Epiphytic Bacteria Inhabiting the Yellow Waterlily *(Nuphar luteum* L.)

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Abstract

Studies on the development dynamics and the physiological properties of heterotrophic bacteria growing on the surface of the yellow waterlily {*Nuphar luteum* L.) during the plants' development cycle were carried out. It was stated that the number of the epiphytic bacteria is different in the examined sections of the plant. The number is higher on bottom parts of petioles and rhizomes but lower on the surface of leaf blades and petioles beneath the leaf blade. The number of the epiphytic bacteria oscillated between 0.54 x 10^6 and 37.85 x 10^6 cells per lg of wet weight of the plant. The gram negative rods dominated among the epiphytic bacteria, the majority of which was slowly growing strains. Among the epiphytic bacteria the most numerous strains were those hydrolyzing fat, starch, and protein; the least numerous were the chitinolytic bacteria.

Keywords: epiphytic bacteria, heterotrophic bacteria, generic composition, physiological properties, macrophytes

Introduction

It is estimated that the number of epiphytic bacteria on the surface of algae is between 10^3 and 10^6 cells/cm^2 [1]. Their number on the surface of tissue plants oscillates between 10^5 and 10^6 cells/cm^2 [2, 3, 4, 5].

Epiphytic bacteria colonizing a plant are food for numerous protozoans, snails, mayfly larvae and *Chironomidae* larvae [6]. For many detritus eaters they are a source of biogenes such as nitrogen, carbon, phosphorus, and sulphur [4]. According to Baker [6], secretions of epiphytic bacteria are crucial nutrition for freshwater snails. Epiphytic microflora also produce growth factors which can be used by macrophytes [6]. The research conducted by Strzelczyk and Mielczarek [7] shows that the metabolic activity of the epiphytic bacteria is higher than that of planktonic and benthic bacterias, and depends on a colonized plant and metabolized substaces.

The objective of this research is identification of the heterotrophic epiphytic bacteria inhabiting the yellow waterlily, determining their number, development dynamics, morphology and some physiological properties.

Materials and Methods

Study Area

The object of microbiological research were epiphytic bacteria colonizing the yellow waterlily *(Nuphar luteum* L.), growing in the littoral zone of Moty Bay, located in the southern part of Jeziorak Lake. This lake belongs to the Hawian Lake District and the Drweca - Vistula catchment area. This is an eutrophic water basin of the channel type, meridionally oriented, which was created during the last glaciation. The lake surface is 32.3 km^2,

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length - 27.45 km, the mean width about 1.2 km, maximum depth - 12.9 m, and average depth 4.3 m. The shoreline is well - developed and consists of numerous bays, including strongly eutrophicated Moty Bay. The water is yellow - green with relatively little transparency (0.8 - 2.0 m) and alkaline reaction (pH 7.8 - 8.5). More detailed data on this lake are given by [8, 9,10, 11].

Sampling

Material for the studies was gathered during the vegetation cycle of the yellow waterlily in summer and autumn of 1999 and in spring, 2000. Research on the epiphytic bacteria comprised strains existing on floating leaves, petioles (15 cm - a petiole's section below a leaf blade and 15 cm in the extinguish sunlight area) and also rhizomes along with adventitious roots. The leaves, petioles' sections and rhizomes' sections (selected with a drag net) were placed in sterile glass jars and transported to the laboratory in an ice container (the temperature inside did not exceed +7°C) where they were immediately subjected to analysis. The time between sampling and analyzing was no longer than 6 hours.

Estimating the Number and Development Dynamics of the Epiphytic Bacteria

In order to estimate the numbers of epiphytic bacteria on the yellow waterlily, the sections of the material being used (10 g) were flooded with 90 ml of sterile buffer water [12] and homogenized in a Unipan type 329 homogenizer for 2 minutes at 4 thousand radiations per minute. Next there was a set of 10-rated dilutions prepared from the obtained homogenate. The total number of the epiphytic bacteria (TVC) was determined by means of spread plates method, inoculating the material on the iron-peptone agar medium according to Ferrer, Stapert and Sokolski [13]. All the seedings were done in three parallel repetitions and the inoculated plates were incubated at a temperature of 20°C. The grown colonies were counted after 3 and 10 days; determining the grown strains as fast - and slowly growing. The results were calculated per lg of wet plant mass.

