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Interactions of azobenzene and microflora in a sandy loam Spodosol

N. Wackerow-Kouzova

Agrophysical Research Institute, 14 Grazhdansky Prospect, St. Petersburg 195220, Russia

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A b s t r a c t. Transformation and degradation of synthetic organic compounds in soil occurs basically under the influence of microorganisms. In the present studies the interactions of soil microorganisms and water insoluble acaricide azobenzene were investigated. Azobenzene is a volatile orange-coloured crystalline substance with the acaricide and insecticide effects. The influence of azobenzene on the biomass of soil bacteria and the succession of heterotrophic bacteria were studied. Microbiological analyses showed that azobenzene in concentration up to 50 mg kg^{-1} of soil did not have any substantial effect on the total number and qualitative composition of soil bacteria.

The spatial distribution of soil microorganisms was studied using a modified glass slide technique of Kholodny-Rossi. Analysis of the microbial distribution on glass slides placed in soil showed an interaction between azobenzene and microbial spatial distribution. A specific microflora developed on the surface of azobenzene particles and caused the degradation of azobenzene crystals into amorphous mass. Accumulation of azobenzene in the microbial colonies was observed. It was shown that the bacteria isolated from the glass slides and soil were able to assimilate azobenzene in the presence of carbohydrates.

K e y w o r d s: microorganisms, soil, azobenzene, biodegradation

INTRODUCTION

One of the serious problems of modern ecology is environmental contamination by xenobiotic compounds. The principal environmental pollutants are pesticides, fertilizers and products of chemical industry. The chemical compounds released into the environment finally enter into soils. The xenobiotics can be rapidly decomposed by the activities of soil microorganisms. However, some of them are not quickly degraded (Gu *et al.*, 2003; Samanta *et al.*, 2002). Many of the synthetic substances are water insoluble and resistant to microbial decomposition. Hydrophobic compounds often persist in nature for disturbingly long periods of time (Knackmuss, 1996) and, therefore, can act in food chains and accumulate in the organisms of animals and man. The water insoluble xenobiotics are distributed in soil usually in the form of separate particles, crystals and films which determine the mechanisms of their translocation and interactions with the soil microflora (Kruglov, 1991).

Therefore, the influence of xenobiotics on the soil quality (Depaolis and Kukkonen, 1997; Kodešová *et al.*, 2003; Margesin *et al.*,, 2000) and on the soil microflora (Kouznetsov *et al.*, 2004; Kozdroj and van Elsas, 2001; Margesin *et al.,* 2000) is actively studied. Azobenzenes are of a considerable interest among such compounds. Azocompounds are released into the environment in the form of pesticides, dye stuffs, and as the products of aniline-based herbicides transformations. Results indicated that aniline-based herbicides are initially hydrolysed and then condensed under the influence of soil microorganisms (Simmons *et al.*, 1987; Simmons *et al.*, 1989). Condensation reactions may lead to the formation of increasingly complex molecules such as azoxybenzenes and azobenzenes.

In soil, azodyes can be transformed by ligninolytic fungi and micromycetes (Chivukula and Renganatan, 1995; Goszczynsky *et al.*, 1994; Novotný *et al.*, 2004). Bacteria assimilate the azodyes under aerobic (Zissi *et al.*, 1997) and anaerobic conditions (Carliell *et al.*, 1995; Zissi and Lyberatos, 1996).

The objectives of the present study were to: 1) estimate the effects of azobenzene on the diversity of heterotrophic soil bacteria; 2) evaluate the influence of azobenzene on the spatial microbial distribution in a sandy loam soil; 3) investigate the influence of azobenzene on the growth of cultivable soil bacteria, isolate the sensitive and resistant to azobenzene microbial forms; 4) estimate the ability of selected cultivable microorganisms to affect azobenzene transformation.

*Corresponding author's e-mail: wknd@list.ru

MATERIALS AND METHODS

Disturbed samples of a sandy loam Spodosol (according to FAO classification) were collected from the depth of 0-20 cm on arable plots in St. Petersburg region (Russia). Organic matter content and pH_{KCl} of soil were equal to 21 g C kg^{-1} soil and 6.3, respectively. Soil samples were incubated at a moisture content of 50% of full saturation and at 28°C for 6 months. The initial concentration of azobenzene in the soil was 50 mg kg^{-1} soil. The microbiological analysis was carried out after 0, 7, 30, 60 and 120 days of incubation. The program of statistical data processing of one-factorial experiment 'Diana3', version 1.02, was used for an estimation of the influence of azobenzene on the bacterial complex of the soil.

