

POTENTIALLY PATHOGENIC AND ANTIBIOTIC RESISTANT BACTERIA IN THE DANUBE DELTA AQUATIC ECOSYSTEM

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Abstract. Human and animal pathogenic and potentially pathogenic bacteria are constantly released into water waste system and from there to the environment. These microorganisms are able to mix acquiring new resistance by horizontal gene transfer (shuffling of genes and mobile genetic elements). At the same time, antibiotics are released into water and might exert selective activities as well as ecological damage in water communities, resulting antibiotic resistance. Water constitutes not only a way of dissemination of antibiotic resistant organisms among human and animal populations because drinking water is produced from surface water, but also a route by which resistance genes are introduced in natural bacterial ecosystem. Pollution of water resources is a major risk to human health and water quality throughout the world especially when the aquatic ecosystem is a part of a protected area. The main goal of the present paper was to monitor the occurrence of antibiotic resistant bacteria in the Danube Delta aquatic ecosystem. The experimental study was performed in 2014 on St. Gheorghe branch, the oldest branch of Danube Delta. The surface water samples were seasonally collected from eleven control sections with anthropogenic potential risk that could influence the water quality. The fecal indicators such as coliforms and enterococci were quantitatively assessed and antibiotic resistant bacteria were identified by disc diffusion method. Results indicated that the density of Gram-negative bacteria was higher than density of enterococci during the monitoring period. The isolated strains exhibited high rates of β -lactam-resistance especially to ampicillin and amoxicillin plus clavulanic acid and similar resistant phenotypes were consecutively identified from the same control section.

Keywords: bacteria, antibiotic, water, ecosystem.

AIMS AND BACKGROUND

The normal biological activity of microorganisms depends on external environmental conditions. In good environmental conditions bacteria thrive and in stringent conditions microorganisms compensated by a high adaptation capacity. Depending on the characteristic habitats, natural aquatic environments are divided

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into groundwater and surface water. Microbiota composition varies as a function of water class and depends mainly on salt and organic compounds concentration, turbidity, temperature and pH, concentration sources and water dynamism. Classically, in the aquatic environments, bacterioplankton is separated into two main groups: bacteria attached to aggregates and free-living bacteria^{1,2}. In open waters away from soil, sediments or presence of other organisms, microorganism density rarely reaches the limit of 106 CFU/ml, considered as significant for an effective contribution to the ecosystem. Most bacteria do not live free in natural waters but related to plankton or to various debris or aggregates. Free-living bacteria are rarely native freshwater often being in transit from one location to another. Freshwater are less interconnected and therefore, microorganisms have many features in common with the ground microorganisms. Fast flowing waters like rivers, frequently transported the microorganisms from sediment³. Natural waters receiving waste waters resulting from human activities alter the original water quality. Exceeding the limits of regeneration and dilution capacities of aquatic ecosystems, pollutants from both points and diffuse sources are spreading rapidly through the water current^{4,5}.

Fecal pollution indicators exist in humans and animals in most parts of the world. There is a lot of controversy over the survival and transmission of the fecal coliform group into streams and rivers⁶. Fecal source tracking is emerging as a reliable discipline to understand the outcome of sanitary value microorganisms in the most ecosystems. The fecal pollution of water from a health/sanitary point of view is the contamination of water with disease-causing organisms (potentially pathogenic or pathogenic organisms) that may inhabit the gastrointestinal tract⁷.

In order to protect the public health, detecting the sources of these microbes and their changes in surface water is important for an effective water treatment. Pathogens are primarily contained in fecal materials derived from human, livestock and wild animals and they mostly get into surface water through sewage discharge, agriculture, urban and storm water runoff. Numbers of coliform bacteria and enterococci are widely used as microbiological indicators for fecal pollution, so those bacteria can serve as parameter to provide basic information on surface water quality. In general, the levels of fecal bacteria are higher in commercial, residential and agricultural lands, but lower in forest areas⁸.

Microbial water quality is traditionally monitored using culture-based techniques that selectively promote the growth of bacterial indicators of fecal pollution. Currently used indicators are easy to grow and they are often present in higher numbers than pathogens. The high level of nutrients and pathogens from fecal pollution, introduced into aquatic environments, can have a negative impact on the receiving biota and overall ecosystem health. The Danube which is the second longest river in Europe flows through several countries from where, it receives discharges of agricultural, industrial and urban effluents. Moreover, it is a theatre

for heavy navigation, which is another major problems for human health and environmental quality⁹.

