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SOIL BIOLOGY

Translocation of Bacteria from Animal Excrements to Soil and Associated Habitats

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Abstract—The population dynamics of *Salmonella enterica* var. Typhimurium MAE 110 *gfp*, *Escherichia coli* O157:H7 *gfp*, and *Pseudomonas fluorescens* 32 *gfp* were investigated in their introduction to cattle excrements and subsequent entering the soil, plants of cress (*Lepidium sativum* L.), and migration through the gastroenteric tract of French snails (*Helix pomatia* L.). The survival of these bacteria in the excrements and soil was investigated at cyclically changing (day–night, 25–15°C) and constant (18°C) temperatures. The cyclically changing temperature adversely affected the survival of *E. coli* O157:H7 *gfp*, and *P. fluorescens* but did not influence *S. enterica* var. Typhimurium. All the bacteria and, especially, the analogues of enteropathogens showed high survival in the cattle and snail excrements, soil, and on the plants under the gradual decrease in their population. On the cress plants grown in a mixture of cattle excrements and soil, an increase in the number of the introduced bacteria was observed.

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INTRODUCTION

The investigation of microorganisms' propagation over natural media—soil, composts, plant residues, and plants—and the determination of the risk of pathogens entering humans and animals remains a topical problem [1, 3]. Noncomposted cattle excrements (CE) are often applied as fertilizers. If these CE contain pathogenic microorganisms such as *Salmonella* spp., *Escherichia coli*, and some others, a threat of contaminating not only the soil and water but also the plants (particularly, vegetables) grown on the soil emerges [7, 9, 15, 25]. The fate of the microorganisms entering the environment with animal excrements is determined by many factors: the moisture, the temperature, the nutrients, the composition and activity of the native microbial community, etc. [11, 12, 14, 25].

Under natural conditions, the soil temperature follows the day–night cycleunder the alternation of day and night. The daily temperature fluctuations in storage heaps of manure are noticeable in the surface layer (unless the self-heating of the heap is developed). The same phenomenon is possible in manure under range cattle management, when, in each particular place, the volume of excrements is small. Under these conditions, the effect of the daily temperature fluctuations on the survival of bacteria in the excrements, soil, and on plants should be significant. Therefore, the regularities of the survival of bacteria, particularly of enterobacteria, are important to know not only under constant temperature but also under the cyclically changing temperatures that take place in nature. There are many works on the survival of bacteria under constant temperature [7, 14, 25], but investigations in conditions close to the natural ones were never conducted [16, 20, 23].

A traditional methodological approach to investigate the survival of microorganisms in natural substrates is their introduction (inoculation) to a particular substrate and the observation of their population dynamics. The dynamics of populations of saprotroph microorganisms in their introduction to one substrate have been well studied [2, 8]. There are only a few works on the survival of microorganisms in their sequential displacement to a second or a third substrate [7, 22]. The introduction of microorganisms at rather high doses allows revealing if the stabilization of their number takes place in the first or succeeding substrates or there is only the following regularity: a more or less fast but constant decrease in the population of the microorganisms or their total disappearance. The last phenomenon is unlikely to happen in nature, since, in this case, enteropathogens would have disappeared from ecosystems long ago.

The aim of this work was to study the population dynamics of one saprotrophic and two enteropathogenic bacteria in cattle excrements at cyclically changing and constant temperatures and their introduction into the soil with CE and their appearance on plants and in the excrements of invertebrates.

MATERIALS AND METHODS

The microorganisms and the media for their growing and identification. Genetically labeled bacteria that are able to synthesize green fluorescent protein (GFP) were the objects for this study. *Salmonella enterica* var. Typhimurium MAE 110 *gfp* (further named *S.* Typhimurium *gfp*) was received from Dr. Yuta Remling (Microbiological Center, Royal Institute, Stockholm, Sweden), *Escherichia coli* O157:H7 *gfp* was received from the Laboratory of A. van Bruggen (University of Wageningen, the Netherlands), and *Pseudomonas fluorescens* 32 *gfp* was supplied by Dr. R.J. Saler (Faculty of Biology, Arkansas University, USA). The investigations were performed with antibiotic-resistant and avirulent strains (the genes of virulence were removed).

