Universitat de Girona

IMPACT OF CHLOROPHENOLS AND HEAVY METALS ON SOIL MICROBIOTA: THEIR EFFECTS ON ACTIVITY AND COMMUNITY COMPOSITION, AND RESISTANT STRAINS WITH POTENTIAL FOR BIOREMEDIATION

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Joan Cáliz Gelador

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Universitat de Girona

La Dra. Genoveva Montserrat Bueno, professora del departament de Biologia de la Universitat de Girona i el Dr. Xavier Vila Portella, professor titular del departament de Biologia de la Universitat de Girona,

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La tesi exposada a continuació es presenta com a compendi de publicacions, la qual està formada per dos articles publicats i un tercer en revisió, així com un quart article que està en preparació. En base a les condicions establertes per la Comissió d'Autorització de Defensa de Tesis Doctorals en la sessió de 16 de Desembre de 2009, a continuació es mostra que es compleixen els requeriments sol·licitats. Els índexs de qualitat de les revistes es detallen segons dades de SCI 2009 (índex d'impacte i rang dins àrea temàtica de la revista).

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Summary; Resum; Resumen

Summary

This thesis is part of a broader research project that aims to assess the effect of several priority organic and inorganic pollutants in relation to two different Mediterranean soils. The main goal of this project is to determine the ecological risk concentrations of each pollutant, as well as to analyse the factors that influence on their effective concentrations, in order to predict the impact of these pollutants on different soil ecosystems and to provide scientific data for the regulation of the soil protection polices. According to this purpose, the influence of pollutants has been revealed by assessing activity and composition of the microbial community of these soils without any prior history of exposure to such toxic compounds. This study has been performed by means of different ecotoxicity tests to analyse the impact of the pollutants from different perspectives.

The organic pollutants selected for the experiments are 2-monochlorophenol (MCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP), whereas the inorganic pollutants are Cr(VI), Cd and Pb. Both Mediterranean soils, which have been classified as Calcaric Regosol (CRe) and Haplic Arenosol (HAr), respectively, have been chosen because of their different physicochemical properties. To highlight the impact of pollutants in relation to the response of the indigenous microbial populations, both soils have been studied in microcosms incubated under controlled laboratory conditions. Activity and composition of the microbial community have been assessed during several weeks, respectively, by respirometric methods and PCR-DGGE molecular approach. Moreover, culture-plating methods have been used to isolate microorganisms from contaminated microcosms, which have been finally identified and characterized in relation to their resistance and capacity to degrade or transform the pollutants, in order to infer their potential usefulness for bioremediation applications.

The respirometric techniques and the molecular approach have shown a significant effect of the pollutants on the soil microbial communities, thus indicating that not only the activity but also the abundance of several microbial groups are influenced by the added toxic compounds. These results suggest that DGGE could be more useful than respirometry to detect ecotoxicological effects of organic pollutants that can be used as C sources, but the respirometric techniques seem to be more sensitive than the molecular approach to show the effects of heavy metals. The toxicity of chlorophenols has shown that generally increases with the increasing degree of chlorination. However, the toxic effects of MCP and TCP are stronger in

the soil HAr than in the soil CRe, while the impact of PCP is weaker in the soil HAr even when compared with MCP and TCP (regarding the highest concentrations), because the particular properties of each soil influence differently on the fate of chlorophenols. Regarding the inorganic pollutants, Cr causes toxic effects at the lowest added amounts in comparison to other heavy metals tested, but Pb should be considered the most toxic when considering the soluble fraction instead of the total concentration. Therefore, bioavailability has been shown to dramatically influence the impact of the heavy metals, and even a different potential toxicity ranking can be achieved if their effective concentrations are considered. These findings point out that bioavailability is a key factor controlling the effective concentration of the pollutants that impacts the soil microbial populations, which must be strongly considered in ecotoxicological assessments since any change in the soil system could modify the fate of the pollutants and lead to stronger toxic effects.

The persistence of several microbial populations has been detected by both DGGE analysis and culture-plating in highly polluted microcosms of the two different Mediterranean soils, which evidence the capacity of the indigenous soil microbiota to survive in the presence of pollutants without a previous adaptation or contact with them. Moreover, some microorganisms have revealed to cope with both organic and inorganic pollutants, thus indicating they could play an important role protecting the soil system even in co-contamination events. Some of these emerging microorganisms that have been identified by DGGE analysis in highly polluted soil microcosms have been isolated by culture methods as well. However, several microorganisms can only be detected by one of the approaches, and thus, both of them have been shown as complementary methods to detect resistant microbiota.

Resistant microorganisms have shown to be specific for each kind of soil, thus suggesting that they are the best suitable for the respective environmental conditions. This statement points out the importance of considering the microbial features and the soil characteristics before designing bioremediation strategies by means of bioaugmentation, in order to achieve a good performance of the process. Several of the isolated microorganisms have shown to resist high concentrations of the pollutants and the capacity to degrade TCP and/or PCP, or to reduce the concentration of Cr(VI). Among them, some strains phylogenetically related to different species of *Streptomyces* have been found to resist and reduce high concentrations of Cr(VI), and *Paenibacillus taichungensis* and two different species of *Bacillus* have revealed as new members of the group of *Firmicutes* to be able to degrade chlorophenolic compounds. They are gram-positive spore-forming bacteria and commonly distributed in soils. These singular features confer them a strong

interest as soil autochthonous microbiota with potential for soil bioremediation applications.

Resum

La present tesi doctoral forma part d'un projecte de recerca més ampli, que pretén avaluar l'efecte de diversos contaminants orgànics i inorgànics vers dos sòls típicament mediterranis. L'objectiu principal del projecte és la determinació de la concentració de risc ecològic corresponent a cadascun dels contaminants assajats, de manera que en permeti la predicció de l'impacte en els diversos ecosistemes edàfics, així com l'obtenció de dades científiques útils per a l'establiment de normatives relatives a la protecció del sòl. Amb aquest propòsit, l'impacte dels contaminants s'ha estudiat en relació a l'activitat i a la composició de la comunitat microbiana dels dos sòls sense història prèvia de contaminació amb cap dels contaminants assajats. L'estudi s'ha complementat amb diferents assajos ecotoxicològics, amb l'objectiu d'analitzar l'impacte dels contaminants des de diverses perspectives.

Els contaminants orgànics que s'han seleccionat són el 2-monoclorofenol (MCP), el 2,4,6-triclorofenol (TCP) i el pentaclorofenol (PCP), mentre que els contaminants inorgànics són el Cr(IV), el Cd i el Pb. Els sòls escollits tenen propietats fisicoquímiques ben diferenciades, i han estat classificats com a Calcaric Regosol (CRe) i Haplic Arenosol (HAr), respectivament. Els sòls s'han estudiat en microcosmos incubats sota condicions controlades de laboratori per tal d'evidenciar la resposta de la comunitat microbiana indígena a l'impacte dels contaminants. S'ha valorat l'activitat i la composició de la comunitat microbiana al llarg de diverses setmanes d'incubació, ja sigui a partir de la respirometria o de mètodes moleculars com la PCR-DGGE dels àcids nucleics extractats. A més, s'ha portat a terme el cultiu de microorganismes a partir de determinades mostres per tal d'aïllar soques procedents dels microcosmos contaminants, les quals s'han identificat i caracteritzat en relació a la resistència i la capacitat de degradar o transformar els contaminants, per tal d'avaluar el seu potencial ús en bioremediació.

La respirometria i les tècniques moleculars han mostrat la presència d'alteracions en les comunitats microbianes dels sòls, de manera que es pot concloure que no només l'activitat, sinó també l'abundància de les diferents espècies que formen part de la comunitat microbiana es veu afectada per la presència dels compostos tòxics considerats. Els resultats porten a pensar que la tècnica molecular DGGE és més indicada que la respirometria quan s'avalua l'efecte de compostos orgànics, però la respirometria sembla millorar la detecció dels efectes causats per metalls pesants. La toxicitat dels clorofenols augmenta amb el grau d'halogenació del compost considerat. No obstant això, els efectes derivats del MCP i del TCP són més intensos en el sòl HAr que en el sòl CRe, mentre que l'impacte causat pel PCP és menor en el sòl HAr fins i tot en comparació amb el MCP i el TCP (a les concentracions més elevades), ja que les diferents propietats de cada sòl assajat donen lloc a

un comportament particular i diferent del contaminant en cada cas. En relació als contaminants inorgànics, el Cr dóna lloc a efectes tòxics a concentracions inferiors en comparació als altres metalls assajats, però el Pb resulta ser el més tòxic si és la concentració de metall disponible la que es té en compte, i no pas la total. Per tant, la biodisponibilitat té una gran importància en l'impacte que causen els metalls pesants, de manera que si es té en compte, l'ordre de toxicitat de l'efecte que causen canvia substancialment. Els resultats presentats indiquen que la biodisponibilitat és un factor clau que controla l'impacte de les concentracions efectives que donen lloc a efectes en la comunitat microbiana del sòl, i que ha de ser considerada en les avaluacions de risc ecotoxicològic. Qualsevol petit canvi en el sistema edàfic, i per tant en la biodisponibilitat dels contaminants, pot modificar el seu comportament i produir efectes tòxics més greus.

Mitjançant l'anàlisi per PCR-DGGE, s'ha evidenciat la persistència de diverses comunitats microbianes en els sòls mediterranis que s'han assajat amb concentracions elevades de contaminants, cosa que posa de manifest la capacitat de la població indígena d'un sòl per resistir la presència de contaminants sense un contacte o adaptació prèvia. A banda d'aquest fet, s'ha vist que alguns microorganismes són capaços de resistir tant contaminants orgànics com inorgànics, de manera que poden representar un paper de gran importància si es pretén la restauració d'ambients edàfics co-contaminats. Alguns dels microorganismes emergents, que han estat identificats a partir de l'anàlisi per DGGE de mostres altament contaminades, s'han obtingut, a més, en cultiu pur mitjançant sembra i incubació en medis sòlids. Malgrat això, alguns microorganismes només han estat identificats mitjançant una de les aproximacions, ja sigui per DGGE o bé per cultiu en placa, de manera que ambdues tècniques esdevenen complementàries.

La microbiota resistent ha demostrat ser específica de cada tipus de sòl, de manera que es pot considerar que aquests microorganismes són els més adients per a les respectives condicions ambientals. Aquest fet posa de manifest la necessitat de considerar les particularitats microbiològiques i les característiques del sòl com a passos previs al disseny d'un procés de bioremediació, especialment quan es pretén utilitzar el bioaugment de la població microbiana, per tal d'assolir el màxim d'eficàcia en el tractament. Alguns dels microorganismes aïllats han demostrat ser resistents a concentracions elevades de contaminants, degradar TCP i/o PCP, així com reduir la concentració de Cr(VI) aportada. Entre aquests bacteris, algunes soques d'*Streptomyces* han mostrat la capacitat de resistir y reduir altes concentracions de Cr(VI), així com una soca de *Paenibacillus taichungensis* i dues soques del gènere *Bacillus*, les primeres dins del grup dels *Firmicutes* que han evidenciat la seva capacitat de degradar compostos clorofenòlics. Tots ells són grampositius i esporulats, de freqüent presència al sòl. Aquestes singularitats els confereixen gran interès com a soques autòctones del sòl amb potencial per a processos de bioremediació.

Resumen

La presente tesis doctoral forma parte de un proyecto de investigación más amplio, que pretende evaluar el efecto de distintos contaminantes orgánicos e inorgánicos respecto a dos suelos típicamente mediterráneos. El estudio se ha efectuado a partir de diferentes tests ecotoxicológicos, para así analizar el impacto de los contaminantes desde diversas perspectivas. El objetivo principal del proyecto es la determinación de la concentración de riesgo ecológico correspondiente a cada uno de los contaminantes ensayados, de manera que permita la predicción del impacto en los distintos ecosistemas edáficos, así como la obtención de datos científicos útiles para el establecimiento de normativas relativas a la protección del suelo.

Los contaminantes orgánicos que se han seleccionado son el 2-monoclorofenol (MCP), el 2,4,6-triclorofenol (TCP) y el pentaclorofenol (PCP), mientras que los contaminantes inorgánicos son el Cr(IV), el Cd y el Pb. Los suelos escogidos tienen propiedades físico-químicas diferenciadas, y se han clasificado como Calcaric Regosol (CRe) y Haplic Arenosol (HAr), respectivamente. De acuerdo con los objetivos propuestos en la tesis, el impacto de los contaminantes se ha estudiado en relación con la actividad y la composición de la comunidad microbiana de dos suelos sin historia previa de contacto con ninguno de los contaminantes ensayados. Los suelos se han estudiado en forma de microcosmos incubados en condiciones controladas de laboratorio para así poner de manifiesto el impacto de los contaminantes en relación con la respuesta de la comunidad microbiana indígena. Se ha valorado la actividad y la composición de la comunidad microbiana a lo largo de unas semanas de incubación, ya sea a partir de la respirometría como de métodos moleculares de tipo PCR-DGGE de los ácidos nucleicos extractados. Además, se ha llevado a cabo la siembra en placa de determinadas muestras, para así aislar microorganismos procedentes de los microcosmos contaminados, los cuales se han identificado y caracterizado en relación a la resistencia o a la capacidad de degradar o transformar los contaminantes y, consecuentemente, investigar su potencial uso en bioremediación.

La respirometria y las técnicas moleculares han mostrado la presencia de efectos en las comunidades microbianas de los suelos, de manera que se puede concluir que no sólo la actividad, sino también la abundancia de las diferentes especies que forman parte de la comunidad microbiana se ve afectada por la presencia de los compuestos tóxicos considerados. Los resultados llevan a pensar que la técnica molecular DGGE está más indicada que la respirometría cuando se evalúa el efecto de compuestos orgánicos, pero la respirometría parece mejorar la detección de los

efectos causados por metales pesados. La toxicidad de los clorofenoles aumenta con el grado de halogenación del compuesto considerado. Sin embargo, el efecto derivado del MCP y del TCP en el suelo HAr es más intenso que en el suelo CRe, mientras que el impacto causado por el PCP es menor en el suelo HAr incluso en comparación con el MCP y el TCP (a las concentraciones más elevadas), ya que las diferentes propiedades de cada suelo ensayado dan lugar a un comportamiento particular y diferente del contaminante en cada caso. Teniendo en cuenta los contaminantes inorgánicos, el Cr da lugar a efectos tóxicos a concentraciones inferiores en comparación a los otros metales, pero el Pb resulta ser el más tóxico si es la concentración de metal disponible la que se tiene en cuenta, y no la total. Por tanto, la biodisponibilidad tiene gran importancia en el impacto que causan los metales pesados. Así pues, si se tiene en cuenta, el orden de toxicidad del efecto que causan los contaminantes resulta ser diferente de cuando no se considera. Los resultados presentados indican que la biodisponibilidad es un factor clave que controla el impacto de las concentraciones efectivas que dan lugar a efectos en la comunidad microbiana del suelo, y que ha de ser considerada en las evaluaciones de riesgo ecotoxicológico, ya que cualquier pequeño cambio en el sistema edáfico, y por tanto en la biodisponibilidad de los contaminantes, puede modificar el comportamiento de estos, y producir efectos tóxicos más graves.

Mediante el análisis por PCR-DGGE, se ha evidenciado la persistencia de diferentes comunidades microbianas en los suelos mediterráneos que se han ensayado con concentraciones elevadas de contaminantes, de manera que se pone de manifiesto la capacidad de la población indígena de un suelo para resistir la presencia de contaminantes sin un contacto o adaptación previa. Además, se ha visto que algunos microorganismos son capaces de resistir tanto contaminantes orgánicos como inorgánicos y, por tanto, pueden representar un papel importante si se pretende la restauración de ambientes edáficos co-contaminados. Algunos de los microorganismos emergentes, que han sido identificados a partir del análisis por DGGE de muestras altamente contaminadas, se han obtenido en cultivo puro mediante siembra e incubación en medios sòlidos. A pesar de ello, algunos microorganismos sólo han sido identificados mediante una de las dos aproximaciones empleadas, ya sea por DGGE o bien por cultivo en placa, por ello ambas técnicas son complementarias a la detección de microbiota resistente.

La microbiota resistente ha demostrado ser específica de cada tipo de suelo, y se puede considerar que estos microorganismos son los más adecuados para las respectivas condiciones ambientales. Este hecho pone de manifiesto la necesidad de considerar las particularidades microbiológicas y las características del suelo como

pasos previos al diseño de un proceso de bioremediación, especialmente cuando se pretende utilizar el bioaumento de la población microbiana, para así conseguir el máximo de eficacia en el tratamiento. Algunos de los microorganismos aislados han demostrado ser resistentes a concentraciones elevadas de contaminantes, degradar TCP y/o PCP, así como reducir la concentración de Cr(VI) aportada. Entre estas bacterias, algunas cepas de *Streptomyces* han demostrado capacidad para resistir y reducir elevadas concentraciones de Cr(VI), así como una cepa de *Paenibacillus taichungensis* y dos cepas del género *Bacillus*, las primeras dentro del grupo de *Firmicutes* que han puesto en evidencia su capacidad de degradar compuestos clorofenólicos. Todas ellas son grampositivas y esporuladas, y de frecuente presencia en el suelo. Estas singularidades les confieren gran interés como cepas autóctonas del suelo con potencial para procesos de bioremediación.

General introduction

Pollution by a wide array of chemical compounds with different features and properties is one of the most relevant environmental damages to ecosystems produced by human activity, which could derive in a serious hazard for the living organisms. For this reason, the environmental protection polices must ensure the health of ecosystems through the establishment of measures for limiting the use of toxic compounds, as well as the control and restoration of contaminated sites. Likewise, environmental research should contribute to develop appropriate methods to assess ecotoxicological effects of pollutants and predict their impact on ecosystems, in order to define concentrations of ecological risk and establish protocols for use and treatment of toxic compounds. Removing pollutants from the environment in an ecologically responsible, safe, rapid, and cost-effective way must be a priority for management agencies. Therefore, environmental research is focused on the improvement of strategies for cleaning and restoration of contaminated ecosystems.

