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Study of the hydrocarbon-oxidizing activity of bacterioplankton in the Moldavian section of the Prut River

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Abstract
The paper presents the results of study of the Prut River heterotrophic bacterioplankton. The bi-disciplinary technique was used to assess its hydrocarbon-oxidizing activity, namely: microbiological research was carried out in parallel with hydrochemical investigation of the natural water samples and samples enriched with diesel fuel. The Moldovan-Romanian transboundary section of the Prut River includes various ecological zones, such as the Costești-Stanca Reservoir, the mouth of the Jijia River, the Prut floodplains with the relict Beleu Lake (where oil extraction is carried out despite the fact that it is a protected area of the scientific reserve "Prutul de Jos") and the Prut River mouth with the Giurgiulesti International Free Port. The microbial community of fluvial bacterioplankton corresponds to the specific conditions of each these biotopes, being the most reproducible and adaptable component of the biota. The results obtained in the study of the Prut River bacterioplankton demonstrate the presence of hydrocarbon-adapted microorganisms with a good potential to utilize petroleum products in their metabolic processes.

Keywords: Prut River, heterotrophic bacterioplankton, hydrocarbon-oxidizing microorganisms

1. INTRODUCTION

Oxidation of organic substances (including hydrocarbons from fuels and lubricants) by aquatic microbiota is attended by consumption of dissolved oxygen. The decrease in oxygen concentration after *n* days of incubation in a hydrocarbon-enriched sample compared to a natural water sample can serve as a convenient method for assessing the potential ability of aquatic microorganism to biooxidation of such contaminants [1].

Heterotrophic bacteria that degrade petroleum hydrocarbons are widely distributed in nature, which makes it possible to use them for bioremediation of oil-contaminated biotopes. An overview of the literature provides insight into which representatives of this group of microorganisms can use petroleum products as a carbon and energy source: *Pseudomonas sp*., *Pseudomonas aeruginosa*, *Rhodococcus ruber*, *Geobacillus thermodenitrifican*, *Rhodococcus sp*., *Alcanivorax sp*., *Gordonia sihwensis*, *Achromobacter xylosoxidan*, *Aeribacillus pallidus*, *Mycobacterium sp*., *Bacillus sp*., *Enterobacter sp*., *Staphylococcus sp*., *Citrobacter sp*., etc. [2]. The listed taxa of bacteria are not a specific physiological group of microorganisms (MOs[1]) adapted for the biodegradation of exclusively petroleum hydrocarbons. Possessing a wide range of enzymatic apparatus, they can switch to the use of the organic substrate that is most available under the given habitat conditions. In aquatic ecosystems (not associated with the oil extraction process), the main source of oil pollution is shipping, industrial

[1] Abbreviations MO and MOs are used by the European Food and Safety Authority (EFSA) [3]

wastewater discharges and storm water run-off from roads and petrol stations. In the composition of bacterioplankton communities of such ecosystems, heterotrophic bacteria from the genera *Bacterium*, *Pseudomonas*, *Mycobacterium*, *Micrococcus*, *Pseudobacterium* and *Rhodococcus* showed the ability to oxidize oil hydrocarbons [3]. The fact that hydrocarbon-oxidizing MOs are an integral part of heterotrophic microbiota of aquatic ecosystems contaminated with petroleum hydrocarbons is confirmed in many research works [4–5].

The study is part of the AQUABIO project, implemented by researchers from the Centre of Hydrobiocenoses and Ecotoxicology within the framework of the State Program "Environment and climate change" (2020–2023). The AQUABIO project covers a wide range of ecosystem problems relevant for the main rivers of the Republic of Moldova (Dniester and Prut) under anthropogenic impact and climate changes, namely: functioning of hydrobiocenoses, migration and impact of pollutants, prevention of harmful effects on ecosystems, etc. In this regard, the following task was formulated: 1) to analyze the seasonal and spatial dynamics of the abundance of heterotrophic MOs, including hydrocarbon-oxidizing bacteria; 2) study of the capacity of the Prut River bacterioplankton to respond to the presence of hydrocarbons in the aquatic habitat by mobilizing oxidative activity.

