RESEARCH ARTICLE

Assessing Resistance of the Microbial Community in Soils to Pollution with Antibiotics

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Abstract

Objective: The study is aimed at assessing resistance of the microbial community in soils (on the example of typical chernozem) to contamination with antibiotics of various chemical nature and spectra of activity (benzylpenicillin, ampicillin, streptomycin, oxytetracycline, tylosin, farmasinum, tromexin, aliseryl, nystatin, and input). **Methods:** The comprehensive study of microbiocenosis in soil included determination of the total number of bacteria by the method of fluorescent microscopy with staining of the samples with acridine orange, determination of the abundance of viable microorganisms of various ecological trophic groups with the use of the methods of sowing on solid nutrient substrate. Reaction of the substrate (pH) was determined by the potentiometric method in soil suspension 1:2.5. **Results:** The research has shown that chernozem contamination with antibiotics reduces the total number of bacteria, decreasing the number of individual ecological trophic groups of microorganisms, and changes their ratio. Contamination with antibiotics results in significant reduction in the number of ammonifying bacteria, which are represented most widely of the various ecological trophic groups of soil microorganisms. Most studied microbiological parameters get reduced when the concentration of antibiotics reaches 100 mg/kg of soil. The maximum influence of antibiotics is noticed within the first 10 days from the moment of contamination. The degree of antibiotics influence is determined by their nature, concentration, and time of exposure. Antibacterial antibiotics have a more inhibitory action on the microbial community, compared to the fungicidal ones. The effect of antibiotics on soil microbial coenosis has prolonged nature. **Conclusions:** By the degree of soil inhibition with microorganisms, antibiotics form the following line: Ampicillin > benzylpenicillin ≥ streptomycin > tylosin ≥ farmasinum > nystatin > tromexin > aliseryl. The degree of resistance to antibiotics, the studied microorganisms of chernozem formed the following line: Bacteria g. Azotobacter > micromycetes > amylolytic bacteria > ammonifying bacteria. The number of ammonifying bacteria is the most informative microbiological indicator of contamination with antibiotics. The indicator of g. Azotobacter bacteria abundance is uninformative in case of contamination with antibiotics.

Key words: Antibiotic resistance, antibiotics, biodiagnostics, bioindication, pollution, soil microbiocenosis

INTRODUCTION

Today, the changes in the microbial community of various types of Russian soils on pollution by heavy metals, oil and oil products,[1-3] pesticides,[4] and electromagnetic effects of various nature[5,6] are quite well studied. Unlike pesticides that have long been used in agriculture, antibiotics have not aroused interest as potential environmental pollutants. However, due to their intensified use in all spheres of agriculture, the problem of natural ecosystems pollution with antibiotics is now given special attention. In terms of industrial production and economic indicators, antibiotics take the first place in the amounts of all pharmaceutical products. Currently, ecotoxicity of antibiotics in the environment has not been studied sufficiently.[7]

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The Central Office for Environment Protection of Germany studied the scale of using antibiotics in the countries of the European Union (EU). It turned out that the main consumers of antibiotics are livestock breeding industries. One of the first countries to prohibit the use of antibiotics in animal husbandry was Sweden, followed by Switzerland, the Netherlands, and several other European countries. In Russia, however, fodder antibiotics are not prohibited. In the EU, there are still no environmental standards for tetracycline and sulfonamides.

The main sources of antibiotics into the environment are pharmaceutical industry, veterinary, and human medicine. Currently, livestock and crop production in many countries use huge amounts of antibiotics both to stimulate animal growth and as a preventive measure. Used antibiotics move through the food chains and get accumulated in plants, particularly in fruits and vegetables.[7-9]

Antibiotics are used for the treatment of bacterial diseases in animals, and as a feed additive. Doctors of veterinary medicine in various countries practice introduction of antibiotics into the fodder for a long period of animals fattening.[10]

Antibiotics ingress into the soil due to using manure[11] and waste water[12] as fertilizer for agricultural lands. Every year, increasingly different concentrations of antibiotics are found in wastewater, soils, ground, and drinking water.[8,9] Locally in soils antibiotics of the tetracycline group from trace quantities up to 900 mg/kg, macrolides up to 800 mg/kg[13] are found. Getting into soil, antibiotics can have an overwhelming effect on various groups of microorganisms, and influence growth and development of plants.[14] Numerous experimental studies[15] showed that most antibiotic substances used in agriculture got adsorbed and penetrated into the tissue of plants through vegetative organs. The toxic effects of antibiotics were very diverse.

