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Humic Substances as Source of Carbon and Nitrogen for Heterotrophic Bacteria Isolated from Lakes of Different Trophy

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Abstract

The ability of planktonic and benthic bacteria isolated from the oligo-mesotrophic lake Jasne and the highly eutrophized, alloiotrophic lake Stęgwica, to utilize humic substances was studied. It was found that all the bacteria used in the experiments were able to utilize lake humus. The most intensive development of bacteria was observed in the presence of humic substances which were the only source of nitrogen, it was weaker when the humic substances were the only source of carbon, and it was weakest when they were the only source of both nitrogen and carbon. The ability of planktonic and benthic bacteria isolated from lakes of different trophy to utilize humic substances is similar.

Keywords: humic substances, biodegradation, planktonic bacteria, benthic bacteria, heterotrophic bacteria.

Introduction

Humic substances make a group of organic compounds occurring in various environments: soil, sea and inland waters, sediments, peat-bogs, and ground waters [1, 2]. They differs in each of these environments in respect to their development, structure and role in nature. The specific structure of humic substances, different in each environment, makes it difficult to define the role they play in ecosystems. Because of their high carbon content, they provide a reservoir of that substance in the biosphere [3]. According to Moran and Hodson [4], humic substances constitute 25 - 65 % of dissolved organic carbon in surface water and up 90 % in swamps and peat-bogs. Humic substances occurring in water and in bottom sediments of lakes are characterized by extremely high biochemical and physiological activity. They affect directly or indirectly the abiotic environment as well as the structure and metabolic processes of aquatic organisms, including many microorganisms [5]. It is supposed that one of the most important ecological functions of humus is the ability to absorb in specific situations in ecosystems excessive amounts of organic carbon, as well as other compounds and then to return them at times of organic component shortages [6].

According to Steinberg [7] after de Haan, bacteria oxidizing humic substances activate enzymes which participate in oxidizing lactic acid. Blondou [8] suggests that peroxidases localized at the cell's periphery take part in humus degradation. In has also been found that there exists an intermediate way of humic substance biodegradation, through cometabolism of their particles with substances of the so-called labile pool of dissolved organic carbon, e.g. with glucose, amino acids, lactates [7]. According to Moran and Hodson [4], decomposition of aquatic humic substances may be brought about by synergism between biological and photochemical processes. According to Kieber [9, 10] and Mopper [11], photooxidation related to the effect of UV rays turns the dissolved humic substances into biologically accessible components by forming non-humic molecules of low molecular mass, or causes a chemical modification of the core of those substances making them liable to decomposition.

Although humic substances have been the object of much attention, our knowledge of their role and importance as well as participation of microorganisms in their formation, decomposition and utilization is still scarce and fragmentary. That is why the object of the present work was to study the capability of aquatic bacteria to utilize humic substances classified as diffi-

cult to access, while constituting a significant source and reservoir of carbon and nitrogen in the biosphere.

Materials and Methods

Study Area

The material for study was obtained from the water and bottom sediments of lake Jasne and lake Stęgwica, which are a part of the Iława Lakeland.

Lake Jasne is classified as oligo-mesotrophic. It is 10.5 ha in area with a mean depth of 8.13 m and maximum depth of 21.0 m. The lake basin is formed in sandy ground, poor in soluble substances. The lake shores, without a major littoral zone, are covered with young pine forest on one side with mixed pine-beech forest with some alder on the other. The lake is characterized by high water transparency reaching 9-11 meters and low water pH ranging from 4.0-4.8.

Lake Stęgwica is classified as alloiotrophic, highly eutrophized with signs of hypertrophy. It is 47.5 ha in area, with a maximum depth of 2.0 m. The lake shores are surrounded by fields and meadows. The littoral zone is overgrown with a wide belt of aquatic macrophytes dominated by *Phragmites australis (Cav.) Trin. ex Stendel, Typha angustifolia L., Typha latifolia L.* and *Nuphar luteum L.* The lake water is characterized by low transparency (about 50 - 80 cm), the water pH ranging from 8.0 to 8.5, and its brown colour is due to high humic substances content sometimes called "sweet humus".