2. EXPERIMENTAL

Water samples were collected in the winter, spring and summer seasons 2023 at the Costesti, Braniste, Sculeni, Leuseni, Cahul, Cislita-Prut and Giurgiulesti stations on the left bank of the Prut River, which are long-term monitoring sites established by the Laboratory of Hydrobiology and Ecotoxicology for its research programs. At the sampling site, measurements of the main physico-chemical parameters were performed: water temperature, pH value, conductivity, dissolved oxygen concentration. The remaining investigations (hydrochemical and microbiological) were carried out under laboratory conditions.

Microbiological samples were collected in sterile glass bottles of 500 mL from the near-surface water layer (0.25 m depth). In these samples, heterotrophic bacterioplankton and MOs capable of growing on a culture medium enriched with diesel fuel as the only source of carbon and energy were determined. Representatives of both groups were detected at the level of colonies by the dishes pour-plate method, followed by incubation at 22°C [6]. The colonies were counting in accordance with the national standard SM SR EN ISO 6222:2014 [7]. The results of counting the culturable microorganisms were expressed in "colony forming units" (CFU/mL).

In hydrochemical samples, biochemical oxygen demand after 5 days of incubation (BOD_5) in a natural and hydrocarbon-enriched samples was determined according to the standard method EN 1899-2 for undiluted samples [8]. The method simulates the natural process of self-purification of water bodies due to biochemical oxidation of organic substances by microbiota. To exclude oxygen consumption by phyto- and zoo- potamoplankton during sample incubation, water was siphoned into incubation flasks through a silicone hose, the opening of which was protected by a plankton net with a mesh size of 45 µm. The main equipment used were: incubator INCUCELL-111, cooled incubator FOC 120E, colony counter COLONY STAR (Scanner + Tablet Computer Standard), germicidal device MEDIVENT 60F.

3. RESULTS AND DISCUSSION

The abundance and composition of heterotrophic bacterioplankton in river water bodies is determined by a combination of many factors, both natural (ecoregion, climate, hydrological regime, geological structure of the river bed, state of bottom sediments, specification of river biota, etc.) and anthropogenic (disturbance of the natural watercourse by hydro-technical constructions, pollution by household, industrial and pharmaceutical wastes, etc.). According to published data, the abundance of heterotrophic bacterioplankton in the Costesti – Giurgiulesti section of the Prut River varies in a wide range from 40 to 12800 (CFU/mL) [9]. The highest number was confined for the summer season, i.e., summer water temperatures. However, weather conditions can disrupt this trend, for example, in the case of spring floods or a significant decrease in river flow during hydrological droughts, which currently occur in the Republic of Moldova not only in summer, but also in spring and autumn. Due to severe drought in September 2015, the water level in the Costesti-Stanca Reservoir dropped by 2 m, and therefore the operation of the Costesti Hydro Power Plant was stopped for several months (for the first time since its start-up in 1978).

In the presented work, the number of heterotrophic bacterioplankton and hydrocarbon-oxidizing MOs in the studied section of the Prut River varied within 100 – 9500 and 40 – 900 (CFU/mL), respectively. In the graphs (Fig. 1 – 3), their number is presented in % ratio due to the large range of scatter of absolute values.

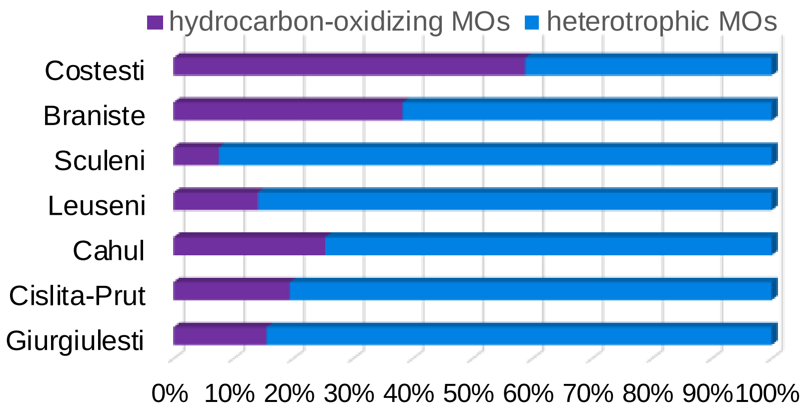

Fig. 1. Prut River, February 2023: proportion of hydrocarbon-oxidizing MOs in relation to heterotrophic bacterioplankton