METHODS

The work provides comprehensive assessment of sustainability of the microbial community in soils to contamination with antibiotics of various chemical nature and spectra of action. Bactericidal antibiotics (benzylpenicillin, ampicillin, and streptomycin), bacteriostatic ones (oxytetracycline, tylosin, and farmasinum), growth promoters in animal husbandry (tromexin and aliseryl), and fungicides (nystatin and input) were used in the study. The object of this study was the microbial coenosis in ordinary chernozem of the southern European facies of carbonate thick weakly humous heavy loam on yellow-brown loess-like loams. Ordinary chernozems (Voronic Chernozems Pachic) occupy the vast plains of the Azov-Kuban plain within the territory of Krasnodar Krai, and the southern part of the Rostov region, according to the new classification - migration and segregation soils.[16]

Chernozems have been chosen as the object since these soils are characterized by high abundance, diversity of microorganisms, and their high activity.[17] Besides, the chernozems area is the major agricultural area of the country. More than half of arable soils are represented by chernozems, where grain, oilseeds, and technical crops are grown, etc. Soils were picked for laboratory model studies from the Botanical Garden of the Southern Federal University, Rostov-on-Don (topsoil, 0–25 cm).

Several model experiments were setup to solve the set tasks. In the first series of model experiments, air-dry soil samples were contaminated with solutions of medical antibiotic - benzylpenicillin, veterinary antibiotic - farmasinum, and their mixture with fungicidal antibiotic - nystatin - in a wide range of concentrations between 1 and 1000 mg/kg soil. Changes in the microbiological parameters were studied 0, 60, and 120 days after contamination. The used range of concentrations was chosen from the literature data, according to the residual quantities of antibiotics detected in the environment.[18]

In the second series of model experiments, the range of antibiotics used as contaminants was expanded (medical - ampicillin, streptomycin, and veterinary tylosin, tromexin, aliseryl, as well as their mixtures with input fungicide) at the concentration of 500 mg/kg of soil. This concentration was chosen according to the results of earlier reconnaissance studies.[19,20] Soil microbiocenosis was studied 3, 30, and 90 days after contamination. The soil not contaminated with antibiotics was the reference. All soil samples were incubated at 20–25°C and optimum moisture (60% of normal capacity).

Laboratory analytical study was performed at the Department of Ecology and Natural Resources of the Southern Federal University, with the use of methods commonly used in ecology, biology, and soil science.[21]

The comprehensive study of soil microbiocenosis included determination of the total number bacteria with the method of fluorescent microscopy with samples staining by acridine orange. Bacterial cells glowing green were counted on ZEISS inverted microscope, AXIO Vert. A1 model with a 450–490 nm wavelength filter.

In addition, abundance of viable microorganisms of various ecological trophic groups was assessed by methods of seeding appropriate dilutions on solid nutrient substrate: Ammonifying bacteria were determined on meat peptone agar, amylolytic bacteria - on starch and ammonia agar, abundance of micromycetes was determined on Czapek's medium. Using the method of fouling lumps on the Ashby medium, abundance of g. Azotobacter bacteria was assessed.

A combination of methods of inoculation onto dense media and direct luminescent microcopying makes it possible to characterize microbial succession in the soil.

Reaction of the substrate (pH) was determined by the potentiometric method in soil suspension 1:2.5.

The integral biological activity indicator has been calculated. It characterizes the intensity and direction of the microbiological processes in the soil as a whole. This method of calculation allows to combine relative values of various indicators, absolute values of which cannot be aggregated since they have different measurement units.

The results of the study were statistically processed with the use of statistical package Statistica 10.0 and Excel. Main indicators of variation statistics have been calculated: Average ± mean error(M ± m), standard deviation (s), coefficient of variation, etc.

