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Impact Assessment of Soil Contamination with Antibiotics (for Example, an Ordinary Chernozem)

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Abstract: The environmental behavior of antibiotics is not well known and the precise environmental risk assessment is not practical. We studied the impact antibiotics (benzylpenicillin, pharmasin and nystatin) at different concentrations (100 and 600 mg kg^{-1}) on population densities of microorganisms and enzymatic activity of ordinary chernozems in model experiments. The applied doses of antibiotics had definite suppressing effects on population densities of microorganisms (up to 30-70% of the control) and on the soil enzymatic activity (20-70% of the control). The correlation analysis of the obtained data revealed positive correlation of enzymes of two studied classes (dehydrogenase, invertase) with a number of micromycetes (r = 0.63, r = 0.65, respectively), catalases with ammonifying bacteria (r = 0.73) and the return correlation of phosphatase with amylolytic bacteria (r = -0.80). The effect of antibiotics on the biological properties of the chernozem lasted for a long time. The studied parameters were not completely recovered in 120 days.

Keywords: Benzylpenicillin, Pharmasin, Nystatin, Ordinary Chernozem, Microorganisms, Biological Properties

Introduction

Tons of pharmacologically active substances are used annually in human and animal medicines for treatment and prevention of illness (Dıraz-Cruz *et al*., 2003; Sarmah *et al*., 2006). Antibiotics get into ecosystems in the initial form or in the form of some metabolites that mainly remain biologically active (Sarmah *et al*., 2006). In contrast to pesticides, antibiotics did not attract interest as potential contaminants until recently (Thiele-Bruhn, 2003). Antibiotics enter the soil mostly with manure (Kemper, 2008; Zhao *et al*., 2010) and sewage water (Golet *et al*., 2003) applied as fertilizers. Various antibiotics are found in ground and waste water and in soil (Ramaswamy *et al*., 2010; Pereira *et al*., 2012; Sirés and Brillas, 2012; Zhou *et al*., 2013). Once in the environment, like any other organic chemicals, their efficacy depends on their physio-chemical properties, prevailing climatic conditions, soil types and variety of other environmental factors.

Residues of pharmaceutical antibiotics have already been discovered in soils in concentrations of up to 300 ng g^{-1} for tetracyclines and 11 ng g^{-1} for sulfonamides (Hamscher *et al*., 2005; Höper *et al*., 2002). High concentrations of tetracyclines have been detected in Turkey and Spain soils, up to 0.5 and 0.2 mg kg^{-1}, respectively (Andreu *et al*., 2009; Karci and Balcioglu, 2009). Concentrations of 20 mg L^{-1} of tetracycline and 1 mg L^{-1} of chlortetracycline have been detected in liquid manure or in swine lagoon (Campagnolo *et al*., 2002). Additionally, various antibiotics are naturally formed in soils, though the concentrations and types of antibiotics getting into soils and water from external sources differ from those of the natural background (Zhang *et al*., 2009). Antibiotics can also be introduced to agricultural land through irrigation with reclaimed wastewater, since they have been frequently detected in the raw and treated sewage wastewaters (Renew and Huang, 2004; Yang *et al*., 2005; Gulkowska *et al*., 2008). Microorganisms become resistant to the antibiotics that are widespread in the environment. Bacterial resistance has been a big issue in terms of human and animal health (Nakatani *et al*., 2012). While it is possible that antibiotics can find their way into the environment from a variety of sources, whether or not there are adverse effects to human, terrestrial and aquatic ecosystems is not well understood.

The goal of this study was to investigate the effect of various pharmaceutical antibiotics on the abundance of microorganisms and enzymes activity in ordinary chernozem.

This study is a part of a series of scientific studies on the effect of biologically active substances on the biological indicators of soil in southern Russia.

Materials and Methods

Antibiotics

We took widely used in medicine and animal husbandry bactericidal (benzylpenicillin), bacteriostatic (pharmasin/tylosin) and fungicidal (nystatin) antibiotics to study their impacts on the soil microbiota and enzymatic activity. Benzylpenicillin is an antibiotic of the biosynthetic penicillin group. It produces bacteriocidal effects by the inhibition of bacterial cell wall synthesis. Benzylpenicillin is active against gram-positive and gram-negative bacteria, anaerobic spore-forming rods. Pharmasin is a drug widely used in veterinary medicine; it contains tylosin as the active compound. Tylosin is an antibiotic of macrolide group; it is active against most gram-positive and some gram-negative bacteria. Nystatin is polyene antifungal antibiotic. It affects pathogenic fungi and yeast-like fungi of genus *Candida*, as well as *Aspergillus* and is inactive against bacteria.