New regulations for soil protection have been developed during the last decade. The basis and emphasis of these regulations have moved progressively from the quantification of pollutants and soil quality levels to risk-based limits (Swartjes et al., 2008). Spanish legislation regarding soil pollution establishes the Generic Reference Levels (GRL) for a list of substances and different soil uses. The classification of soils as polluted or non-polluted is based on those GRL, and it is dependent on the soil use. If protection of ecosystems is considered the main priority, ecotoxicological tests on the polluted soil as described in the OECD (Organisation for the Economic Cooperation and Development) guidelines for the testing of chemicals, or similar, must be performed (Ministerio de la Presidencia, 2005). The OECD guidelines recommend several standard soils for ecotoxicity assays. These soils have specific properties that make them especially sensitive to pollutants to produce conservative estimates for tolerable limits in ecotoxicological assays (OECD, 2000). However, different soils may vary in their ability to cope with pollutants by influencing on their bioavailability or due to the soil status itself. The involvement of the indigenous microbiota in contact with pollutants is also an important issue in order to understand the toxic effects in the soil system, as well as to develop adequate bioremediation strategies. Consequently, it is important to know the effects of toxic compounds on the microbiota in different kinds of soils without previous contamination (Konopka et al., 1999; Roane et al., 2001; Jackson et al., 2005; Viti et al., 2006). In this sense, more research is necessary to improve the

prediction of the actual toxicity of contaminants and update the legislation in soil pollution.

1. The soil system

Soil is the naturally occurring, unconsolidated mineral and organic material at the earth's surface that provides an environment for living organisms (Paul, 2007). During formation, soils develop horizontal layers or horizons (Figure 1) that look different from one another and vary in thickness depending on the intensity of the soil-forming factors. The horizon nearest the soil surface that is organic-matter-enriched is called A horizon, and it is the most propitious for the development of living organisms.

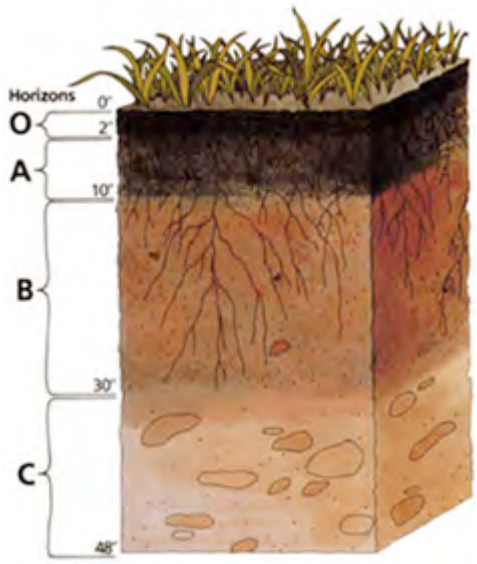
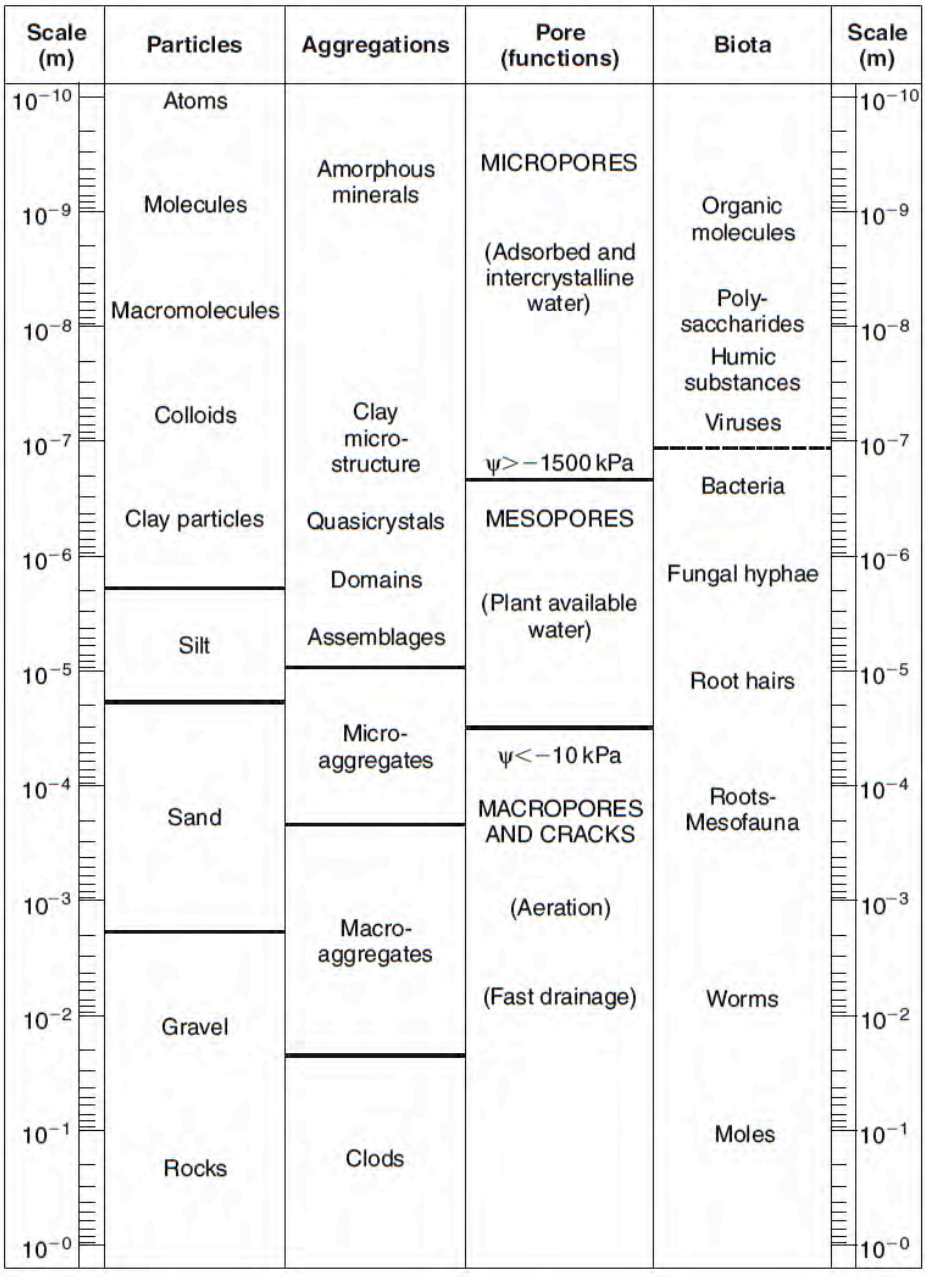


Figure 1. Exemple of soil horizons

Dimensions of physical features commonly encountered when considering the soil habitat range from meters (pedon, soil landscape, and watershed), down through a few millimeters (the fine-earth fraction), to a few micrometers (microorganisms and clay minerals) and nanometers (humic molecules) (Table 1). The larger mineral particles may affect the physical attributes of a soil, and thus, only particles <2mm in diameter are used for soil definition. The proportion of different size classes of these particles in soil is referred as soil texture, and terms such as sandy loam, silty clay, and clay loam are textural classes used to identify the soil's texture. When investigating a field site, considerable insights into the behaviour and properties of the soil can be inferred from its texture, so it is often one of the first properties to be measured. Typically, the individual mineral particles in surface soils are coated and glued together with colloidal organic matter and encrusted with inorganic

cements forming spatial clusters within the matrix known as aggregates or peds. Soils are composed of highly variable aggregates, derived from a wide range of parent materials that exist within innumerable landscapes, and exposed to diverse climates, which have formed in concern with the development of the complex communities of living organisms that make up the biosphere.

Table 1. Spatial dimensions of features commony encountered in describing the soil habitat (Paul, 2007)



Soils provide such a tremendous range of microhabitats that supports an enormous biomass with an estimated 2.6×10^{29} prokaryotic cells alone, and harbour much of the Earth's genetic diversity. A single gram of soil can contain kilometres of fungal

hyphae and more than 10^9 bacterial cells, organisms belonging to tens of thousands of different species (Paul, 2007). The microhabitat (Figure 2) includes the physical location where a particular organism resides, as well as the characteristics of the habitat (e.g. pore space, water content, oxygen, potential redox, temperature, pH) that influence the growth, activities, interactions and survival of other organisms found in it. Micro-zones of good aeration may be only millimeters from areas poorly aerated. Areas near the soil surface may be enriched with decaying organic matter and other nutrients, whereas the subsoil may be nutrient poor; the soil solution in some pores may be highly acidic, others more basic, depending on soil mineralogy and biological activity. Temperature and water contents of surface soil can vary widely from that of subsoil; and the microenvironment of the surfaces of soil particles, where nutrients are concentrated and water films vary in thickness, can be very different from those of soil pores.

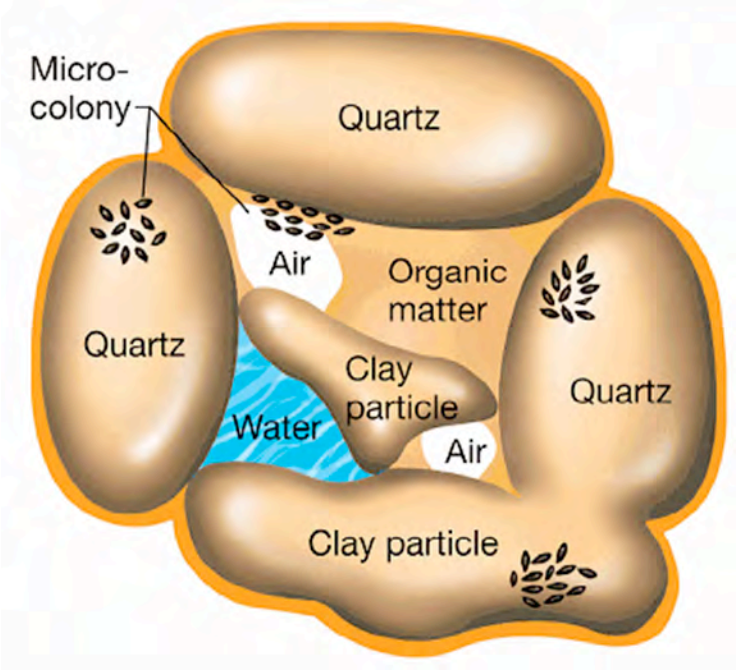


Figure 2. Soil aggregate composed of minerals, organic matter and microcolonies of microorganisms attached to particles (Madigan *et al.*, 2004).

2. Environmental toxicology and toxicity testing

Environmental toxicology is a multidisciplinary science devoted to the study of the harmful effects of chemical, biological and physical agents on living organisms, which can be simplified to the understanding of only three basic functions after the introduction of a xenobiotic into the environment (Landis and Yu, 2004). These functions are the fate and distribution of the introduced chemical with the

environment after the initial release; the interaction of the chemical with the site of action; and the impact of this molecular interaction in the ecosystem. If environmental toxicologists could define appropriate functions that would describe the transfer of an effect from its interaction with a specific receptor molecule to the effects seen at the community level, it would be possible to predict accurately the effects of pollutants in the environment. However, a suitable understanding of these functions is still far.

Many kinds of compounds can exhibit environmental toxicity. The most commonly discussed and researched are pesticides and industrial chemicals because of the large amounts transported and used. Metals from mining operations and manufacturing, and occurring as contaminants in lubricants, are also released to the environment. Crude oil and the petroleum products derived from the oil are a significant source of environmental toxicity because of their persistence and common use in an industrialized society. However, it is not only the presence of a compound that poses a toxicological threat but the relationships between its dose in contact with an organism and its biological effects that determine which environmental concentrations are harmful. Moreover, there are many factors that modify the toxicity of pollutants, which include physicochemical properties, mode of exposure, time, environmental factors, interaction, biological and nutritional factors.

Over the years, a variety of test methods or guidelines have been standardized to evaluate the toxicity of chemicals or mixtures. These protocols are available from the American Society for Testing and Materials (ASTM), the Organization for Economic Cooperation and Development (OECD), and the National Toxicology Program (NTP), and are available as U.S. Environmental Protection Agency (USEPA) publications, the Federal Register, and often from the researchers that developed the standard methodology. There are a large number of toxicity tests that have been developed in environmental toxicology because of the large variety of species and ecosystems that have been investigated. However, it is possible to classify the tests using the length of the experiments relative to the life span of the organism and the complexity of the biological community. Several tests have been focused on single-species, such as acute toxicity test with *Daphnia spp.*, algal growth, frog embryo teratogenesis assay, etc. Multispecies toxicity tests are usually performed in aquatic or terrestrial microcosms. A wide variety of this kind of tests can be performed, such as standardized aquatic microcosm, mixed flask culture, FIFRA microcosm, soil core microcosm, soil in jar, etc. Microbiology can help to the environmental toxicologist to assess the impact of pollutants in the microbial communities, as well as to understand their fate and transformation.

2.1. Soil microbial activity

Estimation of soil microbial activity comprises all the biochemical reactions catalysed by microorganisms. Some processes, such as respiration and heat output, can be conducted by most soil microorganisms whilst others, such as nitrification and nitrogen fixation, can only be conducted by a restricted number of microbial species. Soil respiration has been used frequently for the assessment of the side effects of toxic compounds (Montserrat et al., 2006; Martí et al., 2007; Coello et al., 2009). By aerobic respiration, that is oxidation of organic matter by microorganisms, oxygen functions as the end acceptor of the electrons. The end products of the process are carbon dioxide and water. The metabolic activities of soil microorganisms can therefore be quantified by measuring the CO_2 production or O_2 consumption (Nannipieri et al., 1990). Different parameters can be used to estimate soil respiration. The basal respiration is defined as the respiration without the addition of organic substrate to soil, which can be followed even for long periods of time. Soil respiration can be determined by using simple techniques such as the incubation of soils in jars, where CO_2 can be adsorbed in NaOH traps and determined by HCl filtration, changes in electrical conductivity of the NaOH solution, or use gas chromatography or infrared spectroscopy. The consumption of O_2 can be calculated with the Warburg instrument by the means of an electrorespirometer or gas chromatography. Substrate-induced respiration (SIR) is another parameter for the estimation of soil activity by determining aerobic respiration in the presence of an added substrate such as glucose, amino acids, etc. SIR shows changes in a few hours.

2.2. Soil microbial community composition

In recent years, molecular methods have supposed an advance to the study of microbial community structure and function, since most microorganisms are not able to grow by using standard laboratory culture methods (Amann et al., 1995). It is thought that culturability index of microorganisms present in environmental samples is ranging 0.1-10% of the total community (Amann et al., 1995). Therefore, analysis of the microbial community by culture methods is far to represent the naturally occurring microbial diversity. Different molecular approaches are appropriate to each type of analysis. Community profiling techniques produce fast surveys, which provide a phylogenetic profile of the microbial populations present at a particular site. Thus, these techniques can be used to assess the community composition across space, through time, down pollution gradients, or under various treatments. Methodologies that provide molecular fingerprints are generally based

on physical procedures to separate DNA, after PCR (polymerase chain reaction) or RT-PCR (reverse transcriptase PCR) amplification of concerning genes. It is the case of SSCP (Single-stranded-conformation polymorphism), RAPD (Randomly amplified polymorphism), DGGE (Denaturing gradient gel electrophoresis), RFLP (Restriction fragment length polymorphism) and T-RFLP (Terminal restriction fragment length polymorphism).

3. Ecotoxicity in soils: bioavailability

The response of soil microbiota to the presence of pollutants can be related to the microbial populations present in the soil, the concentration of the pollutants and also to the properties of the soil itself (Pu and Cutright, 2007). All these features can modify not only the pollutant bioavailability but also the stimulation capacity of the microbiota (Cho et al., 2000). Therefore, the effects of pollutants on soil ecosystems are not only dependent on the specific toxic compounds and concentration, but also on the type and physiological status of the impacted soil.

Considering ecotoxicity, it is important to recognize that the toxic effects of a pollutant depend not only on the properties and amounts of the chemical compound itself, but also upon the amount of pollutant to which organisms are actually exposed. If a pollutant is present in soil but is not bioavailable, then it can not cause toxic effects to the biota. Bioavailability can be defined as the amount of contaminant present that can be readily taken up by living organisms, e.g., microbial cells (Maier, 2000). Many factors influence bioavailability and, thus, quantification is extremely complex. Solubility in water is one of several factors that significantly impacts bioavailability, which depends on soil characteristics itself and chemical properties of pollutants. Since organisms often come into contact with toxic compounds in the aqueous phase, solubility in water is often used as an approximate measure of the bioavailable fraction of pollutants (Lazzaro et al., 2006). In addition to solubility, bioavailability also depends upon the localized distribution of the pollutant within the environmental matrix. The fate of pollutants depends to a large degree on the chemical properties of the toxic compounds and environmental factors, which determine how it will partition to various compartments, or phases, of the environment. Partitioning is a general phenomenon that describes the tendency of a pollutant to exist between phases at equilibrium. The compartments of the environment of relevance to partitioning are air, water, soil and sediment, as well as biota. Once a pollutant is released into soil, there are a number of possible fates: soil sorption, leaching (to surface water or groundwater), volatilization to the atmosphere, plant root uptake, etc.

