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EXPERIMENTAL ARTICLES

The Dynamics of the Size and Structure of the Soil Bacterial Complex in the Presence of Azobenzene

N. D. Koneva and Yu. V. Kruglov

All-Russia Research Institute of Agricultural Microbiology, sh. Podbel'skogo 3, Pushkin 8, St. Petersburg, Russia

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Abstract—Azobenzene exerted no significant effect on the dynamics and the species composition of the saprophytic soil bacterial complex, which remained almost the same as in the control and was characterized by the predominance of *Curtobacterium* sp., *Arthrobacter globiformis*, and *Bacillus megaterium* in all stages of succession. Some heterotrophic bacteria were found to be able to accumulate azobenzene. *Bacillus cereus* and *Bac. polymyxa* degraded azobenzene during their cultivation in nutrient media.

Key words: azobenzene, accumulation, heterotrophic bacteria, biodiversity.

Soil contamination, which adversely affects the biocenoses of soils and their quality, is now a challenging ecological problems. Many xenobiotics are persistent compounds and, migrating along food chains, may exert toxic effects on domestic animals and humans.

Among persistent xenobiotics, various aromatic azo compounds, such as textile azo dyes and pesticides are great environmental hazards [1]. However, problems resulting from the interaction of azo compounds and soil microorganisms have been poorly studied.

The aim of the present work was to study the effect of the acaricide azobenzene on the dynamics and the structure of the soil bacterial complex in the process of its succession.

MATERIALS AND METHODS

Soil samples were taken from the arable A horizon (2.1% humus; pH 6.3) of a well-cultivated, light loamy, soddy podzolic soil in the Leningrad region. The experiments were performed using 500-ml vessels with 400 g of soil containing azobenzene at a concentration of 50 mg/kg. The soil samples were wetted to 50% of the total moisture capacity and incubated at 26°C. After 1, 7, 30, 60, and 120 days of incubation, the aliquots of the soil suspension were inoculated onto the standard glucose–peptone–yeast extract agar medium [2] for microbiological analysis. The isolated bacteria were identified according to their morphological, physiological, and biochemical properties [3, 4]. The biodiversity of the bacterial complex was estimated by the Simpson index [5]. The data obtained were statistically processed at significance level $P = 0.05$ [6].

The effect of azobenzene on bacterial growth was estimated using a glucose–peptone agar medium. For this purpose, petri dishes with pieces of azobenzene-impregnated filter paper at their bottoms were filled with 15 ml of the agar medium. The amount of azobenzene in the filters was either 3.5 or 10.0 mg. The agar medium was inoculated with bacteria using a metal 26-cell replicator with one empty, asymmetrically disposed cell, which served to control the proper position of the replicator. The replicator cells contained 0.1-ml aliquots of the analyzed bacterial suspension. The degradation of azobenzene was studied using a liquid mineral medium of the following composition (g/l): yeast extract, 0.2; K_2HPO_4, 1.6; KH_2PO_4, 0.4; NH_4NO_3, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeCl_3$, 0.02; and glucose, 10.0. The initial concentration of azobenzene was 50 mg/l. Azobenzene and the products of its transformation were extracted from the culture liquid with benzene and quantified by direct colorimetric analysis on an FEK photoelectrocolorimeter at $\lambda = 440$ nm and by thin-layer chromatography on Silufol silica gel plates using benzene as a mobile phase [7, 8].

RESULTS AND DISCUSSION

The data presented in Fig. 1 show that the dynamics of the saprophytic populations in soil in the presence and absence of azobenzene were almost the same during a 4-month incubation period: the bacterial populations tended to decrease within the first 60 days of incubation and then to remain constant. The decrease in the bacterial population densities could be described by an exponential curve, which is typical of systems tending to be in equilibrium [9].

The size of bacterial populations in soil is regulated by two factors: the growth potential inherent in a given population (internal factor) and environmental conditions (external factor) [10]. In our case, the decrease in the bacterial populations was most likely associated with the exhaustion of energy-providing substances that are easy to metabolize or the impairment of their qualitative composition, while the relative stability of the system was not affected by azobenzene.

