doi:10.2478/v10014-010-0019-8

COBISS: 1.01
Agris category code: P30, P34

ANTIBIOTIC-RESISTANT SOIL BACTERIA IN HIGH-ALTITUDE (5000-6000 m) SOILS OF THE HIMALAYA

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Received August 16, 2010; accepted December 03, 2010.
Delo je prispelo 16. avgusta 2010, sprejeto 03. decembra 2010.

Antibiotic-resistant soil bacteria in high-altitude (5000-6000 m) soils of the Himalaya

In this study, low-carbon soils collected from an altitude transect from 5000 m to 6000 m were adopted as a simple model system with lower interaction complexity. This could help disentangle the basic environmental factors shaping the abundance and distribution of expressed resistance traits in culturable portion of fast growing heterotrophic strains. Improved plate counts were performed at 4 °C using 0.01 diluted nutrient broth supplemented with cold soil extract as a general media and additionally supplemented with antibiotics Ampicillin, Erythromycin, Kanamycin and Tetracyclin. A number of colonies (500) isolated from six locations were also tested separately for their antibiotic resistance. The results show that these high-altitude cold soils contained bacterial populations culturable at 4 °C in the range of 106 cells / g that were resistant to the four antibiotics and their various combinations tested in this study. The highest prevalence of resistance was observed in vegetated soils, whereas almost two orders of magnitude lower abundance of resistant cells was cultured from barren soils. Redundancy analysis showed that vegetation, soil carbon and pH were successful in explaining the interaction between environmental parameters and various culturable fractions of cold soil bacteria used in this study.

Key words: microbiology / bacteria / antibiotics / resistance / high-altitude / soil / interaction model

Na antibiotike odporne bakterije v visokogorskih tleh Himalaje (5000-6000 m)

V študiji sem uporabil vzorce tal z nizko vsebnostjo organskega ogljika iz višinskega transekta 5000 m-6000 m kot poenostavljen modelni sistem z nizko kompleksnostjo interakcij. Ta bi lahko pomagal razumeti osnovne okoljske dejavnike, ki uravnavajo porazdelitev in obseg izraženih rezistenčnih lastnosti gojljivega dela hitro rastočih heterotrofnih sevov. Izboljšano štetje na ploščah sem izvedel pri 4 °C na 0,01 koncentriranem hranilnem bujonu, dopolnjenim s hladnim ekstraktom tal, kot splošnim gojiščem, ki sem ga dopolnil z posameznimi antibiotiki (ampicilin, eritromicin, kanamicin in tetraciklin). Večje število izolatov (500) iz šestih lokacij sem prav tako testiral ločeno na njihovo odpornost na antibiotike. Ugotavljal sem tudi povezavo med okoljskimi dejavniki ter porazdelitvijo odpornih sevov in splošnega gojljivega deleža talnih bakterij. Rezultati kažejo, da visokogorska hladna tla vsebujejo pri nizkih temperaturah gojljive bakterijske populacije (106 / g), ki so odporni na posamezne antibiotike in razne njihove kombinacije, uporabljene v tej študiji. Poraščena tla imajo največji delež odpornih bakterij, skoraj dva reda manjši pa je prisoten v golih tleh. Statistična analiza je pokazala, da vegetacija, organski ogljik ter pH uspešno razložijo interakcijo med okoljskimi dejavniki in posameznimi gojenimi deleži bakterij, izoliranih iz hladnih tal.

Ključne besede: mikrobiologija / bakterije / antibiotiki / rezistenca / visokogorje / tla / model interakcij

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1 INTRODUCTION

The influence of the wide spread and long term use of antibiotics on the prevalence of resistance traits in the environment is still poorly understood although antibiotic resistance has been recognized as a global public health concern (for review see e.g. Allen *et al.,* 2010; Nwosu, 2001). In this respect, understanding the role of environmental bacteria as resistance gene reservoir is one of the key problems (Demaneche *et al.,* 2008; Riesenfeld *et al.,* 2004; Nwosu, 2001). Many studies have shown the existence of a considerable pool of resistance genes in agricultural soils, fish farm sludges and waters, in isolated human populations even in the absence of an obvious selection pressure (Allen *et al.,* 2010). In addition, metagenomic studies have identified novel resistance genes, a much wider diversity of known genes belonging to various resistance gene families and novel genes coding molecules and enzymes involved as potentiators of microbial resistance (Demaneche *et al.,* 2008; Riesenfield *et al.,* 2004; Allen *et al.,* 2010).

