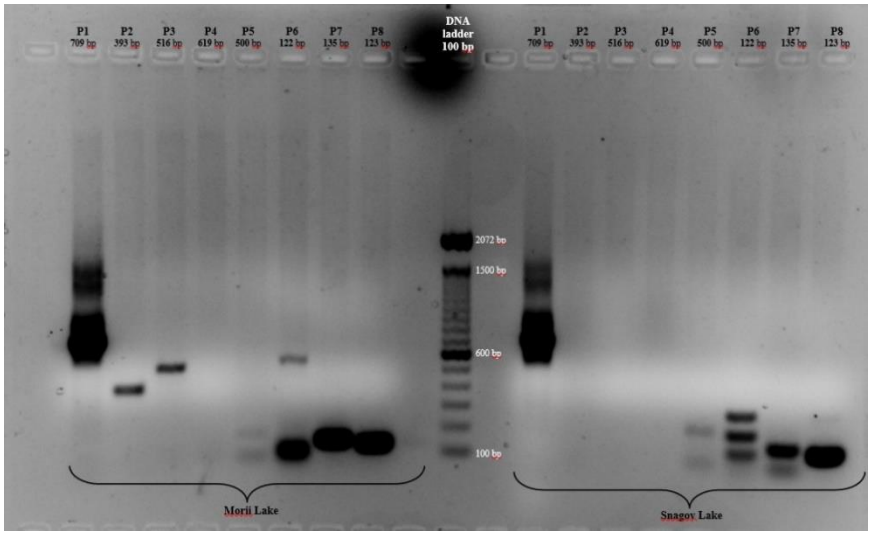


Fig. 7. Products of PCR in agarose gel electrophoresis for environmental DNA isolated from Snagov and Morii lakes (P1 – UBP, P2 – blaSHV, P3 – TEM, P4 – OXA, P5 – CTX-M, P6 – BEP-OprM, P7 – BEP-AB-TolC, P8 – BEP-Acra).

The analysis of the restriction fragments obtained from the use of enzymes clearly demonstrated molecular differences between the samples in the two lens systems (Figure 8). As can be seen, one difference is given by the HindIII endonuclease which cuts into three restriction sites the DNA from Lake Morii and only once the DNA from Lake Snagov.

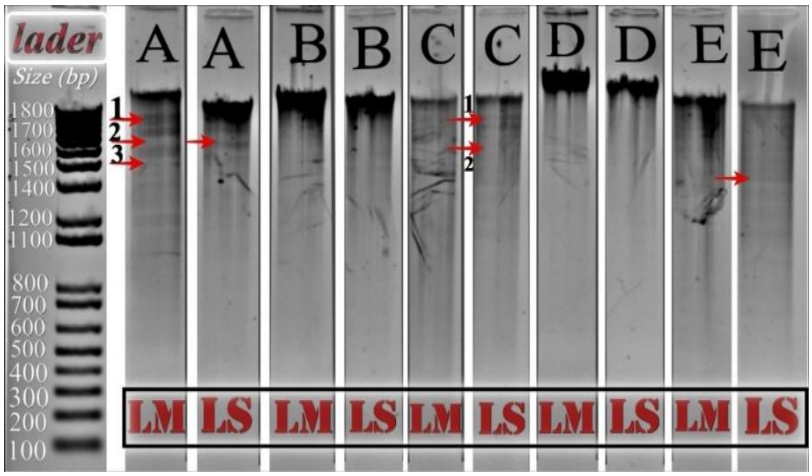


Fig. 8. Restriction enzyme profile of environmental DNA samples isolated from Snagov and Morii lakes

Starting from the differences indicated by the PCR and digestion with restriction enzymes results, specific identification of the bacterial strains present in the two slow ecosystems was performed, targeting in particular bacteria with pathogenic potential.