S. Typhimurium was isolated on a medium containing the following (g/l): yeast extract (5), bacterial peptone (10), agar (17), and distilled water (pH 7.2–7.4). Nalidixic acid (50 mg/l) as an antibiotic was added. After autoclaving, kanamycin (50 mg/l) sterilized by filtration was added. *E coli* O157:H7 *gfp* was isolated on a medium containing the following (g/l): yeast extract (5), bacterial peptone (10), NaCl (10), agar (17), and distilled water (pH 7.2–7.4). After autoclaving, ampicillin (antibiotic, 50 mg/l) sterilized by filtration was added. *P. fluorescens* 32 *gfp* was isolated on a medium containing the following (g/l): bacterial peptone (2), K_2HPO_4 (1.4), $MgSO_4 \cdot 7H_2O$ (1.5), glycerin (15 ml/l), agar (17), and distilled water (pH 7.0–7.2). After autoclaving, kanamycin sterilized by filtration (50 mg/l) and rifampicin (50 mg/l) were added [4]. All the media were sterilized for 30 min at a pressure of 0.5 atm.

For the inoculation to the cattle excreta (CE), the bacteria were grown on the same but liquid media up to reaching the initial stage of their stationary growth phase (~24 h for *S.* Typhimurium *gfp*, 15 h for *E. coli* O157:H7 *gpf*, and 25°C for *P. fluorescens* 32 *gfp*). The temperatures for growing the bacteria were 37°C for *S*. Typhimurium *gfp* and *E. coli* O157:H7 *gfp* and 25°C for *P*. *fluorescens* 32 *gfp*. The collected biomass was once washed with distilled water. Then, it was resuspended in a small amount of distilled water.

Along with the number of introduced bacteria, the population of the native bacteria (copiotrophs, in particular) was determined by the method of plating [21].

The cattle excreta were collected on the Moskovskii konnyi zavod no. 1 farm (Odintsovskii district, Moscow oblast). The ration for the animals included the following (kg/ind./day): green mass (2.5), hay (1), dry combined fodder (3.0), and salt on green mass (40 g). Fresh feces were used in the tests. The initial moisture of the excreta averaged 70%. The CE pH was 7.1.

The soil. The soil (soddy-podzolic) samples were taken from a depth of 0 to 15 cm in the territory of the Botanical Garden of Moscow State University nearby growing plants of sea buckthorn (*Hippophae rhamnoides* L.). The soil was sieved using a 2 mm-meshed sift and stored in polyethylene bags at room temperature and moisture of 6%. The soil contained the following: total carbon (39.6 mg/g), total nitrogen (2.87 mg/g), ammonium nitrogen (3.75 μg/g), nitrate nitrogen (85.5 μg/g), and phosphorus as phosphate ions (17.7 μg/g). The pH of the soil was 6.6. The soil particle-size distribution was the following: clay, 11.65%; sand, 31.8%; and fine silt, 56.55%.

Plants. The capability of the bacteria to colonize plants was studied using cress (*Lepidium sativum*) of the Vitaminnyi variety (producer Agrofirma Aelita); seed germination 92.5%.

Invertebrates. In studying the probability of the bacteria's survival in their passing through the gastroenteric tract, French snails (*Helix pomatia* L.) collected in the Botanical Garden of Moscow State University were used. In the laboratory, the snails were kept in glass vessels at 18–20°C and fed on cabbage leaves and fine carrot slices.

Dynamics of the CE heating at cyclically changing temperature related to the CE mass. The cattle feces (0.5, 1.0, and 2.0 kg) were put into plastic pots; closed with polyethylene film; and, in the center of the substrates in each pot, a thermometer was placed. Three replicates were used. The pots with the substrates were incubated at 25°C for 8 h and at 15°C for 16 h. The control tests were performed at a constant 18°C.

The content of water-soluble organic carbon in the CE, the soil and, in their mixture was determined in water extracts by the bichromate method with recalculation using a calibrated curve plotted after the same reactions with different glucose concentrations.

Inoculation of the cattle excreta with bacteria. The initial bacterial population in the suspension used for the inoculation of the CE with bacteria and in the different substrates was determined under an MIKMED 2 LYUMAM RPO 11 (St. Petersburg) OMO luminescence microscope with the following plating on selective media with the corresponding antibiotics. The dishes with enterobacteria and with pseudomonads were incubated at 37 and 25°C, respectively. The fluorescent colonies were identified and counted under a black-light lamp (Philips, Eindhoven, the Netherlands).