Subsequent to the research conducted within The Joint Danube Survey (JDS), microbiological water quality of the Danube River has been classified on the basis of standard parameters in five classes: little pollution, moderately pollution, critical pollution, strongly pollution and excessively pollution. Based on samples collected from Romania, strongly fecal pollution was observed in the Danube Delta¹⁰.

It is known that the late 20th century was characterised by a significant increase of bacterial infections pathogenicity. It was directly correlated with the use of antibiotics that exert a selective pressure on bacterial populations. Microorganisms with the ability to populate all types of ecosystems can develop antibiotic resistance through multiple genetic transfer mechanisms.

In Romania, one of the most important sources of drinking water is the Danube River, along which there are treatment plants. Unfortunately, some areas from Danube Delta are used as raw surface water for drinking, but without disinfection processes. This is a strong reason to support the aquatic ecosystem quality control. The aim of this paper was to monitor the diversity of fecal bacteria populations in aquatic ecosystem from the Danube Delta.

EXPERIMENTAL

The analyses were seasonal performed in 2014. There were established eleven control sections to cover all St. Gheorghe Branch, the oldest branch of Danube Delta. The sampling points are illustrated in Fig. 1.



Fig. 1. Sampling points on the St. Gheorghe Branch (S1 – Iscacea, S2 – upstream Tulcea, S3 – downstream Tulcea, S4 – Nufaru, S5 – Balteni, S6 – Mahmudia, S7 – Murighiol, S8 – Uzlina, S9 – Ivancea, S10 – St. Gheorghe port, S11 – Black Sea confluence)

The cap of the sterile sampling bottle was removed just before taking an aliquot of water from St. Gheorghe Branch. Gloves were used to avoid any contamination of the sample or researcher. The bottle was plunged downwards below the water surface. All samples were preserved at 4°C then rapidly tested in order to avoid bacterial growth.

The surface water samples were assessed for microbiological contamination level with fecal bacteria as well as their antibiotic resistance. The quantitative analysis was based on most probable number technique. The presumptive test was carried out with a sterile lactose broth test and the confirmation test based on Brilliant Green Lactose broth and oxidase test. 10 ml of both liquid mediums were distributed in small tubes with Durham tube and they were inoculated with 10 ml of surface water. Samples were incubated 24 h at $35\pm 2^\circ\text{C}$ in the presumptive test and positive samples in which accumulated gas and lactic acid were transferred on confirmation mediums for 48 h. The bacteria species were identified with say in words before – if you did not before – (API) system and they were specifically tested on Muller–Hinton medium by disk diffusion method and Clinical and Laboratory Standards Institute (CLSI) recommendations¹¹.

RESULTS AND DISCUSSION

Numerous studies have shown that individual bacterial populations are highly dynamic and they can strongly vary their response in function of two resource availability. Others have shown that shifts in microbial community structure can be related to seasonal cycles in the water source and dissolved organic matter. Similarly, seasonal shifts in water column stability and water temperature may demonstrate an annual pattern of bacterial community variability¹².

The major sources of pollution in the Danube Delta Biosphere reservation are represented by the economic units from the nearby area, and also by the naval transportation¹³. River water for anthropogenic use, transportation and recreation has a major importance for St. Gheorghe Branch. Microbiological contamination from fecal pollution by anthropogenic sources is considered to be a crucial problem¹⁴. The ecological and survival characteristics of bacterial pathogens vary under environmental conditions, indicating probably that no single indicator organism can predict the presence of all fecal pathogens for all types of water. Application of conventional and alternative fecal indicators is critically evaluated in relation to the presence of commonly investigated pathogens¹⁵.

The determination of fecal indicator concentrations along St. Gheorghe Branch within the eleven control sections allowed to draw a picture of a fecal pollution patterns of this area. The results obtained by quantifying the total coliform bacteria and *Enterococcus* sp. are presented in Figs 2a, b.

