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PHYSIOLOGICAL ACTION OF HUMIC SUBSTANCES ON MICROBIAL CELLS

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Summary—By incorporating molecular weight fractions of humic acids of various origins into selective substrates designed for the enumeration of physiological groups of microorganisms, it was found that the presence of humic acids at concentrations of up to 30 mg l^{-1} normally resulted in increased numbers of soil microbes active within a particular physiological group. Observed increases could be as much as 2000-fold. Microbes in an organic humus-rich soil were more stimulated by humic substances than organisms from a sandy soil.

In certain microbes humic substances appeared to induce a change in metabolism, allowing the organisms to proliferate on substrates which previously they could not utilize. Indications were obtained that within the 10–30 mg l^{-1} concentration range lower molecular weight humic fractions (approx. 5500 daltons) were more effective than higher molecular weight material. At higher concentrations the reverse was sometimes noticed. Similarly, fulvic acids at concentrations of up to approximately 50 mg l^{-1} would appear to have a more pronounced physiological effect than humic acids, whereas the latter might be more effective at higher concentrations.

The response of certain physiological groups to humic products of natural origin appeared to be comparable to that of surfactants such as Tween and Brij. This would suggest that the physiological action of humic substances is, at least partly, the result of their surface activity, making the membrane one of the prime targets of the physiological action of humics on living cells.

INTRODUCTION

Humic substances have been shown, by various authors, to affect cellular metabolism in processes such as growth respiration, photosynthesis and nitrogen fixation (Flaig, 1968; Khristeva, 1968; Nechutová and Tichý, 1970; Petrović *et al.*, 1982; Prakash and MacGregor, 1983). In the present study some aspects of the origin of the physiological effect of humic compounds on soil microorganisms were investigated.

MATERIALS AND METHODS

Humic material of two different sources was used in the investigation: (1) humic acids obtained from Aldrich Chemical (Milwaukee, Wisconsin) and (2) humic and fulvic acids derived from a clay loam of the Ste-Rosalie series (St-Hyacinthe, Quebec). The soil had a pH of 6.6, an organic carbon content of 1.85% and a C-to-N ratio of 13.2.

Before use, the Aldrich humic acids were purified by dissolving at pH 10 with 0.5 N KOH under an atmosphere of N_2 at room temperature. After centrifugation at 4500 rev min^{-1} (5700 gravities) for 20 min, the humic acids in the supernatant were precipitated at pH 2.0. This solution was adjusted to pH 7.0, frozen at −20°C, and then allowed to thaw again. After centrifugation and freezing–thawing were repeated until all the material completely dissolved with addition of 0.5 N KOH to pH 10. The humic acids were then precipitated at pH 2.0 and resuspended in a Tris buffer consisting of 30 mM trihydroxymethylaminomethane and 0.15 M KCl adjusted to pH 8.4 and ionic strength $I = 0.15$. Subsequently the humic acids were fractionated using Diaflo ultrafiltration membranes fitted into continuously stirred filtration cells. Fractions were obtained in the molecular weight ranges of between 1000–10,000, 10,000–30,000, 30,000–50,000 and 100,000–300,000 daltons. For simplicity these fractions will be referred to as the 5500, 25,000, 40,000 and 200,000 molecular weight fractions, respectively. By applying during the ultrafiltration procedure, the pressure as prescribed by the manufacturer, and by maintaining the pH and ionic strength of the solutions at the levels previously indicated, it was found that the average particle size of the humic matter fractions from the ultrafiltrates matched well with the average molecular sizes (47%) obtained on the same fractions by using equilibrium ultracentrifugation under conditions described by Posner and Creeth (1972). The concentrates of the four fractions, contained in Amicon ultrafiltration cells fitted with an UM05 Diaflo ultrafiltration membrane (nominal molecular weight cut-off approx. 500 daltons), were thoroughly washed with distilled water until the ultrafiltrates had pH 7.0 and gave a negative Cl test with $AgNO_3$. The humic acids in the form of their K salts were then freeze-dried and stored in the dark at −40°C until further analysis.