Table 1. Number of epiphytic bacteria occuring on surface of the yellow waterlily (x 10^6/g wet plant mass).

Date of sampling	Section of plant			
	I	II	III	IV
Spring 08.05.00	2.75	3.02	9.22	4.33
Summer 03.08.99	10.62	5.30	12.27	37.85
Autumn 13.10.99	0.54	1.60	4.83	1.04

Explantations: I - surface of leaves, II - petiole (15 cm - a petiole section below a leaf blade); III - petiole (15 cm - a petiole section in the extinguish sunlight area); IV - rhizomes along with adventitous roots.

Isolation Bacterial Strains

After the incubation and counting of the bacterial colonies, 25 colonies from each experiment were randomly picked and transferred into a semi-liquid iron-peptone agar medium (5 g of agar/1) and incubated for 6 days at a temperature of 20°C. After checking the cleanliness of the bacterial cultures in the slides coloured with the Gram method, the strains were stored at a temperature of +4°C. The strains were grafted into a fresh iron-peptone medium every two months.

Morphological Studies

The morphology of epiphytic bacteria and their diversification into Gram negative and Gram positive were examined in the slides coloured with the Gram method [14]. For the research, strains were used after 2 and 5 days of the incubation in a liquid iron-peptone medium, at a temperature of 20°C.

The Identification of Epiphytic Bacteria

The bacteria isolated from the surface of the examined plant were identified to genera and groups according to the scheme suggested by Schewan, Hobbs and Hodgkins [15] and data published in papers by Hendrie, Mitchell and Shewan [16], Thornley [17] and Buchanan and Gibbons [18].

Physiological Properties

The isolated epiphytic bacteria were seeded into a series of test media containing various organic compounds. In this research a few properties were taken into consideration: an ability to hydrolise protein, starch, fat, pectin, cellulose, and chitin and an ability to carry out the process of amonification and acidification of a glucose medium. The media used for the research were prepared after Donderski [19, 20] and Lalke-Porczyk [21].

Results

The results of the research on the number of heterotrophic epiphytic bacteria are shown in Table 1. The average number of the bacteria on the surface of the yellow waterlily was from 0.54 x 10^6 to 37.85 x 10^6 cells/ lg of wet plant mass. On the basis of the obtained results, it is possible to draw a conclusion that the number of heterotrophic epiphytic bacteria depends on a season of the year, a stage of the plant's vegetative development, and the examined surface of a plant. The maximal number of epiphytic bacteria was found on the surface of the yellow waterlily's rhizome in summer, but the minimal one was found on the yellow waterlily's leaf blade in autumn. As a rule there were more bacteria on the lower bottom parts of the plant, namely on the surface of the petiole in the extinguish sunlight area and on the surface of the

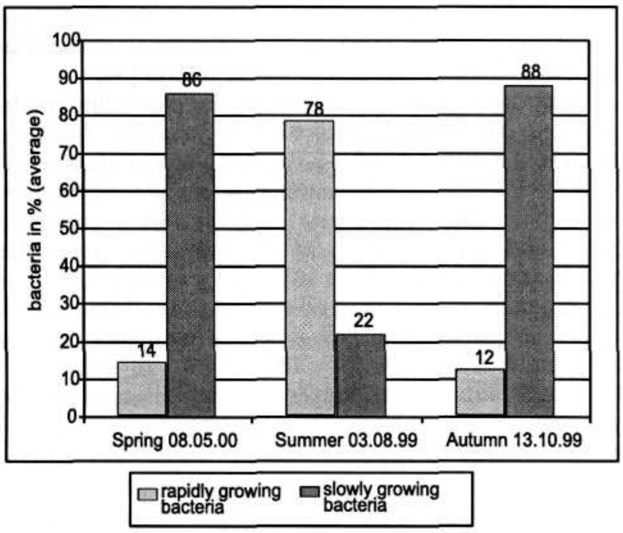

Fig. 1. Development dynamics of epiphytic bacteria growing on surface of the yellow waterlily (average).

rhizome than on the surface of the leaves and the petioles below the leaf blades.

The development dynamics of heterotrophic epiphytic bacteria is shown in Figure 1. The obtained results prove that slowly growing bacteria dominated among the epiphytic bacteria existing on the yellow waterlily. They accounted for 72% - 96% of all the strains examined in autumn. However, rapidly growing bacteria dominated in summer and accounted for 42% - 96% of all the epiphytic bacteria.