RESULTS AND DISCUSSION

Soil contamination with antibiotics and their mixtures with fungicides reduce the number of main ecological trophic groups of microorganisms [Table 1] and change their relations and the structure of the microbial community as a whole. In most cases, there was the direct relationship between the content of antibiotics in the soil and the degree of reduction of microbiological indicators (correlation between antibiotics concentration and the decrease of studied parameters $r = -0.68$–0.86). From the range of picked concentrations of 1–1000 mg/kg, the minimum statistically significant effect of the inhibitory action of antibiotics was observed at the concentration of 100 mg/kg of soil. The maximum impact of antibiotics on the soil microbial coenosis became evident within the first 10 days after contamination.

Reaction of the environment (pH) is an important chemical indicator of soils, on the one hand - due to its influence on the content of humic components, and on the other hand - due to its effect on the activity of soil microbiota.[22] Analysis of contaminated samples showed that antibacterial antibiotics (benzylpenicillin, ampicillin, streptomycin, oxytetracycline, and tylosin) changed the reaction of soil medium to alkaline. The range of pH fluctuations during the entire incubation period (10–120 days) of the reference sample was 7.0–7.2, in samples contaminated with antibiotics - 7.5–8.3, depending on their chemical nature and on antibiotic concentration. Fungicidal antibiotics and growth stimulants (nystatin, tromexin, and aliseryl) slightly changed the medium reaction to acid (pH difference between the reference and contaminated samples was 0.5). Probably, the change of acidity was due to microbial community transformation under the action of antibiotics, and its biochemical effect on the soil. Besides, antibiotics themselves were reduced to chemical components and metabolites, which could also contribute to changes in soils' acidity. Thus, antibiotics disrupt the natural environment of soil microorganisms, which are one of the reasons for changing their number and the structure of microbial coenosis as a whole.

By fluorescent microscopy, a decreased total number of bacteria were detected in case of contamination with antibiotics [Table 2]. Close correlation ($r = -0.85$) was established between antibiotic concentration and the total number of bacteria. The most significant decrease in the total number of bacteria occurred within the first 10 days from the moment of contamination. During the following periods of the research, a tendency to recovery of their numbers was observed. However, despite the observed tendency, even 120 days after contamination, the difference in the number of bacteria in the contaminated and the reference samples were 23% (1000 mg/kg, $P < 0.01$). This prolonged nature of the influence of antibiotics on the microorganisms in soil was associated with the processes of their inactivation in soil. Some of them were inactivated in the soil within the 1st hours after introduction; other could remain unchanged for a long time - several days or even several months, depending on the chemical structure of the antibiotic, and on the properties of the substrate.[23]

Other studies have shown that tetracyclines have the highest rate of accumulation in the soil, followed by sulfonamides and quinolones.[24] In addition, it has been shown that erythromycin, oleandomycin, tylosin, as well as polyether antibiotics, are well degradable in soil under natural conditions. However, their metabolites remain unchanged for a long time and can influence the microorganism's activity. It has also been found that tylosin and some polyether antibiotics have less severe effect on the microorganisms than sulfanilamides and tetracyclines.[25]

Analysis of changes in the abundance of the g. Azotobacter bacteria showed their relative resistance to contamination with antibiotics. Changes in the abundance of bacteria were observed only during the first periods of exploration (10 days). Compared to the reference, the decrease in the abundance of bacteria was 30–40% after contamination with ampicillin and streptomycin, and 50–60% after soil contamination with a mixture of ampicillin + input and streptomycin + input. During further periods of the study, changes of the abundance of this bacteria genus were not observed. 100 days after contamination, abundance of bacteria recovered to the reference values. With that, g. Azotobacter bacteria were more sensitive to contamination with oil and oil products, heavy metals, ionizing radiation, than other groups of soil microorganisms.[5,26]