Measurement or estimation of bioavailability is problematic, as it is affected by many individual and interacting conditions related to soil and water chemistry, pollutant chemistry and partitioning, biological transformation and concentration factors (Hund-Rinke and Kördel, 2003). The techniques currently being researched can be categorized broadly as direct or indirect and biological or chemical (Lanno et al., 2004), although truly direct chemical methods are not possible since only organisms can determine whether a chemical is bioavailable or not. Direct biological techniques measure the actual amount of a pollutant taken up by a target organism, thus making them the most accurate measure of bioavailability. Indirect biological techniques provide a quantifiable effect of a pollutant, such as lethality, enzyme induction or inhibition, and reproductive effects, but the concentration remains unknown. A novel approach relies in the use of genetically modified microorganisms to detect and quantify specific pollutants (Brandt et al., 2002; Philp et al., 2004). This type of biosensor is based on the highly specific genetic control mechanisms used by microorganisms to ensure that specific proteins are expressed only when they are needed, for example, for the detoxification of a particular toxic substance. Indirect chemical methods usually involve the extraction of a fraction of the pollutant (metals or organics) from a soil, the extractability being equated to bioavailability and defined by the chemical itself, the nature of the extractant(s), and the experimental conditions applied.

4. Assayed pollutants

U.S. EPA developed a list of the common industrial pollutants that pose an imminent threat to public health and the environment (Table 2). The list contains a large number of organic compounds and several inorganic compounds. The major criterion used in selecting priority pollutants are human toxicity, presence of significant concentrations on land affected by post or current industrial use, toxicity in plants and animals, potential for bioaccumulation and biomagnification, mobility in the environment and persistence. Among a list of 275 substances, the heavy metals considered in this study (Cr, Cd and Pb) are in the first 20 positions of the ranking, while pentachlorophenol (PCP), 2,4,6-trichlorophenol (TCP) and 2-chlorophenol (MCP) are in the position 45, 86 and 245, respectively.

Table 2. The priority list of hazardous substances, sorted by rank of toxicity, detailed for the first 50 pollutants (http://www.atsdr.cdc.gov/cercla/07list.html).

2007 Rank	Substance name	Points	2005 Rank	2007 Rank	Substance name	Points	2005 Rank
1	ARSENIC	1672.58	1	26	BENZIDINE	1114.24	26
2	LEAD	1534.07	2	27	AROCLOR 1248	1112.20	27
3	MERCURY	1504.69	3	28	CYANIDE	1099.48	28
4	VINYL CHLORIDE	1387.75	4	29	AROCLOR 1242	1093.14	29
5	POLYCHLORINATED BIPHENYLS	1365.78	5	30	AROCLOR	1091.52	62
6	BENZENE	1355.96	6	31	TOXAPHENE	1086.65	30
7	CADMIUM	1324.22	8	32	HEXACHLOROCYCLOHEXANE, GAMMA-	1081.63	32
8	POLYCYCLIC AROMATIC HYDROCARBONS	1316.98	7	33	TETRACHLOROETHYLENE	1080.43	31
9	BENZO(A)PYRENE	1312.45	9	34	HEPTACHLOR	1072.67	33
10	BENZO(B)FLUORANTHENE	1266.55	10	35	1,2-DIBROMOETHANE	1064.06	34
11	CHLOROFORM	1223.03	11	36	HEXACHLOROCYCLOHEXANE, BETA-	1060.22	37
12	DDT, P,P'-	1193.36	12	37	ACROLEIN	1059.07	36
13	AROCLOR 1254	1182.63	13	38	DISULFOTON	1058.85	35
14	AROCLOR 1260	1177.77	14	39	BENZO(A)ANTHRACENE	1057.96	38
15	DIBENZO(A,H)ANTHRACENE	1165.88	15	40	3,3'-DICHLOROBENZIDINE	1051.61	39
16	TRICHLOROETHYLENE	1154.73	16	41	ENDRIN	1048.57	41
17	DIELDRIN	1150.91	17	42	BERYLLIUM	1046.12	40
18	CHROMIUM, HEXAVALENT	1149.98	18	43	HEXACHLOROCYCLOHEXANE, DELTA-	1038.27	42
19	PHOSPHORUS, WHITE	1144.77	19	44	1,2-DIBROMO-3-CHLOROPROPANE	1035.55	43
20	CHLORDANE	1133.21	21	45	PENTACHLOROPHENOL	1028.01	45
21	DDE, P,P'-	1132.49	20	46	HEPTACHLOR EPOXIDE	1027.12	44
22	HEXACHLOROBUTADIENE	1129.63	22	47	CARBON TETRACHLORIDE	1023.32	46
23	COAL TAR CREOSOTE	1124.32	23	48	AROCLOR 1221	1018.41	47
24	ALDRIN	1117.22	25	49	COBALT	1015.57	50
25	DDD, P,P'-	1114.83	24	50	DDT, O,P'-	1014.71	49

4.1. Haloaromatic compounds: chlorophenols

Halogenated aromatic compounds are widely distributed in the environment as a result of their widespread use as herbicides, insecticides, fungicides, solvents, fire retardants, pharmaceuticals and lubricants. Several of these chemicals cause considerable environmental pollution and human health problems due to their persistence and toxicity. Chlorinated compounds are used more frequently than fluorinated or brominated compounds. Phenol was the first antiseptic, but chlorophenols (CP) possess far higher antimicrobial activity and acidity than phenol. CP were subsequently used as biocidal agents. PCP is very recalcitrant and persistent in the environment and it has limited aqueous solubility, except in alkali solution, where it forms a soluble salt. It has long been used as a wood preservative and is a biocide of impressive effectiveness, being fungicidal, bactericidal, algicidal, insecticidal, molluscicidal and herbicidal (Paulus, 1993). Other less substituted CP have been used in a variety of industries as intermediates in pesticide synthesis, dyestuffs and pharmaceutical products. These biocides compounds represent a hazard for soil microbial communities, and may influence the turnover of nutrients and soil fertility (Chaudri et al., 2000).

The fate of CP is especially difficult to predict because of their physicochemical properties (Table 3) that make their behaviour quite dependent on the environmental characteristics. Solubility, volatility and hydrophilicity of CP decrease with increasing the degree of chlorination, while their inherent toxicity increases (Van Agteren et al., 1998). CP may form ionized species (Figure 3), depending on

the pH (different pKa values), acquiring different physicochemical properties and leading to diverse behaviours (Van Agteren et al., 1998). Their pKa values are within the range of naturally occurring soil pHs, leading to a diversity of CP ionization states in such environments (Shimizu et al., 1992). Depending on the soil pH, each compound may ionize, rendering it more polar and water-soluble and less volatile, or it may remain in the non-ionized form. These non-ionized forms differ in their vapor pressure, solubility, polarity, Kow, etc. Therefore, the ionization state of the molecule in a particular soil will establish its tendency to move, sorb or transform in the soil. This tendency will influence the bioavailability of the compound and the corresponding effects on the biota and environment when the pollutant reaches the soil (Van Beelen, 2000).

Table 3. Main physicochemical properties of MCP, TCP and PCP (Van Agteren et al., 1998).

	MCP	**TCP**	**PCP**
pKa	8.6	7.0	4.7
Water solubility 20ºC (mg L^{-1})	28,000	800-900	14
Log Kow	2.16	3.4-3.8	5.0
Vapor pressure 25ºC (mm Hg)	2.4	0.06	0.00011
Henry constant, K (atm $\cdot m^3 mol^{-1}$)	$1.43x10^{-5}$	$9.2x10^{-6}$	$2.45x10^{-8}$

The persistence of CP in soil has been attributed to the absence of degrading microorganisms, but an increasing number of observations indicate that transformation potential is wide-spread in favorable environmental conditions (Stanlake and Finn, 1982; D'Angelo and Reddy, 2000; Nordin et al., 2005), including both anaerobic and aerobic conditions (McAllister et al., 1996; Field and Sierra-Alvarez, 2008). Several soil microorganisms have the potential to degrade halogenated compounds similar to CP, since some of them are naturally occurring in the soil (Gribble, 1994). Biodegradation of CP has been even reported in soils at high concentrations and without previous contact with the pollutants (McGrath and Singleton, 2000; Sanchez et al., 2004; Mahmood et al., 2005). Several bacterial genera are already known to have CP-degrading capacity, such as *Pseudomonas* (Shah and Thakur, 2002; Evans et al., 2004), *Sphingomonas* (Kharoune et al., 2002; Yang et al., 2006), *Flavobacterium* (Saber and Crawford, 1985; Steiert et al., 1987), *Arthrobacter* (Stanlake and Finn, 1982; Nordin et al., 2005) and *Burkholderia* (Kharoune et al., 2002; Webb et al., 2010). Fungal species with this capacity are *Trametes versicolor* (Tuomela et al., 1998), *Lentinula edodes* (Okeke et al., 1997), *Anthracophyllum discolour* (Rubilar et al., 2007) and other *Agaricomycetes*.

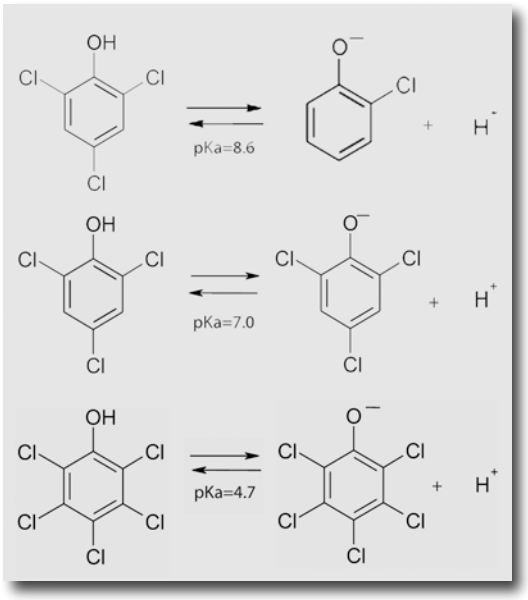


Figure 3. Molecular forms of MCP, TCP and PCP

Dechlorination is the first critical step in the biodegradation of CP, and two different mechanisms can be distinguished. In anaerobic conditions, biodegradation of CP is performed by a reductive dehalogenation mechanism. CP are used as terminal electron acceptors in anaerobic respiration. Few microorganisms use this strategy, which is well known in the gram-positive *Desulfitobacterium* and also in the gram-negative sulfate-reducing bacteria *Desulfomonile tiedjei* (Mohn and Kennedy, 1992). In aerobic conditions, CP are generally transformed by oxidative dehalogenation mechanism. Many aerobic bacteria can degrade CP by using oxigenases that initiate the attack on the aromatic ring (Tomasi et al., 1995); mono- and dichlorophenols are exclusively metabolized through the chlorocatechols pathway, while highly chlorinated CP are metabolized through the hydroquinones pathway. White-rot fungi used the enzymes of the ligninolytic complex to degrade many phenolic compounds (Bollag et al., 1988) such as CP (Anke and Weber, 2006).

4.2. Heavy metals: Cr, Cd and Pb

Pollution caused by heavy metals is a worldwide phenomenon. Among the many heavy metals, lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), zinc (Zn), and copper (Cu) are of most concern, although the last three metals are essential trace elements for animal and human nutrition. These metals are widely used in industry, particularly in metal-working or metalplating, and in products such as batteries and electronics. They are also used in the production of jewellery, paint pigments, pottery glazes, inks, dyes, rubber, plastics, pesticides and even in medicines. Therefore, huge amounts of metals are released to air, water and soil

ecosystems. Heavy metals are highly toxic because, as ions or compound forms, they are usually soluble in water and may be readily absorbed into living organisms. After absorption, these metals can bind to vital cellular components such as structural proteins, enzymes and nucleic acids, and interfere with their functioning. In humans, some of these metals can cause severe physiological and health effects, even in small amounts.

Heavy metals may form cations or anions in soils, and some of them in different oxidation states. Cd and Pb can only exist as divalent cations. However, Cr can have several oxidation states, but in soils the most stable and common forms are trivalent Cr(III) and hexavalent Cr(VI) (Fendorf, 1995), which have different chemical properties and toxicity. Hexavalent chromium is water-soluble, highly toxic and mutagenic to most organisms and carcinogenic for humans, while trivalent chromium is essential (in low concentrations) for human and animal nutrition, relatively water-insoluble and less toxic than Cr(VI) (Francisco et al., 2002). At acidic pH values, the chemical reduction of Cr(VI) favoured the predominance of Cr(III), thus decreasing its bioavailability and mobility (Richard and Bourg, 1991). The microbial reduction of Cr(VI) to Cr(III) may also occur directly through enzymatic activity (Losi et al., 1994; Lovley and Coates, 1997) or indirectly through producing a compound that can reduce Cr(VI) (Lovley, 1993; Fendorf and Li, 1996). The mechanisms through which bacterial strains reduce Cr(VI) to Cr(III) are variable and species dependent (McLean et al., 2000). This ability has been found in several genera including *Pseudomonas*, *Micrococcus*, *Bacillus*, *Achromobacter*, *Microbacterium, Arthrobacter* and *Corynebacterium* (McLean et al., 2000; McLean and Beveridge, 2001; Pattanapipitpaisal et al., 2001; Megharaj et al., 2003; Viti et al., 2003).

In general, heavy metals may be incorporated into silicates or oxides, complexed to the soil organic matter, or adsorbed to exchange sites of permanent or variable charge. Soils polluted with heavy metals are frequently assessed according to the total content. However, because of the variety of binding and transport mechanisms that are specific to each individual element, and because of the many soil properties influencing those mechanisms, mobile fractions have to be considered to obtain a realistic view of the actual contamination of the soil (Lazzaro et al., 2006), the risk of groundwater pollution (Bruemmer et al., 1986) and the inhibition of the soil microbiota (Angle et al., 1993). Differences in metal bioavailability can greatly have effects on toxicity and stress microbial populations (Roane et al., 2001), thus leading to a huge diversity of situationsa, according to the metals and soil types involved.

It is well established that metals persist indefinitely in soils, where they may interfere in the soil properties (mainly related to microbiota) and potential uses. However, microbial populations thriving in a polluted environment usually develop or acquire resistance capabilities, which allow them to survive and keep soil functions. These mechanisms include the formation and sequestration of heavy metals in complexes, the production of exopolysaccharides and the chemical reduction to less toxic species and direct efflux of metal out of the cell (Hernandez et al., 1998; Rasmussen and Sørensen, 1998; Sundar et al., 2011). Bioremediation strategies can take advantage of these processes to improve environmental health. In this sense, microbial-based metal remediation techniques usually rely on the ability of some microorganisms to resist and detoxify metals (Roane and Pepper, 1999).

5. Bioremediation and soil restoration

The major reasons for the control of environmental pollution and the consideration of restoration of contaminated sites are, first and foremost, public health concerns; second, environmental conservation; and finally, the cost of decontamination. Water and soil are essential to agriculture and industry, which has to be kept free of pollutants. Maintenance of safe potable water supplies is a major health concern around the world, and for most countries is a finite resource. Contaminated soil can also become a contaminated water problem if the toxic compounds migrate to groundwater. Therefore, there is a critical need for cost-effective technologies to help to remove environmental pollutants.

There are many treatments for cleaning up contaminated soils which can include flushing contaminants out of the soil by using water or chemical solvents, or air; destroying the contaminants by incineration; encouraging natural organisms in the soil to break them down; or adding material to the soil to encapsulate the pollutants and prevent them from spreading. Depending on the nature of the problem, it may be necessary to excavate the contaminated soil and move it to a site for its safe disposal and treatment (*ex situ* approach), or it can also be treated in the same place (*in situ* approach). Bioremediation is a cost-effective strategy for detoxifying contaminated environments that can be applied in both types of approaches, which relies upon the activity of microorganisms with capacity for biodegradation or biotransformation of the toxic compounds (Häggblom and Valo, 1995). Microbiology may not only help the environmental toxicologist to understand the fate and transformation of environmental pollutants but also may provide the efficient tools to clean up and restore an ecosystem.

Biostimulation in context of bioremediation aims at enhancing the activities of indigenous microorganisms, which are able to metabolize the offending contaminant, by the addition of nutrients, oxygen, water, etc. For bioaugmentation, the inoculation of specific strains or consortia of microorganisms in the contaminated site is considered to improve the degrading or transforming capacity of the system for a specific pollutant. Therefore, biostimulation leads to an increase of catabolic potential, while bioaugmentation does by increasing genetic diversity. Biostimulation is a much simpler and less costly approach that is recommended for the transformation of naturally occurring pollutants such as petroleum mixtures. In contrast, it must be noted that bioaugmentation may be required for the biodegradation of more recalcitrant and new synthesized xenobiotic pollutants. The use of microorganisms for bioaugmentation requires culturable specific strains that must be able to perform high degradation or transformation activities of pollutants in particular contaminated sites (Mera and Iwasaki, 2007). However, these easily culturable microorganisms may have a low adaptability to soils with different characteristics than the environments from where they were isolated (Pu and Cutright, 2007). Original acclimated microbial populations may be more appropriate for bioremediation, since they are naturally resistant to changes in the environment and predation (Barbeau et al., 1997; Beaulieu et al., 2000). It is thus strongly interesting to achieve a wide array of isolated microorganisms, obtained from environments with dissimilar features, which can be used in bioremediation purposes in different situations.

Extensive laboratory studies have shown the capability of various organisms to remediate contaminated soil and water. More research, however, needs to be achieved to determine the applicability and practicability of utilizing these microorganisms in contaminated field sites.

6. A framework of the project and procedures

This thesis is part of a broader research project focused to assess the effects of several organic and inorganic pollutants (detailed in section 3.1 and 3.2, respectively) in relation to two Mediterranean soils. The soils have been chosen considering their different properties. One of them, which has been classified as Haplic Arenosol (HAr), corresponds to the properties of the standard soil proposed by the OECD for ecotoxicity testing in terrestrial environments.

The influence of pollutants in the soil microcosms has been analyzed by monitoring the respirometric activity (cumulative respiration and substrate induced respiration), as an estimation of the heterotrophic microbial activity. Changes in the composition of the soil microbial community have been assessed by PCR-DGGE analysis of 16S rRNA gene fragments amplified from extracted nucleic acids. The toxicity assessment includes the performance of other tests conducted from the soil extracts, such as the algal growth inhibition and terrestrial plant seedling emergence and growth inhibition. The purpose of these analyses is to obtain the dose-response relationships of each pollutant and predict its potential impact on environments with similar properties. The fate of pollutants after their addition to the soil microcosms is also considered for the discussion of the results.