Among the morphological types of the epiphytic bacteria inhabiting the yellow waterlily Gram negative rods dominated (Figure 2). On average they accounted for 56%-80% of total bacteria. The pleomorphic forms were also relatively numerous. Their average number was from 15% to 36%. The Gram positive cocci and bacilli were the smallest group among the epiphytic bacteria, their presence was only observed in spring samples.

The results concerning the identyfication of the bacteria inhabiting the yellow waterlily are shown in Table 2. On the basis of the conducted research one can draw a conclusion that the generic composition of the epiphytic bacteria vegetating on the yellow waterlily depends on the examined plant's surface and season of the year.

Table 2. Generic composition of epiphytic bacteria inhabiting surface of the yellow waterlily (average - bacteria in per cent).

Genus or group of bacteria	Spring (08.05.00)	Summer (03.08.99)	Autumn (13.10.99)
Pseudomonas	6	9	11
Flavobacterium – Cytophaga	29	28	33
Aeromonas – Vibrio	2	12	8
Enterobacteriace	30	12	15
Arthrobacter – Corynebacterium	17	3	14
Bacillus	10	1	4
Alcaligenes	1	8	8
Achromobacter	5	27	9

Explantations: I - surface of leaves, II - petiole (15 - cm section below the leaf blade), III - petiole (15 - cm section in the extinguish sunlight area), IV - rhizomes along with adventitous roots.

Most of the strains examined in all the periods belonged to the groups of *Flavobacterium-Cytophaga* and *Enterobacteriaceae.* Another relatively numerous group; from spring to summer, was *Pseudomonas.* Pleomorphic bacteria belonged to the *Arthrobacter-Corynebacterium* group were considerable accounted in spring and autumn. In summer, *Achromobacter* genus occered in a great number. The physiological properties of the yellow waterlily epiphytic bacteria have been shown in Figure 3. From this data it appears that the biggest groups

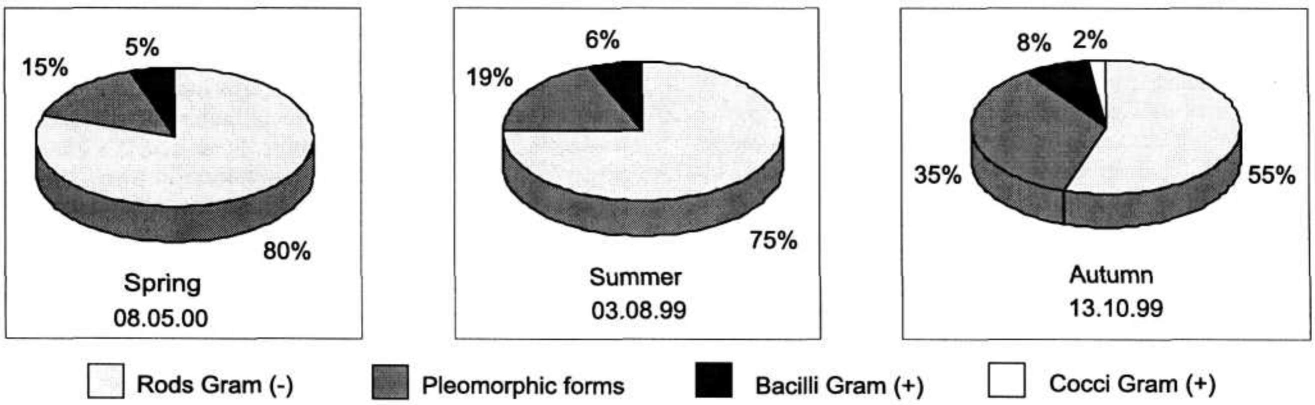

Fig. 2. Morphological types among epiphytic bacteria inhabiting surface of the yellow waterlily (average).

