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# Contamination of Soil by Copper Affects the Dynamics, Diversity, and Activity of Soil Bacterial Communities Involved in Wheat Decomposition and Carbon Storage▽†

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**A soil microcosm experiment was conducted to evaluate the influence of copper contamination on the dynamics and diversity of bacterial communities actively involved in wheat residue decomposition. In the presence of copper, a higher level of $CO_2$ release was observed, which did not arise from greater wheat decomposition but from a higher level of stimulation of soil organic matter mineralization (known as the priming effect). Such functional modifications may be related to significant modifications in the diversity of active bacterial populations characterized using the DNA stable-isotope probing approach.**

Microbial communities consist of a complex assemblage of species at a given time in a given space, each having different metabolic characteristics and physiological requirements and driving at least one of the multiple reactions involved in organic matter transformation. Understanding the relationship between microorganisms, organic matter, and environmental parameters (such as agricultural practices in the agrosystem) is thus of pivotal importance for better evaluation of carbon dynamics in terrestrial ecosystems and for addition of an ecological approach to carbon cycle models (11). Metal contamination in agricultural soils could affect such carbon dynamics (12). Exposure to metal stress has been shown to shape the native microbial community structure by selecting metal-resistant populations (13). This can significantly affect the capacity of the soil microbial communities to degrade organic matter, thereby leading to a loss of soil fertility and modifications in the balance between $CO_2$ release and long-term C storage (3). All these considerations show the importance of studying the relationship between organic matter, metal, and microbes at the community level in order to elucidate the mechanisms controlling the impact of heavy metals on soil functions.

The aim of this study was to evaluate the extent to which contamination of soil could modify the dynamics, diversity, and activity of bacterial populations in soil involved in wheat residue decomposition and the repercussions in terms of carbon dynamics in soil. A microcosm experiment in which $^{13}$C-labeled wheat residues (95.8% $^{13}$C; C/N ratio, 63.4) were incorporated into two soil samples originating from the same field experiment, subjected to significant copper contamination (Cu-contaminated soil) or not contaminated (pristine soil) (14), was carried out. The soil used was a calcareous silty-clay soil (pH 7.8) from the experimental farm of INRA Epoisse (Burgundy, France). It was collected in November 2004 from the surface layer (0 to 5 cm) and sieved at 4 mm. The total copper content of the surface layer of the contaminated plot was about 214 mg $Cu \cdot kg^{-1}$ soil, compared with 29.3 mg $Cu \cdot kg^{-1}$ soil for the pristine soil. Microcosms were set up by placing 10 g (dry weight) of soil in 150-ml hermetically sealed plasma flasks. Soil samples were supplemented with sterile water to reach 80% of the maximum water-holding capacity.

Briefly, $^{13}$C-labeled wheat was incorporated into the soil microcosms and incubated for 28 days to carefully monitor both residue decomposition and modifications of the bacterial community involved, as previously described (1). The evolution of total $CO_2$ and $^{13}CO_2$ release and the bacterial community dynamic structure were assessed throughout the incubation. DNA and RNA were extracted from the soil microcosms, and nucleic acid fractions enriched in $^{13}$C were separated using density gradient ultracentrifugation, as previously described (1). The genetic structure of the bacterial community was determined from the $^{12}$C and $^{13}$C fractions by using the DNA fingerprinting technique known as bacterial automated ribosomal intergenic spacer analysis (B-ARISA) after 0, 7, 14, and 28 days of incubation. A molecular inventory of the bacterial taxonomic diversity in copper-contaminated soils was performed by elaborating 16S rRNA clone libraries on $^{12}$C- and $^{13}$C-labeled RNA after 14 days of incubation, as previously described by Bernard et al. (1). The results were compared with those obtained in a previous study of pristine soils (1).