The cattle excreta with suspensions of the corresponding bacteria were mixed in firm polyethylene bags. The volume of the suspensions was selected so that the final CE moisture did not exceed 90%. The cattle excrements (500 g) were put into plastic vessels (0.7 l) that were closed with polyethylene film and incubated in the dark at cyclically changing temperatures as mentioned above. The inoculation of the CE was conducted 4 days after the incubation in a thermostat at cyclically changing temperatures.

The mixing of the CE with the soil. In every particular case, the moment of mixing the CE containing the introduced bacteria with the soil was determined experimentally after the introducers reached their pop-

ulation density (the plateau on the curve) in the substrate analyzed. As a rule, this occurred within 2 to 3 weeks. Then, half of the pot contents was taken and mixed with the soil in a 1 to 6 ratio (425 g of soil and 75 g of CE); in the recalculation based on the dry weight, the ratio was 1 to 16. We considered this first stage of the tests as a short-term one. The rest of the CE samples was incubated further at the same conditions, and the number of the introduced bacteria was periodically estimated. These samples were mixed with the soil after several weeks. This stage of the tests was called the long-term stage. The CE moisture before the mixing was 68%, and the soil moisture was reduced to 14%. The moisture of the CE—soil mixture after the mixing amounted to about 15%.

The growing of the plants and the determination of the *gfp*-bacteria population on the cress plants. Cress seeds (0.5 g) were sown in pots containing the CE–soil mixture with the introduced bacteria. The pots were closed with polyethylene film and placed under a fluorescent lamp for 16 h. Five days after the appearance of the seedlings, the plants were counted in each pot. Then, 30 plants (the aboveground parts) were cut. The cut plants were carefully washed with sterile distilled water, and, after their concentration by centrifuging, the number of *gfp*-containing bacteria was determined in the suspension obtained. A luminescence microscope and plating on a selective nutrient medium were used.

For counting the bacterial population on the cress roots, 30 roots were taken out of the soil, the soil particles were carefully removed by shaking, and they were washed three times with sterile distilled water. The rhizosphere material was obtained in this way. The mass of the rhizosphere soil was measured after its drying to a constant weight. In the supernatant liquid, the population of *gfp*-bacteria was determined using the plating method.

The feeding of the French snails on the cress plants. In each plastic pot, one actively feeding snail was placed. Before the performance of the tests, the snails were not fed for 24 h. In each pot were 50 plants cut in the same pots where the bacterial population in the phylo- and rhizosphere was determined. In 24 h, the excrements were collected once. The number of colony-forming units (CFU of the *gfp* bacteria/g of excreta) was counted.

The statistical analysis. The experiments were repeated three times; three replicates were used in each variant. The average values were used. The survival of the bacteria, including their survival in the tests at constant and cyclic temperatures, was compared using the *t*-test. The statistical analysis was performed with help of the Excel program.

RESULTS AND DISCUSSION

The dynamics of the CE heating at cyclically changing temperatures in different CE volumes. The temperature regime of the tests was chosen based on the analysis of three year data on the mean summer day and night temperatures in Moscow oblast (Taldom district) with an excess of the night temperature by 3°C. The duration of the experiments on the determination of the rate and extent of the CE heating related to the incubated volume amounted to 10 days. The most steadily repeated results were only obtained 216 h after the experiment's beginning (the data are not presented). In all the variants, the dynamics of the temperature in the CE differed from the fluctuations of the air temperature. However, in the 0.5 kg CE mass, the fluctuations of the temperature ranged within intervals close to the assigned 25°C in the daytime and 15°C at night. When the CE masses were 0.1 and 2.0 kg, some excess or weak heating of the substrates took place. In the CE with a mass of 0.1 kg, its temperature changed, on the average, between +18 and +26°C, but it did not change according to the assigned regime of +15 to +25°C. The CE mass of 2.0 kg warmed up only to 24°C for 8 h in the variant with incubation at 25°C. In this connection, the CE mass of 0.5 kg was selected for further experiments.

The dynamics of the labeled bacteria population in the CE. In the experiments on the survival of enteropathogens in natural substrates, a rather high initial number of the introduced bacteria (~10^{10} cells/g of dry CE) was used. Such an approach was stipulated by several reasons. First, excrements and, moreover, soil and plants are not the optimal media for the active growth of enteropathogens. Therefore, the dynamics of their populations on these substrates may differ from that of native saprotroph microorganisms [2, 8]. The contradictory published data on this problem [17, 18, 24, 25] confirm this fact. Second, the majority of the previous studies revealed a fast decrease in the number of introducers, both saprotrophs and enteropathogens, up to their complete disappearance during several weeks (more rarely, during several months) [6, 10, 11]. Third, the use of a high inoculation density is needed to equal the odds of the introduced bacteria in their competition with the representatives of the native saprotroph microbial community, which is characterized by a high number of microorganisms. According to the published data [19], the number of nonpathogenic *E. coli* cells in CE can be up to 10^8.