A simple model system composed of low-carbon soil altitude transect from 5000 m to 6000 m was adopted. Low plant diversity and short growing season in one of the most remote and human least directly impacted regions served as a system with lower interaction complexity that could help disentangle the basic environmental factors shaping the distribution and abundance of expressed resistance traits in culturable portion of fast growing heterotrophic strains. Improved plate counts for this environment were performed at 4 °C using 0.01 diluted nutrient broth supplemented with cold soil extract as a general media. In addition, a number of colonies (500) isolated from six locations were tested for their antibiotic resistance. The distribution of antibiotic strains was correlated to environmental parameters recorded and described before (Stres *et al.,* 2010).

2 MATERIALS AND METHODS

2.1 GENERAL CULTURABILITY

The soils and physical-chemical and various biological characteristics of the six soil samples were described before (Stres *et al.,* 2010). Shortly, soils were collected on the south facing slope of high alpine ridge descending from Drohmo peak (6980 m), the Kanchenjunga Himal, Nepal. The abundance of the culturable fraction of heterotrophic microbial community was assessed using plate counts according to approach described by Hashimoto and Hattori (1989) and Janssen *et al.* (2002) and modified as described below. The soil dilution series was prepared in 1 g / L MgSO4 buffer and three replicates per dilution were plated on the following three different oligotrophic complex media for each sample: 0.01 strength Nutrient Broth (Difco) in salt solution solidified with 1% agar (NB-A) supplemented with cold soil extract (Ley *et al.,* 2001; Janssen *et al.,* 2002; Olsen and Bakken, 1987). A colony - forming curve (CFC) (Hashimoto and Hattori, 1989) was generated for each soil by counting newly visible colonies over a 14 week incubation period at 4 °C and plotting the cumulative number of colonies at each time point. Only the counts after three weeks were used for calculations in the present work.

2.2 GENERAL RESISTANCE TO ANTIBIOTIC COMPOUNDS.

The 10 g portions of soil samples were resuspended in total volume of 100 mL of sterile salt solution (Ley *et al.,* 2001) and the cells were stripped from soil particles at 200 rpm for 20 min. Decimal serial dilutions were prepared and 100 ^L were inoculated on NB-A plates supplemented with one of the four antibiotic compounds (Ampicillin (50 ^g / mL), Tetracycline (20 ^g / mL), Kanamycin (20 ^g / mL), Erythromicin(15 ^g / mL) and incubated at 4 °C. Plates were inspected for well - spaced colonies (distance > 5 mm) after 3 weeks as no additional colonies appeared after 4 week incubation and 95% confidence intervals were calculated.

To verify the antibiotic resistance of the strains appearing after longer incubation periods, a subset of randomly selected colonies (n = 50) from each sample was restreaked on the same plates supplemented with single antibiotic compound.

2.3 ANTIBIOTIC RESISTANCE OF ISOLATED STRAINS

In order to obtain a more conservative estimate on resistant fraction within the culturable portion of bacteria, strains obtained from the first cultivation experiment without antibiotic compounds were tested separately for their antibiotic resistance. Cultures were plated on the same media they were isolated on, but supplemented with one of the four antibiotics as above.

2.4 STATISTICAL ANALYSES

The antibiotic resistance of isolated strains obtained in this study and environmental parameters (Stres *et al.,* 2010) served as input data in linear constrained ordina-

tion, redundancy analysis (RDA) with forward selection that was used to create an environmental model explaining the variability in response variables (antibiotic resistance patterns, abundance of resistant colonies, general abundance of culturable cells). The Monte Carlo permutation test (999 permutations) was applied to compute the significance of hypothetical relations using CANOCO V 4.5 (Biometris) (Leps and Smilauer, 2002).

3 RESULTS AND DISCUSSION

The abundance of resistant CFU to four antibiotics used ranged from lowest 10^2 to 10^6 CFU / g soil in barren and plant covered soils, respectively. Antibiotic resistant CFU determined at 4 °C were almost 100 times more abundant in plant covered (5200 m, 5400 m, 5600 m) than in barren soils (5000 m, 5800 m, 6000 m), despite rather similar number of culturable cells in these soils (Fig. 1).