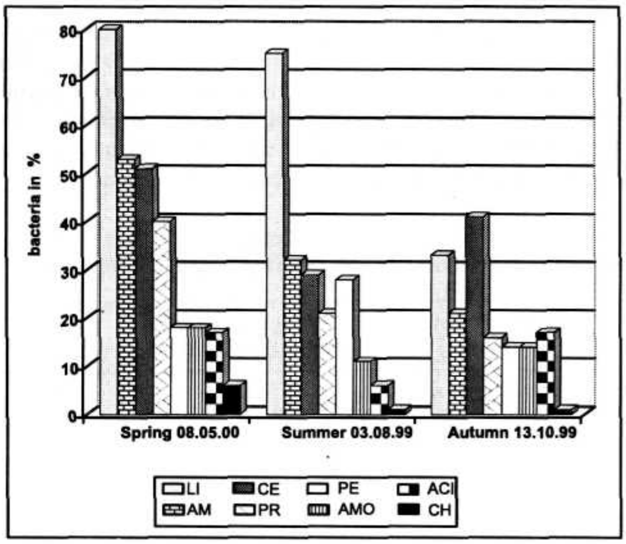

Fig. 3. Physiological groups of epiphytic bacteria isolated from surface of the yellow waterlily (average)
Explantations:
LI - lipolytic bacteria, AM - amylolytic bacteria, CE - celulolytic bacteria, PR - proteolytic bacteria, PE - pectinolytic bacteria, AMO - bacteria earring out process of ammonification, ACI - bacteria earring out process of acidification of glucose medium, CH - chitinolytic bacteria

among the yellow waterlily epiphytic bacteria were hydrolysing strains: fat (32% - 83%), starch (12% - 65%), and protein (6% - 48%). The least numerous groups among the epiphytic bacteria were those hydrolyzing chitin (1% - 6%), acidifying a glucose medium (6% - 17%), and carrying out the process of ammonification (11% - 18%). The most numerous epiphytic bacteria hy drolyzing fat, starch and protein were found in spring.

Discussion

The heterotrophic bacteria present in natural environment make the biggest group of organisms which are active in the processes of decomposition of organic matter. Besides the benthic and planktonic bacteria, the epiphytic bacteria, in terms of organic substances decomposition in water basins play an important role. According to Strzelczyk and Mielczarek [7], they are more metabolic active than the other bacterial groups.

The research on the number of the heterotrophic epiphytic bacteria inhabiting the yellow waterlily shows that it is seasonally changeable and depends on an inspected part of the plant. The maximal number of these bacteria was found in summer and the minimal one in autumn.

Niewolak [22] and Olah [23] explain the summer maximum due to increased content of organic substances, secreted by living plants and an increase of water temperature. An increase of the allochtonous pollution, resulting from heavier tourist activity, can occasionally evoke the number of bacteria in summer. A considerable decrease of the bacteria number in spring could have resulted from a little concentration of nutrients and water temperature. Moreover, a big number of the bacteria are eaten by animals in winter season [24].

This research points out that the heterotrophic epiphytic bacteria inhabiting the yellow waterlily fluctuate from 0.54 x 10^6 to 37.85 x 10^6 cells/lg of wet plant mass. Lalke - Porczyk [21], examining the number of the heterotrophic epiphytic bacteria from the yellow waterlily's lower and upper surfaces of the leaf blade, found the number of 0.7 x 10^7 to 7.5 x 10^7 cells/1 g of dry plant mass. On the other hand, Conover and Sieburth [25] claim that the number of bacterial cells per lg of dry mass of *Sargassum natans* is between 2.0 x 10^1 and 2.0 x 10^5 cells.

The literature data suggest that the bacteria are irregularly distributed over the plant surface [5]. This research also proves this phenomenon. There was a greater number of bacteria found on the bottom sections of the examined plant, namely on the surface of the petiole in the extinguish sunlight area and on the rhizome's surface, than on the petiole below the leaf blade and directly on the leaf blade's surface. Lalke-Porczyk [21] found significant differences in the number of heterotrophic epiphytic bacteria between upper and lower surface numbers of the yellow waterlilys; that is, from 1.50 x 10^8 to 118 x 10^{12} cells/lg of dry plant mass on the upper parts, and from 1.30 x 10^5 to 8.80 x 10^7 on the lower parts. The development of the bacterial microflora on the upper side of water floating leaf blades was definitely influenced by strong solar radiation, including UV radiation, and considerable changes of water temperature connected with water vaporization from the plant's surface. The microflora development on the underface of the leaf blades was boosted by constant contact with water, the lack of direct solar radiation, and the secretion of the food substance by numerous secretion hairs [21]. It is common knowledge that the number of the planktonic bacteria in the above-deposit water layer is usually higher as compared with the upper water layers. This can cause more rapid settlement of the bacteria and their higher number on the surface of rhizomes in the above-deposit water, namely on the rhizome and the extinguish sunlight area's petiole, than on the surface of the leaf blade or the petiole directly below the blade.