Bacteria that are often identified in the environment were the objects of our investigations. *E. coli* is an indicator of fecal environmental pollution. *Salmonella* spp. and *E. coli* O157:H7 bacteria are capable of existing as either saprotrophs or parasites. They also develop successfully in the gastrointestinal tract (GIT) of humans and animals and can be a reason for serious diseases. Thus, the bacteria used in the studies can be considered cosmopolitans [25]. Probably, owing to this circum-

Table 1. The survival of the bacteria investigated in the short-term and long-term experiments in the CE and CE–soil mixtures

Test variant		*t*-test values		
		S. Typhimurium *gfp*/ *E. coli* O157:H7 *gfp*	*S.* Typhimurium *gfp*/*P. fluorescens* 32 *gfp*	*E. coli* O157:H7 *gfp*/ *P. fluorescens* 32 *gfp*
Short-term	CE $t = 2.57$	2.13	*3.67*	*3.15*
	CE–soil $t = 2.78$	1.76	*3.39*	*3.97*
Long-term	CE $t = 2.31$	2.02	*2.92*	*2.55*
	CE–soil $t = 3.18$	*11.47*	*17.88*	*3.24*

Note: Here and further, the significant differences in the *t*-test values are in italics.

stance, no statistically significant differences in the dynamics of the survival of these bacteria in the CE and CE–soil mixture were revealed under the different temperature regimes of the incubation (Table 1). However, the comparison of the dynamics of each particular bacterium only in the CE and the CE–soil mixture in the incubation at constant and changing daily temperatures has revealed some differences (Fig. 1, Table 2). The *E. coli* O157:H7 *gfp* and, especially, *P. fluorescens* 32 *gfp* bacteria survived better at the constant temperature. The survival of *S.* Typhimurium MAE 110 *gfp* was not related to the changes in the temperature as evidenced by the data of the *t*-test (Table 1). The cyclically changing temperature regime is the most natural; therefore, only the results obtained under these conditions will be discussed further.

Immediately after the mixing of each particular introducer with the CE, the bacterial population was, on the average, 10^{10} cells/g of dry CE mass. With time, the number of *Escherichia coli* O157:H7 and *P. fluorescens* 32 *gfp* gradually decreased, and, in 15 days, it amounted to 5×10^7 cells/g of dry CE mass for *P. fluorescens* 32 *gfp* and 3.0×10^7 cells/g of dry CE mass for *E. coli* O157:H7 *gfp*. The number of *S.* Typhimurium