There was no discernible effect of particular antibiotic compound on the abundance of resistant CFU within particular soil sample, however, the levels of resistant colonies were significantly different ($P < 0.01$) between barren and vegetated soils. The percentages of resistant bacteria varied from 0.01 to 15% in barren soils, median 2%. Surprisingly, the number of antibiotic resistant and the number of culturable bacteria appeared to be equal in plant covered soils, suggesting that all culturable bacteria were also resistant to antibiotics. This is surprising as the values reported in this study are one to two orders of magnitude higher than those reported for transgenic and control corn fields for Ampicillin resistance. In addition, the prevalence of Ampicillin resistant bacteria in undisturbed prairie soil ranged from 54.4% to 69.9% (Demaneche *et al.,* 2008), representing half of the prevalence found in this study. The results of the two studies could represent a simple gradient from intensive agricultural practice through undisturbed prairie to simplified more extreme natural vegetated environment where antibiotic resistance could represent a novel competitive advantage. However, whether these strains are more exposed to antibiotic producing strains or are only exposed to better conditions for gene exchange can not be resolved. Seemingly the question, whether the antibiotics used in this study serve as activators of specific biochemical path-

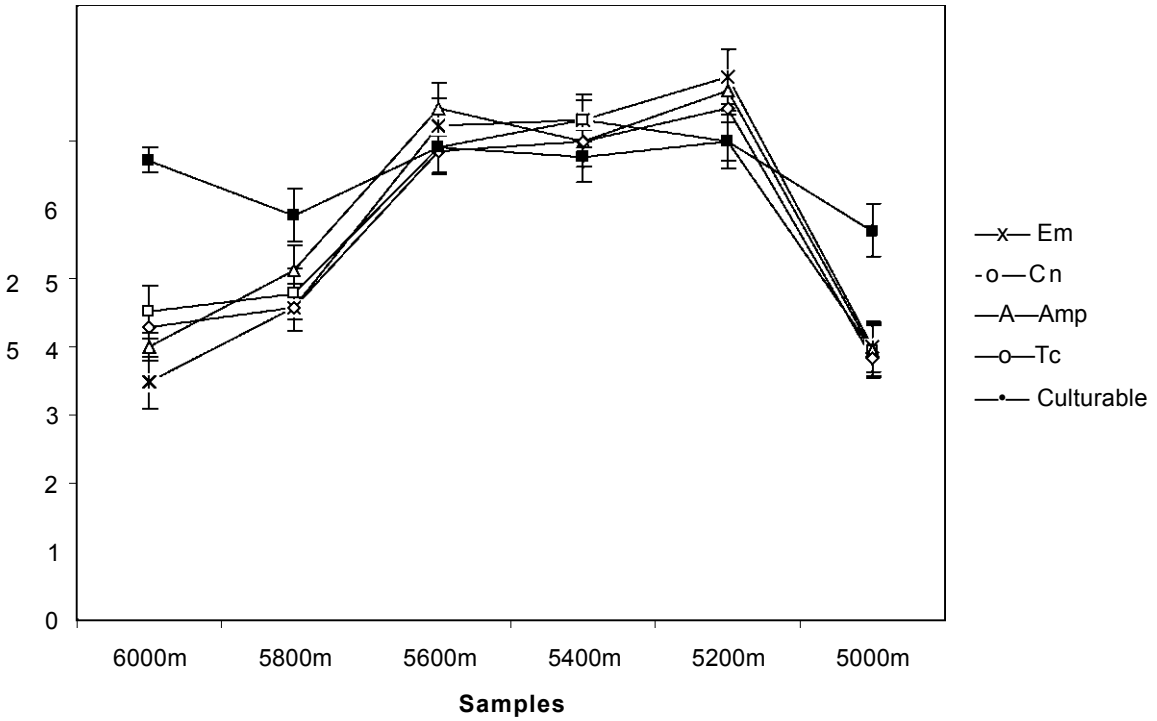

Figure 1: *Colony counts of heterotrophic and antibiotic resistant strains emerging on 0.01 NB-A plates supplemented with single antibiotic compound at 4 °C. Error bars represent 95% confidence intervals. Amp - Ampicillin; Em - Erythromycin; Kn - Kanamycin; Tc - Tetracycline.*

Slika 1: *Število heterotrofnih in na antibiotike odpornih konij, ki so zrasle na gojišču 0,01 NB-A s posameznimi antibiotiki pri 4 °C. Oznake napak predstavljajo 95 % intervale zaupanja. Amp - ampicilin; Em - erithromicin; Kn - kanamicin; Tc - tetraciklin.*

ways, signaling molecules in quorum sensing or just as a simple carbon source can not be answered at this time.