Culture-plating methods have also been used to isolate microorganisms from contaminated microcosms, which have been finally identified as a complementary approach to PCR-DGGE analysis for the detection of resistant microbiota. Representative isolated strains have been characterized in relation to their resistance and degradation/transformation capacity of the pollutants, in order to infer their potential usefulness for bioremediation applications.

The results derived from all these investigations have been discussed from different perspectives and some of them have already been published in scientific reviews. The toxicity assessment of each group of pollutants (organic and inorganic), according to the soil type and considering all toxicity tests, has been discussed in order to establish the dose-response relationships depending on the soil properties (Martí et al., 2011; Martí et al., submitted).

The studies reported in this thesis aim to assess the response of the indigenous soil microbiota in contact with the pollutants, as well as the identification and characterization of resistant microorganisms that could be useful for bioremediation.

7. General objectives of the study

Assessment of the capacity of the native microbiota of natural Mediterranean soils that have not been previously in contact with organic (chlorophenols) and inorganic (Cr, Cd and Pb) pollutants to cope with potential contamination events by these toxic compounds.

Evaluation of the usefulness of both respirometric and molecular (PCR-DGGE) techniques to assess the impact of the pollutants in soil microcosms.

Detection, isolation, identification and characterization (in relation to pollutants resistance and degradation/transformation capabilities) of soil microorganisms that can be potentially useful for bioremediation processes in these environments.

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Chapter I

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The microbiota of an unpolluted calcareous soil faces up chlorophenols: Evidences of resistant strains with potential for bioremediation

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ABSTRACT

To highlight the effects of a variety of chlorophenols (CP) in relation to the response of an indigenous bacterial community, an agricultural Mediterranean calcareous soil has been studied in microcosms incubated under controlled laboratory conditions. Soil samples were artificially polluted with 2-monochlorophenol (MCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP), at concentrations ranging from 0.1 up to 5000 mg kg^{-1}. Both activity and composition of the microbial community were assessed during several weeks, respectively, by respirometric methods and PCR-DGGE analysis of extracted DNA and RNA. Significant decreases in soil respirometric values and changes in the bacterial community composition were observed at concentrations above 1000 mg kg^{-1} MCP and TCP, and above 100 mg kg^{-1} PCP. However, the persistence of several active bacterial populations in soil microcosms contaminated with high concentration of CP, as indicated by DGGE fingerprints, suggested the capacity of these native bacteria to survive in the presence of the pollutants, even without a previous adaptation or contact with them.

The isolation of potential CP degraders was attempted by culture plating from microcosms incubated with high CP concentrations. Twenty-three different isolates were screened for their resistance to TCP and PCP. The most resistant isolates were identified as *Kocuria palustris, Lysobacter gummosus*, *Bacillus* sp. and *Pseudomonas putida*, according to 16S rRNA gene homology. In addition, these four isolates also showed the capacity to reduce the concentration of TCP and PCP from 15% to 30% after 5 d of incubation in laboratory assays (initial pollutant concentration of 50 mg L^{-1}). Isolate ITP29, which could be a novel species of *Bacillus*, has been revealed as the first known member in this bacterial group with potential for CP bioremediation applications, usually wide-spread in the soil natural communities, which has not been reported to date as a CP degrader.

1. Introduction

Pollution by a wide array of chemical compounds with different features and properties is one of the most relevant environmental damages to ecosystems produced by human activity. Therefore, environmental protection and restoration must take account of the ecosystems health status respect to main pollutants, such as chlorophenols (CP), and develop remediation strategies to improve them. Likewise, environmental research should contribute to improve their knowledge and develop the techniques necessary for cleaning polluted ecosystems, which are mostly based on the activity of microorganisms with capacity for biodegradation or biotransformation of pollutants.

CP are widely used either as synthesis intermediates in polymers, dyes and pesticides or as biocides themselves. These compounds are included in the priority pollutants lists, for example those of USEPA (http://www.atsdr.cdc.gov/cercla/07list.html), which have recommended restricted use to minimize their further accumulation and toxicity effects in the ecosystems. Solubility, volatility and hydrophilicity of CP decrease with increasing the degree of chlorination, while their inherent toxicity increases (Van Agteren et al., 1998). CP may also form ionized species, depending on the soil pH (different p*K*a values), acquiring different physicochemical properties and leading to diverse behaviors in soils. This makes the prediction of their fate in soils difficult, especially considering the wide range of physicochemical properties of contaminated soils that may be involved in the behavior of the added pollutants. Since several chlorophenolic compounds are commonly used, their presence in the environment is concerning and widespread. The response of soil microbiota to the presence of these pollutants is

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considered to be related to the microbial populations present in the soil, the concentration of the pollutants and also to the properties of the soil itself (Pu and Cutright, 2007). All these features can modify not only the pollutant bioavailability but also the stimulation capacity of the microbiota (Cho et al., 2000). Therefore, the impact of CP on soil ecosystems is not only dependent on the specific compound and concentration, but also on the type and physiological status of the polluted soil. In this sense, the influence of these pollutants in Mediterranean soils, commonly located in dry climate areas and with calcareous origin, is not well known.

The persistence of CP in soil has been attributed to the absence of degrading microorganisms, but an increasing number of observations indicate that transformation potential is wide-spread in favorable environmental conditions (D'Angelo and Reddy, 2000), including both anaerobic and aerobic conditions (McAllister et al., 1996; Field and Sierra-Alvarez, 2008). Several soil microorganisms have the potential to degrade halogenated compounds similar to chlorophenols, since some of them are naturally occurring in the soil (Gribble, 1994). CP biodegradation has been even reported in soils at high concentrations and with no previous contact with the pollutants (McGrath and Singleton, 2000; Sanchez et al., 2004; Mahmood et al., 2005). However, a strong inhibition in microbial activity has also been found when CP compounds are present in soil, even at low concentration (Chaudri et al., 2000).

The isolation of potential CP degraders from soil can be a source of biotechnologically active organisms to be used in the detoxification of contaminated environments by transforming or degrading pollutants in bioremediation processes (Otte et al., 1994; Dechesne et al., 2010). Nevertheless, it is commonly accepted that isolated bacteria have a low adaptability in soils of different characteristics from those where they were isolated. On the contrary, original acclimated microbial populations may be more appropriate for pollutant biodegradation, since they are naturally resistant to changes in the environment and predation (Barbeau et al., 1997; Beaulieu et al., 2000). It is thus strongly interesting to achieve a wide array of isolated microorganisms, obtained from environments with dissimilar features, which can be used in bioremediation purposes in different situations. For the isolation of resistant and degrading microbial strains, culture plating has been successfully applied to obtain pollutant biodegraders, including CP and other organic compounds, from different environments (Saber and Crawford, 1985; Mitsevich et al., 2000; Yang et al., 2006; Hilyard et al., 2008). Most of the CP degraders that have been isolated to date belong to genus *Pseudomonas* (Kiyohara et al., 1992; Männistö et al., 1999; Shah and Thakur, 2002; Evans et al., 2004), *Sphingomonas* (Männistö et al., 1999; Beaulieu et al., 2000; Kharoune et al., 2002; Yang et al., 2006), *Flavobacterium* (Saber and Crawford, 1985; Steiert et al., 1987; Männistö et al., 1999) and *Arthrobacter* (Stanlake and Finn, 1982; Nordin et al., 2005). Although members of *Firmicutes* are commonly present in soil, they have not been described as chlorophenol degraders (Field and Sierra-Alvarez, 2008). However, the particular ecological features of members of this group confer them an especial interest to be used properly in bioremediation processes.

The central aim of this research was to assess the involvement of an indigenous bacterial community in a typical calcareous Mediterranean soil by artificial CP pollution and isolate native strains that could be useful for bioremediation processes. Pollution was produced through the addition of 2-chlorophenol (MCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) to soil microcosms without previous contact with this kind of compounds. The influence of pollutant amendments was analyzed by monitoring the Cumulative Respiration, as an estimation of the soil physiological status, and assessing the composition of the microbial community during the incubations by PCR-DGGE analysis of 16S rRNA gene fragments amplified from extracted DNA and RNA. Culture-plating methods have been used to isolate bacteria able to resist CP from contaminated microcosms, which have been finally identified and characterized in relation to TCP and PCP resistance and degradation capacity, in order to infer their potential usefulness for bioremediation applications.

2. Material and methods

2.1. Soil sampling and microcosm incubations

Experiments were performed with an agricultural soil without any prior history of exposure to the organic pollutants assayed. The soil was collected in July 2007 from the superficial layer (A horizon) of a site located in the Mediterranean area of Santa Bàrbara, Tarragona (UTM: 31 T 286500E 4509303N). After sampling, the soil was sieved (<2 mm) and stored by air-drying conservation method until the forthcoming experiments. This soil was classified as calcaric regosol (FAO-UNESCO, 1998) developed on sedimentary materials. It was a fine-textured and alkaline (pH 8.2) soil, which contained carbonates (24%) and 1.66% organic carbon. This soil type is very common in the Mediterranean area and it has adequate C/N ratio (9.44) to perform respirometric assays.

The contaminants selected for the experiment were 2-monochlorophenol (MCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) (Sigma–Aldrich GmbH, Steinheim, Germany). The contamination of the soil samples was performed with aqueous solutions of MCP (highly soluble) or with sterilized washed sand, previously mixed with TCP or PCP (OECD, 2000) to avoid solvent-derived effects on the microcosms if the pollutants were added in organic solutions. Thus, PCP was added to fine siliceous sand (100 μm particle size) as a methanolic solution and after evaporation of the methanol aliquots of the contaminated sand, 2 g of sand per 50 g of soil were added.. In the case of TCP, addition of the pollutant to sand was performed by solid–solid dilution to avoid losses caused by evaporation in the methanol elimination process, due to the higher volatility of TCP. Aliquots of TCP-contaminated sand were added to soil as described for PCP-contaminated sand. Clean sand in the same proportion was also added to soil samples to be used as controls.

After a pre-incubation at the field soil humidity (8.7% dry weight), 50 g of soil sample was treated separately with 0.1, 1, 10, 100, 1000 and 5000 mg kg^{-1} MCP, TCP or PCP, and without pollutant (control). In order to perform respirometric analysis, soil samples were placed in closed reactors (500 mL) with a water content equivalent to the 60% of the soil water holding capacity (WHC) and incubated in the dark at 25 °C during 28 d. The water added to reach the WHC was deionised and sterilized previously. Control and polluted-soil microcosms destined to molecular analysis (PCR-DGGE) were incubated likewise, parallel to the incubations designed to respirometric measurements, but harvested and sampled destructively after 7, 14 and 28 d. Non-incubated soil samples without pollutant were used as initial controls. After each harvest, soil samples were stored at −80 °C until extraction of nucleic acids. All incubations performed for respirometric and molecular analysis were done in triplicate.

2.2. Respirometric analysis of microbial activity

The heterotrophic aerobic microbial activity was analyzed by respirometry. Microcosms processed with this purpose were incubated in manometric respirometers as described above, which allow the determination of sample oxygen consumption (Oxitop®, WTW). The cumulative oxygen consumption (Cumulative Respiration, CR) was periodically registered throughout the incubation period. To determine the statistical significance of the differences between

treated samples and controls, an ANOVA followed by Duncan's *post hoc* test ($p < 0.05$) (SPSS 16) was done.

2.3. Nucleic acid extractions

Nucleic acids were extracted from 0.5 g of soil samples using the FastDNA® SPIN Kit for Soil and the FastRNA® Pro Soil-Direct Kit (Bio101, Carlsbad, USA), for DNA and RNA respectively. Previously, the optimal amount of soil sample was experimentally determined from the relationship between the weight of processed soil samples and the concentration of extracted nucleic acids (data not shown) to accomplish a high nucleic acid yield while avoiding saturation of the extraction procedure. Prior to extraction itself, soil samples were suspended in the buffers to be used in the first step of the respective extraction protocol and homogenized with a hand-held blender (DIAX 900, Homogenizer tool G6, Heidolph, Kelheim, Germany) for 5 min. Soil-slurries were shaken three times, to increase nucleic acid extraction recovery, for 30 s at 5.5 m s^{-1} in a FastPrep-24 instrument (MP Biomedicals, Inc.). Samples were left on ice for 5 min between each round of shaking. Nucleic acids extraction was performed following the manufacturer procedures. The integrity of nucleic acids was checked by agarose gel electrophoresis and the amount and purity of DNA and RNA were determined using a NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDrop, Technologies, Inc., Wilmington, DE). DNA and RNA extracts were stored at −80 °C until processed.

2.4. PCR amplification

All chemicals and *Taq* polymerase used for the PCR-amplification of nucleic acids were provided by Applied Biosystems (Foster City, CA). Partial 16S rRNA gene fragments covering the variable V3 to V5 regions were obtained by PCR amplification of DNA templates in a 9700 GeneAmp thermal cycler (Applied Biosystems, Perkin-Elmer, CA) using the *Bacteria* universal primers 357F (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Muyzer et al., 1993; Muyzer et al., 1995). A 44-bp GC-rich clamp sequence was added to the 5′ end of primer 357F to allow further separation of PCR products by DGGE (Muyzer and Smalla, 1998). Each PCR mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphate (dNTP), 0.5 μM of each primer and 1.25 U of Ampli*Taq* polymerase. DNA (~20 ng) was added as template for the PCR reactions in a final volume of 50 μL ddH_2O. The thermocycling conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, and a final elongation at 72 °C for 10 min. PCR amplification products were checked by agarose gel electrophoresis. PCR products with the correct size and similar yields (~100 ng μL^{-1}) were used for DGGE analysis.

Reverse transcription of extracted RNA to complementary DNA (cDNA) was performed with the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) following the manufacturer-recommended procedures. cDNA synthesis was carried out in 20 μL reaction volumes with the reverse *Bacteria* universal primer (907R, 1 μM) and RNA template (~100 ng). RNA extracts were processed to remove eventual genomic DNA contamination following the protocol indicated in the RT-kit manual. Control reactions were run to check for DNA contamination of RNA extracts. PCR amplification was done, as described above, using 2–4 μL of cDNA as template.

2.5. DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) was performed using an INGENY PhorU system (Ingeny International BV, Goes, The Netherlands). PCR products were separated in 6% (w/v) polyacrylamide gels prepared with a 35–75% urea-formamide vertical gradient, according to the instructions of the manufacturer [100% denaturant agent contains 7 M urea and 40% deionised formamide (McCaig et al., 2001)]. Electrophoresis was performed for 12 h with 1× TAE buffer at 60 °C, at a constant voltage of 160 V. Gels were stained for 30 min with SybrGold (1:10,000; Invitrogen Molecular Probes, Eugene, OR) and visualized under UV excitation. Bands of interest were chosen after detailed analysis of fingerprint images and excised from gels. DNA from excised DGGE bands was rehydrated in 50 μL of sterile ddH_2O, eluted after incubation at 65 °C for 30 min and reamplified using 2 μL of the eluate with the PCR conditions and the corresponding primers (without GC clamp) previously described. PCR products were stored at −80 °C until sequencing.

Digital images were analyzed by using the GELCompar II v.6.1 software package (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Comparison between samples loaded on different DGGE gels was completed using normalized values derived from control samples common in all of them. Calculation of the pair-wise similarities of densitometric profiles was based on Pearson's correlation coefficients with an optimization of 2%. Cluster analysis based on this similarity matrix was done by UPGMA (unweighted pair-group method with arithmetic averages).

2.6. Isolation and DNA extraction of chlorophenol resistant bacteria

Soil samples contaminated with 1000 mg kg^{-1} MCP, 1000 mg kg^{-1} TCP, 100 and 1000 mg kg^{-1} PCP respectively, were chosen for the isolation of resistant bacteria, after 28 d of incubation at the same conditions described above for respirometric and molecular analysis. Samples consisting of 1 g of freshly soil from each microcosm were suspended in 100 mL of sterile Ringer's solution (Scharlab, Barcelona, Spain) in 250 mL conical flasks and dispersed by stirring for 20 min at 200 rpm The resultant suspensions were serially diluted and plated in triplicate on solid 10-fold diluted Luria–Bertani medium (Sigma–Aldrich GmbH, Steinheim, Germany) supplemented with MCP, TCP or PCP to final concentrations of 50, 100, 500 and 1000 mg L^{-1}. Plates were incubated up to a week at 25 °C in the dark. Between 10 and 15 colonies were selected from each soil microcosm according to their different morphology. Pure cultures were obtained after repetitive inoculation in fresh medium and saved for further identification and characterization.

Nucleic acids were extracted from colonies picked up directly from agar plates using Wizard™ Genomic DNA Isolation Kit (Promega, Madison, WI, USA). The isolation of DNA was done following the manufacturer's indications. The amount of DNA was quantified using a NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDrop, Technologies, Inc., Wilmington, DE) and stored at −80 °C until processed. Partial 16S rRNA gene fragments were amplified by PCR using the *Bacteria* universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGTTACCTTGTTACGACTT-3′) (Lane, 1991). The PCR mixture was prepared as previously described. The thermocycling conditions were: 94 °C for 5 min, followed by 10 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 2 min, 25 cycles with annealing temperature step down to 50 °C, and a final elongation at 72 °C for 10 min. PCR amplification products were checked by agarose gel electrophoresis. Products of the correct size were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and stored at −80 °C until sequencing.

2.7. DNA sequencing and data analysis

The PCR-amplified DNA products, obtained from the DGGE bands and the bacterial isolates, were sequenced by Macrogen

Inc. (Seoul, South Korea). Primers 357F and 907R were used in order to sequence the PCR-products from the DGGE bands. Primers 27F and 1492R were used to sequence the 16S rRNA gene of the isolates, in combination with the universal primer Eb787F (Baker et al., 2003) as reverse and forward, respectively.