Sampling

Samples of water and bottom sediments for study were taken in the spring (10. 05), summer (3. 08), autumn (25. 11) 1995 and in the winter (23. 01) of 1996 in the pelagic zone of the lakes. Water for microbiological studies was taken by means of a Ruttner sampler from a depth of 1 m and poured into sterile glass bottles. The water designated for isolating humic substances was collected into polyester containers 25 l in volume and acidified with HCl (36.5 %) to pH 2.0. Bottom sediments were collected by means of tube sampler of the authors' own construction, and their upper layer down to 20 cm deep was transferred to sterile glass jars. The samples of water and bottom sediments designed for microbiological studies were transported to the laboratory in a container with ice, the temperature inside being about 7 °C.

Isolating Humic Substances

For the isolation of humic substances from water the method described by Thurman and Malcolm [12] was used. The water cleared of larger particles of suspension by filtering it through a nitrocellulose filter, with 0.45 μm pore size (Sartorius), was passed through a sorption column filled with ion exchange resin "Amberlite" XAD 8 (Fluka). The humic substances bound by the sorbent were washed away with 0.25 M NH_4OH. The eluate was condensed in a vacuum evaporator at 45°C to a volume of 100 cm^3. In order to isolate humic substance from the bottom sediments, they were subjected to 16-hour extraction with 0.1 M NaOH at 40°C. Then the sediment was centrifuged at 15,000 rpm for 10 minutes. The supernatant thus obtained was a solution of humic substances occurring in bottom sediments, which was then condensed as above. The concentration of the humic substances in the solutions was determined by the gravimetric method, normalizing the data for 1 dm^3 water or 1 g bottom sediments of the lakes under study.

Isolating Bacterial Strains

The bacteria for study were isolated using the spread plates method and the iron-peptone agar medium after Ferrer, Stapert and Sokolski [13]. The samples of water and bottom sediments, diluted with sterile buffer water [14], were plated on the surface of the medium and incubated at 20°C for 10 days. Then 25 strains of planktonic and benthic bacteria were random by isolated from each sample and transferred to a semi-liquid iron-peptone medium. The strains, after being checked for purity, were stored in a refrigerator at 4°C for further studies. The strains were transfered every two months on fresh semi-solid iron-peptone medium.

Identification of Bacteria

The bacteria were identified to the genus or group based on Bergey's Manual of Determinative Bacteriology [15] and on the diagnosis diagram proposed by Shewan et al. [16].

Utilization of Humic Substances

Ten strains (5 planktonic and 5 benthic) from each lake, isolated in the seasons under study, were used in the experiment. The bacteria were multiplied on slants iron-peptone medium at 20°C for 72 h, then washed off with 5 cm^3 sterile physiological salt solution (0.85 NaCl). The suspension thus obtained was centrifuged at 15,000 rpm for 10 minutes and the bacterial sediment washed twice with physiological salt solution. Next the washed bacteria were resuspended in the same solution adjusting the suspension absorbency to obtain 0.15 at light wave-length λ=560 nm, this corresponding to 10^8 bacterial cell per cm^3. The measurements were carried out using a Spekol 221 spectrophotometer (Carl Zeiss, Jena). The following media were inoculated by 0.1 cm^3 of bacterial suspension thus prepared:

- medium 1 - basic mineral medium B containing : KNO_3 - 0.5g; NH_4NO_3 - 0.5g; K_2HPO_4 - 1.0g; $MgSO_4$ - 0.4g; $CaCl_2$ - 0.2g; $FeCl_3$ - 0.01g; NaCl - 0.1g; glucose - 0.1g; casein hydrolyzate - 0.1g; distilled water - 1 dm^3

- medium 2 - basic mineral medium supplemented with humic substances at 10 mg×dm^3 concentration
- medium 3 - modified mineral medium B without glucose and casein hydrolyzate, containing humic substances at 10 mg×dm^3 concentration as sole source of carbon
- medium 4 - modified mineral medium B without KNO_3, NH_4NO_3 and casein hydrolyzate, containing humic substances at 10 mg×dm^3 concentration as sole nitrogen source
- medium 5 - modified mineral medium B without KNO_3, NH_4NO_3, casein hydrolyzate and glucose, containing humic substances at 10 mg×dm^3 concentration as sole source of carbon and nitrogen.