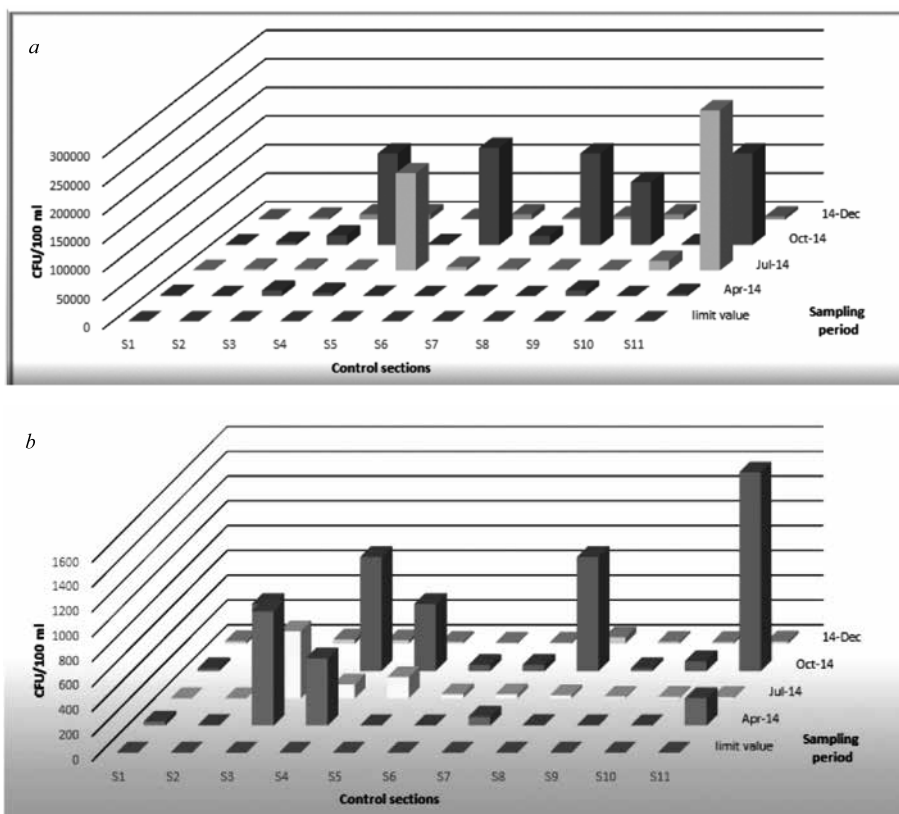


Fig. 2. Spatial and temporal variation of coliform bacteria (a) and *Enterococcus* (b) density in surface water from St. Gheorghe Branch during 2014; measure units are CFU/100 ml; on the ordinate axis are the sampling points and the abscissa are sampling campaigns

The highest coliforms densities recorded in surface samples from S3 (downstream Tulcea) and S9 (Ivancea) in the first campaign carried out in April 2014, and S5 (Balteni) and S11 (Black Sea Confluence) in July 2014. Significant amounts of coliform bacteria density were observed in S4 (Nufaru), S6 (Mahmudia), S8 (Uzlina), S9 (Ivancea) and S11 (Black Sea confluence) in October 2014.

Enterococci density was recorded in high-value in surface samples from S3, in the first three campaigns of 2014. In October 2014, the number of *Enterococci* populations significantly increased from previous months and it can be observed high values in S4 (Nufaru), S5 (Bălteni), S8 (Uzlina) and S11 (Black Sea confluence).

The number of fecal indicators decreased in the winter, likely influenced by the sharp drop in temperature.

Most of *Enterobacteriaceae* strains from water samples presented resistance to ampicillin + clavulanic acid and ampicillin in April 2014. *Citrobacter freundii*

(Fig. 3) strains resistant to tetracycline were identified with a higher frequency and *Pseudomonas fluorescens* resistant to all tested antibiotics was identified in S9 (Ivancea).



Fig. 3. *Citrobacter freundii* isolated from S10 (St. Gheorghe Port) water sample and identified with API 20E; it was resistant to amoxicillin+clavulanic acid, ceftazidime, ampicillin, chloramfenicol, nalidixic acid and tetracycline

High values of *Enterococcus* density was observed in S3 (downstream Tulcea) and S4 (Nufaru) and there were identified antibioresistant *Aerococcus viridans* (Fig. 4).

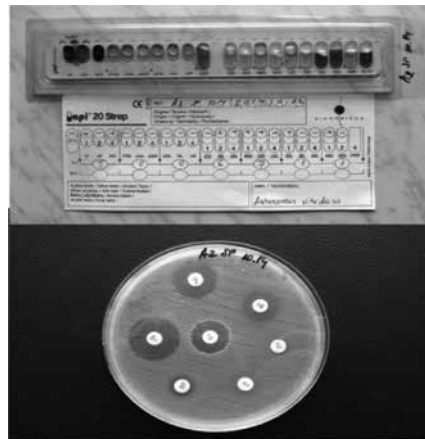


Fig. 4. *Aerococcus viridans* isolated from S2 (downstream Tulcea) water sample and identified with API Strep; it was resistant to ampicillin, gentamicin and tetracycline

The coliform density was remarkable in July 2014 in S5 (Balteni) and S11 (Black Sea confluence). All identified and tested strains were β -lactams resist-

ant, but there are some strains that presented resistance to other antibiotic types such as: *Proteus mirabilis* (Fig. 5) resistant to thrimetoprim sulphametoxazol and *Enterobacter cloacae* resistant to thrimethoprim sulphametoxazol, nalidixic acid, tetracycline, chloramphenicol, gentamycin and erythromycin.