Soil

Deep heavy loamy calcareous low-humus ordinary chernozem developed from yellow-brown loess like loams in the Botanical Garden of the Southern Federal University (Rostov-on-Don) was studied. Such soils are typical of the south of European Russia and have special significance in ensuring food security of the country (Val'kov *et al*., 2008). Soil samples for model experiments were taken from the plow horizon (0-25 cm). The width of chernozem humus horizon is about 80 cm, the soil texture is hard-loamy soil, the medium reaction is 7.7, humus content is 4.1%, total nitrogen (by Kjeldahl)-0.25%; mobile phosphorus (by Machigin)-28.8; total potassium (by Berzelius)-2.06%.

Experimental Test Procedure

Air-dried soil samples were treated with solutions of benzylpenicillin and pharmasin and with their complexes with nystatin in concentrations of 100 and 600 mg kg^{-1} soil. These concentrations were taken on the basis of literature data on residual quantities of antibiotics in the environment (Chander *et al*., 2005; Hu *et al*., 2010) and according to the results of preliminary studies. Within the dose range of 10-600 mg kg^{-1}, the minimum effect was observed at concentrations of 100 mg kg^{-1} and the maximum effect took place at concentrations of 600 mg kg^{-1} (Akimenko *et al*., 2013a; 2014a; 2014b). All the samples were incubated at 20-25°C in dark place in order to avoid quick destruction of antibiotics at the optimum moisture content (60% of the field water capacity). Soil not subjected to treatment with antibiotics was used as the control. The changes in population density of microorganisms and enzymatic activity were studied after 10, 60 and 120 days of incubation. Analytical determinations of the biological properties were performed in four replicates.

Statistics were calculated using Statistica 6.0. Significant differences were accepted at $p<0.05$, $p<0.01$ and $p<0.001$. Spearman's rank correlation coefficient was calculated.

Microbial Activity Tests

Multiple studies of the microbial cenosis in the ordinary chernozem included the determination of population density of microorganisms by the method of deep inoculation from corresponding dilutions onto solid nutrient media: Ammonifying bacteria on Meat Infusion Agar (MIA), amylolytic bacteria on Starch and Ammonia Agar (SAA), micromycetes on acidified Czapek's medium, nitrogen-fixing bacteria (genus Azotobacter) on an Ashbymedium (soil plaque method) (Zvyagintsev, 1991).

Enzymes Activity Tests

The catalase activity was measured by the Galstyan method; the dehydrogenase activity, by the Galstyan method modified by Khaziev; the phosphatase activity, by the modified method of Galstyan and Arutyunyan; and the invertase activity, by the modified colorimetric method of Khaziev (Kazeev and Kolesnikov, 2012).

Results

Antibiotics may change the bacterial diversity and resistance of soil communities by increasing selection pressure in favour of resistant phenotypes (Čermák *et al*., 2008). The results obtained in this study demonstrate (Fig. 1 and 2) that all the studied concentrations of antibiotics have a suppressing effect on population densities of soil microorganisms; the same result was obtained in (Colinas *et al*., 1994). At the same time, there are data attesting to astimulating effect of antibiotics in low concentrations (0.1-0.5 mg kg^{-1}) on microorganisms (Schmitt *et al*., 2004). Close correlation was found between concentrations of antibiotics and the change in population densities of the studied groups of soil microorganisms (r = -0.68-0.86). Other studies demonstrated the dependence of the effects of antibiotics on soil microbiota on their concentrations (Herron *et al*., 1998). Thus, it was found that soil contamination with sulfachloropyridazine inhibited the soil microbiota, though further rise in the concentration of this antibiotic caused the development of resistance in microorganisms (Schmitt *et al*., 2004). The changes in the population density of ammonifying bacteria in the ordinary chernozem under the impact of antibiotics are shown in Fig. 1A.