Ammonifying bacteria were most sensitive to introduced antibiotics, unlike other studied groups of microorganisms. Similar results were obtained by other researchers.[27,28] Using correlation analysis, close relationship was found between the concentration of antibiotics and changes in the number ammonifying bacteria ($r = -0.80$). Similarly, other studies have shown that the degree of antibiotics effect depends on their concentration, the higher the dose is, the stronger the effect is.[29] For example, it has been shown that the introduction of sulfachloropyridazine into the soil during the first periods of the study results in inhibition of the number of microorganisms, while the subsequent increase in the concentration of antibiotics develops resistance of microorganisms.[30] Ampicillin, streptomycin, and tylosin (500 mg/kg) reduce the number of ammonifying bacteria by more than 50% of the reference. Streptomycin has the most significant effect (reduction by 85% of the reference, $P < 0.001$). Fungicide has no significant veracious impact on the number of ammonifying bacteria. However, in combination with medical and veterinary antibiotics, the number of ammonifying bacteria reduces to a greater extent, thus indicating synergism of antibiotics and fungicides and an increase in their negative impact. Other researchers obtained similar results in combinations with antifungal preparations and found the synergistic effect of mixtures of antibiotics.[31] The reasons of these effects still remain unclear.

Amylolytic bacteria have been more resistant to the antibiotics under study than ammonifying bacteria. Ampicillin and streptomycin veraciously do not reduce the number of amylolitics during all periods of the research, and the trend of increasing the number of bacteria can be traced, compared

Table 1: The influence of contamination with antibiotics (100 mg/kg) on the main ecological trophic groups of soil microorganisms

Day	Reference	Benzylpenicillin	Benzylpenicillin+nystatin	Farmasinum	Farmasinum+nystatin
The number ammonifying bacteria, mln. CFU/g of soil					
10	3.01±0.10	1.0±0.07***	2.08±0.30**	1.62±0.26**	0.88±0.04***
60	2.96±0.06	2.87±0.01	2.79±0.05	2.32±0.05*	2.62±0.04*
120	2.94±0.06	2.87±0.01	2.73±0.02	2.23±0.02**	2.56±0.01*
The number amylolytic bacteria, mln. CFU/g of soil					
10	2.96±0.06	1.88±0.11***	1.38±0.06***	1.76±0.07***	1.31±0.01***
60	2.93±0.08	2.87±0.01	2.77±0.04	2.72±0.05	2.60±0.02*
120	2.93±0.08	2.87±0.01	2.63±0.04	2.88±0.07	2.59±0.07*
The number of micromycetes, thousands CFU/g of soil					
10	1.98±0.08	1.68±0.06*	1.01±0.05**	1.75±0.07	0.72±0.03***
60	1.84±0.02	1.87±0.07	1.52±0.02	1.77±0.07	1.36±0.02**
120	1.84±0.02	1.82±0.02	1.48±0.01*	1.77±0.07	1.34±0.01**

Veracious differences from the reference: *P<0.05, **P<0.01, ***P<0.001 at n=4

Table 2: The dynamics of changes in the total number of bacteria in chernozem contaminated by tylosin, bln/g

Day 10				
Indicators of variation statistics	**Reference**	**10 mg/kg**	**100 mg/kg**	**1000 mg/kg**
M±m	11.40±0.01	10.1±0.08	6.60±0.22**	3.90±0.05***
s	0.02	0.30	0.90	0.20
CV,%	0.31	3.38	13.46	0.21
% of reference		89	58	35
Day 60				
M±m	11.72±0.14	10.59±0.11	8.63±0.17*	6.63±0.13**
S	0.50	0.40	0.70	0.50
CV,%	4.65	4.01	8.03	8.11
% of reference		90	74	57
Day 120				
M±m	11.89±0.04	11.02±0.05	9.60±0.07*	9.12±0.12**
s	0.20	0.20	0.30	0.50
CV,%	1.31	1.67	2.95	5.12
% of reference		93	81	77

Veracious differences from the reference: *P<0.05, **P<0.01, ***P<0.001 at n=4 to the reference. However, the veterinary antibiotic tylosin, as well as complexes of antibiotics with fungicide reduces the number of amylolytics; at the same time, with increasing the duration of incubation in contaminated samples, the number of bacteria reduces more and more significantly. Other studies have shown that antibiotics such as sulfonamide, tetracycline, and trimethoprim have an inhibitory effect on the number of amylolytics.