The Ste-Rosalie soil was treated for 2.5 h with 0.1 N HCl, carbon extracted with 0.1 N KOH–0.1 M $Na_4P_2O_7$ solution (Kononova, 1966) for 24 h under an atmosphere of N_2 at room temperature. The alkaline extract was separated from the residual soil by centrifugation at 4500 rev min^{-1} (5700 gravities) for 30 min. The humic acids (precipitate) and fulvic acids (supernatant) were separated by acidification of the

extract to pH 2.0. One-half of the humic acids obtained by centrifugation at 4500 rev/min was then fractionated into molecular weight fractions as described for Aldrich humic acids. The other half was purified, washed and freeze-dried without any molecular weight fractionation.

The fulvic acid solution, after adjustment to pH 2.0 was passed over Amberlite XAD-8 resin previously purified and activated by refluxing in a Soxhlet apparatus with methanol for 2 days. The adsorbed fulvic matter was eluted using a Tris buffer of the same composition as mentioned before, after which the eluate was concentrated over a Diaflo UM05 ultrafiltration membrane. Washing and freeze-drying procedures were the same as mentioned for humic acids.

The physiological effect of the humic substances was studied on the physiological groups of amylolytic and proteolytic microorganisms and denitrifiers originating from the Ap horizon of two types of soil, a sandy soil and an organic soil (Table 1). The selective basic substrates and conditions of incubation used for the microorganisms were those described by Pochon and Tardieux (1962). Fulvic or humic acids were added to the basic substrates before autoclaving (20 min at 115°C). This treatment had no apparent detrimental effect on the physiological properties of the humic products under investigation. Five-fold dilution series of the soils were incubated five-fold in the selective growth media designed for the physiological groups under investigation. For each sample this procedure was done in triplicate. After suitable incubation period the presence or absence of microbial activity related to the particular physiological group was determined in the incubated substrates by means of appropriate chemical tests. In the case of, for instance, starch decomposers or nitrifiers, aliquots of the substrates were investigated for the disappearance of starch or the appearance of nitrates respectively. For each dilution the number of positive tubes in the five replicates was determined and the

Table 1. Characteristics of soils used as source of microorganisms

	Sandy soil	Organic soil
pH	5.7	7.0
Organic matter (%)[a]	0.72	50.88
Carbon (%)	0.42	29.00
Nitrogen (%)	0.02	1.80
Ratio C-to-N	21	16

[a] Data expressed on a dry weight basis.