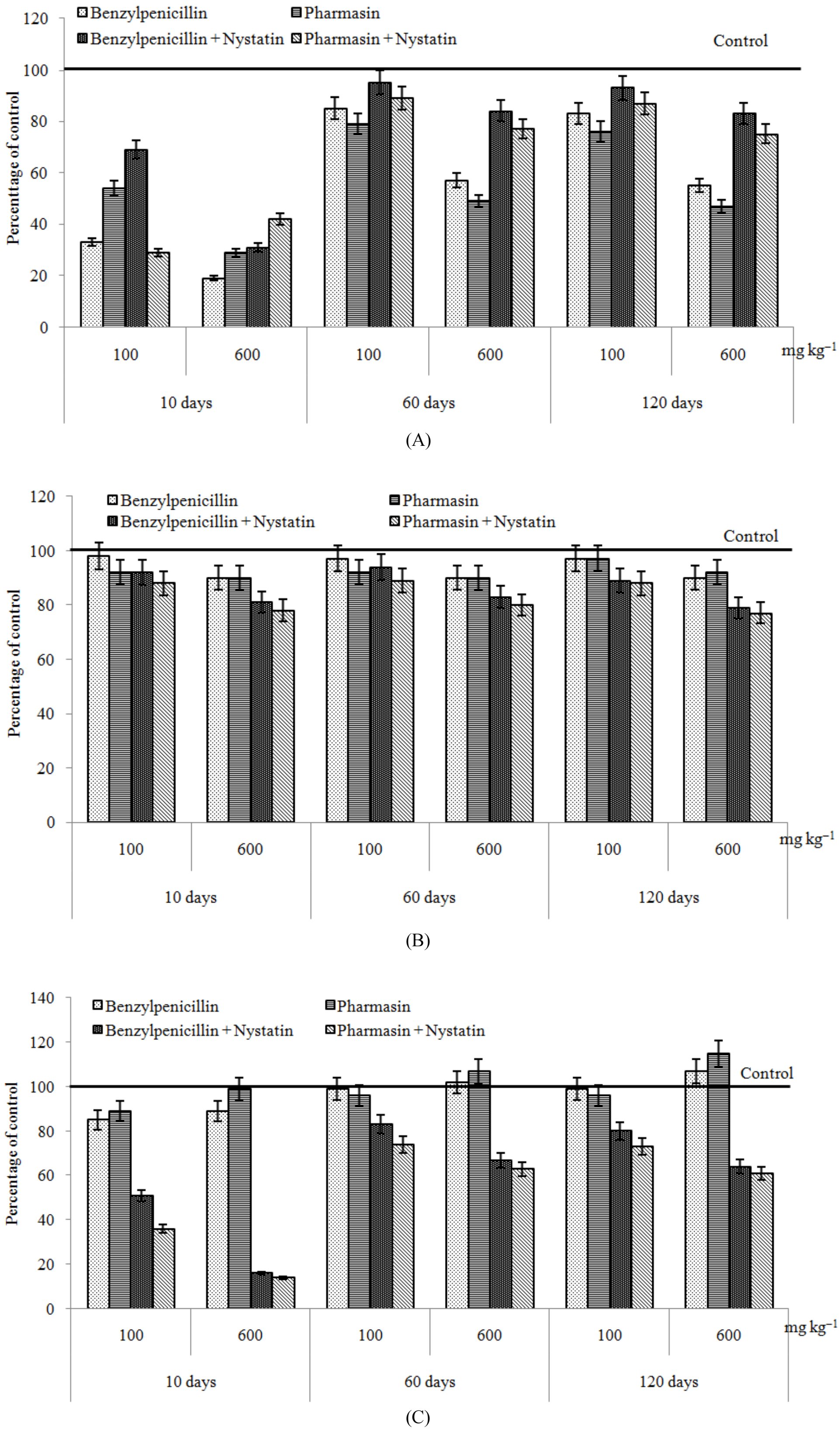


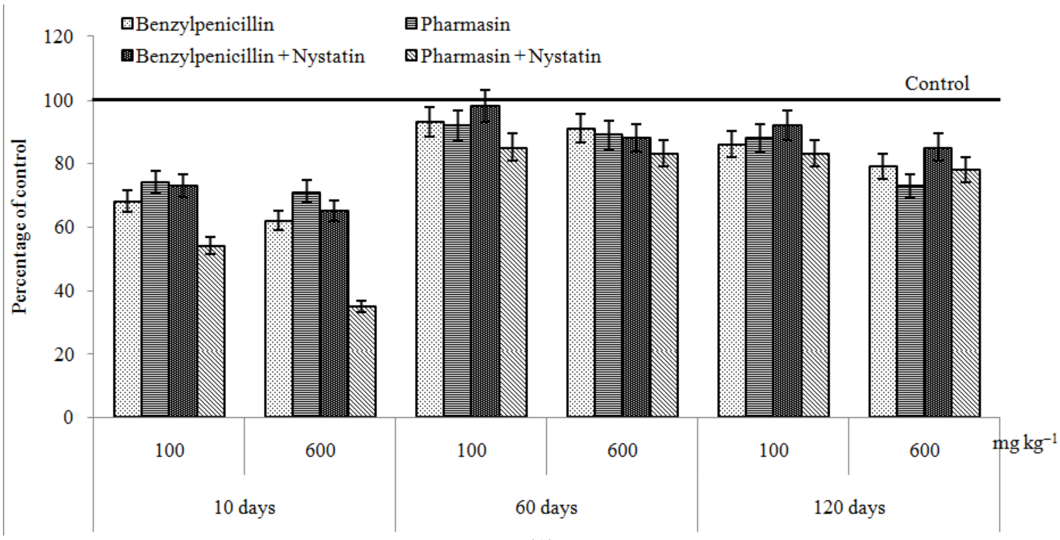
Fig. 1. Effect of antibiotics on the abundance of soil microorganism A (ammonifiers) B (amylolytic bacteria) C (micromycetes)

The complex of pharmasin and nystatin had the maximum inhibiting effect on ammonifiers in concentration of 100 mg kg^{-1}; the complex of benzylpenicillin and nystatin in the same concentration had the minimum effect. Other researchers noted a decrease in the inhibiting effect onammonifiers in the presence of fungicidal preparations and a synergetic effect of the mixtures of antibiotics with similar action (Barlow *et al*., 2008; Brown and Balkwill, 2009). The reasons for such effects remain unknown. On the contrary, upon higher concentrations (600 mg kg^{-1}), the decrease in population density of ammonifying bacteria under the impact of antibacterial antibiotics was more pronounced than that under the impact of their complexes with fungicidal preparation. The restoration of population density of bacteria was observed after 60 and 120 days of the experiment. It was virtually complete upon application of antibiotics in minimum concentration, 75-85% of the control in the case of maximum concentrations of antibiotic complexes ($p<0.05$) and 45-58% ($p<0.001$) for antibacterial antibiotics. Similar data on the dynamics of the number of ammonifying bacteria were obtained in model experiments with different types of radiation (Denisova *et al*., 2005; 2011; Denisova and Kazeev, 2008). When contaminating with relatively small doses (up to 1 Maximum Permissible Concentration (MPC) of heavy metals (Kolesnikov *et al*., 2011; 2013) and oil and petroleum products (Kolesnikov *et al*., 2006; 2010a), the population density of microorganisms in increased in the first days from the moment of contamination (up to 1 month), then decreased and did not restore completely even after 360 days. However, the effects of antibiotics on soil microorganisms had a more prolonged character in comparison with the effects of high temperature sterilization (Akimenko *et al*., 2013b) and pesticides (Kazeev *et al*., 2010). In researches with pesticide atrazine (Moreno *et al*., 2007) it is shown that in general, an increase in the measured microbiological and biochemical parameters with atrazine concentration in soil was observed. The increase in microbial activity with atrazine pollution was noticeable after lengthy incubation. Thus, upon the soil sterilization with high temperature, the number of microorganisms was restored to the control values already on the 60th day.