The pH of the media was adjusted to its value "in situ" in the lakes under study. All the inoculations were carried out in triplicates. Immediately after inoculation of the media and mixing up the contents of the Erlenmayers flasks, 1cm^3 of the suspension was taken from each of them and fixed with formalin so that its final concentration was 2%. A similar course of action was taken after six days of culturing at 20°C. In those samples, the numbers and biomass of the bacteria were determined by direct counting and by measuring cells on membrane filters (Milipore GTBP, pore size 0.22 μm) stained with acridine orange [17] and viewed in an epifluorescence microscope Jenalumar (Carl Zeiss, Jena).

The ability to utilize humic substances by the same bacterial strains was also estimated by measuring the intensity of their development (turbidity) in the media as above. The results were read after six days of culturing at 20°C, by measuring the absorbency by means of a spectrophotometer Spekol 221 (Carl Zeiss, Jena), at wave-length λ=560 nm, in comparison with a blind sample which was an unoculated medium of appropriate composition.

Statistical Analysis

The results of studies were subjected to statistical analysis determining the level of significance by means of the t-test. The calculations were executed using the programme CSS:STATISTICA.

Results

The results of studies of the effect of humic substances on the development of bacteria have been presented in Figures 1-3 and Tables 1-3. All the strains studied showed the highest increase in numbers and biomass when cultured in the basic mineral medium B supplemented with humic substances. The bacteria isolated from lake Jasne exhibited greater intensity of development reflected in a greater number of cells in the same length of time than those isolated from lake Stęgwica. Less intensive development of bacteria was noted in media where the humic substances constituted the sole source of carbon, of nitrogen or of both carbon and nitrogen. The bacterial strains used in the experiment showed better development in the medium containing humic substances as the sole source of nitrogen than in the medium where humus was the sole source of carbon (Figure 1). Only some strains of the genus *Micrococcus sp.* utilized humic substances as a source of carbon more intensively as a source of nitrogen (Table 1). All the strains studied were found to be capable of utilizing humic substances as the sole source of carbon and nitrogen jointly; however, most strains in such cultures showed a much lower increase in numbers than in media in which humic substances were the only source of carbon or nitrogen.

The effect of humic substances on the biomass of bacterial cells varied (Figure 2, Table 2). A phenomenon frequently observed in cultures on the basic mineral medium B supplemented with humic substances, particularly pronounced in strains of the genus *Bacillus sp.* and of the group *Arthrobacter-Corynebacterium*, was the increase in the cells mean that volume, which was reflected in a greater biomass of the bacteria. In all the remaining cultures (media) the values of the cells mean that volumes in most strains were nearly the same. That is why the curves presenting the changes in biomass reflect those changes in numbers of the bacteria in those cultures.

The results of spectrophotometric measurements of light absorbency in the bacterial cultures on media supplemented with humic substances are in line with those obtained by the direct count method and by measurement of bacteria on membrane filters. All strains showed most intensive development on the basic mineral medium B supplemented with humic substances. It appears that their development was two or three times as intensive as of bacteria on mineral medium B without humic substances used as a control (Figure 3). Analyzing bacterial development in the medium where humic substances were the only source of carbon, it appears that only *Micrococcus sp.* strains showed a significantly higher intensity of development on just medium than on media in which humic substances were the source of nitrogen or both carbon and nitrogen (Table 3). It follows from the data that most bacteria were developing better in the medium in which humic substances were the sole source of nitrogen and showed only little ability to utilize carbon from the humic substances as its source. The poorest development of virtually all strains was observed in cultures on media in which the sole source of both carbon and nitrogen were humic substances. That suggests that humic substances as a sole source of both carbon and nitrogen are difficult to access, and are generally utilized to only a low degree.