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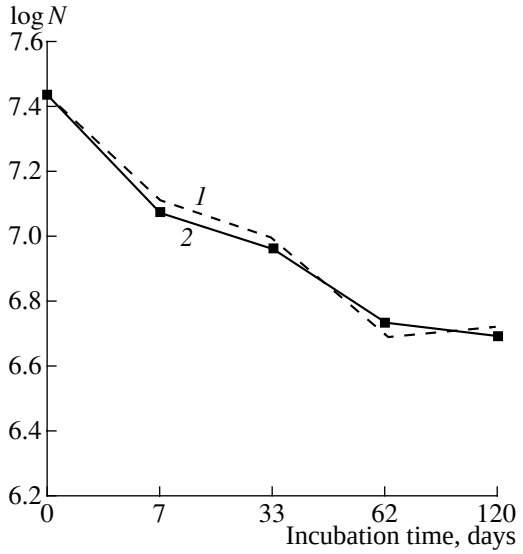

Fig. 1. Dynamics of the population of heterotrophic bacteria in the soddy podzolic soil in the (*2*) presence and (*1*) absence of azobenzene.

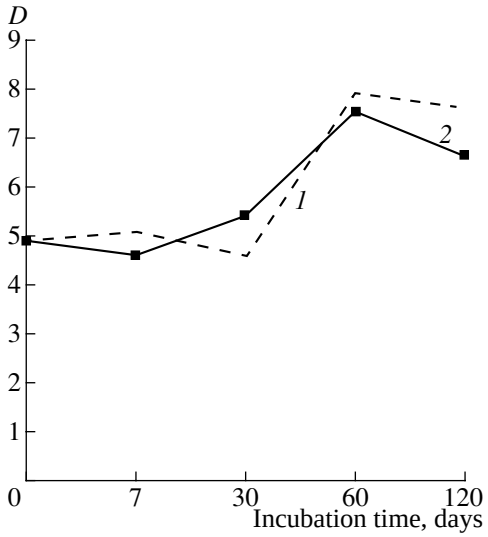

Fig. 2. Dynamics of the diversity index of the soil bacterial complex in the course of its succession: (*1*) in the absence of azobenzene and (*2*) in its presence.

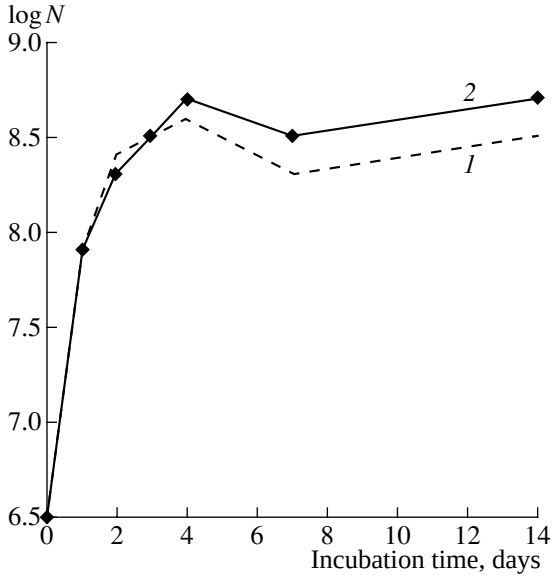

Fig. 3. Growth of *Bac. polymyxa* in the (*2*) presence and (*1*) absence of azobenzene.

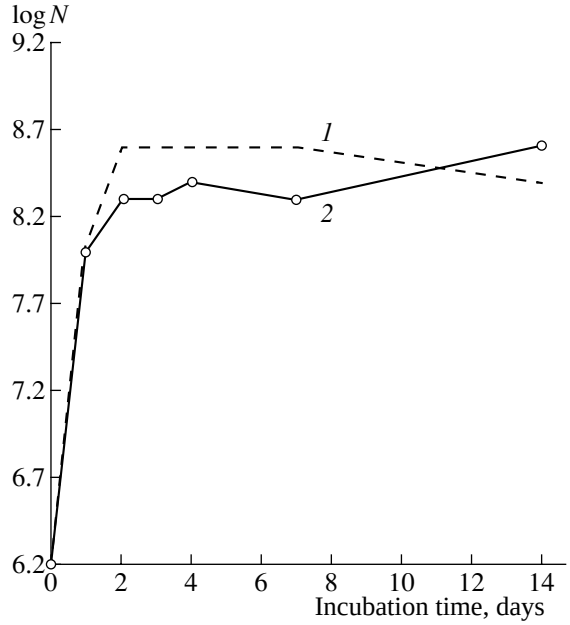

Fig. 4. Growth of *Bac. cereus* in the (*2*) presence and (*1*) absence of azobenzene.