All retrieved 16S rRNA gene sequences were checked for chimera detection using the Bellerophon tool (Huber et al., 2004). Sequences were properly aligned using the online automated aligner SINA (SILVA Incremental Aligner) available at SILVA website (http://www.arb-silva.de/; Pruesse et al., 2007). Alignments were imported into the ARB software package (http://www.arb-home.de/; Ludwig et al., 2004) and loaded with the SILVA 16SrRNA-ARB-compatible database (SSURef-102, February 2010). Phylogenetic trees were constructed by maximum likelihood (RAxML) analyses using reference sequences and sequences of the isolates longer than 1200 bp. Subsequently, the shorter sequences obtained from DGGE bands were added by applying the parsimony tool implemented in ARB, thereby maintaining the overall tree topology. Closest relative bacteria were identified based on the phylogenetic tree affiliations, and sequence similarities were calculated using the ARB distance matrix tool. Partial and almost complete sequences of the 16S rRNA gene derived either from DGGE bands or bacterial isolates were deposited in GenBank.

2.8. Characterization of resistant bacteria and potential biodegraders

Isolates were incubated at 25 °C during one week in the medium described by (Francisco et al., 2010), in order to test for their resistance level to TCP and PCP. The medium was slightly modified from the original and contained 100 mL L^{-1} of salt solution (in g L^{-1}: $CaCl_2{\cdot}2H_2O$ 0.79, NaCl 0.08, KNO_3 1.03, $NaNO_3$ 6.98, $MgSO_4{\cdot}7H_2O$ 1.00, nitriloacetic acid 1.00), 10 mL L^{-1} of trace elements (in mg L^{-1}: $FeSO_4{\cdot}7H_2O$ 200.0, $ZnSO_4{\cdot}7H_2O$ 10.0, $MnSO_4{\cdot}H_2O$ 3.0, H_3BO_3 30.0, $CoSO_4{\cdot}7H_2O$ 24.0, $CuSO_4{\cdot}5H_2O$ 1.0, $NiCl_2{\cdot}6H_2O$ 1.7, $NaMoO_4{\cdot}2H_2O$ 3.0, EDTA 500.0), yeast nitrogen base (YNB) 1%, HEPES 25 mM and 0.5% glucose as carbon source. The pH was adjusted to 8 using NaOH. To prepare solid plates, 15 g L^{-1} of agar was added to the medium. After autoclaving, chlorophenols were added at concentrations of 10, 50, 100 and 200 mg L^{-1} TCP or PCP. Resistance of the isolates was checked by qualitative observation of the colony development in polluted plates in comparison to the controls without pollutant.

The most resistant isolates were checked for their capacity to biodegrade TCP and PCP. Isolates were previously grown in the medium described above in order to obtain the inocula for the biodegradation incubations (initial concentration about 4×10^7 cells mL^{-1}). Experiments were conducted in 120 mL serum bottles capped with Teflon-lined screw taps. Degradation of chlorophenols was tested in 25 mL of the same medium, but supplemented with 0.05% glucose and TCP or PCP to a final concentration of 50 mg L^{-1}. Controls without inocula were set up likewise. Incubations were done on an orbital shaker (150 rpm) at 25 °C for 5 d. All experiments were done in triplicate. Growth was measured at the end of the incubations by registering optical density (OD) at 600 nm. Finally, cultures were fixed by mixing with methanol (1:1) and filtered (0.2 μm nylon membrane filters GNWP, Millipore Iberica S.A.U, Spain), prior to HPLC analysis.

HPLC analyses were performed to determine CP concentrations using a Perkin-Elmer series 200 with autosampler. A Phenomenex Luna C_{18} analytical column (150 × 2.1 mm i.d., 5 μm) with a binary mobile phase, A (0.1% acetic acid in water) and B (acetonitrile), following a linear gradient. The elution gradient was carried out with the following proportions (v/v) of the phase B (t (min), %B): (0, 10), (5, 75), (5.5, 100), (8, 100), (8.5, 10), (15, 10). Detection was performed with a mass selective spectrometer API 150EX (PE Sciex, Concord, Ontario, Canada) equipped with a turbo ion spray ionizing in negative mode.

3. Results

3.1. Respirometric assessment

No inhibitory effects were detected at 0.1, 1 and 10 mg kg^{-1} CPs assayed. On the contrary, a significant increase in the respiration rate was detected at 100 mg kg^{-1} MCP and TCP compared to controls (Fig. 1). The lowest concentration of the pollutants, producing a significant reduction of the oxygen consumption, corresponded to 1000 mg kg^{-1} for MCP and TCP and to 100 mg kg^{-1} for PCP. At higher concentrations, all the pollutants showed strong inhibitory effects (>85% inhibition respect to the soil control respiration). These results were used as preliminary tests to select treatments for a throughout analysis of the changes in the microbial community composition by PCR-DGGE. Chosen samples were those amended with 100, 1000 and 5000 mg kg^{-1} for MCP, 100 and 1000 mg kg^{-1} for TCP, and 10, 100 and 1000 mg kg^{-1} for PCP. The treatments with the highest concentration that showed no inhibitory effect on respirometry tests and those with concentrations that resulted in an inhibitory effect were included within this set of samples.

3.2. DNA- and RNA-based DGGE fingerprints of soil samples

Changes in the microbial community composition of soil microcosms were analyzed by PCR-DGGE to determine the effects of the pollutants and the concentrations which produce different degrees of alteration. Taking into account DNA-derived data, soil samples clustered in four distinct groups, according to similarities in the densitometric DGGE profiles (Fig. 2). In agreement with the respirometric assays, the DGGE fingerprints of control experiments clustered together with soils containing mostly low concentrations of pollutants (100 mg kg^{-1} MCP and TCP, and 10 mg kg^{-1} PCP) in group I. These samples subclustered at a similarity level above 80% in relation to the added contaminant revealing small changes derived from the application of the experimental procedures. Despite this observation, we suggest that these differences do not alter significantly the general effect of the pollutant at higher concentration due to two main reasons. First, all samples obtained from the initial stages of the incubation amended with the highest concentration of the pollutants (5000 mg kg^{-1} MCP and 1000 mg kg^{-1} TCP and PCP) were also clustered together within group I. Second, similarity values between group I and those containing the soil samples highly altered in the microbial community

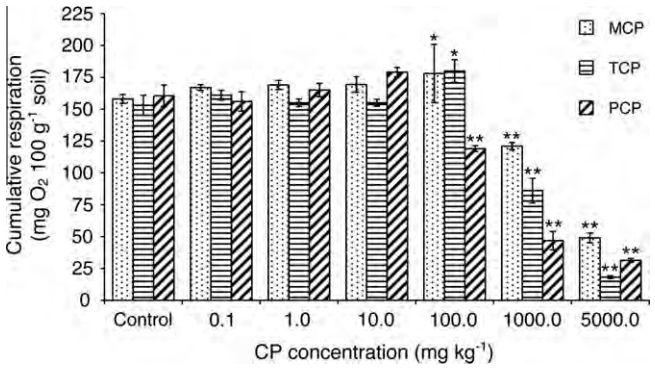

Fig. 1. Cumulative oxygen consumption obtained from the Cumulative Respiration (CR) assays of the polluted microcosms after 28 d of incubation. Variation coefficient is indicated around the mean values. (*) Significant stimulation differences compared to controls ($p < 0.05$). (**) Significant inhibition differences compared to controls ($p < 0.05$).

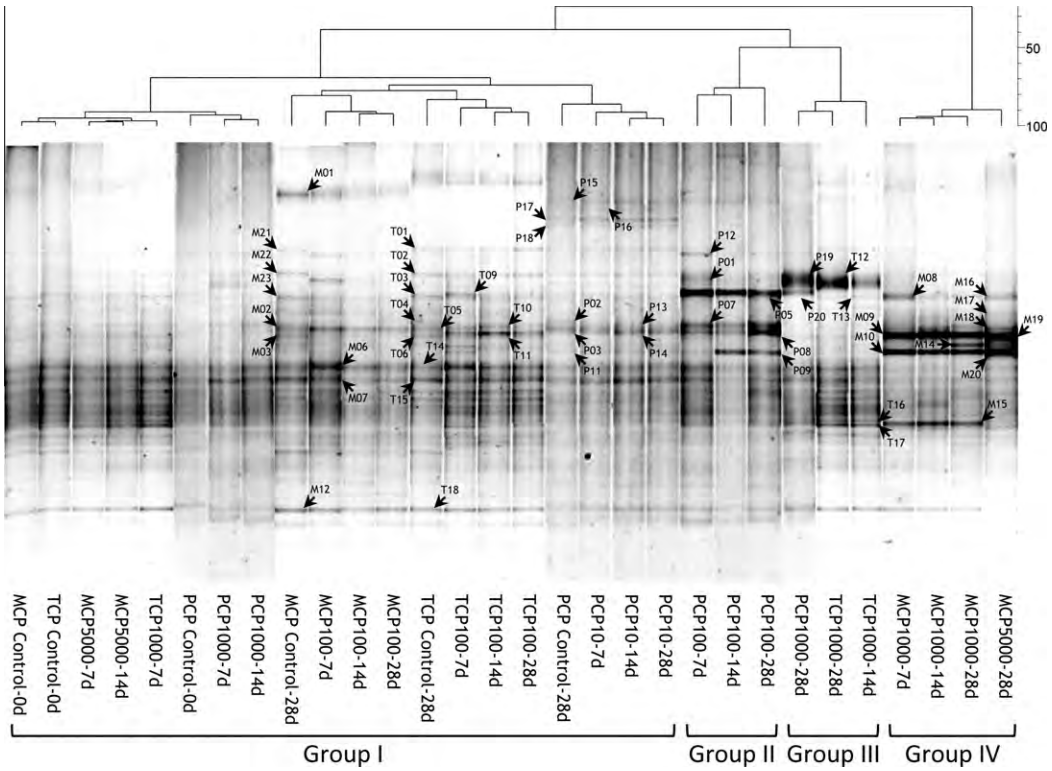

Fig. 2. DNA-based DGGE fingerprints of partial 16S rRNA gene fragments obtained with *Bacteria* universal primers, as they have been grouped in a dendogram by cluster analysis. Arrowheads indicate the bands that were excised and sequenced. Bands codes: M, T and P indicate whether the bands were recovered from MCP-, TCP- or PCP-treatments, respectively. The dendogram is based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method. Codes for soil samples indicate consecutively the pollutant used in the treatment (MCP, TCP or PCP), its concentration and the period of incubation (in days).

composition felt below 40% indicating a significant effect of high pollutant concentrations.

Three additional groups could be distinguished according to the GELCompar analysis. Groups II and III included samples amended with 1000 mg kg^{-1} TCP or PCP (from the end of the incubation period) and 100 mg kg^{-1} PCP, respectively. Group IV included the MCP-polluted samples. Observable changes involve the disappearance of certain bands, probably related to pollutant sensitive bacteria, and the appearance of several new bands indicating the development of supposedly resistant bacteria as dominant populations. At intermediate pollutant concentrations (1000 mg kg^{-1} MCP and 100 mg kg^{-1} PCP) these changes were usually detected after 7 d of incubation and persisted throughout the incubation period. However, at the highest pollutant concentrations, potentially resistant bacteria did not appear until 14 d of incubation at 1000 mg kg^{-1} TCP and 28 d at 5000 mg kg^{-1} MCP and 1000 mg kg^{-1} PCP.

The analysis of RNA-DGGE fingerprints leads to similar results considering the samples with low CP concentrations that clustered in group I, together with the control soil samples, at a similarity level above 85% (Fig. 3). The samples included in group II and III were also clustered as in the DNA-DGGE fingerprint analysis. However, soil samples amended with 5000 mg kg^{-1} MCP and 1000 mg kg^{-1} PCP at 14 d of incubation were included in group III, thus indicating that potentially resistant bacteria could be detected before by RNA-DGGE analysis than by DNA-DGGE. Samples polluted with MCP showed few similarities among them, but from DNA analysis these fingerprints resulted in a high similarity.

3.3. Phylogenetic identification of sensitive and resistant bacterial populations

Both DNA- and RNA-based PCR-DGGE approaches have been used to identify most representative bacterial populations of the soil microcosms. The analysis of the extracted rRNA has been specially considered in this study to target the metabolically active bacteria; theoretically, they should contain a higher number of ribosomes than dormant or non-growing cells (Wagner, 1994; Felske and Akkermans, 1998; Kerkhof and Kemp, 1999; Nicol et al., 2004). Eighty-nine relevant bands were excised from DNA- and RNA-based DGGE gels and treated for further PCR reamplification and sequencing (Figs. 2 and 3, respectively). From them, 72 bands produced useful sequences, without ambiguous positions, and were used for identification purposes (Table 1). Most sequences yielded very high similarity values with previously published sequences. Some of the DGGE bands resulted in identical sequences despite appearing in different positions of the gel (i.e., bands M16/17/18/19/20, T02/03, P01/05/12, R13/14 and R17/18/19, in Figs. 2 and 3), probably due to variable melting behaviors or the presence of multiple ribosomal gene copies in a single organism (Prat et al., 2009). On the other hand, some bands appearing in different samples at the same gel position resulted in different sequences (e.g. band P02 compared with P07 and P03 compared with P08, in Fig. 2).

Several DNA-DGGE bands, present in control fingerprints, were lost at high pollutant concentrations, suggesting the presence of pollutant sensitive bacteria. Sequences retrieved from these bands showed high homologies (>98.4%) with members of the *Nitrosomonadaceae* (M22, M23, T02 and T03), the *Cytophagaceae* (P17 and P18) and the *Gemmatimonadaceae* (M12 and T18). Other sequences appeared to be close to *Bacillus boroniphilus* (P11), *Bacillus asahii* (P02, P03, P13 and P14), *Rubrobacter* sp. (M07 and T15) and *Flavisolibacter ginsengisoli* (P15 and P16). Considering the detection of sensitive bacteria, DNA-DGGE profiles did not show the same results as RNA-DGGE profiles. The most significant difference concerns to several bands that yielded sequences similar to *B. asahii*, which disappeared in the former but were repeatedly detected as

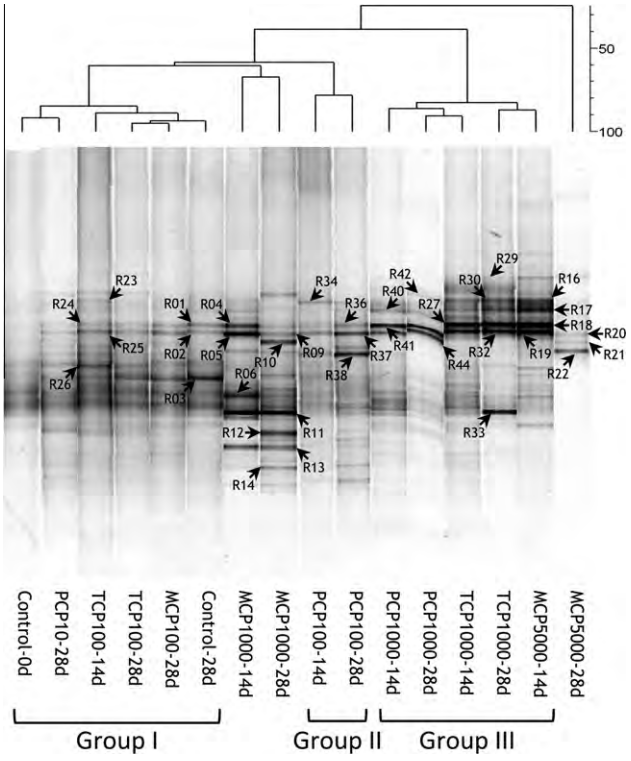

Fig. 3. RNA-based DGGE fingerprints of partial 16S rRNA gene fragments obtained with *Bacteria* universal primers, as they have been grouped in a dendogram by cluster analysis. Arrowheads indicate the bands that were excised and sequenced. The dendogram is based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method. Codes for soil samples indicate consecutively the pollutant used in the treatment (MCP, TCP or PCP), its concentration and the period of incubation (in days).

metabolically active in the latter at high pollutants concentration (bands R04, R17, R18, R19, R27, R32, R41 and R44).

In contrast, almost all the bands belonging to potentially pollutant resistant bacteria were detected in both DNA and RNA analyses. In MCP-treated soils, they included bands M08, M09, M10, M16, M17, M18, M19, M20, R05, R09, R20 and R22 (an uncultured *Planococcaceae*), bands M14 and R10 (*Brevibacillus levickii*) and bands M15 and R11 (*Georgenia ferrireducens*). In all cases, homologies with previously published sequences were higher than 98.5%. All of them appeared at 1000 mg kg^{-1} MCP, but bands M16, M17, M18, M19, M20, R20 and R22 were also detected at 5000 mg kg^{-1} MCP. Moreover, the rRNA analysis revealed additional information about the active members of the bacterial community found at high pollutant concentrations, indicating a selective enrichment of certain phylotypes. That is the case of band R21 (*B. levickii*), which also appeared at a MCP concentration of 5000 mg kg^{-1} after 28 d of incubation, whereas bands R12 (*Micromonospora sagamiensis*), R13 and R14 (both corresponding to *Thermomicrobia* members) were only detected at 1000 mg MCP kg^{-1}.

Given TCP- and PCP-treated soils, several bacteria can be considered to resist both pollutants. Potentially resistant bacteria, found in both DNA and RNA-derived DGGE gels, include bands T09, T13, P01, P05, P12, P20, R23, R30 and R34 (uncultured *Burkholderiaceae*), bands T12, P19, R29 and R42 (*Pseudomonas putida*), bands T11, P08, R25 and R37 (uncultured *Sphingomonadaceae*) and bands T10, P07, R24 and R36 (*Lysobacter gummosus*). In all cases, homologies with previously published sequences were higher than 97.1%. Only the bands related to uncultured *Burkholderiaceae* and *P. putida* were detected at the highest concentration of both pollutants (1000 mg kg^{-1}), although the former was not found in RNA approach at this PCP concentration. Finally, bands P09 and R38 were detected exclusively at 100 mg kg^{-1} PCP and sequences showed 99.3% homology with *Methylibium petroleiphilum*, while band R33 (*G.* ferrireducens) was found at 1000 mg kg^{-1} TCP in RNA-DGGE analysis.