Comparing the development of bacteria in all the media studied, it is seen that the level of significance

(p) was lower than 0.05, and in most cases amounted to 0. That is evidence of a statistically significant difference in the utilization of humic substances by aquatic bacteria as source of carbon, nitrogen, or both carbon and nitrogen. It also follows from the statistical analysis that there is no statistically significant correlation between the development of bacteria in the test media and the trophy of the lake from which they have been isolated, since in all the cases analyzed the significance level was higher than 0.05. Similar results have been obtained when comparing the development of bacteria isolated from the water and from the bottom sediments of the two lakes. Only in the case of the basic mineral medium B without humic substances were there found significant differences (significance level 0) between the development of benthic bacteria and that of planktonic bacteria. In mineral medium B supplemented with humic substances and in media where the humic substances were the sole source of carbon, nitrogen and both carbon and nitrogen the significance level was greater than 0.05, which indicates that there are no differences in the utilization of humic substances by the two groups of bacteria.

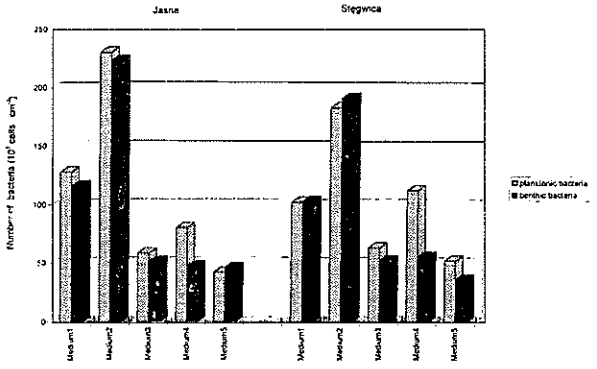


Fig. 1. Effect of humic substances as a source of carbon and nitrogen on numbers of heterotrophic bacteria.

Discussion

Studies on humic substances have demonstrated that they constitute the most resistant part of organic matter, and Visser's [18] results even prove that they are inaccessible to bacteria. However, later studies carried out by Tranvik and Sieburth [19] and by Moran and Hodson [4], reveal the important biological role of humic substances as nutrient substrate for aquatic microorganisms. According to those authors, cultures of bacteria isolated from lake water and kept in lake water supplemented with humic substances collected from the same lake, increased in numbers and biomass, which is evidence of their ability to carry out biodegradation of those substances. Our work on the growth and the development of heterotrophic bacteria isolated from the water and the bottom sediments of lakes Jasne and Stęgwica (cultured on five different media in which humic substances were the sole source of carbon, nitrogen and both carbon and nitrogen, and in which the bacteria were found to grow in number) confirm the ability of heterotrophic bacteria to decompose those substances and utilize them as a source of nutrient elements. Visser's [18] results seem to contradict the above statements and even suggest the inaccessibility of humic substances to bacteria. However, that divergence may result from differences in the procedure of isolating the humic substances, which is not without effect on their structure. The technique of extraction of the humic substances may on the one hand cause their partial disintegration and increase their liability to microbiological decomposition but, on the other, it may make them completely inaccessible and unassimilable [18, 20].

In our experiment, the microorganisms cultured in basic mineral medium B with glucose and amino acids as well as humic substances exhibited very intensive development, reflected in the increase in their numbers and biomass. In media where the only source of carbon are humic substances and there is no supplementary source of energy (glucose and casein hydro-

Table 1. Increase of bacteria numbers in presence of humic substances as an only source of carbon and nitrogen.

Name of bacteria	Inoculum	Number of bacteria**				
		Medium 1	Medium 2	Medium 3	Medium 4	Medium 5
Arthrobacter-Corynebacterium	6.8*	121.5	190.5	54.2	72.1	32.2
Flavobacterium-Cytophaga	7.1	123.2	209.8	55.3	94.5	47.5
Micrococcus	6.5	112.8	224.6	48.5	42.3	32.7
Bacillus	9.1	112.9	195.3	64.3	85.8	58.5
Pseudomonas	6.9	114.3	187.9	45.7	68.1	38.7
Achromobacter	6.7	97.6	222.7	45.8	68.8	43.8
Enterobacteriaceae	7.7	121.0	218.7	58.0	37.2	40.8
Alcaligenes	6.2	93.2	166.5	55.6	41.3	7.7
Chromobacterium	8.7	82.3	137.3	61.6	74.0	60.4