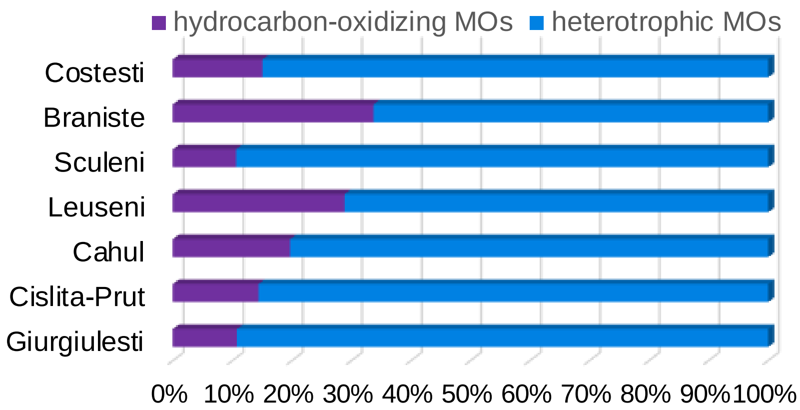

Fig. 2. Prut River, April 2023: proportion of hydrocarbon-oxidizing MOs in relation to heterotrophic bacterioplankton

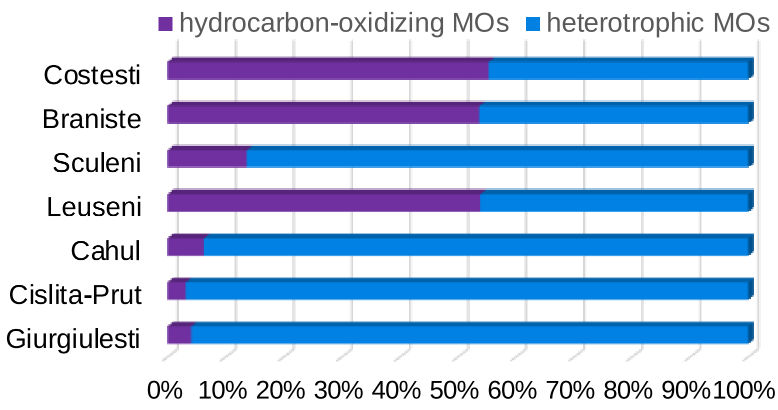

Fig. 3. Prut River, July 2023: proportion of hydrocarbon-oxidizing MOs in relation to heterotrophic bacterioplankton

The presence of hydrocarbon-oxidizing MOs in navigable river sections has been confirmed by scientific studies [3]. For 25 years, the Lower Prut was not navigable. But in 2013, navigation was resumed. Now barges with construction materials and other cargoes run from Cahul to the Giurgiulesti Port. Heterotrophic bacterioplankton responds to this by increasing the proportion of hydrocarbon-oxidizing MOs, which is clearly visible in winter samples when water level is low (Fig. 1).

In addition to this, the lower section of the Prut River crosses the oil-bearing horizon, located mainly on the territory of Romania. There is no petroleum extraction directly in the riverbed, but there are more than 20 boreholes in the floodplain section of the river – at the Beleu Lake. During spring or rain floods, runoff from floodplain area flows into the Prut River, enriching the river bacterioplankton with allochthonous lake microflora adapted to the presence of hydrocarbon in the water. This river site is located between Cahul and Cislita-Prut stations of our research area.

The Giurgiulesti sampling point is situated in close proximity to the confluence of the Prut River into the Danube. This is where the Giurgiulesti International Free Port (with its Oil Products Terminal) is located. Depending on weather conditions and hydrological situation, Danube water reflux is observed at this station, affecting both physico-chemical and microbiological parameters of instantaneous samples. Taking into account that Oil Products Terminal is located directly on the bank, it is to be expected that the microbiota of this river site should be adapted to hydrocarbon pollution.

Our long-term studies of the Prut River ecosystem show that the section downstream of the Costesti HPP is a potential zone for adaptation of river microbiota to hydrocarbons. This is quite logical if you consider that the most important components of the Electrical Power System are Transformer Stations. Transformer oil, which serves in transformers as a heat-removing medium and an integral part of the insulation, is a petroleum product. During the operation of stations, oil enters the environment: onto the soil, into groundwater, washed away by precipitation into the river, and ends up in bottom sediments, where it accumulates. Bottom layers in such areas are a zone of adaptation of microbiota to hydrocarbons. As a result of turbulent processes in watercourses, there is a constant exchange of bacteriobenthos and bacterioplankton communities. In this way, the bacterioplankton at the Braniste station is replenished with hydrocarbon-adapted microflora (Fig. 4).