Analysis of the morphological, physiological, and biochemical properties of the 25 strains that were isolated in the present study allowed us to assign them to 11 bacterial species (Table 1). It can be seen that two to three bacterial species comprising up to 60% or more of the total bacterial population were dominant at different stages of the succession. In the first 30 days of incubation, the population was dominated by *Curtobacterium* sp. and *Arthrobacter globiformis*, while *Bac. megaterium* and *Micrococcus varians* dominated after 60 days, and *Bac. megaterium* and *Curtobacterium* sp. dominated after 120 days of incubation. It is evident that the relative abundance of the sporogenic bacteria *Bac. megaterium*, *Bac. cereus*, and *Bac. licheniformis* increased and that of *A. globiformis* decreased in the process of succession. After four months, *A. globiformis* was no longer among the dominant bacteria.

The addition of azobenzene to the soil did not essentially affect the succession of the bacterial complex except that, after 120 days, the relative abundance of *A. globiformis* decreased to comprise as little as 4% (three times less than in the control), while *Curtobacterium* sp. and *Bac. megaterium* dominated, comprising almost 50% of the population of heterotrophic bacteria.

Table 1. The structure of the soil bacterial community in the course of its succession (% of the total population)

Species	Days				
	1	7	30	60	120
Arthrobacter globiformis	26/26	20/24	15/17	15/12	12/4
Arthrobacter uratoxydans	5/5	8/6	5/7	6/10	6/8
Bacillus cereus	6/6	6/4	8/10	11/11	14/10
Bacillus licheniformis	0/0	1/<1	1/1	4/3	5/4
Bacillus megaterium	9/9	8/11	15/11	17/19	15/21
Bacillus polymyxa	<1/<1	1/<1	1/1	2/1	1/2
Curtobacterium sp.	33/33	35/36	40/34	11/16	23/24
Micrococcus varians	13/13	14/9	5/11	17/18	7/11
Pseudomonas facillis	5/5	4/4	6/5	11/6	12/13
Pseudomonas stutzeri	<1/<1	2/1	3/1	2/2	2/1
Xanthomonas campestris	2/2	1/4	2/3	4/3	3/2

Note: Data in the numerators and denominators of the fractions refer to the control and the experiment, respectively.

Figure 2 illustrates changes in the bacterial diversity of the soil [5]. In the course of succession, the diversity index of the bacterial complex increased due to the decrease and equalization of the populations of the dominant bacterial species. The small difference between the experiment and the control allows the conclusion to be drawn that heterotrophic bacteria in the soddy podzolic soil are resistant to azobenzene. To explain the high tolerance of soil bacteria to azobenzene, one should take into account its extremely poor solubility in water, due to which the contact between azobenzene and the soil bacteria is negligible.

Table 2 shows how azobenzene introduced into agar medium affected the growth of some bacterial species isolated from the soil. It can be seen that azobenzene inhibited the growth of *A. globiformis*, *A. uratoxydans*, and *Curtobacterium* sp. and stimulated the growth of *Bac. cereus* and *Bac. polymyxa*. In the presence of azobenzene, the colonies of *A. uratoxydans*, *Bac. cereus*, and *Bac. megaterium* turned orange-yellow and produced more slime. The orange-yellow color of the colonies was most likely due to the accumulation of azobenzene in them, as follows from the tin chloride test [7]. Therefore, some soil bacteria can accumulate azobenzene and, hence, may be involved in the translocation and transformation of this compound in soil. The ability of bacterial colonies to accumulate azobenzene was confirmed in the experiments in which bacteria and azobenzene were separated by a layer of agar or air. In the former case, a piece of azobenzene-impregnated filter paper was placed at the bottom of a petri dish, overlain with an agar layer onto which bacteria were inoculated, and incubated until the colonies grew. In the latter case, the agar medium was inoculated with bacteria, the petri dish was inverted, and fine azobenzene crystals were placed onto the lid of the inverted dish from the inside. In both cases, the grown colonies were orange-yellow and the reaction for azobenzene was positive. The ability of azobenzene to diffuse through the agar

