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The effect of humic substances isolated from a variety of marine and lacustrine environments on different microorganisms*

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Abstract

The antimicrobial activity of twelve preparations of humic substances isolated from sea water, marine bottom sediments and lake water was examined. Humic substances from marine bottom sediment samples were fractionated into humic and/or fulvic acids. The susceptibility of 11 strains of anaerobic, 8 strains of aerobic bacteria and 2 strains of yeast-like fungi to preparations of humic substances, humic and/or fulvic acids was determined employing the plate dilution technique in Brucella agar (anaerobic bacteria), Mueller-Hinton agar (aerobic bacteria) and Sabouraud agar (yeast-like fungi). Concentrations from 150 to 600 μg ml^{-1} of the preparations examined inhibited the growth of numerous microorganisms (Table).

The results obtained seem to indicate that humic substances are involved in the self-purification of sea and lake waters.

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1. Introduction

Humic substances (HS) found in bottom sediments, sea and lake waters, soils, peat and coals, resulting from the decomposition of organic matter, particularly dead plants, consist of a mixture of complex macromolecules with polymeric phenolic structures (Flaig et al. 1975).

Taking into consideration the known fragments of the structure of HS we came to the conclusion that they should demonstrate an antimicrobial activity. In this publication we report the examination of antimicrobial properties of twelve preparations isolated from sea water, marine bottom sediments and lake water. Unfractionated HS were isolated from sea and lake water samples. HS from marine bottom sediment samples were fractionated into humic acids (HA) insoluble, and/or fulvic acids (FA) soluble in acidified water (pH 2).

The plate dilution technique in agar was used to determine the susceptibility of 11 strains of anaerobic and 8 strains of aerobic bacteria as well as 2 strains of yeast-like fungi to preparations of HS, HA and/or FA.

2. Material and methods

2.1. Material

Samples of HS were isolated from the following media:

- sea water (Southern Baltic): Gdańsk Deep, 104 m (No. 1); Gulf of Gdańsk surface water (No. 2 and No. 3),
- marine sediments (Southern Baltic): Gdańsk Deep, 0–3 cm layer (No. 4), 3–6 cm layer (No. 5), 6–9 cm layer (No. 6), 3–4 cm layer (No. 7), 13–14 cm layer (No. 8) and 21–22 cm layer (No. 9),
- surface lake water (Norway): Lake Skjevatien (No. 10), Lake Trohoringen (No. 11) and Lake Hollevudmyra (No. 12).

HS, HA and/or FA were isolated from the samples according to the procedure given in the literature (Pempkowiak 1989, Pempkowiak et al. 1994).

The following strains of microorganisms were tested:

- Anaerobic bacteria
  Standard strains: *Bacteroides fragilis* ATCC 25285, *Bacteroides vulgatus* ATCC 8482, *Bacteroides ovatus* ATCC 8483, *Fusobacterium nucleatum* ATCC 25585, *Peptostreptococcus anaerobicus* ATCC 27337, *Propionibacterium acnes* ATCC 11827, *Clostridium perfrigens* ATCC 13124.

Strains isolated from the human intestinal tract: *Peptostreptococcus productus*, *Actinomyces bovis*, *Clostridium difficile*, *Clostridium septicum*.

- Aerobic bacteria
  Standard strains: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacillus cerus* ATCC 10876, *Bacillus subtilis* NTCT 8236, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606.
- Yeast-like fungi
  Candida albicans, *Candida glabrata*.

3. Methods

Isolation of HS from water samples. HS were isolated from water according to a standard procedure based on sorption on Amberlite XAD-2 resin, followed by desorption with ammonium hydroxide. Water was filtered through Whatman GF/F filters, acidified to pH 2.0 with concentrated HCl, and passed through a glass column (3×35 cm) filled with Amberlite XAD-2 resin. HS absorbed onto the Amberlite were eluted with 0.5 mol dm^{-3} NH_4OH. The excess ammonium hydroxide was removed in a rotary evaporator and the concentrated HS solution was freeze dried and stored in a dessicator.

Isolation of FA and HA from sediment samples. Humic substances from solid samples (sediments) were isolated according to a traditional alkaline extraction. A 1:20 w/v ratio of a sample to 0.2 mol dm^{-3} NaOH was utilized. The extractions, each lasting 16 hr, were repeated 5 times. After each extraction the solution was centrifuged. Combined extracts containing HS (HA and FA) were acidified. The precipitate of HA was separated from the solution containing FA by centrifugation, dissolved in 20 cm^3 of 0.1 mol dm^{-3} NaOH and again centrifuged at 30 000 g for 30 min. The solution of HA thus obtained was acidified (pH 2). Precipitated HA were centrifuged, freeze dried and stored in a dessicator until the analyses were performed. The acidified extract containing FA was passed through a glass column (1.5×20 cm) containing Amberlite XAD-2 resin. Adsorbed FA were then desorbed with 0.5 mol dm^{-3} NH_4OH, preconcentrated in a rotary evaporator (40 °C), freeze dried and stored.

The susceptibility of microorganisms to humic substances was determined using the plate dilution technique in agar. In the case of anaerobic bacteria it was Brucella agar supplemented with 5% defibrinated sheep's

blood. In the case of aerobic bacteria, Mueller-Hinton agar, and in the case of the yeast-like fungi Sabouraud agar were used. HS were dissolved in sterile distilled water (immediately before the experiment) to obtain the following concentrations: 600, 300 and 150 μg ml^{-1}. The plates were inoculated using Steers multipoint inoculator. The inoculum contained 10^6 CFU/spot. In each experimental series, the growth of strains on the culture medium without the HS was checked. Incubation of plates with anaerobic bacteria was performed at 37 °C (310 °K) for 48 h in anaerobic jars containing a mixture of 10% CO_2, 10% H_2 and 80% N_2 in the presence of a palladium catalyst and an indicator of anaerobiosis. In the case of the aerobic bacteria and yeast-like fungi, the incubation of plates was performed at 37 °C for 24 h (aerobic conditions). The concentration at which no macroscopic growth of the microbes could be detected on the medium was regarded as the lowest concentration inhibiting the growth of microorganisms (MIC).