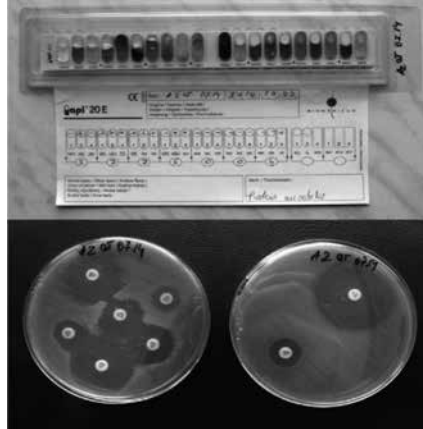


Fig. 5. *Proteus mirabilis* isolated from S2 (downstream Tulcea) water sample and identified with API NE; it was resistant to ampicillin and gentamicin

The highest value of *Enterobacteriaceae* strains recorded in October 2014 was in the water sample collected from S6 (Mahmudia), where it was identified *Salmonella* spp. (Fig. 6) resistant to β -lactams and thrimethoprim sulphametoxazol, chloramphenicol, nalidixic acid and tetracycline. Although the number of coliforms in S7 (Murighiol) was not remarkable, it was identified antibioresistant *Enterobacter gergoviae*.

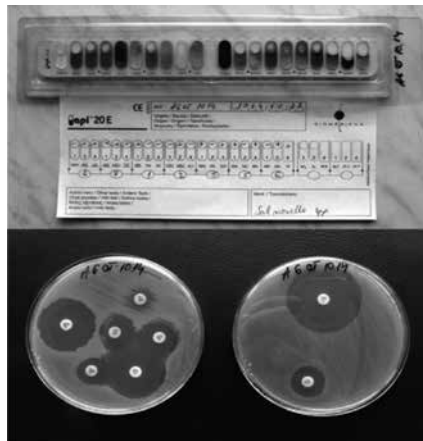


Fig. 6. *Salmonella* spp. isolated from S6 (Mahmudia) water sample and identified with API 20E; it was resistant to β -lactams and ampicillin and gentamicin

As regards an analysis of the curve of the density of fecal indicators, it can see the same trend of development both for coliforms and *enterococci* during 2014 (Fig. 7).

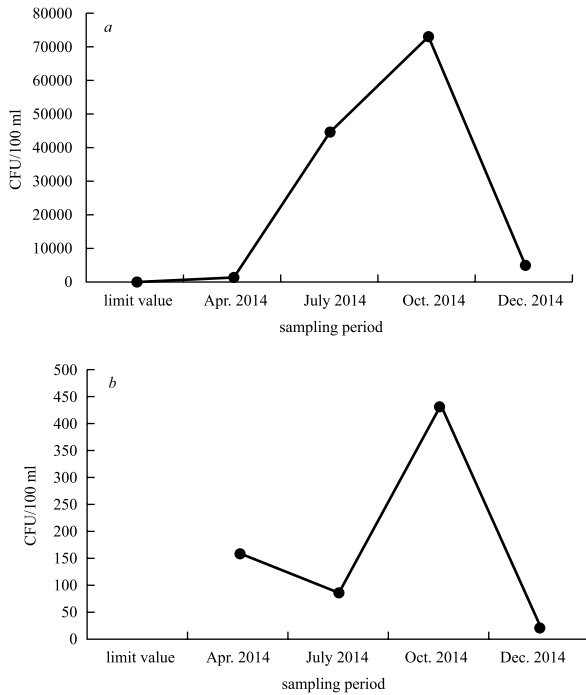


Fig. 7. Temporal variation of coliform bacteria (a) and *Enterococcus* (b) density during 2014

CONCLUSIONS

The processes of anthropic and socio-economic activities carried out in the Danube Delta causes physicochemical and microbiological contaminations in both aquatic ecosystem. Microbiological pollution can become extensive and complex as microorganisms can penetrate into all ecosystems due to their great adaptability and high rate of dissemination and infection. Infections difficult to treat are facilitated when microorganisms develop antibiotic resistance. Both coliform bacteria and *Enterococcus* densities in surface water recorded high values in July and October 2014 and the lowest values recorded in the winter campaign. Some antibiotic resistant bacteria were identified.

The results have shown the presence of fecal microorganisms in the surface water of St. Gheorghe Branch in quantities that impose the application of environmental securing measures to avoid the propagation of communicable diseases which can cause water borne outbreaks to the community exploiting the water for domestic purposes.

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