The conducted research proves that the dominating morphologic form among the yellow waterlily's epiphytic bacteria is rods gram negative (from 56% to 80% of the examined strains). According to Donderski [19], this morphological type can account for up to 96% of all the heterotrophic bacteria in Jeziorak Lake. Many research works point to biologically active substances (secreted by numerous algae) as an inhibiting factor for the growth and development of the gram positive bacteria [25, 27, 28], which can explain their domination in water basins, especially during the blooming of the algae and cyanobacteria.

Changing environmental conditions undoubtedly affect the physico-chemical parameters of lake water and also the population growth of bacteria.

The research has proved that the dominating microorganism groups inhabiting the yellow waterlily are slowly growing strains. The rapidly growing bacteria dominated only in summer. The quantity fall of rapidly growing strains is probably connected not only with the decrease of easily available food substance, but also with the water temperature fall, which, in turn, reduces microorganism activity and prolongs the longevity of some populations.

The heterotrophic bacteria in water basins consist of populations of different physiological groups with biochemically varied activity [19, 20, 29]. As a consequence of these groups' activity, the organic substance occurring in the water ecosystem is mineralized to inorganic compounds. The microorganisms use both monomeric - molecular compounds, easily available for bacteria food material, the content of which is rather small though, in the metabolic processes and polymeric high - molecular compounds, exogenously breaking them down to monomeric - molecular ones.

This research has examined the yellow waterlily's epiphytic bacteria ability to break down some large - particled substances, which occur in natural waters.

The measured results point to the fact that the most numerous group among the epiphytic bacteria is lipolytic bacteria accounting for 32% -83% of the examined strains. Lipids produced both by plants and by animals are one of the most important polymeric groups occurring in fresh waters [30]. According to Arts [31] bacteria can actively cumulate fat compounds and then use them as energy sources. Lipides can also perform a function of mechanical and thermal isolators. Organisms, such as green algae, cyanobacteria [32] and copepods can considerably increase a number of lipolytic bacteria because of the fact that they cumulate enormous amounts of fat in their cells [31]. The research conducted by Donderski and Strzelczyk [33], and Lalke - Porczyk [21] also prove that the lipolytic bacteria are one of the most numerous bacterial groups occurring in Jeziorak Lake.

Starch is a natural ingredient of water plants. Active bacteria use it as a source of energy and carbon. The research on starch decomposition leads to a conclusion that amylolytic bacteria account for a relatively numerous bacterial group [34, 35]. The number of amylolytic bacteria increases linearly along with the increase of carbohydrates concentration [36]. This can possibly justify a fairy high percentage of amylolytic bacteria in Jeziorak Lake, which is of eutrophic character. On average, 12%-65% of the bacterial strains demonstrated an ability to hydrolize starch during the period of the experiment; in addition starch was decomposed most effectively in spring and summer. A good ability to decompose starch by epiphytic bacteria was also proved by the results of the research by Donderski and Strzelczyk [33], and Lalke - Porczyk [21], who found 10%-71% of the amylolytic bacteria among the strains inhabiting the yellow waterlily surface.

Except for starch, one of the best-decomposed large - particle compounds was protein. The epiphytic bacteria with proteolitic abilities accounted for 6%-48% of all examined heterotrophic bacteria. Suigita [34] claim that bacteria decomposing protein in water basins can account for 70% up to 100% of the total bacteria number. The wide distribution of proteolitic bacteria in the water of Jeziorak Lake is confirmed by the research conducted by Paluch and Niewolak [37], Donderski [20] and Lalke - Porczyk [21]. According to Murdyk [29], so big a distribution of bacteria decomposing protein is connected with the fact that the main ingredient of the organic matter in water basins, along with carbohydrates, is just protein, polipeptides, and amino - acids. Their source is the excretion of phytoplankton, macrophytes and zooplankton, and their remains. The greatest proteolitic activity during this research was observed in spring and autumn. The weak autumn activity can possibly be attributed to low temperatures. According to Helemake and Weyland [38], the optimal proteases synthesis temperature is 18°C.