Spatial distribution of microorganisms in soil was studied using a glass slide technique modified by Kruglov (1991). Glass slides of 2x5 cm were covered with a thin layer of agar medium without a carbon source or with starch. Then pesticide crystals were placed on the agar surface and afterwards agar was dried in a sterile oven at 40°C. The glass slides were placed in the soil and incubated at 28°C for three months. Five replicates were used in the experiments. Glass slides were regularly removed from the soil and fixed in vapours of OsO_4. Then they were stained in a 5% carbolic solution of erythrosine. The slides were examined by a microscope and documented on microphotographs.

To isolate microorganisms, nutrient agar of the following composition was used (g l^{-1}): 1 of K_2HPO_4, 1 of yeast extract, 1 of peptone, 1 of glucose. The Ashby's medium (Tepper *et al.*, 1993) without a nitrogen source was used for isolating N_2-fixing bacteria (g l^{-1}): 0.2 of K_2HPO_4, 0.2 of $MgSO_4 \times 7H_2O$, 0.2 of NaCl, 0.1 of K_2SO_4, 5 of $CaCO_3$, 20 of mannitol. The morphological and biochemical identification of the bacteria was conducted according to the Bergey's Manual of Systematic Bacteriology (1984-1989).

The effect of azobenzene on the growth of the bacteria was studied using a replication technique. Replicate filter papers (5 per treatment) were placed in Petri dishes and then 20 ml of fused agar medium was added. The concentration of azobenzene was 3.5 and 10 mg per filter.

Azobenzene degradation was studied in a liquid medium supplemented with 200 mg l^{-1} azobenzene. The cultures were grown in 250 ml flasks shaken at 28°C and 100 rpm for one month. As *trans*-azobenzene is water-insoluble, it was dissolved in ethanol (1 ml l^{-1} medium) before addition to the nutrient medium. Nitrogen-fixing bacteria were tested in the Ashby medium. The mineral medium for bacilli has a chemical composition as follows (g l^{-1}): 1.6 of K_2HPO_4, 0.4 of KH_2PO_4; 0.5 of NH_4NO_3; 0.2 of $MgSO_4$ x $7H_2O$; 0.02 of $CaCl_2$; 0.02 of $FeCl_3$; 0.2 of yeast extract; 10 of glucose. Azobenzene was extracted from the culture liquid with two volumes of benzene and determined by photocolorimetry at λ=440 and by thin-layer chromatography on silicagel plates ('Silufol').

RESULTS AND DISCUSSION

The effects of azobenzene on the diversity of heterotrophic soil bacteria

Microbiological analyses showed that azobenzene in concentration up to 50 mg kg^{-1} of soil did not markedly affect the total number and qualitative composition of culturable bacteria. The following heterotrophic soil bacteria were identified by phenotype: *Arthrobacter globiformis, Arthrobacter uratoxydans, Bacillus cereus, Bacillus licheniformis, Bacillus megaterium, Bacillus polymyxa*, *Curtobacterium* sp., *Micrococcus varians, Pseudomonas facilis, Pseudomonas stutzeri, Xanthomonas campestris*. At the initial incubation, *Curtobacterium* sp., *A.globiformis*, *M.varians* and *B. megaterium* dominated in the soil. After one week of soil samples incubation the number of *M. varians* decreased in a variant with azobenzene in comparison with the control (Fig. 1); the coefficient of factor influence was 0.76. After 120 days of incubation, A. *globiformis* was not found among the dominant species in this trial (Fig. 2); the coefficient of factor influence was 0.98.

Therefore, azobenzene did not produce any significant effect on heterotrophic cultivable bacteria in sandy loam Spodosol. It was shown that hydrophobic substances can be accumulated by various components of the soil and become inaccessible for the microorganisms (Sarnaik and Kanekar, 1995).

Fig. 1. Influence of azobenzene on the bacterial complex of the soil (7 days of incubation). Data are average values of 15 replicates: 1 – *Curtobacterium* sp., 2 – *A. globiformis*, 3 – *M. varians*, 4 – *B. megaterium*, 5 – *P. facilis*, 6 – *B. cereus*, 7 – *X. campestris*, 8 – *A. uratoxydans*, 9 – *B. polymyxa*, 10 – *P. stutzeri*, 11 – *B. licheniformis*.