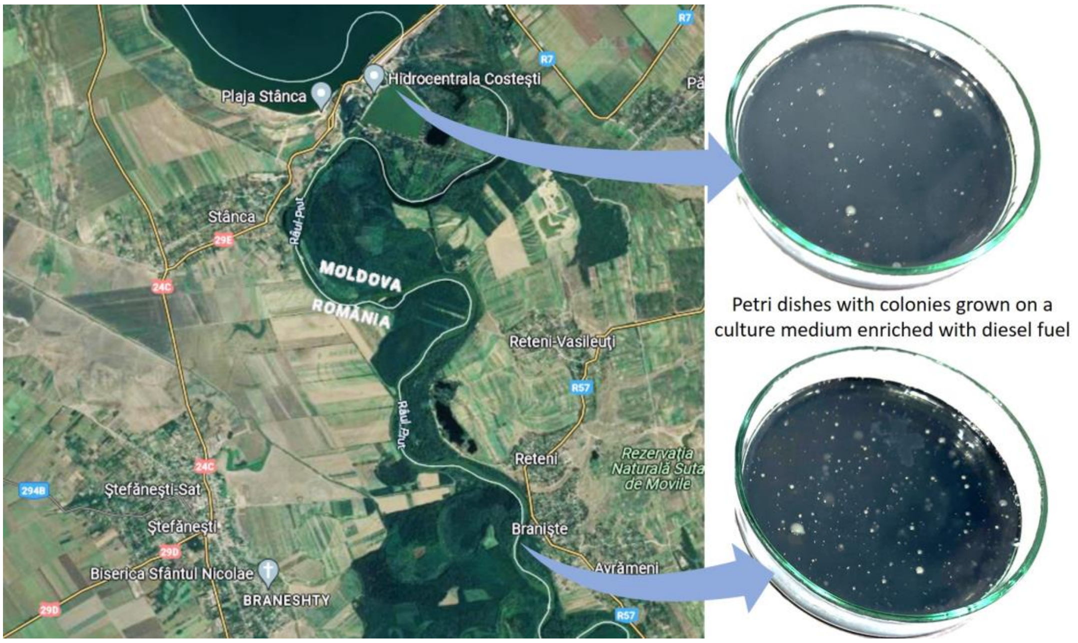

Fig. 4. Colonies of hydrocarbon-oxidizing MOs (black background) detected in water samples collected in April 2023 at the Costesti and Braniste stations

In our studies of the Dniester River ecosystem, it was shown that there is a correlation (negative, strong: R = -0.74) between the number of hydrocarbon-oxidizing bacteria in a water sample and the BOD_5 parameter [10]. The same correlation was found in the present study for the Costesti – Giurgiulesti section of the Prut River: weaker in summer (R = -0.21) and stronger in winter (R = -

0.70). Graphical analysis of this phenomenon is presented in Figure 5 and means the following: in those sections of the river where the abundance of this group of heterotrophic bacterioplankton is high, the BOD value is already low. And vice versa: where the number of hydrocarbon-oxidizing MOs is low, the BOD is still high. Given the fact that the BOD parameter serves as an indicator of organic pollution, we have the reason to conclude about the important role of this group of microorganisms in the self-purification processes of the Prut River. This chart also demonstrates a significant number of hydrocarbon-oxidizing MOs at the Leuseni station, where the congestion of vehicles at the border crossing point Leuseni-Albita has a direct impact on both terrestrial and aquatic habitats.