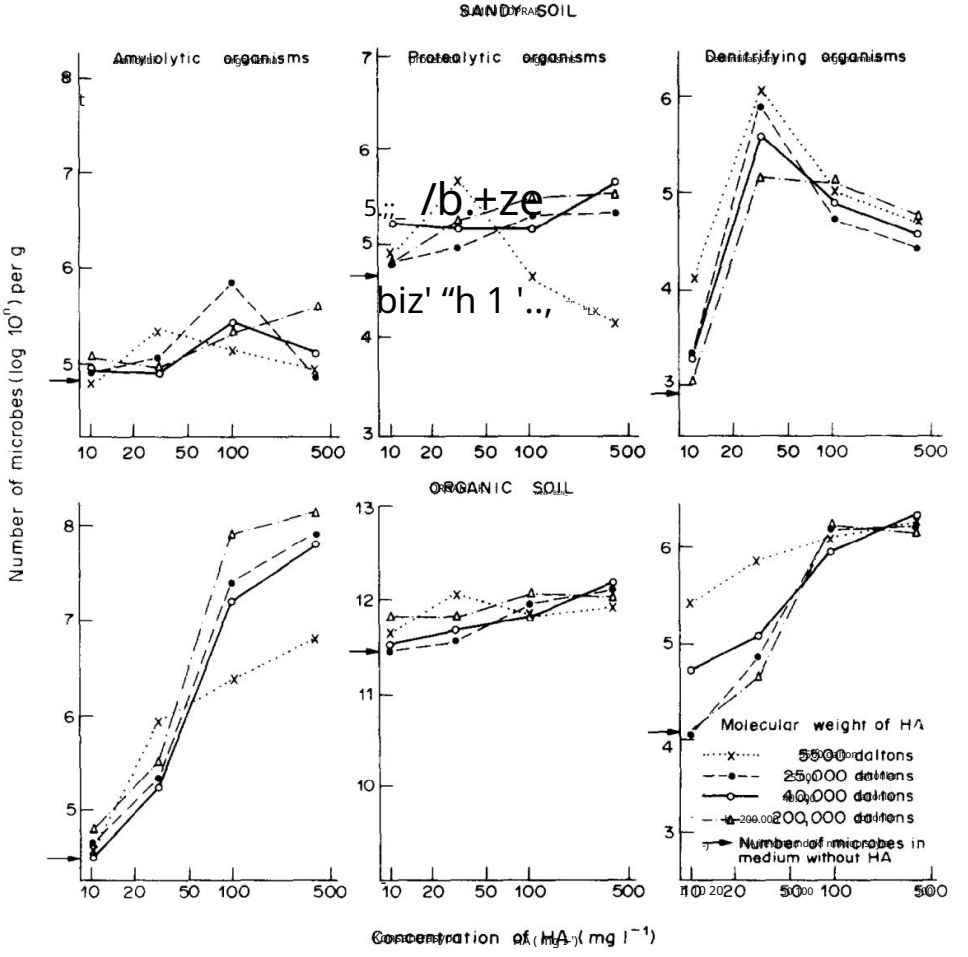

Fig. 1. Effect on amylolytic, proteolytic and denitrifying microorganisms from two types of soils of molecular weight fraction of Aldrich humic acids incorporated at various concentrations in selective culture media.

concentration of the viable organisms active within the physiological group under investigation was then estimated by the most probable number (MPN) method. When the coefficient of variation of the three MPN values was higher than 30%, the outer value was respectively the mean was eliminated. In case the coefficient of variation of the remaining two values was still larger than 30%, all the data were rejected and the analysis was repeated.

For study of the ATP content of microorganisms the method described by Jakubczak and Leclerc (1986) was followed (extraction of ATP with DMSO). ATP was measured on a LKB 1250 luminometer. The protein content of the microbes was determined by means of Folin phenol reagent (Lowry *et al.*, 1951).

RESULTS AND DISCUSSION

By incorporating molecular weight fractions of Aldrich humic acids into selective substrates designed for the enumeration of ammonifying, proteolytic and denitrifying microorganisms, it was found that in practically all instances humic acid concentrations of up to 30 mg l^{-1} had a stimulating effect on the physiological groups from a sandy as well as an organic soil (Fig. 1). It would appear that within this concentration range microbial counts were higher in the presence of lower molecular weight fractions (approx. 5500 daltons) than with higher molecular weight material.

For the organisms from the sandy soil the low molecular weight humic acids were on the whole less stimulating, and might in some instances even be toxic at concentrations exceeding the 30–50 mg l^{-1} range. With respect to the highest molecular weight fraction tested (approx. 200 000 daltons), a plateau or a maximum in the number of organisms of both soils was normally obtained at a humic acid concentration of around 100 mg l^{-1}.

Micro-organisms from the organic soil appeared, in general, to be less sensitive to high concentrations of humic acids than organisms from the sandy soil. No noteworthy toxic effects were noticed at humic acid concentrations of even as high as 500 mg l^{-1}. Organisms from the organic soil therefore seemed to be better adapted to high concentrations of humic products in their environment, and to benefit more from them than organisms from the sandy soil.