3.4. Isolation and identification of the resistant bacteria

Between 10 and 15 isolates were selected from each soil microcosm supplied with different amounts of MCP, TCP or PCP, based on the colony morphology. 16S rRNA gene sequencing was used for the identification of the isolates (Table 2). Most isolates showed above 98% similarity to sequences of cultured bacteria. However, isolates ITP29 and ITT11 (Fig. 4) exhibited similarities of 99.5% and 97.4%, respectively, with uncultured bacteria, but less than 97% similarity with the closest cultured bacteria. The obtained isolates distributed in three divisions: *Firmicutes* (65%), *Actinobacteria* (26%) and *Gammaproteobacteria* (9%). While isolates related to *Firmicutes* and *Actinobacteria* were obtained from soils amended with all kinds of chlorophenols, those related to *Gammaproteobacteria* were only obtained from TCP- and PCP-polluted soils.

3.5. Isolates characterization of CP resistance and degradation

Twenty-three different isolates were tested in culture plates with different amounts of TCP or PCP to estimate their resistance (Table 2). A single representative from each group, including isolates with identical 16S rRNA gene sequences, was chosen for resistance tests to TCP and PCP. MCP resistance was not tested since its high volatility could lead to inaccurate results (Van Agteren et al., 1998). Most of the isolates were found to grow in the presence of 100 and 200 mg TCP L^{-1}, but some of them were able to tolerate PCP concentrations above 50 mg L^{-1}.

The resistant strains to both, TCP and PCP, were identified as *Kocuria palustris* (ITT09), *L. gummosus* (ITP09), *Bacillus* sp. (ITP29) and *P. putida* (ITP30). All selected strains showed the capacity to reduce the concentration of the added chlorophenolic compounds in different degrees (Fig. 5). Growth at the end of the incubations leads to similar OD values for all of them (from 0.45 to 0.55). Highest degradation capacities accounted for a reduction of 15–30% (8–12 mg L^{-1}), in comparison to controls, in a 5 d period, from an initial CP concentration of 50 mg L^{-1}. Higher degradation activities were found for TCP compared to PCP. Among the analyzed isolates, *Bacillus* sp. (ITP29) and *L. gummosus* (ITP09) highlighted a suitable degrading capacity of both pollutants, while P. putida (ITP30) also show a significant PCP degradation rate.

4. Discussion

4.1. Resistance of the soil microbial community to different degrees of CP pollution

Two methodologies were used to assess the influence of chlorophenols (MCP, TCP and PCP) on the microbial community of a previously unpolluted Mediterranean soil. The first approach, based on respirometric measurements, has shown the effects of pollutants on microbial activity. The cumulative soil respiration, commonly used as an estimation of the aerobic activity of the heterotrophic microbiota in toxicity assessments (Strotmann et al., 2004; Plaza et al., 2005; Montserrat et al., 2006; Martí et al., 2007; Coello et al., 2009) has been a useful technique to evaluate the overall soil state. The second approach, based on a PCR-DGGE analysis of both DNA and RNA, has revealed its capability to infer changes in the complexity and activity of the microbial community in the experimental soil microcosms. PCR-DGGE has been widely used to ana-

Table 1
Closest matches of 16S rRNA gene sequences obtained from DNA- and RNA-DGGE gels. Bands codes: M, T and P indicate whether the bands were recovered from MCP-, TCP- or PCP-treatments, respectively; R indicates the bands derived from RNA analysis. Closest bacterial species, sequence similarities (%) and affiliations were retrieved according to ARB phylogenetic analysis.

Bands[a]	Acc. num.	Closest bacterial species	%[b]	bp[c]	Affiliation	DNA-DGGE bands	RNA-DGGE bands	Response[d]
		Firmicutes						
P11	FR666815	*Bacillus boroniphilus* (AB198719)	98.9	550	*Bacillaceae*	P11	–	S
P03	FR666799	*Bacillus asahii* (AB109209)	99.5	550	*Bacillaceae*	P02/03/13/14	R01/02/04/17/18/19/27/32/41/44	S/R
M10	FR666810	Uncultured bacterium (EU134657)	99.3	535	*Planococcaceae*	M08/09/10/16/17/18/19/20	R05/09/20/22	R
M14	FR666812	*Brevibacillus levickii* (AJ715378)	98.5	550	*Paenibacillaceae*	M14	R10/21	R
		Actinobacteria						
R12	FR666819	*Micromonospora sagamiensis* (GQ163461)	100	531	*Micromonosporaceae*	–	R12	R
M15	FR666811	*Georgenia ferrireducens* (EU095256)	99.8	535	*Bogoriellaceae*	M15	R11/33	R
M07	FR666797	Uncultured *Rubrobacter* sp. (EU341239)	98.4	551	*Rubrobacteraceae*	M07, T15	R03	S
		Chloroflexi						
R13	FR666820	Uncultured bacterium (AM935607)	98.9	527	*Thermomicrobia*	–	R13/14	R
		Alphaproteobacteria						
P08	FR666813	Uncultured bacterium (EU133463)	99.8	524	*Sphingomonadales*	T11, P08	R25/37	R
		Gammaproteobacteria						
P19	FR666809	*Pseudomonas putida* (EF529517)	100	549	*Pseudomonadaceae*	T12, P19	R29/42	R
P07	FR666818	*Lysobacter gummosus* (AB161361)	97.1	550	*Xanthomonadaceae*	T10, P07	R24/36	R
		Betaproteobacteria						
P05	FR666805	Uncultured bacterium (AB488171)	99.6	548	*Burkholderiaceae*	T9/13, P01/05/12/20	R23/30/34	R
T02	FR666798	Uncultured *Nitrosomonadaceae* (EF018627)	98.4	550	*Nitrosomonadaceae*	M22/23, T02/03	-	S
P09	FR666814	*Methylibium petroleiphilum* (AF176594)	99.3	548	*Burkholderiales*	P09	R38	R
		Bacteroidetes						
P15	FR666800	*Flavisolibacter ginsengisoli* (AB267477)	98.9	539	*Chitinophagaceae*	P15/16	–	S
P17	FR666801	Uncultured *Bacteroidetes* (EU979040)	99.6	540	*Cytophagaceae*	P17/18	–	S
		Gemmatimonadetes						
M12	FR666794	Uncultured *Gemmatimonadetes* (EF612383)	99.6	541	*Gemmatimonadaceae*	M12, T18	–	S

[a] A single representative band from each group, including those with identical 16S rRNA gene sequences, used for phylogenetic identification.
[b] Similarity percentage.
[c] Base pare length.
[d] (S) Indicates that the phylotypes have been considered as sensitive, since the corresponding bands were present in the control fingerprints but disappeared in the polluted microcosms. (R) Indicates that the phylotypes have been considered as resistant, since the corresponding bands appeared in the fingerprints of polluted-soil microcosms.

Table 2
Phylogenetic affiliation of the bacterial isolates based on 16S rRNA gene analysis and characterization of their resistance to TCP and PCP. Isolates codes indicate the soil microcosms procedence: ITM, ITT or ITP for microcosms polluted with MCP, TCP or PCP, respectively. Closest cultured bacterial species and sequence similarities (%) were retrieved according to ARB phylogenetic analysis.

Isolate[a]	Acc. num.	Closest cultured bacterial species	%[b]	Soil microcosms procedence of isolates[c]				Resistance[d]							
								TCP ($mg \cdot kg^{-1}$)				PCP ($mg \cdot kg^{-1}$)			
				MCP-1000	TCP-1000	PCP-100	PCP-1000	10	50	100	200	10	50	100	200
		Actinobacteria													
ITT12	FR667170	*Georgenia ferrireducens* (EU095256)	98.3	ITM09/12/15	ITT12/13	–	–	++	++	++	++	++	–	–	–
ITM10	FR667171	*Citricoccus alkalitolerans* (AY376164)	98.1	ITM10/11/14	–	–	–	++	++	++	–	+	–	–	–
ITT09	FR667173	*Kocuria palustris* (Y16263)	100	–	ITT09	–	–	++	++	++	++	++	+	–	–
ITP10	FR667177	*Arthrobacter crystallopoietes* (X80738)	100	–	–	–	ITP10	++	++	++	+	–	–	–	–
ITT16	FR667186	*Arthrobacter agilis* (X80748)	99.6	–	ITT16	–	–	+	–	–	–	–	–	–	–
ITP08	FR667175	*Rhodococcus pyridinivorans* (AF173005)	99.7	–	–	–	ITP08	++	++	++	+	+	–	–	–
		Firmicutes													
ITT01	FR667164	*Bacillus licheniformis* (CP000002)	99.9	ITM01	ITT01	–	–	++	++	++	–	–	–	–	–
ITT02	FR667165	*Bacillus thuringiensis* (D16281)	100	ITM02	ITT02	–	–	++	++	++	–	+	–	–	–
ITP22	FR667166	*Bacillus megaterium* (AB271751)	100	ITM03	ITT03	ITP22	ITP03	++	++	++	–	+	–	–	–
ITP04	FR667167	*Bacillus mojavensis* (AB021191)	100	ITM04/06	ITT06	–	ITP04/06	++	++	–	–	–	–	–	–
ITP24	FR667168	*Bacillus mycoides* (AF155956)	99.5	ITM05	ITT05	ITP24	ITP05	++	++	+	–	+	–	–	–
ITM08	FR667169	*Terribacillus saccharophilus* (AB243845)	99.2	ITM08	–	–	–	++	++	+	+	+	–	–	–
ITP21	FR667179	*Bacillus endophyticus* (AF295302)	100	–	–	ITP21	–	++	++	+	–	–	–	–	–
ITP23	FR667180	*Bacillus firmus* (D16268)	98.0	–	–	ITP23	ITP02	++	–	–	–	–	–	–	–
ITP27	FR667181	*Bacillus asahii* (AB109209)	99.4	–	–	ITP27	–	–	–	–	–	–	–	–	–
ITP29	FR667182	*Bacillus humi* (AJ627210)	96.9	–	–	ITP29	–	++	++	++	++	++	++	++	++
ITT10	FR667184	*Bacillus circulans* (AY724690)	99.6	–	ITT10	–	–	++	++	++	++	++	–	–	–
ITM13	FR667183	*Oceanobacillus caeni* (AB275883)	99.3	ITM13	–	–	–	++	++	++	++	++	–	–	–
ITT11	FR667185	*Oceanobacillus chironomi* (DQ298074)	96.3	–	ITT11/15	–	–	+	+	+	–	–	–	–	–
ITP26	FR667174	*Paenibacillus lautus* (D78473)	99.4	–	ITT07	ITP26	ITP07/16	++	++	++	+	++	–	–	–
ITT08	FR667172	*Staphylococcus warneri* (L37603)	99.8	–	ITT08	–	–	++	–	–	–	–	–	–	–
		Gammaproteobacteria													
ITP30	FR667178	*Pseudomonas putida* (EF529517)	100	–	ITT17	ITP20/28/30	ITP01/11/12	++	++	++	++	++	++	++	+
ITP09	FR667176	*Lysobacter gummosus* (AB161361)	98.0	–	–	–	ITP09	++	++	++	–	++	++	++	–

[a] A single representative isolate from each group, including those with identical 16S rRNA gene sequences, used for phylogenetic identification. Isolates tested further for their degradation capacity of TCP and PCP are presented underlined.
[b] Similarity percentage (%). All compared sequences are longer than 1400 bp.
[c] The corresponding concentrations of the pollutants assayed are expressed as mg kg^{-1}.
[d] Qualitative appreciation for development of the colonies in comparison to the controls: similar (++), lower (+) and null (–).

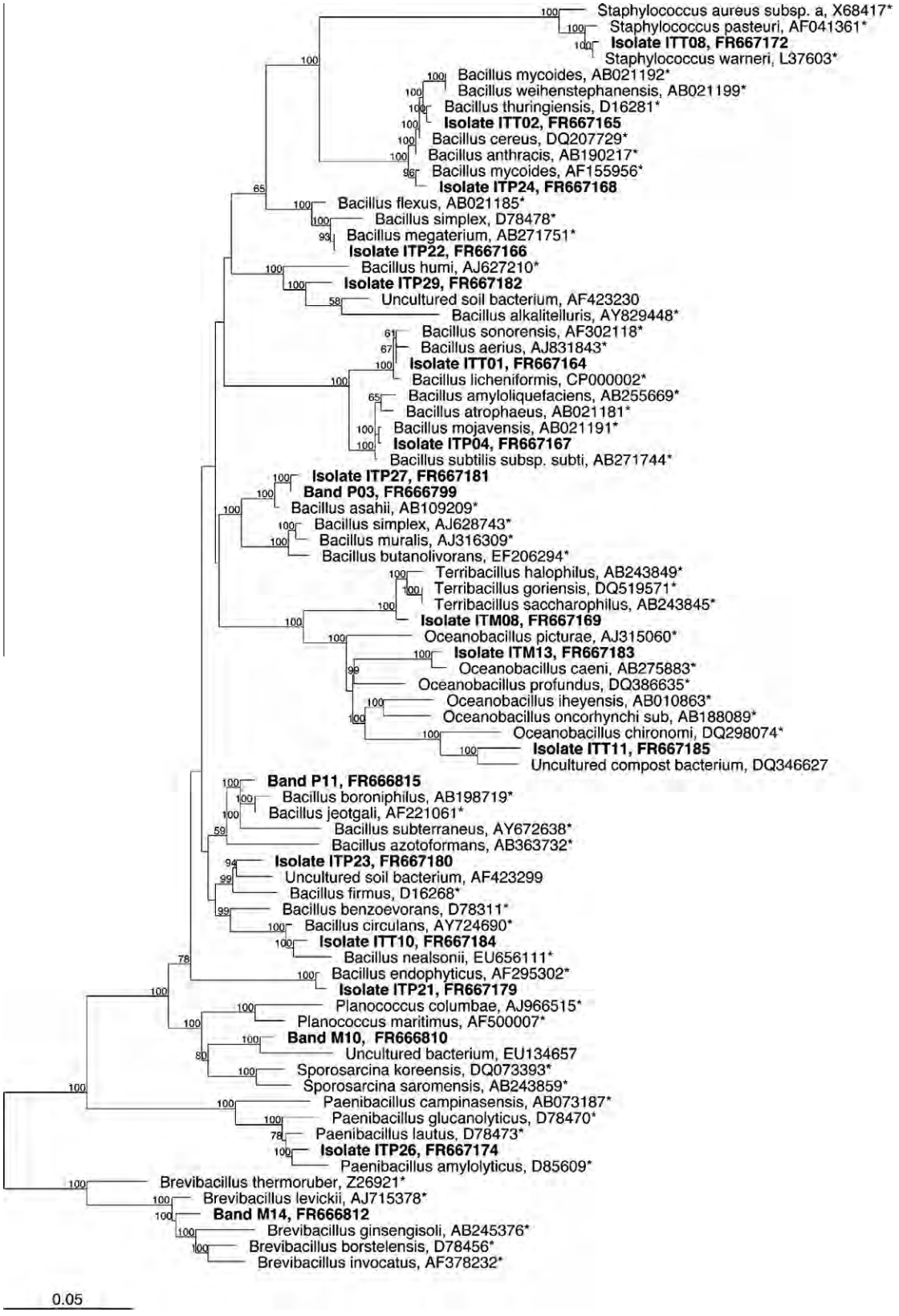

Fig. 4. Maximum likelihood phylogenetic tree calculated for 16S rRNA gene sequences of *Firmicutes* obtained in this study (in boldface) and those of their closest relatives. Bootstrap values >60% are indicated at branch nodes. Representative sequences retrieved from DGGE bands are indicated as "Band" and those obtained from the isolated resistant bacteria as "Isolate". Type strain sequences included in SILVA database are marked with an asterisk (*). The scale bar indicates 5% estimated sequence divergence.

lyze changes in microbial community composition of soils (Edenborn and Sexstone, 2007; Campbell et al., 2009). Both, the respirometric and the molecular approach, showed a significant effect of the pollutants on the soil microbial communities, thus

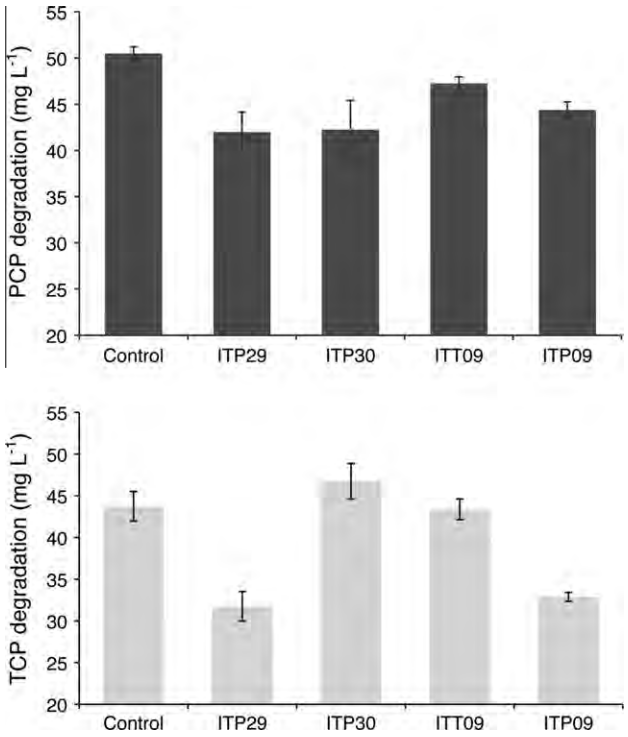

Fig. 5. CP degradation by most resistant isolates (ITT09, ITP09, ITP29 and ITP30) tested in pure cultures amended with 50 ml L^{-1} TCP or PCP. Variation coefficient is indicated around the mean values.

indicating that not only the activity but also the abundance of several bacterial groups were influenced by the added contaminants.