The technical limitations and differences in approaches could be also limiting the comparability of the results between studies, as different approaches to cell stripping, temperature and time of incubation were used next to different carbon source. This highlights the profound inconsistencies in the approaches used to monitor the resistance properties of environmental bacteria as these approaches are not standardized and the data are produced on a range of media, antibiotic concentrations, temperatures and incubation periods (Nwosu, 2001; D'Costa *et al.,* 2006; Allen *et al.,* 2010).

However, it is tempting to speculate that these results indicate that antibiotic resistance is a common trait in this high altitude environment and that plant presence significantly increased the frequency of antibiotic resistance to one and combinations of multiple antibiotics. It also seems that these resistance traits are acquired through different mechanisms than human application and indicate that cold soil bacteria are an important reservoir of antibiotic resistance genes potentially entering water flows during enhanced percolation during snow thaw.

Further, the testing of the strains isolated on NB-A-CSA plates without antibiotic compounds revealed that the vast majority of resistant strains were resistant to three antibiotics, Ampicillin, Kanamycin and Erythromycin (Fig. 2). This is congruent with the recent findings of D'Costa *et al.* (2006) that environmental strains are resistant to multiple antibiotics and also suggests that the distribution of resistance determinants is rather similar among the antibiotic resistance strains from the six samples of the high-altitude cold soils. In addition, the overall abundance of resistant population appears to be modulated exclusively by the presence of plants, despite the similar abundances of culturable bacteria in other barren samples, differences in soil chemistry and plant species (Poa sp., mosses or combination of the two) covering vegetated soils (Stres *et al.,* 2010).

In order to establish which environmental parameters were significantly associated with the observed patterns in antibiotic resistance patterns in the cold soils, RDA analysis was conducted. Environmental characteristics (soil physical and chemical parameters) reported in

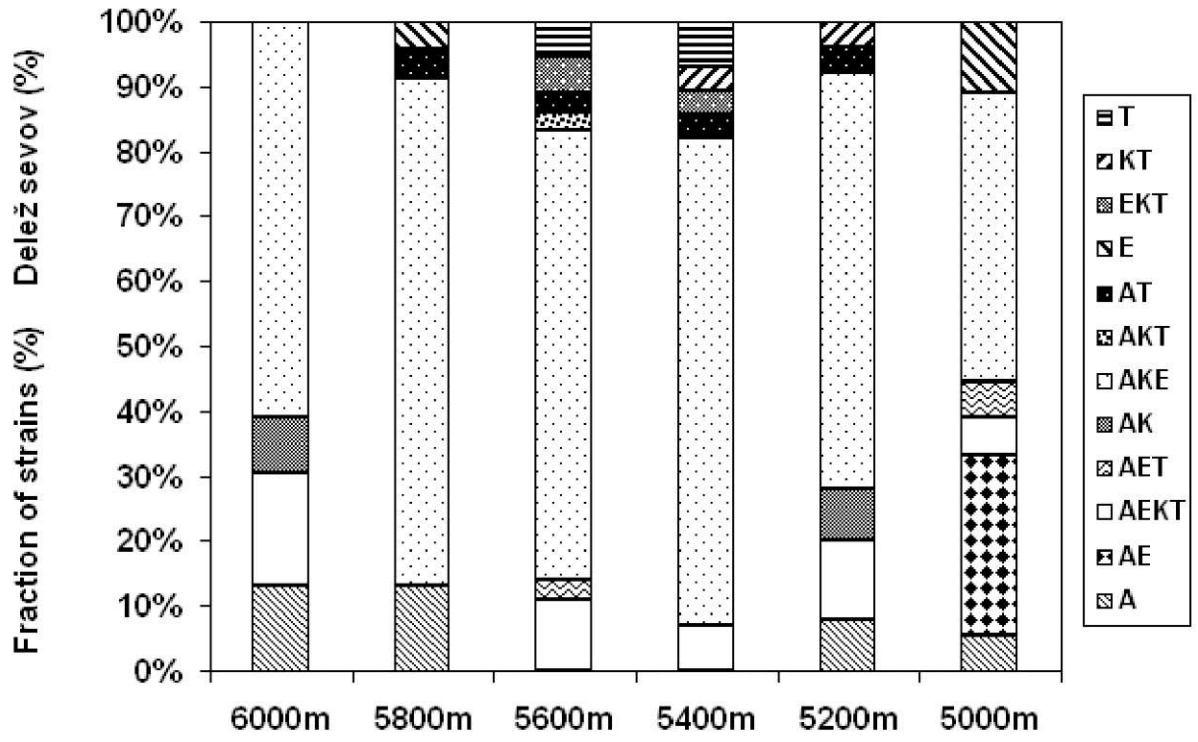

Figure 2: *The distribution of antibiotic resistance determinants in analyzed strains isolated from six composite high-altitude cold-soil samples from 5000 - 6000 m altitude transect. The letters designate the antibiotics and their combinations. A- Ampicillin; E - Erythromycin; K - Kanamycin; T - Tetracycline.*