Explanation: * – number of bacteria x 10^5 per cm^3
** – number of bacteria of inoculum substrated

lyzate) bacteria are less capable of biodegradation and utilization of humic substances as nutrients. According to Steinberg [7], a direct way of humus biodegradation may be cometabolism of its particles with substrates of the so-called labile pool of dissolved organic carbon, e.g. with glucose and amino acids. Those low-molecular compounds exert a positive effect on the utilization of humus, since they constitute a supplementary source of energy in the first , reducing stage of decomposition of humic substances.

A number of studies indicate that the nitrogen component of humic acids is the most accessible to microorganisms [21, 22]. The problem of utilizing the nitrogen contained in the humic acids amounts to a large extent on the ability to disrupt bonds between the phenol core and the amino acids chain [21]. The results of the present work confirm those observations. Nearly all the bacteria studied exhibited much better development in the medium where the sole source of nitrogen were the humic substances than in those where the humic substances were the source of carbon or of both carbon and nitrogen . Only the strain of *Micrococcus sp.* showed a higher degree of utilization of humic substances as a source of carbon than as a source of nitrogen.

Studies of the enzymatic properties of bacteria of the genus *Pseudomonas* and of the group *Arthrobacter-Corynebacterium* have demonstrated that they are best "equipped" with an enzymatic system. Among other enzymes they possess N-galactosidases and polygalacturonases, which decompose aliphatic and aromatic carbohydrates [7, 23, 24]. This considerable diversity and the content of enzymes participating in the decomposition of humic substances make these bacteria the best adapted to carrying out biodegradation of the humic substances. The results of the present experiment with bacteria of the genus *Pseudomonas sp.* and the group *Arthrobacter-Corynebacterium* confirm that observation. Those bacteria developed fastest and most intensively in media where the humic substances constituted the only source of nitrogen or carbon or both those elements together. Our results also point out that strains of other genera, such as *Micrococcus, Bacillus, Achromobacter, Chromobacterium, Alcaligenes and Flavobacterium-Cytophaga* group, are capable of utilizing humic substances as nutrient substrate.

The bacteria used in the present work came from environments of different trophies and different humic substance content. Lake Stęgwica is a highly eutro-

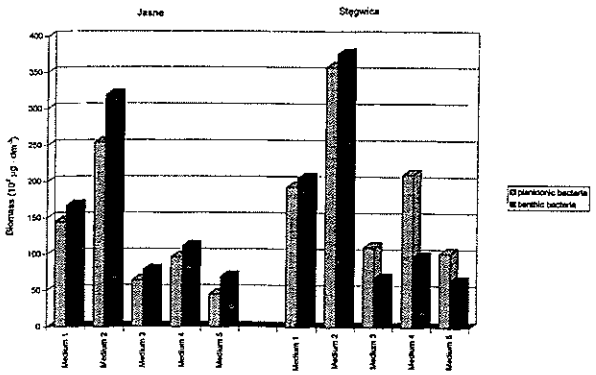


Fig. 2. Effect of humic substances as a source of carbon and nitrogen on biomass production by heterotrophic bacteria.

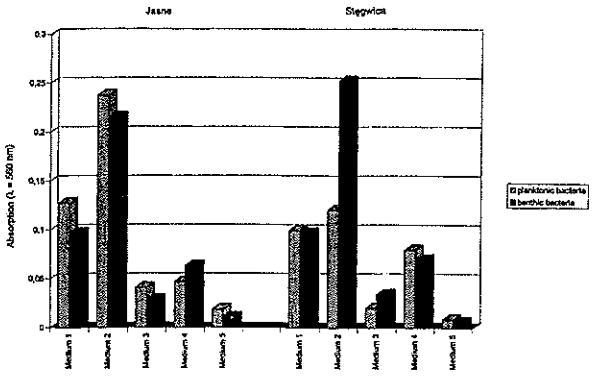


Fig. 3. Effect of humic substances as a source of carbon and nitrogen on intensity of growth heterotrophic bacteria.