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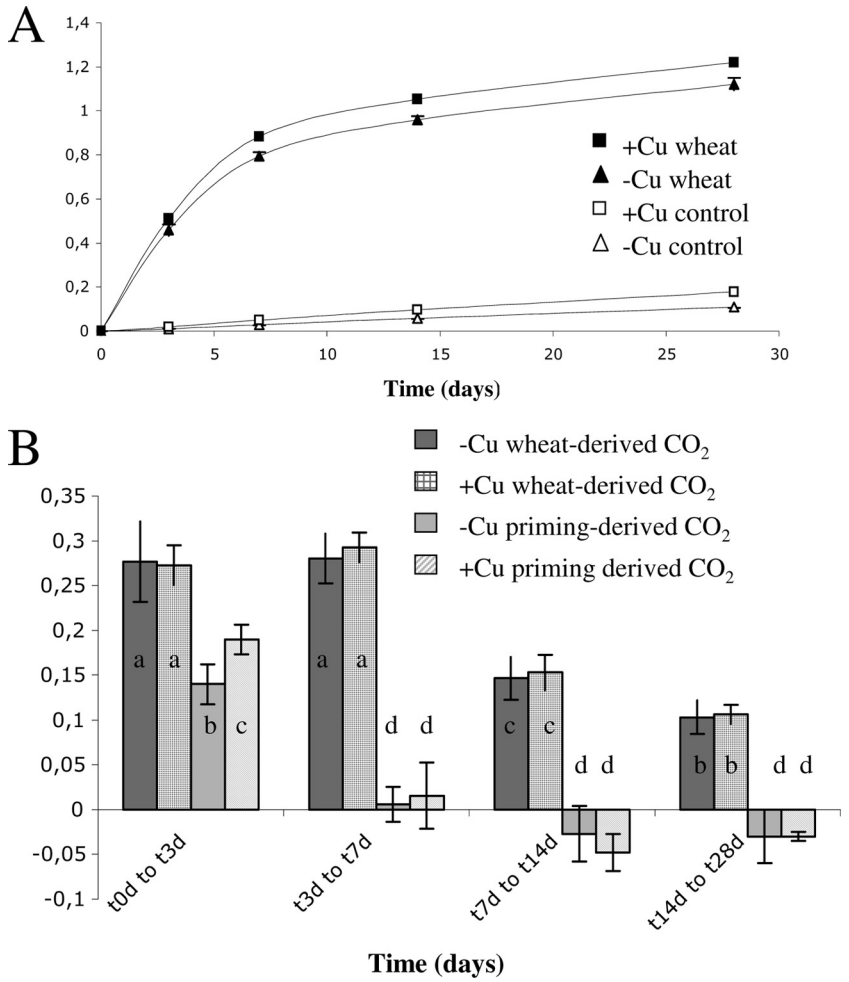


FIG. 1. (A) Total $CO_2$ accumulated during the 28 days of incubation for control (empty symbols) and wheat-enriched (bold symbols) soil microcosms. Squares represent soil collected from a Cu-contaminated parcel, and triangles represent the uncontaminated counterpart. Results are expressed in mg of whole $CO_2$ per gram of soil. Standard deviations were obtained from triplicate microcosms. (B) Noncumulative values of wheat-derived $CO_2$ and priming-derived $CO_2$ evolution, expressed in mg of $CO_2$ per gram of soil. Wheat and priming-derived $CO_2$ values were calculated from measured total $CO_2$ and $^{13}CO_2$ values (see text). Bars denoted by different letters represent significant differences at $P$ values of $\leq 0.05$.

$CO_2$ concentration was determined with an MT1 micro gas chromatograph analytical instrument equipped with a poraPLOT Q capillary column and a solid-state detector. Carbon isotopic enrichment was determined by gas chromatography-isotope ratio mass spectrometry, using a trace gas interface coupled in continuous flow with a VG Isochrom mass spectrometer (Micromass, Manchester, England). The accumulated total $CO_2$ release in the wheat-amended soil microcosms followed classical kinetics, with an exponential increase between days 0 and 7 of incubation, after which a slowdown was observed (Fig. 1A). Copper contamination induced higher levels of total $CO_2$ release in both control microcosms and wheat-amended microcosms (Fig. 1A). Tobor-Kaplon et al. (15) have suggested that metal-adapted populations increase their respiration rate because of a higher stress-induced maintenance cost and that the respiration rates of sensitive populations decrease as a result of intoxication. However, the observed discrepancy did not result from a more intensive mineralization of wheat residue, since no significant difference between Cu-contaminated and pristine soils for the cumulated rates of wheat-derived $^{13}CO_2$ was observed (Fig. 1B). Consequently, total $CO_2$ variations between copper-contaminated and pristine soils could result from an increase in nonlabeled soil organic matter (SOM) mineralization. This would be consistent with the results of our previous study conducted with non-copper-contaminated soils (1), in which a similar increased release of $^{12}CO_2$, originating from SOM mineralization following wheat residue inputs and due to a so-called priming-effect process, was observed (2). Evaluation of the $^{12}CO_2$ release in each soil treatment confirmed an early (up to day 7 of incubation) and resilient priming effect, which was significantly higher in copper-contaminated soil (Fig. 1B). These results might be explained mainly by the mineralization of a part of the living carbon released after the death of copper-sensitive populations (6), but the large amount of accumulated $^{12}CO_2$ released on day 3 (Fig. 1B) supports the hypothesis of SOM mineralization leading to a transitory loss of soil C stock.