Slika 2: *Porazdelitev determinant odpornosti analiziranih sevov izoliranih iz šestih visokogorskih hladnih tal iz transekta med 5000-6000 m nadmorske višine. Oznake napak predstavljajo antibiotike ali njihove kombinacije: A - ampicilin; E - erithromicin; K - kanamicin; T - tetraciklin.*

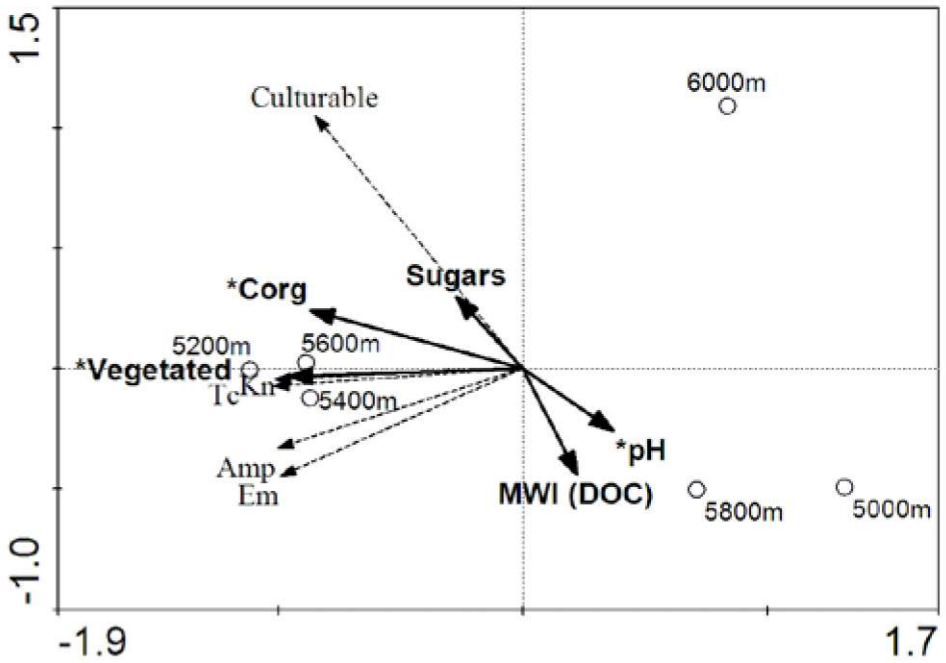

Figure 3: *Results of redundancy analysis (RDA) describing general cultivation success and the abundance of various resistant populations (the response variables) in relation to the sampling localities (empty circles) and environmental parameters (bold arrows). The significant environmental parameters are marked with asterisk (*). Corg - organic carbon; MWI(DOC) - molecular weight index of dissolved organic carbon; Amp - Ampicillin; Em - Erythromycin; Kn - Kanamycin; Tc - Tetracycline.*

Slika 3: *Rezultati statistične analize (RDA) prikazujejo uspešnost gojenja heterotrofnih mikroorganizmov ter različnih odpornih populacij (odzivne spremenljivke - črtkane puščice) v odvisnosti od mest vzorčenja (krožci) ter okoljskih dejavnikov (poudarjene puščice). Signifikantni okoljski dejavniki so označeni z zvezdico (*). Corg - organski ogljik; MWI(DOC) - indeks velikosti molekul raztopljenega organskega ogljika; Amp - ampicilin; Em - erithromicin; Kn - kanamicin; Tc - tetraciklin.*

Stres *et al.* (2010) served as explanatory variables whereas the general culturability and abundance of resistant CFU to each antibiotic (Fig. 1) served as response variables. RDA showed that only three out of 20 measured environmental parameters could explain significantly the variability in the measured abundances of culturable cells and resistant populations. The best predictor of variability in the high-altitude microbial abundance at 4 °C was vegetation, organic carbon and pH, explaining 87.1%, 9.1% and 3% of the data variability (P = 0.002; P = 0.026; P = 0.01), respectively. This environmental model explained 99.2% of variability in abundance of the various culturable fractions explored in this study and 99.7% of species - environment relations. Other environmental parameters tested in this study (Stres *et al.,* 2010) did not produce significant effects and were omitted from Fig. 3 with two exceptions (MWI(DOC), sugars).