As the response of the microbial physiological groups to the humic acids extracted from the Ste-Rosalie clay loam (Fig. 2) was usually comparable to

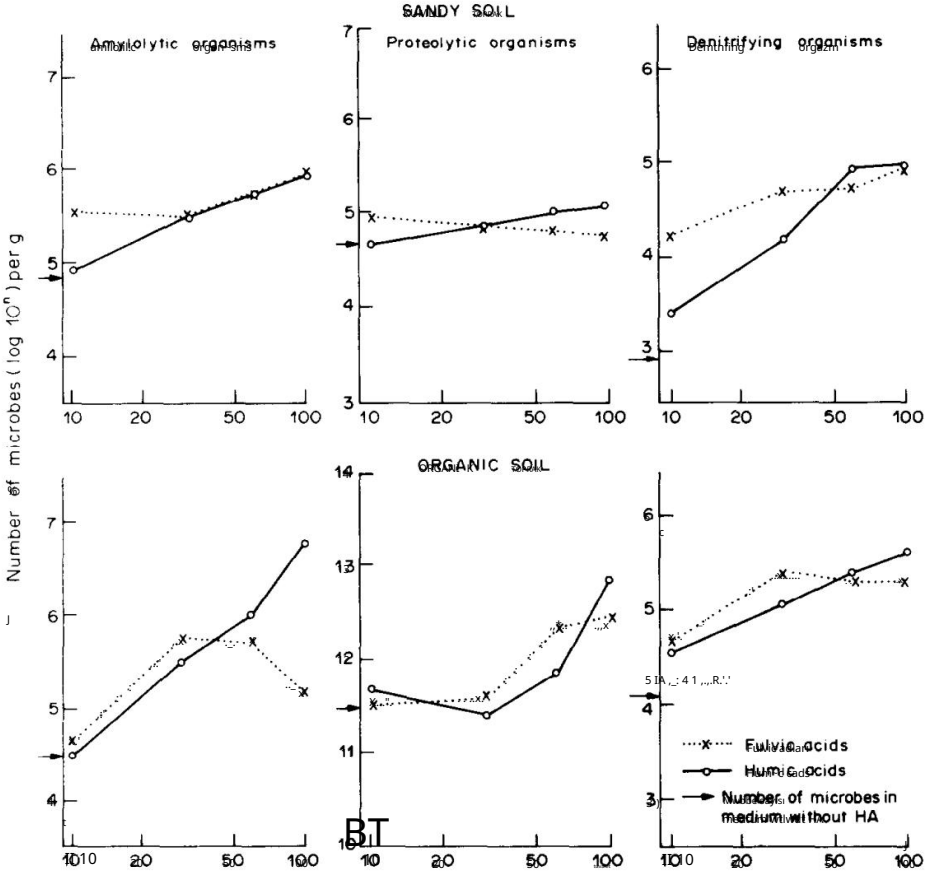

Fig. 2. Effect on amylolytic, proteolytic and denitrifying microorganisms from two types of soils, of fulvic and humic acids from a Ste-Rosalie clay loam, which were incorporated at various concentration in selective culture media.

that observed for the Aldrich material, it would appear that the origin of the humic acids had little effect on their fractionation pattern. Fulvic acids at concentrations of up to approximately 50 mg l^{-1} would appear to have a more pronounced effect than humic acids. For higher concentrations indications are that the reverse is true. This phenomenon, which resembles that of the observed differences between the Aldrich humic acid lower and higher molecular weight fractions, may be at the basis of the controversy encountered in the scientific literature regarding differences in intensity of physiological action by fulvic and humic acids. Some authors maintain that fulvic acids are the more active substance; others have claimed that humic acids have the larger physiological effect (Prakash and Rashid, 1968; Grunda, 1970). The present findings suggest that whereas at lower concentrations fulvic acids are likely to stimulate physiological activity more than humic acids, the reverse appears to be true at higher concentration levels.

In a previous study differences in physiological effect were reported between humic products extracted by pyrophosphate (pH 7) and sodium hydroxide (pH 13), respectively (Visser, 1985). Pyrophosphate-extracted humics were often more effective than alkali-extracted material, particularly at concentrations of between 20–50 mg l^{-1} and gave rise to higher microbial counts. The present findings suggest that the main reason for these differences were the result of the humic acids in the pyrophosphate extract consisting of lower molecular weight material.