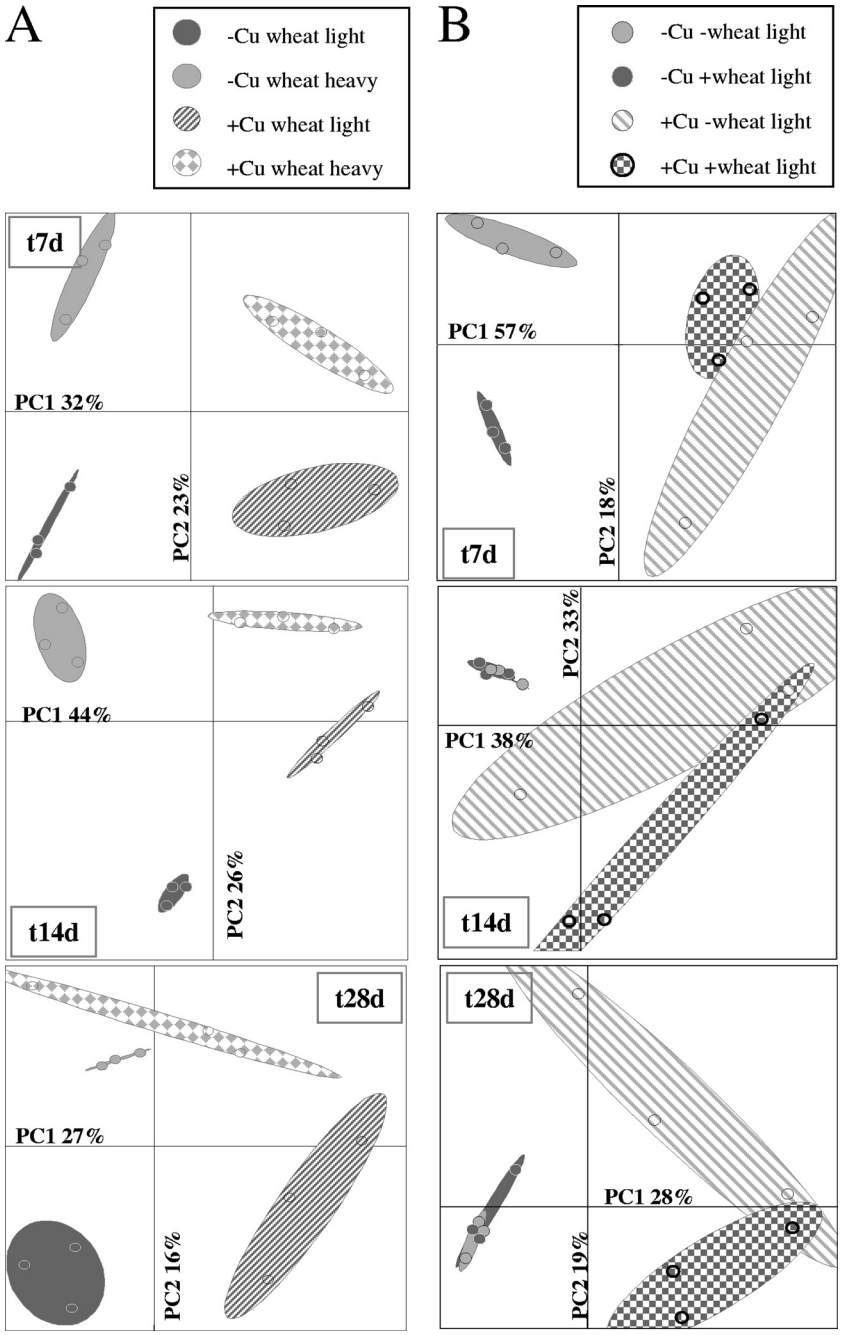


FIG. 2. (A) PCA ordinations of bacterial genetic structure for light and heavy DNA fractions of microcosms filled with natural or Cu-contaminated soil and enriched with $^{13}$C-labeled wheat residue, presented for day 7, day 14, and day 28 separately. Statistical ellipses drawn over the plot replicates represent 90% confidence. (B) PCA ordinations of bacterial genetic structure for light DNA fractions of control and wheat-enriched microcosms filled with natural or Cu-contaminated soil, presented for day 7, day 14, and day 28 separately. Statistical ellipses drawn over the plot replicates represent 90% confidence.