Interestingly, the soil content of reductive sugars (Stres *et al.,* 2010) was directly correlated to general culturability of soil bacteria, however, this correlation was not found statistically significant. This finding is interesting in its own right in understanding of environmental parameters that enable recovery of larger proportions of culturable bacteria from the environmental samples (Janssen *et al.,* 2002; Davies *et al.,* 2005). This approach could provide a different strategy in cultivation approaches, first by analyzing the environmental conditions in various samples and pinpointing the environmental parameters correlated to increased culturabiltiy of microorganisms, with efforts mostly directed to various organic species, which is now much more easily achievable through the use of GC-MS or MALDI-TOF MS. On the other hand, the molecular weight index describing the size of complex organic substances was inversely proportional to general culturability. This is also interesting as the size of this index is inversely proportional to molecular weight, suggesting that the general measure of an average molecular weight in dissolved organic carbon fraction offers a too low resolution to be of any particular value in such cases. On the other hand, RDA showed no significant correlation between patterns of antibiotic resistance (Fig. 2) and environmental parameters. This suggests that the distribution of antibiotic resistance determinants did not differ significantly from a random pattern. Alternatively, the presence of other factors and processes that shape the distribution of particular anti-

biotic patterns, not recorded in this study, could explain these observations.

4 CONCLUSIONS

The high-altitude cold-soils contain at 4 °C culturable bacterial populations that are resistant to the four antibiotics tested in this study. The highest prevalence of resistance to antibiotics was recorded for plant covered soils, where all culturable cells exhibited resistance to antibiotics. On the contrary, almost two orders of magnitude lower abundance of resistant cells was cultured in barren soils. Redundancy analysis showed that vegetation, soil carbon and pH were successful in explaining the interaction between environmental parameters and culturable fractions of cold soil bacteria used in this study.

5 ACKNOWLEDGMENTS

I am indebted to Dr. Jerneja Ambrožič Avguštin for the stimulating discussions during our sabbatical at the Mediterranean Institute for Advanced Studies (IMEDEA) that directed my attention back to my old unpublished results.

6 REFERENCES

Allen H.K., Donato J., Wang H.H., Cloud-Hansen K.A., Davies J., Handelsman J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Rev. Microbiol., 8: 251-259

Davis K.E.R., Joseph S.J., Janssen P.H. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol., 71: 826-834

D'Costa V.M., McGrann K.M., Hughes D.W., Wright G.D. 2006. Sampling the Antibiotic Resistome. Science, 311: 374-377

Demaneche S., Sanguin H., Pote J., Navarro E., Bernillon D., Mavingui P., Wildi W., Vogel T.M., Simonet P. 2008. Antibiotic resistance soil bacteria in transgenic plant fields. Proc. Nat. Acad. Sci., 105: 3957-3962

Hashimoto T., Hattori T. 1989. Grouping of soil bacteria by analysis of colony formation on agar plates. Biol. Fertil. Soils, 7: 198-201

Janssen P.H., Yates P.S., Grinton B.E., Taylor P.M., Sait M. 2002. Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the Divisions *Acidobacteria, Actinobacteria, Proteobacteria,* and *Verrucomicrobia.* Appl. Envir. Microbiol., 68: 2391-2396

Leps J., Smilauer P. 2003. Multivariate Analysis of Ecological Data using CANOCO. Cambridge, Cambridge University Press, UK: 280 p.

Ley E.R., Lipson D.A., Schmidt S.K. 2001. Microbial biomass level in barren and vegetated high altitude talus soils. Soil Sci. Soc. Am. J., 65: 111-117

Nwosu V. 2001. Antibiotic resistance with particular reference to soil microorganisms. Res. Microbiol., 152: 421-430

Olsen R.A., Bakken L.R. 1987. Viability of soil bacteria: optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol., 13: 59-74

Riesenfeld C.S., Goodman R.M., Handelsman J. 2004. Uncultured soil bacteria are reservoir of new antibiotic resistance genes. Env. Microbiol., 6: 981-989

Stres B, Philippot L, Faganeli J, Tiedje J.M. 2010. Frequent freeze-thawcyclesyield diminishedyet resistant and responsive microbial communities in twotemperate soils: a laboratory experiment. FEMS Microb Ecol, doi:10.1111/j.1574-6941.2010.00951.x