The presence of high concentrations of the pollutants produced a decline in the activity of the heterotrophic bacteria, while showing significant changes in the microbial community composition. Our results confirmed that PCP is the most toxic of the assayed pollutants, showing significant effects from 100 mg kg^{-1}. These results agree with those described by Chaudri et al. (2000), who reported a negative impact of PCP at similar concentrations (50–100 mg kg^{-1} PCP) based on analysis of the soil microbial biomass and counts of total aerobic heterotrophic bacteria. Furthermore, Mahmood et al. (2005) described shifts in the microbial community composition of soil samples amended with 200 mg kg^{-1} PCP. Considering both MCP and TCP, our results showed that toxic effects clearly appeared from concentrations over 1000 mg kg^{-1}, although TCP-amended soils led to lower respirometric activity values at this concentration of pollutant than with MCP. Moreover, several resistant phylotypes could be identified at 100 mg kg^{-1} TCP, not detected in control samples, which also evidence the higher influence of TCP than MCP in the soil microcosms.

Different lag phases for the adaptation of the resistant microbial community to the toxic effects of CP were evidenced at 1000 mg kg^{-1}. At this concentration, the apparition of resistant bacteria could be detected from 7 and 14 d in MCP- and TCP-polluted-soil microcosms, respectively, and only at the end of the incubation period for PCP. These evidences also indicate the higher toxic effects of CP with increasing the degree of chlorination.

4.2. Stimulation of soil activity at intermediate CP concentrations

Interestingly, the presence of MCP and TCP at an intermediate concentration (100 mg kg^{-1}) led to an increase on the respirometric activity, thus indicating an unexpected stimulation of the activity. This increase can be due to the biodegradation of these pollutants, as supported by the presence of strains with biodegrading capabilities that were isolated from the same soil (Fig. 5). In this sense, long-term experiments performed by Chaudri et al. (2000) suggested that resistant microbial populations from soil samples, amended with 200 mg kg^{-1} PCP, proliferated by metabolizing the organic pollutant, as well as the carbon liberated by the death of the sensitive populations. However, stimulation of the activity was not detected in our microcosms incubated at 100 mg kg^{-1} PCP or at higher concentrations of any pollutant, probably because these concentrations were high enough to produce strong inhibition effects on the bacteria present in soil. A significant increase of the respiration activity was not either detected at lower concentrations since the extra amount of C was probably not enough to evidence it, as had been already observed by Chaudri et al. (2000).

4.3. Similarities between DNA- and RNA-derived DGGE results

In this study, the influence of chlorophenols on the composition of the soil microbial community has been analyzed by PCR-DGGE of DNA extracts from the soil samples, and the results have been compared with those generated from RNA extracts, which are assumed to better reflect the metabolically active bacteria (Nogales et al., 2001; Girvan et al., 2003). Results were highly similar since most representative phylotypes of the soil microcosms were identified by both DNA- and RNA-derived data, strongly proving they were metabolically active. These results agree with those described by Mahmood et al. (2005) and Nogales et al. (2001) who also reported a good correspondence of the soil community composition when comparing DNA- and RNA-derived data from contaminated environments.

Nevertheless, a few phylotypes were detected by only one of the molecular approaches. Phylotypes detected only by DNA-DGGE analysis could be indicative of quiescent bacteria present in the soil microcosms, since active cells usually contain higher numbers of ribosomes than quiescent cells (Nogales et al., 1999). The contrary situation, exclusively evidenced when new bacterial populations initiate development, might be explained because targeting RNA specially increases sensitivity (Nogales et al., 2001).

4.4. Resistant bacteria to high CP concentrations

The development of presumably resistant bacterial populations, which belonged to 11 different phylotypes according to 16S rRNA gene homology, was evidenced by PCR-DGGE in soil samples amended with high concentration of CP. The molecular approach also revealed the presence of some of these phylotypes common at different treatments. In addition, 23 isolates were obtained from highly polluted microcosms, and most of them were confirmed as especially resistant to high concentrations of TCP, but also of PCP. These results prove the survival of several bacteria in the presence of different CP without a previous adaptation or contact with them, in agreement with several previous studies that reported PCP degradation by an indigenous soil bacterial community at concentrations from 200 to 500 mg kg^{-1} (Andreoni et al., 1998; McGrath and Singleton, 2000; Mahmood et al., 2005).

A few phylotypes detected by PCR-DGGE in soil microcosms could be isolated in pure cultures: four different sequences derived from DGGE bands (M15, P19, P07 and P03) showed identical homology to the 16S rRNA sequences of isolated bacteria (ITT12, ITP30, ITP09 and ITP27, respectively). Three of these isolates were obtained from highly polluted microcosms and identified as *G. ferrireducens* (ITT12), *P. putida* (ITP30) and *L. gummosus* (ITP27), according to 16S rRNA gene homology. The tests carried out to determine the resistance level of these isolates to chlorophenols

are in completely agreement with the isolation source, as well as with the soil microcosm where the identical DGGE bands were detected. Several species of *Pseudomonas* and *Lysobacter ruishenii* have previously been described as resistant bacteria to CP or to other organic pollutants (Kiyohara et al., 1992; Gautam et al., 2003; Wang et al., 2010). On the contrary, *G. ferrireducens* has only been described as a metal resistant bacterium (Cavalca et al., 2010) and, until now, species of this genus have never been reported to be able to resist CP. The fourth isolate was obtained from PCP-polluted soils and identified as *B. asahii* (ITP27) according to 16S rRNA gene homology. However, even though it was detected by DGGE analysis at high pollutant concentrations, only in RNA analysis, no evidence of resistance was found by the performed tests with the isolated strain.

In contrast, several presumably resistant bacteria detected as new DGGE bands, appearing only in polluted soils, could not be isolated. Some sequences (corresponding to bands P05 and P08) appeared to be closely related to those of species of known degrading capacity, as *Paucimonas lemognei* (Elbanna et al., 2004) and *Sphingomonas chlorophenolica* (Beaulieu et al., 2000; Yang et al., 2006). In addition, the sequence retrieved from band P09 showed a high homology to *M. petroleiphilum*, which has been reported to completely degrade the gasoline additive methyl *tert*-butyl ether (Hanson et al., 1999; Nakatsu et al., 2006; Chen et al., 2008). Finally, sequences retrieved from bands M10, M14, R12 and R13, showed a high homology to an unidentified *Planococcaceae*, *B. levickii*, *M. sagamiensis* and an uncultured *Thermomicrobia*, respectively. To our knowledge, these species or closely related bacteria have not been described as CP resistant.

Culture techniques also allowed recovering some other isolates from soil samples with high concentration of the pollutants, not detected in DGGE analysis, since they probably were present at low relative abundance. However, most of these isolates showed low or null resistance to the pollutants, suggesting that they could be able to resist pollutants only in a state of dormancy, such as spore-forming bacteria typical from soil communities. Several strains of *Bacillus* have been described as resistant to different phenol and other antimicrobial compounds in a spore form (Briggs, 1966; Russell, 1990; Sagripanti and Bonifacino, 1996). In contrast, two isolates identified as *Kocuria palustris* and *Bacillus* sp. (ITT09 and ITP29, respectively) showed a high resistance to both TCP and PCP. Additionally, those identified as *Terribacillus saccharophilus*, *Oceanobacillus caeni*, *Bacillus circulans*, *Arthrobacter agilis*, *Rhodococcus pyridinivorans* and *Paenibacillus lautus* (ITM08, ITM13, ITT10, ITT16, ITP08 and ITP26, respectively) showed a high resistance only to TCP. Strains of *Arthrobacter* and *Rhodococcus* have been reported to degrade CP (Stanlake and Finn, 1982; Haggblom et al., 1988; Nordin et al., 2005). *Bacillus circulans* and *Paenibacillus* are known to resist other organic pollutants (Vrdoljak et al., 2005; Wu et al., 2007).

4.5. Bacterial isolates with TCP and PCP bioremediation potential

Isolates showing the highest resistance level to highly chlorinated chlorophenols have been tested for their ability to degrade TCP or PCP. *Bacillus* sp. (ITP29) and *L. gummosus* (ITP09) degrade larger amounts of TCP than PCP, as PCP is a more persistent compound. However, *Kocuria palustris* (ITT09) and *P. putida* (ITP30) are able to degrade PCP but not TCP. It has been described that some bacterial strains are able to degrade PCP but not some of its metabolites (Ruckdeschel et al., 1987; Utkin et al., 1995). Recently, Karn et al. (2010) found that *Kocuria* sp. is able to biodegrade 600 mg L^{-1} PCP from a paper mill. Wang et al. (2010) reported a strain of *Lysobacter ruishenii* able to degrade the phenol derivative chlorothalonil. Several species of *Pseudomonas* have been repeatedly described as CP degraders by many authors (Radehaus and Schmidt, 1992; Resnick and Chapman, 1994).

To date, no CP biodegrader, belonging to the genus *Bacillus*, have yet been described (Field and Sierra-Alvarez, 2008). However, in our work, *Bacillus* sp. (ITP29), which has been isolated from microcosms polluted with high concentrations of PCP, has been confirmed as a TCP and PCP degrader. 16S rRNA gene sequence of this isolate showed less than 97% homology with *Bacillus humi* and *Bacillus alkalitelluris*, which are the closest species. Therefore, this isolate could be a novel species based on the 16SrRNA gene sequence criterion, although further genotypic and phenotypic analysis should be performed in order to confirm it. *Bacillus* are gram-positive spore-forming bacteria, very commonly distributed in soils and slightly known as organic pollutant biodegraders. These singular features confer them a strong interest as possible new bacterial species with potential biotechnological application in soil bioremediation processes, that should be surveyed in future investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.01.016.

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Chapter II

J. Cáliz, X. Vila, E. Martí, J. Sierra, R. Cruañas, M. Antònia Garau, G. Montserrat. “Impact of chlorophenols on microbiota of an unpolluted acidic soil: microbial resistance and biodegradation”. *FEMS microbiology ecology*. Vol. 78, issue 1 (October 2011) : p. 1250-164

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Abstract

The impact of 2-monochlorophenol (MCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) on the microbial community of an acidic forest soil was studied under controlled laboratory conditions by spiking microcosms with the pollutants at concentrations ranging from 0.1 to 5000 mg kg^{-1}. A decrease in the cumulative respirometric values and changes in the bacterial and fungal community composition were detected at 1000 mg MCP kg^{-1}, 100 mg TCP kg^{-1} and 100 and 1000 mg PCP kg^{-1}. However, drastic effects on the microbial community were revealed only at higher concentrations of MCP and TCP, although the toxicity of PCP was expected to be stronger. The acidic condition of the soil presumably reduces bioavailability of PCP, leading to less pronounced effects than the other pollutants. This finding highlights the consideration of pollutant bioavailability in each environment to adequately assess contamination effects. Twenty-two different chlorophenol-resistant and potentially degrading microorganisms were isolated from highly polluted microcosms. The most resistant isolates were related to *Burkholderia arboris, Bacillus circulans, Paenibacillus taichungensis, Luteibacter rhizovicina* and *Janibacter melonis*. These isolates also showed the capacity to reduce the concentration of TCP or PCP between 15% and 35% after 5 days of incubation (initial concentration of 50 mg L^{-1}). The isolate related to *B. circulans* is an atypical case of a member of the *Firmicutes* group for which chlorophenol-degrading capacities have been described.

Keywords:

Bacillus; respirometry; PCR-DGGE; bioavailability; bioremediation

Chapter III

Exposition of a calcareous Mediterranean soil to Cr, Cd and Pb suggests a differential selection of the microbiota due to bioavailability of heavy metals

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Abstract

The involvement of the bacterial community of an agricultural Mediterranean calcareous soil in relation to several heavy metals has been studied in microcosms under controlled laboratory conditions. Soil samples were artificially polluted with Cr(VI), Cd(II) and Pb(II) at concentrations ranging from 0.1 to 5000 mg kg^{-1} and incubated along 28 days. The lowest concentrations with significant effects in soil respirometry were 10 mg kg^{-1} Cr and 1000 mg kg^{-1} Cd and Pb. However, only treatments showing more than 40% inhibition of respirometric activity led to significant changes in bacterial composition, as indicated by PCR-DGGE analyses. Presumable Cr- and Cd-resistant bacteria were detected in polluted microcosms, but development of the microbiota was severely impaired at the highest amendments of both metals. Results also showed bioavailability is an important factor determining the impact of the heavy metals assayed, and even an inverted potential toxicity ranking can be achieved if their soluble fraction is considered instead of the total concentration. Multiresistant bacteria were isolated from Cr-polluted soil microcosms, some of them showing the capacity to reduce Cr(VI) concentrations between 26-84% of the initial value. Potentially useful strains for bioremediation were related to *Arthrobacter crystallopoietes*, *Stenotrophomonas maltophilia* and several species of *Bacillus*.

Introduction

Heavy metals are frequently present in the environment at unusual high concentrations as a consequence basically of the industrial activity. It is well established that they persist indefinitely in soils, where they may interfere in the soil

properties (mainly related to microbiota) and potential uses. However, with few exceptions, there has been little progress in amending environmental regulations to protect soil system from elevated metal concentrations (Giller et al., 2009), despite the USEPA priority list (http://www.atsdr.cdc.gov/cercla/07list.html) considers Cr, Cd and Pb within the group of the first 20 pollutants. Moreover, simultaneous soil contamination by persistent organochlorine pesticides and metals or metalloids is also widely detected in urban and agricultural soils (Van Zwieten et al., 2003). U.S. Environmental Protection Agency considers that co-contaminated sites with organic and metal pollutants comprise 40% of the hazardous waste sites (USEPA, 1997). Consequently, the possibilities of recovering these situations have to be taken into consideration.

Total metal concentrations are often poor indicators of the actual amount of metals in the soil solution to which microorganisms are exposed (Lazzaro et al., 2006b). Therefore, factors such as pH and soil texture, which can strongly influence metal bioavailability, must be taken into account when establishing permissible limits for soil metal concentrations (Giller et al., 2009). Differences in metal bioavailability can greatly affect toxicity and stress microbial populations (Roane et al., 2001) and. since they depend on each metal and soil type, a huge diversity of possibilities is derived.

Several soil studies have reported the influence of heavy metals on microorganisms by affecting their biochemical activities, decreasing biomass and diversity, and changing to a metal-resistant microbial community (Roane and Pepper, 1999; Sandaa et al., 2001; Becker et al., 2006; Bernard et al., 2009). Therefore, microbial populations thriving in a polluted environment usually develop or acquire resistance capabilities, which allow them to survive and keep soil functions. These mechanisms include the formation and sequestration of heavy metals in complexes, production of exopolysaccharides, the chemical reduction to less toxic species and direct efflux of metal out of the cell (Hernandez et al., 1998; Rasmussen and Sørensen, 1998; Sundar et al., 2011). Bioremediation strategies can take advantage of these processes to improve environmental health. In this sense, microbial-based metal remediation techniques usually rely on the ability of some microorganisms to resist and detoxify metals (Roane and Pepper, 1999).

The involvement of microorganisms in contact with pollutants is an important issue in order to understand the toxic effects of metals pollution events in the environment, as well as for developing adequate bioremediation strategies. Consequently, it is important to know the effects of pollutants on the microbiota in different kinds of soils without previous contamination (Konopka et al., 1999;

Roane et al., 2001; Jackson et al., 2005; Viti et al., 2006) An alternative approach to field studies, where environmental parameters can be poorly controlled, are laboratory microcosm experiments, which are simple model ecosystems as close to the nature state as possible (Jessup et al., 2004). Although they can hardly reproduce the complexity of natural conditions, external factors can be more easily controlled (Lazzaro et al., 2006a), identical replicates can be performed and parameters can be standardized to allow the comparison among treatments, soils or conditions.

The main objectives of this research were to use soil microcosms to assess the impact of several heavy metals (Cr, Cd and Pb) on the indigenous bacterial community of a previously unpolluted Mediterranean soil. From these experimental incubations procedures, detection and isolation of metal resistant bacteria were performed, and several strains were tested to assess their usefulness in soil bioremediation processes.

This research is part of a study including the assessment of chlorophenolic pollutants in relation to this soil and even to another soil with acidic properties (Cáliz et al., 2011a; Cáliz et al., 2011b; Marti et al., 2011). This wider focus also allows to analyze the results in the frame of the influence of a wide array of pollutants on different kinds on environments, and to consider the potential of the microbiota to face up to co-contamination events.

*M*aterial and methods

1. Soil sampling and microcosms incubations

Experiments were performed with samples taken from an agricultural soil without any prior history of exposure to heavy metals contamination. Soil samples were collected in July 2007 from the superficial layer (A horizon) of a site located in the Mediterranean area of Santa Bàrbara, Tarragona (UTM: 31 T 286500E 4509303N). They were sieved (<2 mm) and stored by air-drying conservation method until the forthcoming experiments. This soil was classified as calcaric regosol (FAO-UNESCO, 1998) developed on sedimentary materials. It was a fine-textured and alkaline (pH 8.2) soil, which contained carbonates (24%) and only 1.66% organic carbon. This soil type is very common in the Mediterranean area and it has adequate C/N ratio (9.44) to perform respirometric assays.