Soil DNA and RNA were coextracted from three 1-g (dry weight) soil samples per microcosm by following the DNA extraction procedure of Ranjard et al. (14), modified to optimize RNA recovery as described by Bernard et al. (1). The DNA/RNA separation efficiency and the integrity of the purified nucleic acids were verified electrophoretically with agarose gel. Cesium chloride density gradient centrifugation was performed as described by Bernard et al. (1). Total DNA and RNA concentrations in soil extracts and in fractions collected from density gradients were assessed using PicoGreen and RiboGreen staining kits (Molecular Probes, Paris, France), respectively, in accordance with the manufacturer's instructions. Light and heavy fractions were distinguished in accordance with the distribution of DNA along the CsCl density gradient as described by Bernard et al. (1). The density distributions of DNA extracted from Cu-contaminated soil were

similar to those observed for the pristine soil, as were the $\delta^{13}C$ levels measured along the gradients (data not shown). For molecular analysis, the light DNA fraction (not containing $^{13}C$) was considered representative of populations not involved in decomposition of $^{13}C$-labeled wheat, and the heavy DNA fraction (most enriched with $^{13}C$) was considered representative of populations actively assimilating carbon derived from $^{13}C$-labeled wheat residue.

B-ARISA fingerprinting was performed on the $^{12}C$ and $^{13}C$ soil DNA fractions obtained at days 0, 7, 14, and 28 for wheat-amended and nonamended soils, contaminated or not contaminated with copper. Principal component analysis (PCA) of the B-ARISA profiles on days 7 and 14 confirmed the discrimination of contaminated and pristine soils on the PC1 axis, explaining 32% of the variability (14), whereas the heavy ($^{13}C$) and light ($^{12}C$) DNA fractions were separated on the PC2 axis (explaining 23% of the variability) (Fig. 2A). Whatever the soil contamination, significant bacterial community dynamics were observed over the incubation period, suggesting a succession of active populations consecutive to wheat incorporation (8). However, particular active populations were stimulated in contaminated and pristine soils, confirming the hypothesis of Oorts and colleagues (10) on the possible adaptation of microbial communities to metals with maintenance of the potential decomposition ability (functional redundancy). Such an observation might be related to the observed differences in residue mineralization and priming effect intensity described above, especially in the early stage of residue decomposition.

Interestingly, the weaker discrimination between contaminated and pristine soils observed after 28 days (demonstrated by the Monte Carlo test) (Fig. 2A) suggests an enrichment of Cu-sensitive populations that might result from copper ageing in soil (7). At the same time, B-ARISA profiles obtained from the light DNA fractions revealed a significant shift, induced by the wheat amendment, but exclusively in pristine soil (Fig. 2B). This shift might result from the release of extracellular enzymes by fresh-organic-matter (FOM) feeders able to partly depolymerize SOM and thereby stimulate SOM feeders without labeling them (1, 2). The fact that unlabeled populations in the contaminated soil did not seem to be stimulated by fresh organic inputs suggests that the priming effect would mainly be generated by labeled populations via cometabolism processes. $^{13}C$-labeled FOM provides energy to microorganisms to depolymerize recalcitrant SOM (2). On day 14 of incubation, when the genetic structure modifications were the most significant, reverse transcription-PCR amplification and cloning of 16S rRNA genes were carried out on the heavy fraction of the wheat-amended Cu-contaminated soil microcosm and on the light fraction of the wheat-amended pristine soil microcosm as previously described (1). The resulting libraries, [EHCu] (enriched heavy plus Cu), and [CLCu], were compared with those obtained from the heavy and light fractions of the wheat-enriched ([EH] and [EL]) and the nonamended ([CL]) pristine soils already described by Bernard et al. (1) (Table 1).