It should be mentioned that in this study the observed increases in microbial numbers under the influence of humic substances is not likely to have been the result of any disintegrating effect of humic substances on agglomerates of soil particles or clusters of microbial cells. The microbes came into contact with the humic products only after their inoculation into the selective culture medium, and any subsequent disintegration of clusters of potentially active microorganisms or soil particles should therefore not have affected the microbial count as determined by the most probable number (MPN) method. In any case, the humus effect was also observed on suspensions of microorganisms from mixed laboratory cultures. Selective substrates for the culture of microorganisms active within a physiological group are designed to induce maximum growth under optimum culture conditions of all potentially viable organisms belonging to that particular group. The higher microbial numbers observed in the presence of humic compounds can in my view only be the result of either the humics being used as metabolite or cometabolite by accompanying organisms belonging to different physiological groups or the humics inducing in some of the accompanying organisms a change in metabolism permitting them to become capable of also using the provided substrate and consequently raising the total count in the selective culture medium. As shown by Visser (1985) there are no indications that humic additives were used as a metabolite or cometabolite by the organisms. However, several strains of microorganisms could be isolated which were only capable of growing on certain selective substrates if humic acids were simultaneously present.

From experiments in which the physiological action of humic products of natural origin was compared with that of commercially produced sur-

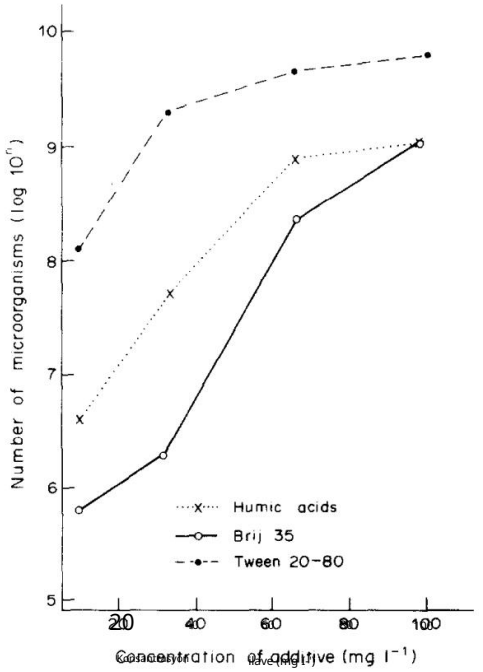

Fig. 3. Effect on amylolytic microorganisms of humic acids from Ste-Rosalie clay loam (mol. wt 25,000 daltons), and some surface active agents, incorporated at various concentrations in the culture medium.

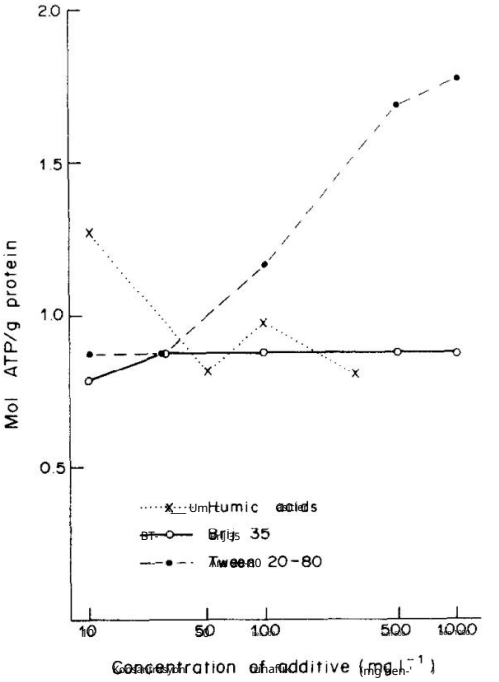

Fig. 4. Effect on ATP content of amylolytic microorganisms of humic acids from Ste-Rosalie clay loam (mol. wt 25,000 daltons) and some model substances, incorporated at various concentrations in the culture medium.

factants such as Tween 20-80 (a mixture of Tween 20, 60 and 80), polyoxyethylene sorbitan monolaurate, monostearate, -monooleate, respectively, or Brij 30 (polyoxyethylene laurylether) quite similar responses were obtained on certain microorganisms such as starch decomposers (Fig. 3). These data strongly suggest that the physiological action of humic acids is, at least partly, the result of their surface activity. In this context it should be mentioned that a more pronounced physiological effect at around the 30 mg l^{-1} concentration level of lower molecular weight humic acid fractions over higher molecular weight material and of fulvic acids over humic acids, concords well with the report by Visser (1982) of higher surface activity of lower molecular weight over higher molecular weight humic fractions and of fulvic over humic acids.