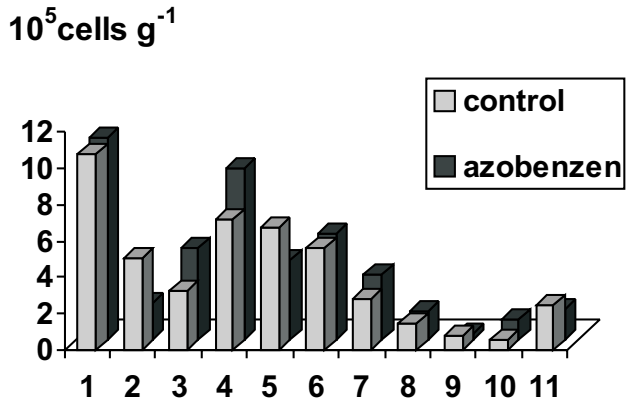

Fig. 2. Influence of azobenzene on the bacterial complex of the soil (120 days of incubation). Data are average values of 15 replicates: 1 – *Curtobacterium* sp., 2 – *A. globiformis*, 3 – *M. varians*, 4 – *B. megaterium*, 5 – *P. facilis*, 6 – *B. cereus*, 7 – *X. campestris*, 8 – *A. uratoxydans*, 9 – *B. polymyxa*, 10 – *P. stutzeri*, 11 – *B. licheniformis*.

Spatial distribution of soil microflora in the presence of azobenzene

The results of the study of spatial distribution of soil microflora in the presence of azobenzene showed that azobenzene was found on the glass slides in the form of orange-coloured crystals and particles. As a rule, the surface of separate particles of this substance was colonised by a single type of microorganisms which first attacked the substrate and formed microcolonies on the surface (Fig. 3a and b). Different particles from the same glass slide often showed various morphological types of microorganisms: small and large rods, cocci and bacteria similar to azotobacter (Fig. 3). The degradation of azobenzene particles occurred during the incubation (Fig. 3c and d). The intensity and character of the growth on glass slides depended on the incubation time and on the content of the metabolizable carbon source on the slides.

Small bacterial cells were found after one month of incubation of the slides with agar medium without the carbon source. The growth of various forms of bacteria around the azobenzene crystals was observed after one