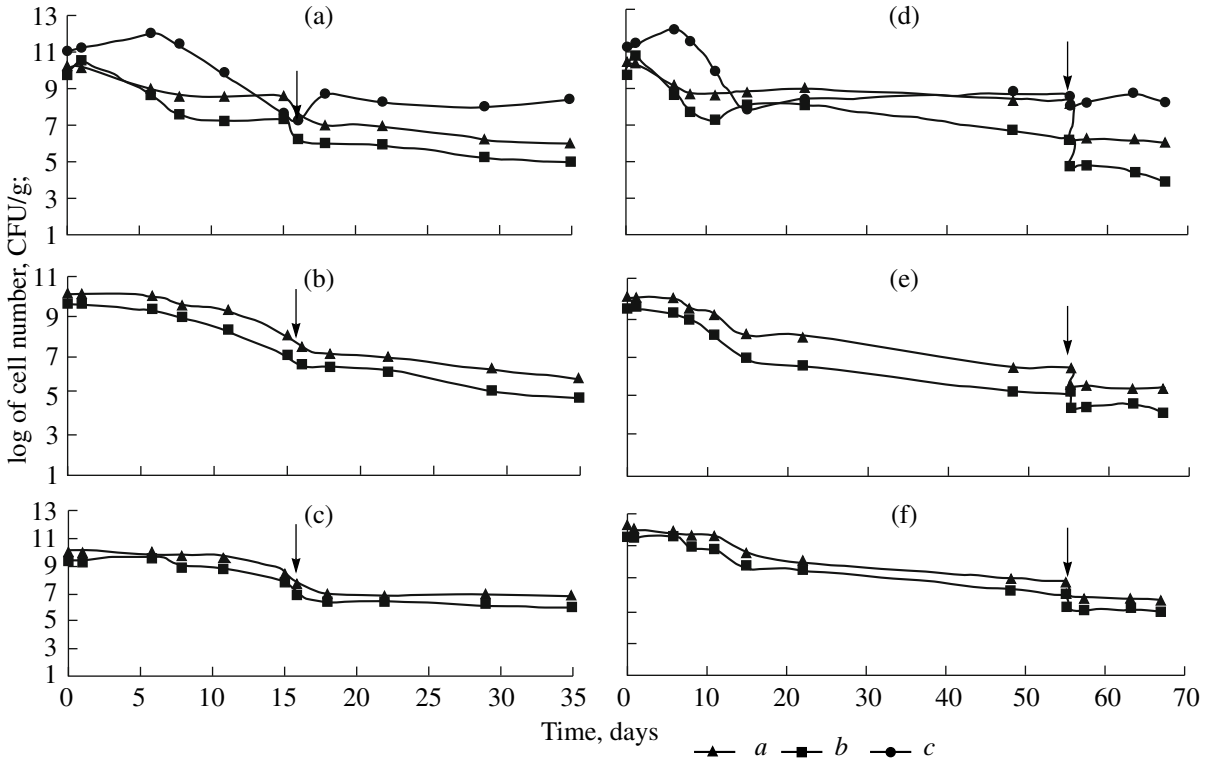

Fig. 1. Dynamics of survival of (a) *S. typhimurium* MAE 110 *gfp*, (b) *E. coli* O157:H7 *gfp*, and (c) *P. fluorescens* 32 *gfp* in their inoculation to CE with the following displacement to soil in the short-term and (d–f) long-term experiments; the arrow shows the time of mixing with the soil. Designations: *a*—cells; *b*—CFU; *c*—native microorganisms (CFU).

Table 2. The *t*-test values characterizing the survival of the bacteria at constant and cyclic temperatures in the short-term and long-term experiments upon the inoculation of the bacteria into the CE and CE–soil mixtures (significance level 0.05)

Test variant		*t*-test values		
		S. Typhimurium *gfp*	*E. coli* O157:H7 *gfp*	*P. fluorescens* 32 *gfp*
Short-term	CE $t = 2.57$	2.05	*3.30*	*5.40*
	CE–soil $t = 2.78$	1.46	1.66	*3.67*
Long-term	CE $t = 2.31$	1.92	*2.68*	*3.50*
	CE–soil $t = 3.18$	*3.95*	*6.47*	*6.08*

MAE 110 *gfp* during the first 8 days after their inoculation also decreased to 2.6×10^8 cells/g of dry CE mass. Then, this population was stabilized and even decreased to some degree. On the 15th day, its number amounted to 3.4×10^8 cells/g of dry CE mass.

The character of the dynamics of the native microorganisms in the CE differed from the dynamics of the introduced bacteria (Figs. 1a, 1d). For the first 5 days, their population increased; then, it decreased drastically without stabilization. This fact is related to the disturbance of the CE substrate as the experiment was prepared. Usually, this disturbance leads to the temporary activation of the metabolism and increases the number of microorganisms in the soil and other substrates [5].

Dynamics of the population of the labeled bacteria in the CE–soil mixture. For studying the dynamics of the bacteria survival in a series of different substrates, their rather high population density is required. Since the populations of *E. coli* O157:H7 and *P. fluorescens* decreased and the *S.* Typhimurium population became stable within 15 days after their introduction, the CE with the introduced bacteria was mixed with the soil (Figs. 1a–1c). In order not to permit a drastic decrease in the number of introduced bacteria below the detected level and to study the dynamics of the survival of the introduced bacteria for a long-term period, the CE mass was divided into two parts. Thus, the dynamics of the population of each bacterium in the CE and the CE–soil mixture were investigated upon short-term and long-term incubation (Figs. 1a–1f).