The genetic diversity of bacteria refers to the variations determined by the genetic organization: the chromosomal structure, the presence or absence of plasmids, determining the real bases of the heterogeneity of a microbial community. In the case of bacteria, genetic diversity is determined, in particular, by the increased potential for plasmid-mediated gene transfer and transposable genetic elements, intraspecific, interspecific and intergeneric. Genetic adaptation has as a substrate the existence of a heterogeneous genetic population of individuals, genetic heterogeneity being the result of the occurrence of mutations, the modification of a large number of nucleotide base pairs and the actions of genetic transfer mechanisms. Mutations are produced by stable changes in the base sequence of a gene, transmissible to offspring. These can be point-like when they involve the modification of a single base and they can alter the action sites of an antimicrobial agent, interfering with its activity or modify a sequence of bases. Mutations always change the genotype, but depending on their nature, they may not be expressed in the phenotype. Depending on the causative agent, mutations are induced by endogenous factors (spontaneous mutations) and exogenous factors (induced mutations). The transfer of genetic information to bacteria means the passage of a piece of DNA from the donor cell to the recipient cell. The most known transfer mechanisms are: genetic transformation, conjugation, transduction, sexduction, transfection and protoplast fusion.

Gene transfer in bacteria has several distinct features: lack of fusion of cells participating in genetic exchange, the unequal quantitative contribution of the two cells, the transfer is unidirectional.

Very often, after transfer, exogenous DNA is degraded by cellular restriction enzymes.

Restriction enzymes are found in most prokaryotic microorganisms but are absent in eukaryotes. The net difference in their distribution is partly explained by the fact that in prokaryotes there is a particularly intense gene flow, without equivalent in the rest of the living world. Restriction enzymes have the role of recognizing and degrading exogenous DNA, limiting its replication and propagation in subsequent cell generations.

Based on metabolic characteristics with Omnilog system, the common and specific bacteria with pathogenic potential were identified (Table 3). The most and diversified strains have been identified in the Morii lake during the summer, which shows that the increase in the density of coliform bacteria is not only determined by the increase in temperature but also by an additional supply of potentially pathogenic bacteria from external sources. Bacterial identification analysis showed a

stability of bacterial populations in Snagov Lake that increase in density with increasing temperature. The only strains with pathogenic potential were *E. coli* and *K. oxytoca*, also present in Lake Morii that proves the existence of spills.

Table 3. Bacterial strains identified form Snagov and Morii lakes during winter and summer 2020.

SNAGOV LAKE	MORII LAKE
Pseudomonas putida	*Escherichia coli*
Shewanella oneidensis	*Klebsiella oxytoca*
Kosakonia cowanii	*Klebsiella pneumonia*
Klebsiella oxytoca	*Enterobacter kobei*
	Klebsiella grimontii
	Plautia stali
	Raoutella ornithinolytica
	Enterobacter bugandensis
	Aeromonas sp
	Citrobacter freundii

Antibiotic sensitivity testing of the bacteria common to the two lakes indicated the presence of resistance only for the strains identified in Morii Lake and Snagov Lake. The *E. coli* strain was resistant to AMP and AMC, and *Klebsiella oxytoca* to AMP, AMC and FEP.

CONCLUSIONS

In this study, a series of microbiology and molecular biology studies were carried out for the samples collected from the natural aquatic environment - Lake Snagov and anthropogenic - Lake Morii, highlighting the adaptation mechanisms both at the level of bacterial communities and at the molecular level induced by anthropogenic pollution. The physical-chemical analyzes were carried out on site having an extremely important role on the development of bacteria.

As a result of the quantitative microbiological analyses, a higher density of total coliform bacteria was revealed in Lake Snagov compared to Lake Morii, but these results were proportional to the density of fecal coliforms. Thus, the densities of thermotolerant coliform bacteria and *E. coli* were lower in Lake Snagov. This phenomenon can be argued with the fact that Lake Snagov is a natural lake, with vast vegetation compared to Lake Morii where the pollution is much higher. The detection of Enterococcus the two lakes highlighted the anthropogenic characteristics at Morii Lake due to the pollution of human activities.

ACKNOWLEDGEMENTS

This work was financially supported by Core Program SMARTWAY, PN 19 04 02 01, contract no. 20N/2019 from the Romanian National Authority for Scientific Research and Innovation CNCS/CCCDI-UEFISCDI.

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Citation: Radulescu, D.M., Banciu, A.R., Stoica, C., Vaideanu, M.A., Novac, L., Pascu, L.F., Nita-Lazar, M., Natural and anthropic aquatic ecosystems – structural differences in bacterial populations, *Rom. J. Ecol. Environ. Chem*., **2022**, 4, no. 2, pp. 87-95.