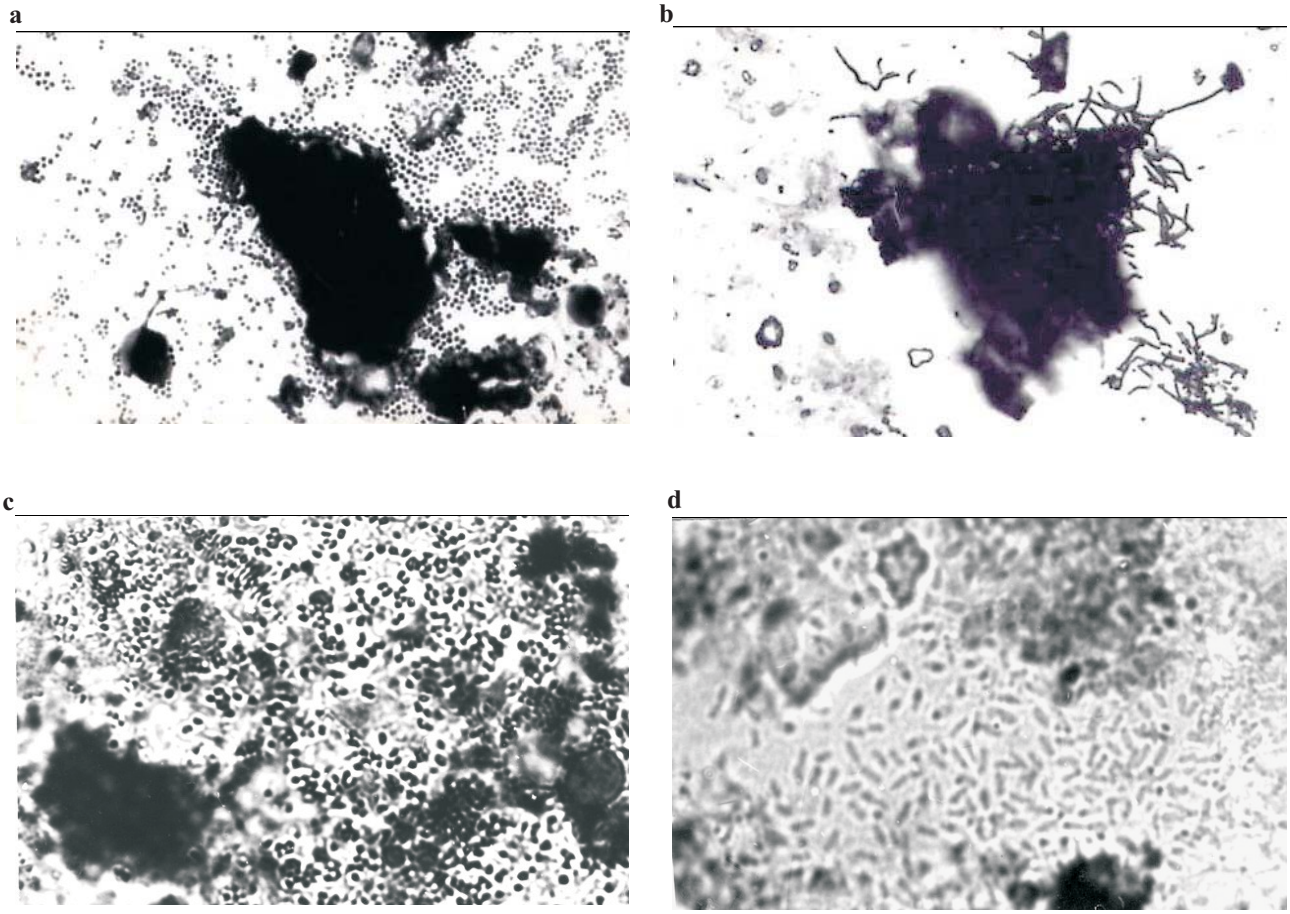

Fig. 3. Microflora developing on azobenzene particles in the soil, x1000: a, b – one week of incubation; c, d – 2 months of incubation.

month of incubation. Fungal and actinomycetes hyphae were observed more seldom and usually developed at some distance from the azobenzene particles.

The intensity of the microbial growth on glass slides was greater in the experiment with starch medium. The various forms of bacteria, and fungal and actinomycete hyphae were observed on the glass slides already after one week of incubation. The bacteria concentrated and formed microcolonies around the azobenzene particles.

After 2-3 months the degradation of large crystals of azobenzene and their transformation into amorphous masses was observed in both trials (Fig. 3c and d). It is assumed that the microorganisms concentrating on the surface of the particles may decompose azobenzene.

Bacteria of different genera were isolated from the glass slides: *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Flavobacterium* and the nitrogen-fixing bacteria – *Azotobacter* and *Bejerinckia*.

Toxic effects of azobenzene on soil microorganisms

The effect of substrate on the bacterial growth was studied on the agar plates. Based on a change in the color of colonies, the results suggested that some microorganisms on the plates accumulated azobenzene (Table 1). The presence of azobenzene in the bacterial colonies was confirmed by qualitative reaction with $SnCl_2$ (Guben-Vehl, 1963). The ability of azobenzene to effect its distribution in the agar medium may be related with its metastability (Haring, 1946; Sharp, 1946). Azobenzene accumulation was also observed in the mycelium of micromycetes isolated from soil. The hyphae of fungi plated on azobenzene-containing medium were coloured in orange under the microscope investigation.

The toxic effect of azobenzene on soil corineform bacteria was found out at their cultivation on nutrient media with the addition of the substance (Table 1). Therefore, these bacteria can be indicators of azobenzene presence in an environment. The inhibitory effect of azobenzene on micromycetes isolated from soil - *Trichoderma lignorum*, *Mucor* sp., *Penicillium* sp. was also noted. The reduction of radial growth rate and a delay of the formation of spores were observed. Toxicity of azobenzene for microorganisms increased with its increasing concentration in the nutrient media.

Earlier it was shown that the Cl-containing azobenzenes were capable of accumulating in tissues of fishes and molluscs (Allinson and Morita, 1995). The azobenzenes were accumulated in tissues of sea mollusc *Indoplanorbis exustus*, but only up to a certain concentration which did not cause toxic action (Allinson and Morita, 1995).