After the mixing of the CE with the soil, the number of introducers decreased due to the dilution, on the average, by an order of magnitude and amounted to 2.9×10^7, 2.5×10^7, and 3.4×10^7 cells/g dry matter for the *S.* Typhimurium *gfp*, *E. coli* O157:H7 *gfp*, and *P. fluorescens* 32 *gfp*, respectively. Later on, in the next 20 days, the population of the introduced bacteria gradually decreased: it was well expressed in the *E. coli* O157:H7 *gfp*, more weakly in the *S.* Typhimurium *gfp*, and it was not observed in the *P. fluorescens* 32 *gfp*. After the mixing with the soil, the population of the native microorganisms first increased and then decreased to some degree; after that, stabilization to some degree took place (Figs. 1a–1c).

Under the longer (up to 55 days) incubation in the CE, the populations of all the bacteria decreased, however, with different rates. The *E. coli* O157:H7 *gfp* population decreased most rapidly (by more than an order of magnitude) according to the counting of the number of CFU or cells; the least reduction was registered for the *Salmonella* population (Figs. 1d–1f).

The mixing of the CE with the soil in the long-term test, as in the short-term one, first caused a decrease in the number of the introduced bacteria, which amounted to 1.2×10^6, 4.0×10^5, and 1.1×10^6 cells/g dry matter for the *S.* Typhimurium *gfp*, *E. coli* O157:H7 *gfp*, and *P. fluorescens* 32 *gfp*, respectively. The further dynamics of the introduced bacteria were somewhat different. The number of CFU in the *Salmonella* and *E. coli* populations decreased, but that of the cells remained constant. In the CE–soil mixture, the opposite regularity was characteristic of the *P. fluorescens* population (Figs. 1d–1f).

The survival of the enterobacteria was similar in the short-term tests with the CE and the CE–soil mixture, as well as in the variant with the CE incubation. On the contrary, for *P. fluorescens* (Table 1), it was different (Table 2).

The number of introduced bacteria on the cress plants and in the French snail excrements. In order to quantify the survival of the bacteria investigated on plants and to estimate the probability of the contamination of vegetables, cress seeds were sown in the CE–soil mixture. Before the sowing, the number of introduced bacteria in this mixture was 8.2×10^5, 7.0×10^5, and 5.0×10^6 cells/g dry matter in the short-term test variant and 9.5×10^5, 3.0×10^5, and 4.7×10^5 cells/g dry matter in the long-term experiment for *S.* Typhimurium *gfp*, *E. coli* O157:H7 *gfp*, and *P. fluorescens* 32 *gfp*, respectively. In the short-term test, the numbers of *S.* Typhimurium and *E. coli* increased to 1.1×10^7 and 2.8×10^6 in the phyllosphere and to 5.9×10^6 and 1.4×10^6 cells/g of dry matter in the rhizosphere, respectively. In the long-term test, their numbers were 4.4×10^6 and 4.3×10^5 in the phyllosphere and 5.4×10^6 and 5.6×10^5 cells/g of dry matter in the rhizosphere, respectively. The *P. fluorescens* population was 2.9×10^6 and 4.2×10^5 in the phyllosphere and 1.5×10^6 and 5.6×10^5 cells/g dry matter in the rhizosphere in the short- and long-term experiments, respectively. Thus, this population has decreased to some extent. The latter