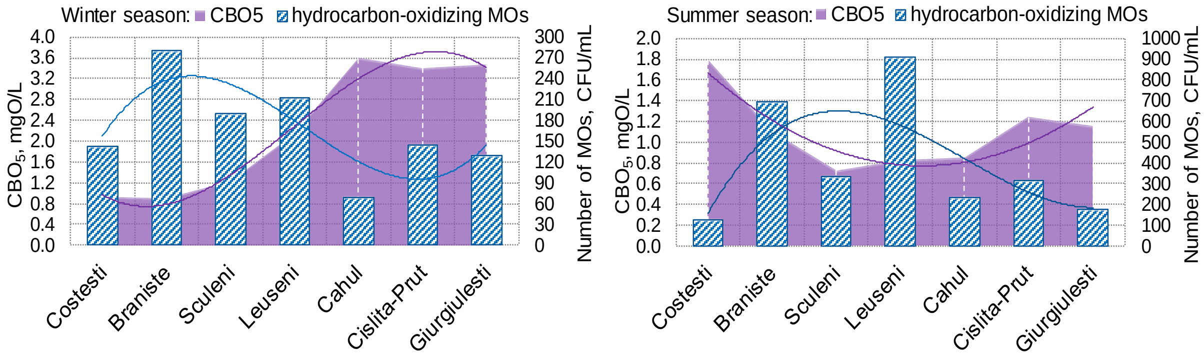

Fig. 5. Graphical analysis of negative correlation for BOD_5 parameter and number of hydrocarbon-oxidizing MOs in the Prut River water samples

To assess the rate of adaptation of heterotrophic bacterioplankton to the biooxidation of hydrocarbons, an experiment was set up with spring and summer water samples from the Lower Prut. The biochemical oxygen demand (BOD_5) of natural samples and samples enriched with petroleum hydrocarbon (PH), diesel fuel in our case, was determined. The experimental conditions ensured incubation of water samples (in flasks with sealed lids, without access to light and oxygen), from which phyto- and zoo-microplankton were removed beforehand. Thus, the decrease of oxygen concentration in the flasks after 5 days of incubation was attributed with high probability to the bioactivity of bacterioplankton. It was selected two sampling points for the experiment, namely: Cislita-Prut and Giurgiulesti (Fig. 6). Results of the experiment are presented in the Table 1.

Fig. 6. Cislita-Prut and Giurgiulesti sites on the Prut River (photo by Bagrin Nina)

Table 1. Biochemical consumption of oxygen by the Prut River heterotrophic bacterioplankton under laboratory experiment conditions

Sampling station	Sampling season	BOD_5 in natural samples, mgO/L	BOD_5 in samples enriched with PH, mgO/L	Oxygen consumed for biooxidation PH, mgO/L
Cislita-Prut	spring	1.89	3.30	1.41
	summer	1.24	4.00	2.76
Giurgiulesti	spring	1.55	3.07	1.52
	summer	1.16	2.93	1.77

The results obtained demonstrate a high ability of the Prut River heterotrophic bacterioplankton to utilize petroleum hydrocarbons in their metabolic processes. In almost all cases, oxygen consumed for PH biooxidation exceeded its consumption for biooxidation of allochthonous organic matter in the samples.

Statistical analysis of this study database shows that the abundance of hydrocarbon-oxidizing MOs correlates very weakly with water temperature (R = 0.28) compared to the total number of heterotrophic bacterioplankton (R = 0.40). And there is practically no correlation between them (R = 0.06). This allows us to conclude that under conditions of anthropogenic load, the natural factor (water temperature) affects the quantitative characteristics of bacterioplancton communities to a lesser extent than the ecological state of the water body.

4. CONCLUSIONS

Aquatic microbiota (bacterioplankton, bacteriobenthos, heterotrophic epiphyte bacteria, etc.) plays a decisive role in the processes of biodegradation of autochthonous and allochthonous organic matter in surface water ecosystems, thus participating in the processes of self-purification and ensuring the cycle of elements in the habitat.

Hydrocarbon-oxidizing bacteria are not a specific group of heterotrophic bacterioplankton. The presence of hydrocarbons in the habitat stimulates part of the microbial community to utilize this organic substrate as well. Therefore, an increase in the number of hydrocarbon-oxidizing MOs in relation to the total number of heterotrophic bacterioplankton can serve as an indicator of hydrocarbon pollution. It should be taken into account that rivers are watercourses. And if an increase in the number of hydrocarbon-oxidizing MOs is detected at a given monitoring site, it can be assumed that pollution could have occurred upstream.

In the Costesti– Giurgiulesti section of the Prut River, the proportion of hydrocarbon-oxidizing MOs in relation to total heterotrophic bacterioplankton ranged from 2 to 55 % during the analyzed period. Seasonal dynamics is not expressed. Along the longitudinal profile of the river, the highest percentage were recorded at the Costesti, Braniste and Leuseni stations.

The negative correlation between the number of hydrocarbon-oxidizing microflora and the BOD value at a certain river section confirms the important role of this group of MOs in the biodegradation of organic pollutants. The high adaptability of the Prut River bacterioplankton to the presence of petroleum hydrocarbons in the water habitat was demonstrated by the results of a laboratory experiment.

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