It is established that many azo-dyes and their intermediate metabolites are carcinogenic for man, but the mechanism of their influence on an alive cell has not been yet studied (Cheung, 1994). The Japanese scientists investigated the ability of 151 chemical compounds to cause the expression of *umu* genes in *Salmonella thyphimurium* TA 1535/pSK1002 and showed that azobenzene possesses rather weak genotoxicity and is capable of causing an induction only after incubation within 2-5 h (Nakamura *et al.*, 1987). It was found that the genotoxicity of azobenzenes increased in the presence of an amino group in molecule (Mori *et al.*, 1986).

T a b l e 1. Growth of the bacteria on the agar plate in the presence of azobenzene

Species	Effect of azobenzene on the growth of the bacteria[1]	Change of the colour of the colonies	Accumulation of the azobenzene
Azotobacter sp.	+	No	No
Arthrobacter globiformis	–	Orange	Yes
Arthrobacter uratoxydans	–	Orange	Yes
Bacillus cereus (№1, №2)	+	Orange	Yes
Bacillus licheniformis	=	No	No
Bacillus megaterium	=	Orange	Yes
Bacillus polymyxa	+	No	No
Bejerinckia sp.	+	No	No
Curtobacterium sp.	–	Orange	Yes
Micrococcus varians	=	No	No
Pseudomonas facilis	=	No	No
Pseudomonas stutzeri	=	No	No
Xanthomonas campestris	=	No	No

[1]The effect of azobenzene on the growth of the bacteria: incentive '+', suppress '–', effect is absent '='.

Azobenzene transformation by heterotrophic bacteria

There are not numerous data on microbial transformation of azobenzene. Thus, a significant part of such works is devoted to the decomposition of azo-dyes. In soils, the azo-dyes can be transformed by the ligninolytic fungi and micromycetes (Chivukula and Renganatan, 1995; Goszczynsky *et al.*, 1994; Novotný *et al.*, 2004) and by heterotrophic bacteria such as *Bacillus* and *Pseudomonas* (Carliell *et al.*, 1995; Sarnaik and Kanekar, 1995; Zissi *et al.*, 1997; Zissi and Lyberatos, 1996).

Five actively growing on medium with azobenzene strains from soil (*B. polymyxa* and *B. cereus* №1) and glass slide (*A. chroococcum*, *Bejerinckia* sp. and *B. cereus* №2) were selected for the study of biotransformation. The transformation of azobenzene by bacteria was studied in a liquid culture in the presence of an easily mineralisable carbon source (glucose for bacilli and mannitol for nitrogen-fixing bacteria).

It was shown that isolated bacteria assimilated azobenzene under co-metabolic conditions. After three weeks of incubation, azobenzene decolourisation was observed. In cultures of *B. cereus* №1, *B. cereus* №2 and *B. polymyxa* the concentration of azobenzene was reduced by 47, 36, and 33% in the medium with glucose, respectively. *Azotobacter* sp. and *Beijerinckia* sp. transformed azobenzene by 30 and 25% in the medium with mannitol.

CONCLUSIONS

1. Pesticide azobenzene in the industrial concentrations did not produce any significant effect on heterotrophic culturable bacteria in sandy loam Spodosol. However, the number of two corineform bacteria *M. varians* and *A. globiformis* decreased in a variant with azobenzene in comparison with the control.

2. Using analysis of the microbial distribution on the glass slides placed into the soil it was concluded that a spatial distribution of microorganisms occurred due to interaction with azobenzene. Chemotaxis may play a significant role in this process. Therefore, a specific microflora developed and concentrated around and on the surface of azobenzene particles and caused the degradation of azobenzene. It is likely that the mechanism of this process is by the co-metabolic action of extracellular enzymes and other products of microbial metabolism (such as hydrogen peroxide, organic acids, alkali, *etc*) released to the surface of the water insoluble xenobiotic particles.

3. The toxic effect of azobenzene on some representatives of soil microflora (corinerform bacteria and some soil micromycetes) was found at their direct cultivation on nutrient media with addition of the substance. Such microorganisms can be indicators of the presence of azobenzene in an environment. It was also revealed that some microorganisms were capable of accumulating azobenzene.

4. It was shown that the bacteria isolated from the glass slides - *A. chroococcum*, *Bejerinckia* sp. and *B. cereus* №2 and soil - *B. polymyxa* and *B. cereus* №1 were able to assimilate azobenzene in the presence of carbohydrates (mannitol or glucose).

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