This comparative analysis confirmed that particular bacterial populations, actively involved in residue degradation, occurred in Cu-contaminated and pristine soils. Major differences resulted from the higher proportions of *Alphaproteobacteria* and *Actinobacteria* in the active fraction in copper-contaminated soil and of *Delta-* and *Gammaproteobacteria* in the pristine soil

TABLE 1. Representation of phylogenetic classes in each clone library[a]

| Characteristic | Data for indicated soil type[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Nonamended | | | | Wheat-amended | | | |
| Cu contamination | − | | + | | − | | + | |
| RNA fraction | Light | | Light | | Heavy | | Heavy | Light |
| Library name[a] | [CL] | | [CLCu] | | [EH] | | [EHCu] | [EL] |
| No. of clones analyzed | 60 | | 56 | | 59 | | 57 | 80 |
| % Class affiliation | | | | | | | | |
| Alphaproteobacteria | 3.3 | | 17.8 | | 3.4 | | 28.5 | 12.6 |
| Betaproteobacteria | 36.6 | | 60.7 | | 64.4 | | 53.5 | 27.8 |
| Gammaproteobacteria | 3.3 (DQ822216, -59) | | 0 | | 13.5 (DQ822272, -285, -290, -299, -302, -321, -326) | | 1.8 (DQ822405) | 5 (EF066534, -590, -591, -595) |
| Deltaproteobacteria | 11.6 (DQ822213, -19, -20, -21, -22, -39, -52) | 5.3 (DQ822331, -34, -69) | | | 3.4 (DQ822294, -316) | | 0 | 8.9 (EF066532, -566, -573, -580, -585, -587, -593) |
| Acidobacteria | 10 (DQ822208, -11, -30, -40, -42) | 3.5 (DQ822343, -77) | | | 6.8 (DQ822286, -320, -324, -327) | | 3.5 (DQ822398, -403) | 10.1 (EF066529, -538, -542, -562, -575, -582, -583, -599) |
| Actinobacteria | 5 (DQ822223, -34, -38) | 1.8 (DQ822363) | | | 0 | | 5.3 (DQ822412, -32, -47) | 6.3 (EF066546, -548, -572, -597, -604) |
| Bacteroidetes | 1.6 (DQ822231) | 1.8 (DQ822366) | | | 6.8 (DQ822269, -303, -309, -319) | | 5.3 (DQ822409, -26, -31) | 5 (EF066554, -556, -574, -601) |
| Candidates | 6.6 (DQ822209, -28, -37, -48) | 0 | | | 0 | | 0 | 10.1 (EF066533, -541, -544, -553, -560, -563, -569, -584) |
| Gemmatimonadetes | 6.6 (DQ822229, -55, -62, -64) | 7.1 (DQ822335, -65, -68, -71) | | | 0 | | 1.8 (DQ822400) | 5 (EF066545, -570, -581, -596) |
| Cyanobacteria | 5 (DQ822226, -43, -63) | 1.8 (DQ822333) | | | 0 | | 0 | 1.3 (EF066530) |
| Planctomycetes | 1.6 (DQ822266) | 0 | | | 0 | | 0 | 0 |
| Verrucomicrobia | 0 | 0 | | | 0 | | 0 | 1.3 (EF066531) |
| Undetermined | 8.3 (DQ822217, -41, -46, -49, -67) | 1.8 (DQ822350) | | | 1.7 (DQ822287) | | 0 | 6.3 (EF066527, -550, -552, -561, -589) |

[a] [EHCu] and [CLCu] clone libraries were determined in this study and compared to the [CL], [EH], and [EL] clone libraries obtained in a previous study (1).
[b] [CL], control light; [CLCu], control light plus Cu; [EH], enriched heavy; [EHCu], enriched heavy plus Cu; [EL], enriched light. Numbers in parentheses are GenBank accession numbers of clones.

(Table 1). Interestingly, copper pollution seemed to have inhibited certain populations known to be fast-growing organisms and to feed on readily available carbon, like the *Gammaproteobacteria*. In contrast, many populations able to degrade polymerized or aromatic compounds, such as *Sphingomonadaceae* and *Actinobacteria* (4, 5), were apparently actively involved in residue degradation in copper-contaminated soils (see Fig. S1 in the supplemental material). The observed *Alphaproteobacteria* included the *Methylobacteriaceae* and *Hyphomicrobiaceae* families, known to be methylotrophs and thus to utilize a one-carbon compound as their sole source of carbon and energy (9). In the context of our study, they could have benefitted from the depolymerization activities of *Sphingomonadaceae* and of *Actinobacteria*.