Table 2. Some characteristics of bacteria growth on glucose–peptone agar with azobenzene

Species	Effect of azobenzene on bacterial growth	Yellow–orange color of colonies	Azobenzene accumulation
Arthrobacter globiformis	–	No	No
Arthrobacter uratoxydans	–	Yes	Yes
Bacillus cereus	+	Yes	Yes
Bacillus licheniformis	=	No	No
Bacillus megaterium	=	Yes	Yes
Bacillus polymyxa	+	No	No
Curtobacterium sp.	–	No	No
Micrococcus varians	=	No	No
Pseudomonas facillis	=	No	No
Pseudomonas stutzeri	=	No	No
Xanthomonas campestris	=	No	No

Note: "+," "–," and "=" stand for "growth stimulation," "growth inhibition," and "no effect," respectively.

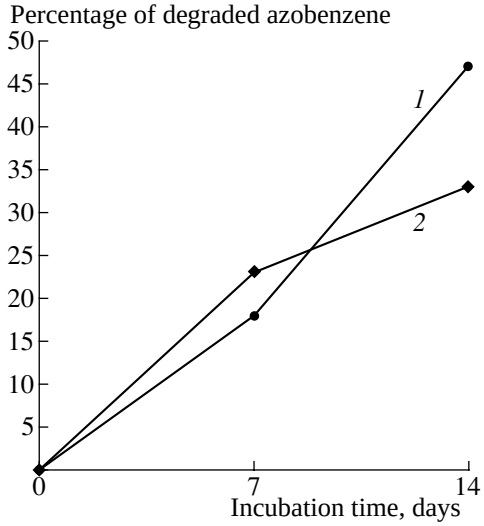

Fig. 5. Degradation of azobenzene by (*1*) *Bac. cereus* and (*2*) *Bac. polymyxa.*

and air is probably related to its metastability [11], due to which in the past this compound was used in agriculture as a fumigant [12]. Because of the metastability, azobenzene may exist in a liquid state at temperatures lower than its melting point. As a result, small droplets of this compound slowly evaporate and the vapor may again crystallize on solid surfaces some distance away. When in the metastable state, compounds, like azobenzene, have a higher molar isobaric potential than in the stable state and are more reactive.

There is limited information in the literature as to the ability of microorganisms to transform azobenzene. The mechanism of its degradation may involve the breakage of the double bond between two adjacent nitrogen atoms in the molecule with the formation of aniline, which can be further mineralized through a number of steps [13–15]. The microbial transformation of azobenzene requires an additional source of carbon and energy (the so-called cometabolism).

The azobenzene-transforming ability of soil bacteria was assessed using two isolates of the spore-forming bacteria *Bac. cereus* and *Bac. polymyxa*. The experiments showed the absence of bacterial growth in a mineral medium with azobenzene as the sole source of energy, indicating that the bacteria did not utilize azobenzene as the energy source. The addition of glucose to the medium appreciably stimulated the growth of *Bac. cereus* and *Bac. polymyxa* both in the presence and absence of azobenzene, suggesting that this compound is not toxic to these microorganisms (Figs. 3 and 4). Figure 5 illustrates the degradation of azobenzene by the bacteria. This process was more intense in the *Bac. cereus* culture, which, as was shown above, can absorb azobenzene from the nutrient medium and from the air. After 14 days of incubation, *Bac. cereus* transformed almost half of the azobenzene initially present in the medium. We failed to reveal aniline or phenol among the products of the azobenzene transformation.

Thus, azobenzene does not essentially affect the composition of the soil bacterial community. Being a metastable compound, azobenzene can readily evaporate and then be absorbed by bacterial cells or adsorb on their surface to undergo subsequent transformation. The investigation of the translocation and degradation of metastable xenobiotics by soil microorganisms is of great ecological interest.

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