The studied antibiotics in concentration of 100 mg kg^{-1} did not exert significant inhibiting effect on the number of amylolytic bacteria (Fig. 1B). Population densities of amylolytic bacteria remained practically the same on the 10th and 120th days of the experiment. Significant inhibiting effects on amylolytic bacteria were observed under concentrations of antibiotics of 600 mg kg^{-1} and complexes of antibiotics were most efficient. It was shown in other studies that antibiotics such as sulfonamide (Thiele-Bruhn, 2003), tetracycline and trimethoprim (Pallecchi *et al*., 2008) in concentrations 300-500 mg kg^{-1} had an inhibiting effect on amylolytic bacteria. Unlikeammonifying bacteria, the population density of amylolytic bacteria practically did not restore by the 120th day of experiment. At the same time, amylolyticbacteria appeared to be more resistant to the impact of studied antibiotics. As anticipated, antibacterial antibiotics (benzylpenicillin and pharmasin) at all the studied concentrations did not cause significant changes in population density of micromycetes in contrast to their complexes with nystatin. As shown earlier (Malygina and Kazeev, 2011), fungicidal antibiotic nystatin had no effects on soil bacteria, but decreased the number of soil micromycetes in concentrations of 100-600 mg kg^{-1}. The suppressing effect of nystatin on the soil fungi was higher in the variants with high nystatin concentrations. The population density of micromycetes under the influence of the complex of antibiotics decreased by 45-55% ($p<0.001$) on the 10th day of the experiment and the population recovery up to 70-80% of the control was observed on the 120th day. However, no complete recovery took place r. Complexes of antibiotics in concentration 600 mg kg^{-1} had significant inhibiting effect on the population density of micromycetes. Inhibiting effects of the complexes of nystatin with benzylpenicillin and pharmasin were practically similar. A tendency for the recovery of micromycetes (up to 60-65% of the control was observed ($p<0.001$). A gradual increase in population density of micromycetes in comparison with the control was observed in variants with antibacterial antibiotics in concentration 600 mg kg^{-1}; in this case, the population density of micromycetes exceeded the control by 10-18% ($p<0.05$) on the 120th day of the experiment. This can be attributed to elimination of competition from bacteria and active colonization of their ecological niches bymicromycetes, or to their strong adaptation, or both (Fig. 1C).

The effect of pharmaceutical antibiotics on bacteria of genus *Azotobacter* was also investigated. Many studies demonstrated that genus *Azotobacter* is highly sensitive to pollution and it was suggested as a test object for the assessment of soil properties at different pollution (Kolesnikov *et al*., 2010b; Denisova *et al*., 2005). However, for all investigated antibiotics at concentration of 100 mg kg^{-1}, statistically significant changes were not detected and the abundance of bacteria decreased by 20% ($p<0.05$) in comparison with the control only with concentrations of complexes of antibiotics of 600 mg kg^{-1}.

Long-term studies have shown the efficiency of the diagnostics and monitoring of soils with biochemical methods, particularly with the help of the indices of soil enzymatic activity. The use of enzymatic activity as a diagnostic parameter is favored by the low error of experiments, simplicity of determination and high sensitivity to external impacts (Galstyan, 1978; 1982; Dadenko *et al*., 2009).


Benzylpenicillin
Pharmasin
Benzylpenicillin + Nystatin
Pharmasin + Nystatin
Control
Percentage of control
0
20
40
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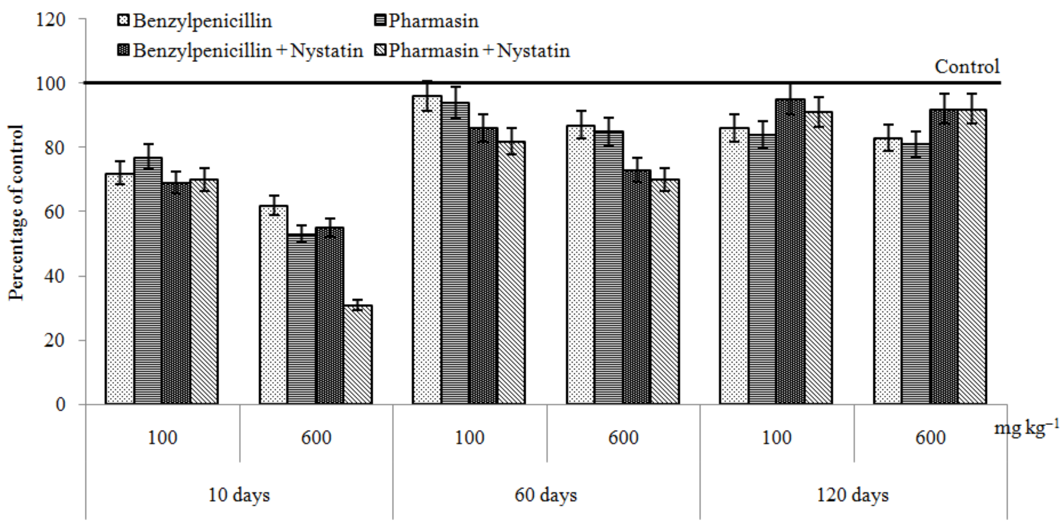
100
120
100
600
mg kg−1
10 days
60 days
120 days