It is known that detergents are able to increase membrane permeability (Gloxhuber, 1974), and humic products, which also show considerable surface activity (Visser, 1982), should act in a likewise manner. Recent work by means of electron microscopy on plant cell sections has shown that at least some humic acids are able to cross cell membranes (S. A. Visser, unpublished). Consequently, humic substances, because of their surface active properties combined with their ability to cross cell membranes, are likely to interact with various intracellular structures and components. Consequently, the previously mentioned ability of certain microorganisms to proliferate in the presence of humic substances on substrates that they cannot normally utilize may very well be the result of an action of the humic matter on certain enzyme precursors or on the initiation of a *de novo* synthesis of normally unavailable enzymes.

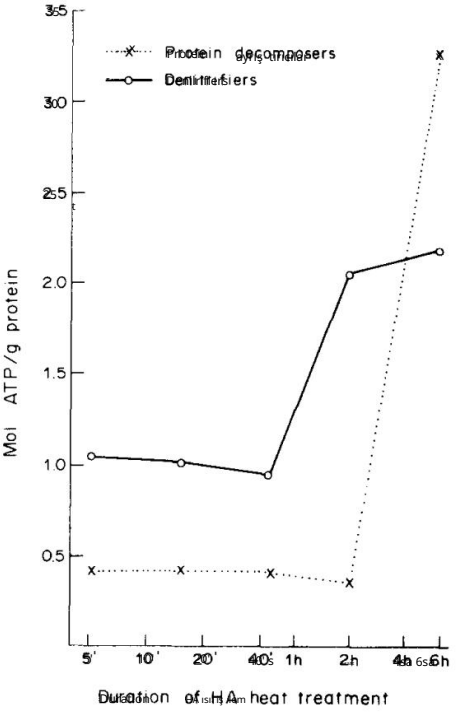

Fig. 5. Influence of heat treatment (ref. humic acids from Ste-Rosalie clay) of humic acids (25 000 daltons) on their effect on the ATP content of proteolytic and denitrifying microorganisms. HA concentration 50 mg l^{-1}.

Indications of another mode of physiological action by humic products were obtained in the course of a study of their effect on microbial ATP levels. Although Tween 20-80 and Brij 35 increased microbial ATP content (Fig. 4) this was not the case for the humic acids. For the commercial detergents the effect was probably linked to an increase in metabolic rate due to a greater availability of nutrients resulting directly or indirectly from a greater permeability of the cellular membrane. It is likely that in the case of humic compounds the favorable action of surface activity on ATP content was counterbalanced by the ability of humics to catalyse the transport of electrons between it and certain thermodynamically normally unavailable electron acceptors or to enhance the rate of already feasible reactions (Zimmerman, 1981). In order to test this hypothesis, the humic acids from the Ste-Rosalie soil were heat-treated in an autoclave at 115°C for up to 6 h. It was then shown that when humic acids which had been heat-treated for 40 min or more, were incorporated in selective culture media, substantial increases in microbial ATP content were observed (Fig. 5). Whereas the type of heat treatment would not have significantly affected surface active properties (which are mainly a reflection of the presence of certain chemical groups), it is likely to have reduced electron transfer capabilities, which are partly linked to the physical structure of the molecule.

Much still remains to be elucidated regarding the physiological effects of humic matter on microorganisms, but as the present study has shown, the additional presence of humic matter in selective culture media can result in much increased counts of microorganisms per gram of soil. Serious consideration should therefore be given to the incorporation of humic products at appropriate concentrations in media intended for the determination of specific microbial activities in terrestrial and aquatic environments.

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