Pectin and pectin compounds, occurring in the cellular space of plants and being a binder that joins cells together, are well decomposed by epiphytic bacteria. There were from 8% to 33% of the yellow waterlily epiphytic bacteria proving an ability to hydrolize pectin. Lalke-Porczyk [21] found out that there were 4%-70% of strains with pectinolitic properties among the strains isolated from the surface of the yellow waterlily leaves. Donderski and Strzelczyk [33] proved that they accounted for only 4% -12% of the examined strains that had been isolated from the water of Jeziorak Lake and 12%-42% in the bottom deposits [20].

Cellulose is a polysaccharide commonly occurring in plant organisms, in which it is a building ingredient of their walls. This research has proved a considerable number of cellulolitic bacteria, making 8%-61% of the total bacteria number. According to Zdanowski [39], the decomposition of cellulose is affected by microbes and environmental factors such as temperature, the basin eutrophisation degree, and the pH of the water. In our climatic zone, cellulose decomposition is the most intensive at higher temperatures from May to October. This research has proved that cellulose decomposition was clearly more effective in spring and summer, when water temperature was relatively high, than in autumn, which was proved by Zdanowski [39].

The gathered data points to the fact that chitin, which is produced in great amounts in the water environment, was the least available compound for microorganisms. The process of chitin metabolisation in water basins can be affected by the high pH of water [39], which has never been lower than 7.6. The optimal pH for chitin hydrolisation, according to Donderski [20], is between 5.0 - 6.0. It is highly probable that so high a pH, reaching even 8.8, can cause the inactivation of chitinases or hinder their synthesis, which leads to lower detectability of the chitinolitic bacteria. Although Lalke - Porczyk [21] found a high percentage of the bacteria hydrolyzing chitin, namely 48% - 52% in Jeziorak Lake.

Ammonification is a very important process, especially in basins where there is frequent algae blooming, which contributes to considerable amounts of protein and amino-acids in water and bottom deposits [41, 24]. During our research we discovered that the number of the epiphytic bacteria able to carry out the process of ammonification was 0% - 36% of all heterotrophic bacteria. Quite a big number of the bacteria able to carry out the process of ammonification leads to a conclusion that amino-acids are a group of compounds which have an immense role to play in the optimal development of water microorganisms [20].

The research data prove that only 4%-42% of the examined strains demonstrated an ability to acidify a glucose medium. The bacteria's poor use of water basin glucose can point to the fact that substrates different than glucose are more commonly metabolized by water microflora, despite the fact that this compound commonly occurs in water environments, both in elemental state and in large-particled compounds of animal and plant remains.

The generic composition of the epiphytic bacteria depended not only on the season of the year, but also on plant section. On average, there were two kinds of bacteria dominating all the other epiphytic bacteria, namely group *Flavobacterium-Cytophaga* and from the family representatives *otEnterobacteriaceae.* There were great numbers of *Flavobacterium-Cytophaga* observed by Mudryk [28] in estuarial lakes, by Morikawa [42] in the River Tamagawa, and Lalke - Porczyk [21] in Moty Bay in Jeziorak Lake. Quite a big representation of this bacterial group not only among the planktonic bacteria but also among the epiphytic ones can be the result of high resistance to solar radiation, especially UV radiation. These bacteria demonstrate a great ability to produce pigments which protect them against the lethal effects of solar radiation [44].

The great number of *Enterobacteriaceae* family, especially in spring and autumn, can suggest that the waters of Moty Bay are polluted with excrements coming from the waterfowl which nest freely in the area.

Arthrobacter-Corynebacterium is a group of organisms which were well represented in spring and autumn. Strzelczyk, Donderski and Mielczarek [44] point to the fact that this group consists of microorganisms occurring more commonly in the bottom deposits and soil rather than in water. These microbes may have been taken away from the bottom deposits by spring and autumn water mixing and colonized the surrounding plants of the yellow waterlily, which can explain their common presence during the water's summer stagnation.

Macrophytes and peryphytes' abilities, including epiphytes, to cumulate biogenic elements, mainly nitrogen and phosphorus, as well as abilities to limit phytoplankton's development through the competition for mineral salts and light, are nowadays more and more used to improve water quality. There are great possibilities of improving water quality offered by introducing artificial bottoms, next colonized by peryphyte organisims, such as protozoans, eelworms, copepods, larvae *Chironomidae* and algae [45]. Unfortunately, knowledge of the epiphytic bacteria inhabiting natural and artificial bottoms is still incomplete. Thus the objective of this research was to find out the data on the number of the bacteria, their potential to decompose polymeric compounds which are ingredients of pollution, and boost eutrophication and degradation of water basins.

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