(A)


Benzylpenicillin
Pharmasin
Benzylpenicillin + Nystatin
Pharmasin + Nystatin
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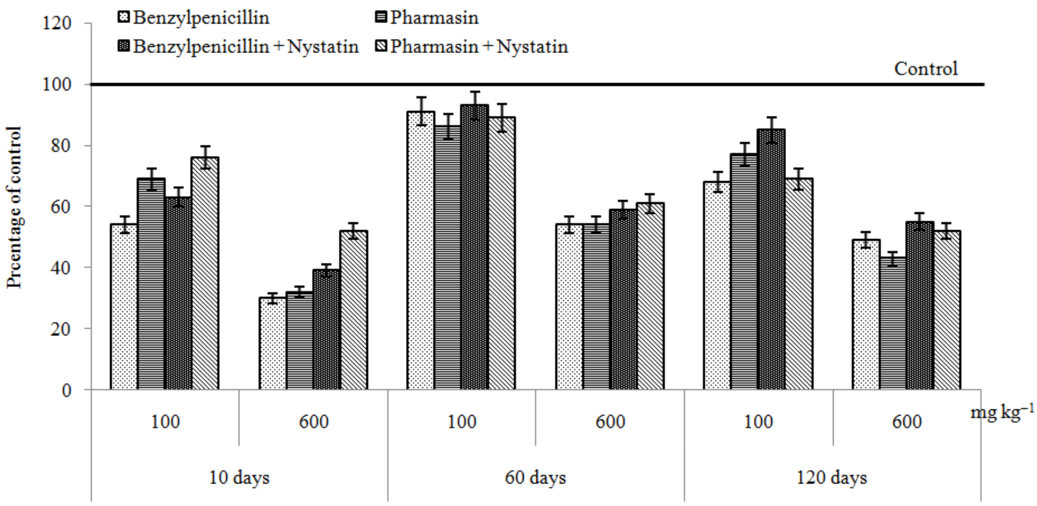
(B)


Benzylpenicillin
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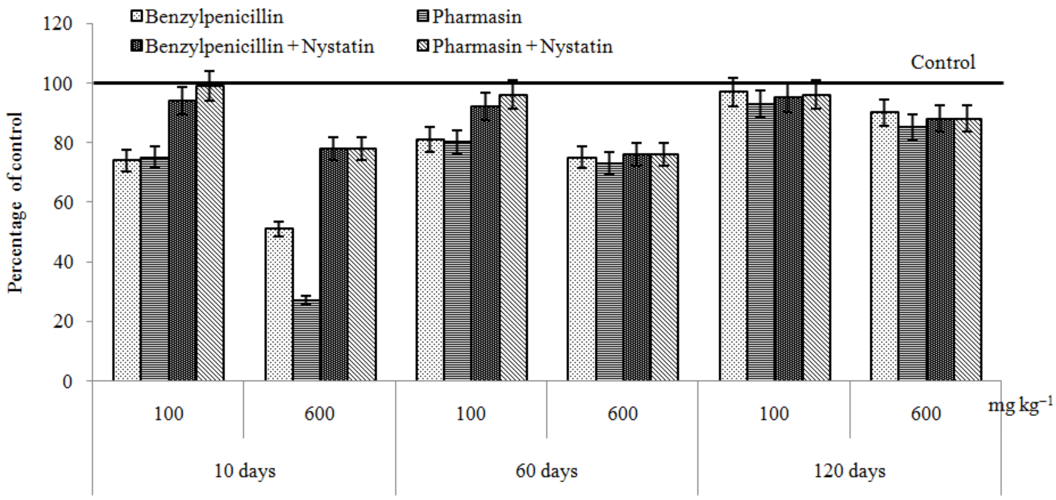
(C)