Antibacterial antibiotics did not have veracious effect on the abundance of micromycetes, unlike their mixtures with fungicides, which reduced the abundance of micromycetes. The maximum effect was ensured by the input fungicide and a mixture of ampicillin + input. Abundance of micromycetes reduced by 43% and 30%, respectively. Similar to the amylolytic bacteria, the number of micromycetes under the action of mixtures of preparations decreased throughout the entire incubation period. In the variants with ampicillin and streptomycin, a slight increase in the number of micromycetes was observed, compared to the reference, and on day 90 of the experiment, the number of micromycetes in the variant with ampicillin exceeded the reference almost twice ($P < 0.05$).

The research has shown that chernozem contamination with antibiotics reduces the total number of bacteria, reduces the number of individual ecological trophic groups of microorganisms, and changes their ratio. Contamination with antibiotics results in a significant reduction in the number of ammonifying bacteria, which are represented most widely of the various ecological trophic groups of soil microorganisms. The abundance of soil micromycetes increases due to the removal of competition with bacteria, and to active colonization of the ecological niche by micromycetes.

The degree of soil ecological disorder[32] may be estimated with the use of the "integrated index of soil biological state" (IIBS). As a result, numerous studies have shown that with the decrease in IIBS by <5%, the soil can perform its normal ecological functions, but if IIBS is reduced by 5–10%, disruption of the information function occurs, by 10–25% - biochemical, physicochemical, chemical, and integral processes are disrupted, by more than 25% - physical functions are disrupted.[26]

With respect to the microbial community in the soil, the integrated index of biological activity (IIBA) was calculated. Introduction of antibiotics and their mixtures with fungicide decreases the integrated index of biological activity of soil. 3 days after contamination with antibiotics and their mixtures with fungicides, IIBA reduces by 28% after the introduction of streptomycin, and by 38% after the introduction of a mixture of streptomycin + nystatin, which is the evidence of disrupted biochemical, physicochemical and chemical functions of soil. On the 30th day of incubation in the variants with antibiotics, a slight IIBA increase is observed, however, in case of preparations complexes, IIBA reduces even more (for example, by 42% after introduction of a mixture of streptomycin + nystatin). On the 90th day of the studies, the similar trend is preserved.

Thus, with increasing the time of incubation of the contaminated samples, an increase of IIBA is observed. However, despite the observed trend of microbiological indicators recovery, the findings indicate violation of soil ecological functions even 120 days after contamination with antibiotics.

The literature is naturally dominated by an optimistic view of the problem with no undesirable effects. However, such questions require more detailed study in various conditions, and on a wide timescale. The necessity of reconsidering the existing ideas about antibiotics is evident; the overly narrow understanding of such compounds as the weapon in fighting other organisms for existence, which is reflected in their name should be rejected, which requires reanalyzing the issue of the ecological role of these signaling compounds.

CONCLUSION

The negative influence of antibiotics and their combinations on the microbial community in the soil has been established. Most studied microbiological indicators decrease on an increase in antibiotics concentration up to 100 mg/kg of soil. In most cases, direct relationship between the content of antibiotics in the soil and the degree of reduction in studied indicators has been observed. The degree of antibiotics influence is determined by their nature, concentration, and time of exposure. Antibacterial antibiotics have a more inhibitory action on the microbial community, compared to the fungicidal ones. By the degree of soil inhibition with microorganisms, antibiotics form the following line: Ampicillin > benzylpenicillin ≥ streptomycin > tylosin ≥ farmasinum > nystatin > tromexin > aliseryl. The number of ammonifying bacteria is the most informative microbiological indicator of contamination with antibiotics. The indicator of the g. Azotobacter bacteria abundance is uninformative in case of contamination with antibiotics. The maximum influence of antibiotics is noticed within the first 10 days from the moment of contamination. After that, a tendency to restoring the population of microorganisms is observed. By the degree of resistance to antibiotics, the studied microorganisms of chernozem formed the following line: Bacteria g. Azotobacter > micromycetes> amylolytic bacteria > ammonifying bacteria. The effect of antibiotics on soil microbial coenosis has prolonged nature. The dynamics of recovery of the number of microorganisms are non-linear.

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