All the clone libraries studied were dominated by *Betaproteobacteria*, with the genera *Acidovorax*, *Aquabacterium*, *Janthinobacterium*, *Methylibium*, *Ramlibacter*, and *Variovorax* represented (see Fig. S2 in the supplemental material). It can therefore be hypothesized that these genera are involved in the degradation of both SOM and fresh residues, regardless of the copper status of the soil. Together, these data suggest that certain populations may adopt different growth strategies in the natural environment, depending on the available carbon source and the surrounding conditions. Our results also suggest that when a few opportunistic populations, like the *Gammaproteobacteria*, were inhibited, copper favored other, slower-growing populations able to cometabolize FOM and SOM and thus potentially increased the priming effect by using a cometabolism mechanism, similar to mechanism 2 described by Fontaine et al. (2). Such a hypothesis could support the greater priming effect observed in the contaminated soils (Fig. 1B).

Our study provides evidence that metals must have only a slight impact on the mineralization of complex FOM due to a high level of functional redundancy at each step in substrate degradation. In contrast, the ability to decompose SOM would be shared by a lower number of species, implying that a small change in microbial diversity induced by a modification of physicochemical soil properties, such as metal concentration, can have a negative or positive impact on the priming effect and thus on carbon storage.

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## REFERENCES

1. **Bernard, L., C. Mougel, P. A. Maron, V. Nowak, J. Lévêque, C. Henault, F. Z. Haichar, O. Berge, C. Marol, J. Balesdent, F. Gibiat, P. Lemanceau, and L. Ranjard.** 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from $^{13}$C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. Environ. Microbiol. **9:**752–764.
2. **Fontaine, S., A. Mariotti, and L. Abbadie.** 2003. The priming effect of organic matter: a question of microbial competition? Soil Biol. Biochem. **35:**837–843.
3. **Giller, K. E., E. Witler, and S. P. McGrath.** 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol. Biochem. **30:**1389–1414.
4. **Gremion, F., A. Chatzinotas, and H. Harms.** 2003. Comparative 16S rDNA and rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ. Microbiol. **5:**896–907.
5. **Hui, L. I., Y. Zhang, I. Kravchenko., X. U. Hui, and C. Zhang.** 2006. Dynamics changes in microbial activity and community structure during biodegradation of petroleum compounds: a laboratory experiment. J. Environ. Sci. **19:**1003–1013.
6. **Kuzyakov, Y., J. K. Friedel, and K. Stahr.** 2000. Review of mechanisms and quantification of priming effect. Soil Biol. Biochem. **32:**1485–1498.
7. **Lejon, D. P. H., J. Lévêque, J. Martins, L. Spadini, N. Pascault, D. Landry, M. J. Milloux, V. Nowak, R. Chaussod, and L. Ranjard.** 2008. Copper dynamics and impact on microbial communities according to soil organic status. Environ. Sci. Technol. **42:**2819–2825.
8. **Lundquist, E. J., L. E. Jackson, K. M. Scow, and C. Hsu.** 1999. Changes in microbial biomass and community composition, and soil carbon and nitrogen pools after incorporation of rye into three California agricultural soils. Soil Biol. Biochem. **31:**221–236.
9. **McDonald, I. R., S. Radajewski, and J. C. Murell.** 2005. Stable isotope probing of nucleic acids in methanotrophs and methylotrophs: a review. Org. Geochem. **36:**779–787.
10. **Oorts, K., H. Bronckaers, and E. Smolders.** 2006. Discrepancy of the microbial response to elevated copper between freshly spiked and long-term contaminated soils. Environ. Toxicol. Chem. **25:**845–853.
11. **Powlson, D. S., P. Smith, and J. U. Smith (ed.).** 1996. NATO ASI series I, vol. 38, p. 429. Springer, Heidelberg, Germany.
12. **Rajapaksha, R. M. C. P., M. A. Tobor-Kaplon, and E. Bååth.** 2004. Metal toxicity affects fungal and bacterial activities in soil differently. Appl. Environ. Microbiol. **70:**2966–2973.
13. **Ranjard, L., L. Lignier, and R. Chaussod.** 2006. Cumulative effects of short term poly-metallic contaminations on soil bacterial community structure. Appl. Environ. Microbiol. **72:**1684–1687.
14. **Ranjard, L., V. Nowak, A. Echairi, V. Faloya, and R. Chaussod.** 2008. The dynamics of soil bacterial community structure in response to yearly repeated agricultural copper treatments. Res. Microbiol. **159:**251–254.
15. **Tobor-Kaplon, M. A., J. Bloem, P. F. A. M. Römkens, and P. C. de Ruiter.** 2005. Functional stability of microbial communities in contaminated soils. Oikos **111:**119–129.