(D)

Fig. 2. The impact of antibiotics on the enzymatic activity of an ordinary chernozem. A (catalase), B (dehydrogenase), C (phosphatase), D (invertase)

We studied the impact of antibiotics on hydrolase (phosphatase and invertase) and oxidoreductase (catalase and dehydrogenase) activities. The results of our experiments arepresented in Fig. 2.

In general, enzymes of the oxidoreductase class were more resistant to antibiotics than enzymes of thehydrolase class. Antibiotics in concentration of100 mg kg^{-1} reliably affected the activity of studied enzymes. The pharmasin-nystatin complex caused maximum inhibition of the catalase activity ($p<0.001$, $n = 4$) on the 10th day of the experiment. Restoration of the activities of catalase, dehydrogenase and invertase up to control values was observed during the incubation period; the phosphatase activity was not restored. All the studied antibiotics in concentration 600 mg kg^{-1} exerted a significant inhibiting action on enzymatic activities. Other researchers demonstrated that antibiotics of tetracycline group in concentration 300 mg kg^{-1} inhibited the activities of catalase and phosphatase by 35-55% of the control (Reichel *et al*., 2014). The maximum decrease in the activities of studied enzymes was observed on the 10th day of the experiment; in the following period, a tendency for restoration of the enzymatic activities was observed. As anticipated, the complexes of antibiotics were most efficient against enzymes. For example, the complex of pharmasin and nystatin inhibited the activities of catalase, dehydrogenase and phosphatase by more than 50% ($p<0.001$, $n = 4$) on the 10th day of the experiment. In other laboratory experiment (Reichel *et al*., 2014), a functional shift was indicated by a four-fold reduced acid phosphatase activity in SDZ-contaminated soil compared to control soil.

The correlation analysis of the obtained data revealed positive correlation of enzymes of two studied classes (dehydrogenase, invertase) with a number of micromycetes ($r = 0.63$, $r = 0.65$, respectively), catalases with ammonifying bacteria ($r = 0.73$) and the return correlation of phosphatase with amylolytic bacteria ($r = -0.80$). It gives the chance to judge a contribution of this or that group of microorganisms in the soil enzymatic pool.

Conclusion

The following regularities were found in our study of the impact of antibiotics on the biological properties of an ordinary chernozem. In general, antibiotics exerted an inhibiting effect on the biological properties of the chernozem and, particularly, on the soil microorganisms. The studied microorganisms formedthe following sequences according to their resistance to antibiotics: Amylolytic bacteria>micromycetes>ammonifying bacteria. Enzymes of the chernozemwere generally more resistant to the antibiotics than the microorganisms. However, they also differedintheir resistance to antibiotics: Among oxidoreductases, dehydrogenase was more sensitive than catalase; among hydrolases, phosphatase was more sensitive than invertase. In general, oxidoreductases were more resistant to antibiotics than hydrolases. With respect toothier resistance to antibiotics, the studied enzymes formed the following sequence: Catalase>dehydrogenase>invertase>phosphatase.

The recovery of the soil biological properties also followed certain regularities. First, it had a nonlinear character, i.e., an increase in the period of incubation did not necessarily lead to a higher recovery of the studied biological properties. This was particularly pronounced for microorganisms in comparison with the enzymatic activity. The recovery pattern of microorganisms after the soil treatment with antibiotics in high concentration (600 mg kg^{-1}) was as follows: Amylolytic bacteria>ammonifying bacteria>micromycetes. The recovery of enzymatic activity had the following pattern: Dehydrogenase>invertase>catalase>phosphatase. In general, the recovery of the biological properties of the ordinary chernozem after the influence of antibiotics depended on the concentrations of antibiotics: The lower the dose, the quicker the recovery. Upon the high concentrations of the antibiotics (600 mg kg^{-1}), some parameters of the biological properties did not recover even after 120 days.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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