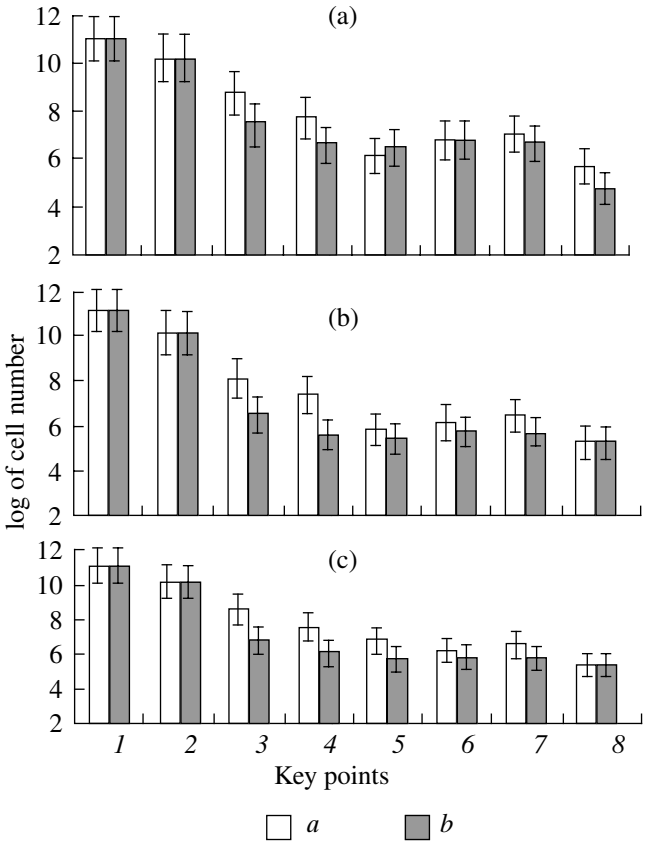

Fig. 2. Dynamics of the survival of (a) *S typhimurium* MAE 110 *gfp*, (b) *E. coli* O157:H7 *gfp*, and (c) *P. fluorescens* 32 *gfp* inoculated into the CE and at the other key points in their displacement to the soil, cress plants, and via the GIT of the snails after their feeding on the cress in the (*a*) short-term and (*b*) long-term experiments. Key points of the tests: *1*—suspension of the bacteria, cells/ml; *2*—cells/g dry CE; *3*—cells/g dry CE before mixing with the soil; *4*—cells/g dry CE–soil mixture; *5*—cells/g dry mixture before sowing of the cress seeds; *6*—cells/g dry cress roots; *7*—cells/g dry cress stalks; *8*—CFU/g dry snail excrements.

fact was unexpected, since some researchers are incline to consider *P. fluorescens* as a typical rhizosphere bacterium (Figs. 2a–2c).

After the feeding of the snails on the infected cress plants, the labeled bacteria were determined in their excrements by the plating method (the inoculation of media) (Figs. 2a–2c). The number of *gfp* bacteria in the snail excrements noticeably decreased as compared to that on the plants and amounted to 4.7×10^5 and 5.2×10^4 CFU/g dry matter for the *S.* Typhimurium, 1.7×10^5 and 1.8×10^5 CFU/g dry matter for the *E. coli*, and 1.8×10^5 and 1.7×10^5 CFU/g dry matter for *P. fluorescens* in the short- and long-term experiments, respectively. However, the determination of these bacteria was significant and showed that invertebrates, namely, French snails, can be active carriers of the enteropathogens in nature. Taking into account the fact that enterobacteria are capable of penetrating into plant tissues to survive there, the risk of spreading infections greatly increases

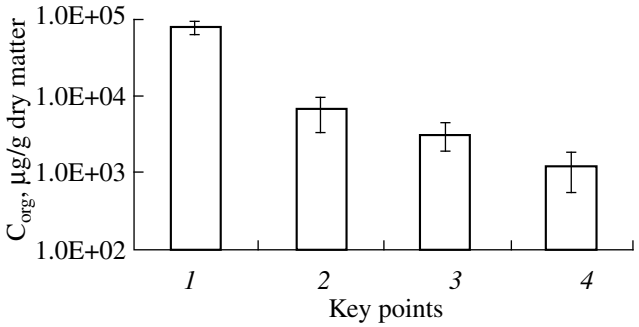

Fig. 3. The concentration of water-soluble organic carbon in the CE and the CE–soil mixture at different stages of the experiment. Key points: *1*—the CE (0 days), *2*—the CE before mixing with the soil (55 days), *3*—the CE–soil mixture (55 days); and *4*—the CE–soil mixture before sowing of the cress seeds (70 days).

[13]. At the same time, our investigations showed that, as the saprotroph bacteria and enterobacteria (with high initial populations) entered the natural substrates, their numbers steadily decreased. One of the reasons for this fact appeared to be the deficiency of nutrients. The dynamics of the water-soluble organic carbon content demonstrate this (Fig. 3). The concentration of water-soluble organic carbon decreased as the bacteria studied migrated via some natural objects: from 7.8×10^4 to 1.2×10^3 μg/g of dry matter in the CE and CE–soil mixture, respectively.

CONCLUSIONS

The studies conducted confirm the possibility of the translocation of microorganisms through a number of interacting natural substrates. In the movement of bacteria to other habitats, one may talk about the cycle of microorganisms [22]. This fact is quite natural, on the one hand, and is not always evident for everybody, on the other. Precisely owing to these migrations of microorganisms, as shown in this work, the majority of enteropathogenic and saprotrophic microorganisms is preserved in nature. Our results show that the conservation and maintenance of microorganism populations in nature are related not only to the fact of how long a microorganism can be in any substrate but also to that of how successfully it can survive under its displacement from one substrate to other ones.

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