Table 2. Increase in bacteria biomass in presence of humic substances as an only source of carbon and nitrogen.

Name of bacteria	Inoculum	Biomass of bacteria**				
		Medium 1	Medium 2	Medium 3	Medium 4	Medium 5
Arthrobacter-Corynebacterium	8.1*	144.4	276.9	46.8	70.8	40.5
Flavobacterium-Cytophaga	13.4	234.1	395.8	91.1	141.1	77.3
Micrococcus	6.6	115.1	212.1	53.5	86.1	33.9
Bacillus	18.8	232.7	407.5	113.7	148.9	103.2
Pseudomonas	13.2	219.1	400.3	72.2	133.9	64.3
Achromobacter	12.6	183.5	405.4	80.4	163.9	74.9
Enterobacteriaceae	3.1	48.4	87.5	23.2	18.9	16.3
Alcaligenes	9.3	139.7	249.7	11.3	74.4	11.2
Chromobacterium	17.4	164.7	276.6	123.2	138.1	120.8

Explanation: * – value of biomass x 10^2 μg per cm^3
** – number of bacteria biomass substrated

Table 3. Intensity of growth heterotrophic bacteria in presence of humic substances as an only source of carbon and nitrogen.

Name of bacteria	Growth in media				
	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5
Arthrobacter-Corynebacterium	0.1211*	0.2254	0.0182	0.0622	0.0147
Flavobacterium-Cytophaga	0.1035	0.2292	0.0112	0.0769	0.0044
Micrococcus	0.1250	0.2212	0.0869	0.0351	0.0261
Bacillus	0.0962	0.2459	0.0443	0.0790	0.0064
Pseudomonas	0.1046	0.2052	0.0160	0.0711	0.0077
Achromobacter	0.0731	0.1264	0.0165	0.0618	0.0098
Enterobacteriaceae	0.1327	0.2556	0.0157	0.0501	0.0054
Alcaligenes	0.0928	0.2460	0.0172	0.0348	0.0008
Chromobacterium	0.0817	0.1619	0.0297	0.0530	0.0136

Explanation: * – value of absorption λ=560 nm

phized,alloiotrophic water body and much richer in humus than the oligo-mesotrophic lake Jasne. It could have been expected that the microorganisms from the two lakes would differ in their ability to utilize humic substances. The bacteria from lake Stęgwica were expected to adapt more quickly and to utilize humic substances better than those from lake Jasne. However, such regularities were not observed. The degree and the rate of utilization of humic substances by the bacteria isolated from the studied lakes were similar. Studies carried out by Tranvik and Hofle [25] also demonstrate a similar efficiency in humic substance degradation by bacteria from oligo- and dystrophic lakes. According to Donderski [26], deep-dwelling organisms from lakes of lower trophy are generally more metabolically active than those coming from lakes of higher trophy. It is suggested that bacteria coming from lakes of lower trophy, and therefore lower organic matter content, are characterized by a greater ability to utilize more diverse compounds than those living in lakes of higher trophy, rich in easily-accessible organic substances.

The results of many authors [7, 18, 19, 21] as well as of the present work indicate that the effect of humic substances on microorganisms has many different aspects. That diverse character of effects makes it difficult to define explicitly the role and importance of humic substances in the lakes themselves as well as for the organisms living in them, including those participating in the formation, transformation and biodegradation of those substances. The present work is a contribution towards the solution of that extremely interesting problem and to a better understanding the role of humic substances as a source of carbon and nitrogen and other elements, and of the capability of utilizing humus components by microorganisms.

Conclusions

1. All the bacteria studied in this work demonstrated an ability to utilize humic substances isolated from the water and from the bottom sediments, or at least some of their fractions.

2. A great majority of the bacteria under study showed better utilization of the humic substances as sole sources of nitrogen than of carbon.

3. The ability to utilize humic substances as nutrient by bacteria isolated from lakes of different trophies is similar.

4. Bacteria isolated from the water and those isolated from the bottom sediments show a similar degree of utilization of humic substances as nutrients.

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