The results are presented in the Table.

4. Results and discussion

The data in the table indicate that humic substances (HS, HA and FA) exhibit varied antibacterial activity.

The following preparations of humic substances exhibited the highest antibacterial effect on anaerobes: fulvic acid No. 5 in the concentration range from 150 to 600 μg ml^{-1} inhibited the growth of 9 of the 11 strains examined; fulvic acid No. 4 inhibited the growth of 7 strains at a concentration of 600 μg ml^{-1}. Similar activity was shown by humic acid No. 7, which inhibited the growth of 7 strains in the concentration range from 150 to 600 μg ml^{-1}. At a concentration of 600 μg ml^{-1} humic substance No. 3 inhibited the growth of 6 anaerobic strains.

The weakest effect on anaerobes was demonstrated by humic substance No. 12. None of the strains was sensitive to concentrations of 150–600 μg ml^{-1}. Moreover, humic substance No. 2 at a concentration of 600 μg ml^{-1} inhibited the growth of only 1 strain.

The strongest effect on the aerobic strains examined was exerted by the following preparations: fulvic acid No. 5 in the concentration range from 300 to 600 μg ml^{-1} inhibited the growth of all 8 strains examined. Fulvic acid No. 4 and humic substance No. 3 were also active. Both preparations at a concentration of 600 μg ml^{-1} inhibited the growth of 4 of the 8 strains. Humic substances Nos. 1 and 2, as well as humic acids Nos. 8 and 9 demonstrated the lowest activity with respect to aerobic bacteria. None of the aerobic strains was sensitive to concentrations of 150–600 μg ml^{-1}.

The results obtained imply that humic substances (HS, HA and FA) were slightly more active to Gram-positive anaerobic and Gram-negative aerobic bacteria. Yeast-like fungi were less susceptible than bacteria (MIC ≥ 600 μg ml^{-1}).

It is noteworthy that the humic substances (both FA and HA) isolated from sediments exhibit stronger antimicrobial properties than HS isolated from water. Moreover, the concentrations of HS in the sediments from which they were isolated exceeded by several orders of magnitude the concentration of HS in surface waters (Pempkowiak 1989). Considering that concentrations of HS in sedimental porewater can be as high as 30 mg dm^{-3}, while the concentration in the Baltic sediments often ranges from 5 to 7% (50–70 g kg^{-1}), it can be concluded that humic substances are capable of influencing the content and composition of microorganisms in the environment.

References

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Table. Susceptibility of microorganisms to humic substances isolated from marine and lacustrine environments

Microbes	**Minimal inhibitory concentration – MIC** [μg ml^{-1}]																																											
	No. 1 HS				No. 2 HS				No. 3 HS				No. 4 FA				No. 5 FA				No. 6 FA				No. 7 HA				No. 8 HA				No. 9 HA				No. 10 HS				No. 11 HS			
	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150	>600	600	300	150
Anaerobic bacteria																																												
Bacteroides fragilis ATCC 25285	+				+				+				+				+				+				+				+				+				+				+			
Bacteroides vulgatus ATCC 8482		+			+					+			+					+			+				+				+				+				+				+			
Bacteroides ovatus ATCC 8483	+				+				+				+				+				+				+				+				+				+				+			
Fusobacterium nucleatum ATCC 25585	+				+					+				+				+				+					+			+				+			+				+			
Peptostreptococcus anaerobicus ATCC 27337	+				+					+				+				+			+					+			+				+					+			+			
Propionibacterium acnes ATCC 11827	+				+				+					+					+		+					+			+				+					+			+			
Clostridium perfringens ATCC 13124		+			+					+				+				+			+					+			+				+				+				+			
Peptostreptococcus productus	+				+					+			+						+		+				+				+					+			+					+		
Actinomyces bovis		+				+				+				+						+		+						+			+				+			+					+	
Clostridium difficile	+				+				+					+					+			+				+				+				+					+		+			
Clostridium septicum	+				+				+					+						+		+				+				+				+			+				+			
Aerobic bacteria																																												
Enterococcus faecalis ATCC 29212	+				+				+				+					+			+				+				+				+				+				+			
Staphylococcus aureus ATCC 25923	+				+					+				+				+			+				+				+				+				+				+			
Bacillus cerus ATCC 10876	+				+				+					+				+			+				+				+				+				+				+			
Bacillus subtilis NTCT 8236	+				+				+					+				+			+				+				+				+				+				+			
Escherischia coli ATCC 25922	+				+				+				+						+		+				+				+				+				+				+			
Klebsiella pneumoniae ATCC 13883	+				+					+			+						+		+				+				+				+					+				+		
Pseudomonas aeruginosa ATCC 27853	+				+					+			+						+		+				+				+				+				+				+			
Acinetobacter baumannii ATCC 19606	+				+					+				+					+			+				+			+				+				+				+			
Yeast-like fungi																																												
Candida albicans	+				+				+					+				+			+				+				+				+				+				+			
Candida glabrata	+				+				+				+				+				+				+				+				+				+					+		

Symbols: HS – humic substances, FA – fulvic acids, HA – humic acids.