After a pre-incubation at the field soil humidity (8.7% dry weight), microcosms with 50 g of soil were treated separately with 0.1, 1, 10, 100, 1000 and 5000 mg kg^{-1}

Cr(VI), Cd(II) or Pb(II), and without pollutants (control). The contamination of soil microcosms was performed with aqueous solutions of K_2CrO_4, $CdSO_4$ or $PbCl_2$. In order to perform respirometric analysis, soil samples were placed in closed reactors (500 mL) with a water content equivalent to the 60% of the soil water holding capacity (WHC) and incubated in the dark at 25 °C during 28 days. Control and polluted microcosms destined to molecular analysis (PCR-DGGE) were incubated likewise, but harvested and destructively sampled after 7, 14 and 28 days. Non-incubated soil samples without pollutant were also used as initial controls. After each harvest, soil samples were stored at -80 °C until extraction of nucleic acids. All incubations performed for respirometric and molecular analysis were done in triplicate.

2. Analyses of metals in the soil solution

Soluble fractions of Cr, Cd and Pb in the soil samples were determined in triplicate from aqueous extracts (DIN, 1984), with subsequent quantification by inductively coupled plasma, using optical detection (ICP-OES) for Cr and mass spectrophotometry (ICP-MS) for Cd and Pb.

3. Respirometric analysis of microbial activity

The aerobic heterotrophic microbial activity was analyzed by respirometry. Microcosms processed with this purpose were incubated as described above in manometric respirometers, which allow the determination of sample oxygen consumption (Oxitop®, WTW). The cumulative oxygen consumption (Cumulative Respiration, CR) was periodically registered throughout the incubation period. To determine the statistical significance of the differences between treated samples and controls, an ANOVA followed by Duncan's *post-hoc* test ($p<0.05$) (SPSS 16) was performed.

Respirometric assay results were used as preliminary tests to select treatments for a throughout analysis of the changes in the microbial community composition by PCR-DGGE. All the treatments with concentrations that resulted in a significant inhibitory effect on respirometry tests and those with the highest concentration of pollutants that showed low or no inhibitory effect were included in the set of samples to be analyzed by molecular methods.

4. DNA extraction

DNA was extracted from 0.5 g of soil samples using the FastDNA® SPIN Kit for Soil (Bio101, Carlsbad, USA), as described in Caliz *et al.* (2011b). Previously, the optimal amount of soil sample was experimentally determined from the relationship between the weight of processed soil samples and the concentration of extracted DNA (data not shown) to accomplish a high nucleic acid yield while avoiding saturation of the extraction procedure. The integrity of DNA was checked by agarose gel electrophoresis and the amount and purity of nucleic acid were determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop, Technologies, Inc., Wilmington, DE). DNA extracts were stored at -80 °C until processed.

5. PCR amplification

All chemicals and *Taq* polymerase used for the PCR-amplification of nucleic acids were provided by Applied Biosystems (Foster City, CA). Partial 16S rRNA gene fragments including the variable V3 to V5 regions were obtained by PCR amplification of DNA templates in a 9700 GeneAmp thermal cycler (Applied Biosystems, Perkin-Elmer, CA) using the *Bacteria* universal primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') (Muyzer et al., 1993; Muyzer et al., 1995). A 44-bp GC-rich clamp sequence was added to the 5' end of primer 357F to allow further separation of PCR products by DGGE (Muyzer and Smalla, 1998). Reaction mixtures and PCR amplification conditions were applied as previously described (Cáliz et al., 2011b). PCR amplification products were checked by agarose gel electrophoresis, and those with the correct size and similar yields (~100 ng μL^{-1}) were used for DGGE analysis.

6. DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) was performed using an INGENY PhorU system (Ingeny International BV, Goes, The Netherlands). PCR products were separated in 6% (w/v) polyacrylamide gels prepared with a 35 to 75% urea-formamide vertical gradient, according to the instructions of the manufacturer (100% denaturant agent contains 7M urea and 40% deionized formamide (McCaig et al., 2001)). Electrophoresis was performed for 12 h with 1X TAE buffer at 60 °C, at a constant voltage of 160 V. Gels were stained for 30 min with SybrGold (1:10,000; Invitrogen Molecular Probes, Eugene, OR) and visualized under UV excitation. Bands of interest were chosen after detailed analysis of fingerprint images

and excised from gels. DNA from excised DGGE bands was rehydrated in 50 μL of sterile ddH_2O, eluted after incubation at 65 °C for 30 min and reamplified using 2 μL of the eluate with the PCR conditions and the corresponding primers (without GC clamp) previously described. PCR products were stored at -80 °C until sequencing.

Digital images were analysed by using the GELCompar II v.6.1 software package (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Calculation of the pair-wise similarities of densitometric profiles was based on Pearson's correlation coefficients with an optimization of 2%. Cluster analysis based on this similarity matrix was done by UPGMA (unweighted pair-group method with arithmetic averages).

7. Isolation and DNA extraction of Cr-resistant bacteria

Soil samples contaminated with 100 and 1000 mg Cr kg^{-1}, after 28 days of incubation at the same conditions described for respirometric and molecular analysis, were chosen for the isolation of resistant bacteria. Suspensions of 1 g of freshly soil from each microcosm in 100 mL of sterile Ringer's solution (Scharlab, Barcelona, Spain) were prepared in 250 mL conical flasks and dispersed by stirring for 20 min at 200 rpm. They were serially diluted and plated in triplicate on solid 10-fold diluted Luria-Bertani medium (Sigma-Aldrich GmbH, Steinheim, Germany) supplemented with K_2CrO_4 to final concentrations of 50, 100, 500 and 1000 mg Cr L^{-1}. Plates were incubated up to a week at 25 °C in the dark. Several colonies were selected from each soil microcosm according to their different morphology. Pure cultures were obtained after repetitive inoculation in fresh medium and saved for further identification and characterization.

Nucleic acids were extracted from colonies picked up directly from agar plates using Wizard™ Genomic DNA Isolation Kit (Promega, Madison, WI, USA). The isolation of DNA was performed following the manufacturer's indications. DNA was quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop, Technologies, Inc., Wilmington, DE) and stored at -80 °C until processed. Partial 16S rRNA gene fragments were amplified by PCR using the *Bacteria* universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') (Lane, 1991). Reaction mixtures and PCR amplification conditions were applied as previously described (Caliz *et al.* 2011b). PCR amplification products were checked by agarose gel electrophoresis. Products of the correct size were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and stored at -80 °C until sequencing.

8. DNA sequencing and data analysis

The PCR-amplified DNA products, obtained from the DGGE bands and the bacterial isolates, were sequenced by Macrogen Inc. (Seoul, South Korea). Primers 357F and 907R were used to obtain the PCR-products from the DGGE bands. Primers 27F and 1492R were used to sequence the 16S rRNA gene of the isolates, in combination with the universal primer Eb787F (Baker et al., 2003) as reverse and forward, respectively.

All retrieved 16S rRNA gene sequences were checked for chimera detection using the Bellerophon tool (Huber et al., 2004). Sequences were properly aligned with the online automated aligner SINA (SILVA Incremental Aligner) available at SILVA website (http://www.arb-silva.de/; Pruesse et al., 2007). Alignments were imported into the ARB software package (http://www.arb-home.de/; Ludwig et al., 2004) and loaded with the SILVA 16SrRNA-ARB-compatible database (SSURef-102, February 2010). The phylogenetic tree was constructed by maximum likelihood (RAxML) analyses using reference sequences and sequences of the isolates longer than 1,200 bp. Subsequently, the shorter sequences obtained from DGGE bands were added by applying the parsimony tool implemented in ARB, thereby maintaining the overall tree topology. The closest relative bacteria were determined according to the phylogenetic tree affiliations and sequence similarities were calculated using the ARB distance matrix tool. Partial and almost complete sequences of the 16S rRNA gene derived either from DGGE bands or bacterial isolates were deposited in GenBank.

9. Characterization of resistant bacteria

Isolates were incubated at 25 °C during one week in the medium described by Francisco et al. (2010), with slight modifications (Cáliz et al., 2011b), in order to test for their resistance to different heavy metals: Cr(VI), Cd(II), Pb(II) and As(V). The pH was adjusted to 7.5 using NaOH. To prepare solid plates, agar was added to the medium to a final concentration of 15 g L^{-1}. After autoclaving, metals were added to the media by using stock solutions of K_2CrO_4, $CdSO_4$, $PbCl_2$ or KH_2AsO_4 to obtain plates with different concentrations of each metal: 0.5, 1, 2 and 4 mM Cr(VI); 0.5 and 2 mM Cd(II); 0,2 and 1 mM Pb(II); 3 and 5 mM As(V). Resistance of the isolates was checked by qualitative observation of the colony development in polluted plates in comparison to the controls without pollutants.

The most resistant isolates were checked for their capacity to reduce the concentration from 0.5 mM Cr(VI) in 100 mL cultures with the medium described above. Experiments were conducted in Erlenmeyer flasks (500 mL) on an orbital shaker (150 rpm) at 25 °C for 48 h. Controls without inocula were set up likewise. Growth was measured at the end of the incubations by registering optical density (OD) at 600 nm. Cr(VI) concentration was analyzed from culture supernatant after the centrifugation of cells using diphenylcarbazide method (American-Public-Health-Association, 1998).

Results

1. Respirometric estimation of microbial activity

The overall state of the soil microbiota was assessed by cumulative respiration analyses. No inhibitory effects were detected at the microcosms incubated with up to 1 mg Cr kg^{-1}. Above this concentration, as higher was the spiked amount of Cr, as lower became the respirometric activity (Figure 1). Considering treatments with Cd and Pb, inhibitory effects were observed at higher concentrations than with Cr, producing a significant reduction of the oxygen consumption from 1000 mg kg^{-1} for both pollutants. Moreover, at the same added amounts of pollutants, lower inhibitions of the respirometric activities were obtained with Pb than with Cd. However, soluble concentrations of both metals were considerably lower than the initially spiked, leading to especially large amounts of unavailable compounds in the treatments with the highest concentrations of pollutants. While most of the added Cr was available (> 90% of the initial concentrations), soluble metal concentrations of Cd and Pb decreased to values below 5.8% and 0.2%, respectively, in the treatments from 10 mg kg^{-1} of each pollutant. These data indicated that respirometric results at concentrations of 288.2 mg Cd kg^{-1} and 0.330 mg Pb kg^{-1} led to similar inhibition levels than 999.7 and 9.065 mg Cr kg^{-1}, respectively. Moreover, a higher inhibition was even detected at 0.980 mg kg^{-1} of Pb than at 3.580 mg kg^{-1} of Cd.

2. DGGE assessment of microbial community composition

Changes in the microbial community composition of soil microcosms were analyzed from the comparison of DGGE fingerprints of 16S rRNA gene fragments, obtained by PCR amplification for bacterial species, to assess the effects of the pollutants and to determine the concentrations that produced different degrees of alteration.

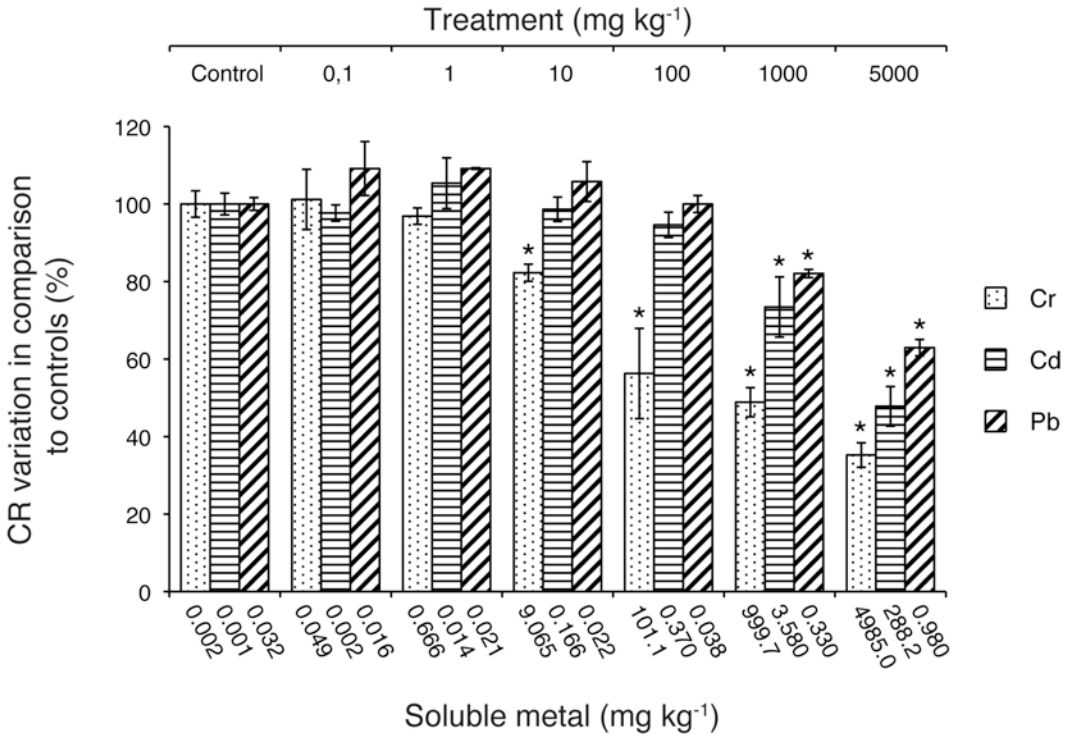


Figure 1. Figure 1. Cumulative oxygen consumption expressed as a percentage in relation to the respective controls, obtained from the Cumulative Respiration (CR) assays of the polluted microcosms after 28 days of incubation. Variation coefficient is indicated around the mean values. Soluble metal concentrations determined for each treatment are also indicated in the X-axis; standard deviations show no relevant differences among these values. (*): Significant inhibition compared to controls ($p<0.05$).

Fingerprints from Cr-amended soil samples were distributed in different groups according to similarities of the densitometric DGGE profiles (Figure 2), although most of the incubations clustered close to those from control samples, at a similarity level above 80%. The fingerprints of the non-incubated and the incubated controls were separated in different groups (group I and group III, respectively), thus indicating that several changes appeared on soil bacterial community as an effect of the incubation itself. The fingerprints of treatments at 10 and 5000 mg Cr kg^{-1}, as well as 1000 mg Cr kg^{-1} at the initial incubation stages also clustered within group I. After 14 and 28 days of incubation, the soil samples at 1000 mg Cr kg^{-1} had a different pattern that was separated by cluster analysis as group II, mainly characterized by the clear appearance of a new band (Cr12). Group IV comprised the samples amended with 100 mg Cr kg^{-1}, which fingerprints were drastically different from those of other groups, as showed by the low level of similarity

around 30%, and dominated by a new band (Cr07). Generally speaking, observable changes involved the disappearance of certain bands, probably related to pollutant sensitive bacteria, and the appearance of several new bands, pointing out the development of presumably resistant bacteria as new dominant populations. At 100 mg Cr kg^{-1}, these changes were detected after 7 days of incubation and persisted throughout the incubation period. However, the bands related to presumably resistant bacteria appeared later at the highest pollutant concentrations (as higher were the concentrations, as later the bands could be detected in the DGGE gel), indicating a slow down in the development of bacterial populations as a consequence of the toxic effects of the metals.

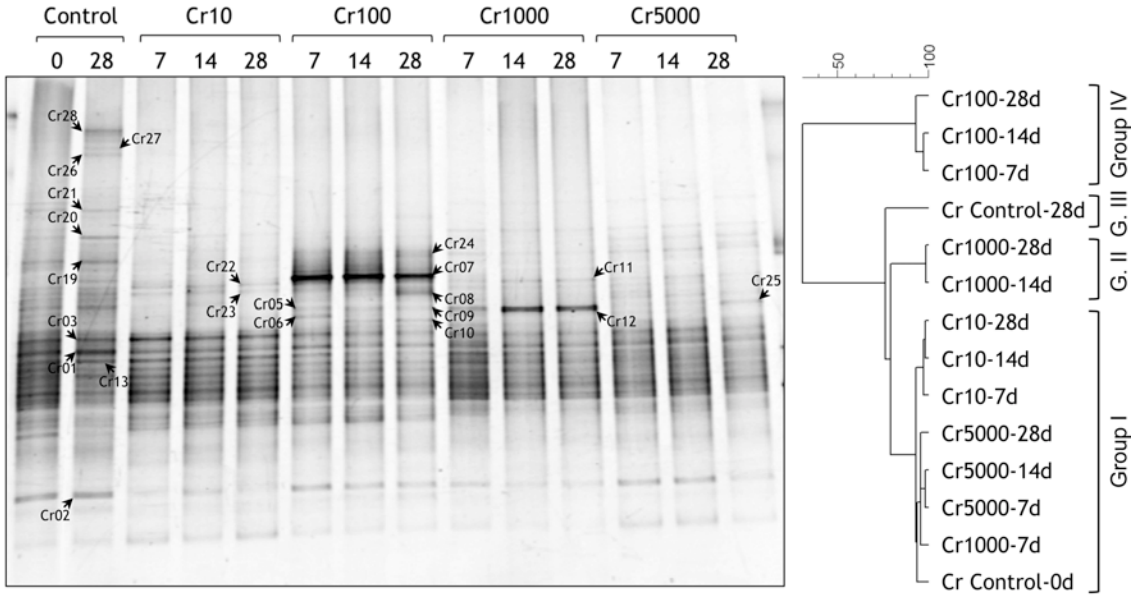


Figure 2. DGGE fingerprints of partial 16S rRNA gene fragments obtained by PCR amplification from Cr-treated soil samples using *Bacteria* universal primers. The concentration of Cr (mg kg^{-1}) and the period of incubation (in days) are indicated for each fingerprint. Arrowheads point out the bands that were excised and sequenced. Fingerprints have been grouped by cluster analysis based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method.

Considering treatments of Cd and Pb, all the samples had a high level of similarity among them (above 80%). However, several groups can be distinguished (Figure 3 and 4). As in Cr experiments, some differences could also be detected between the soil samples of the non-incubated and the incubated controls, since their fingerprints clustered separately (groups I and II, respectively). While fingerprints of treatments at 100 mg Cd kg^{-1} and 5000 mg Pb kg^{-1} were clustered close to the

respective incubated controls, the treatment at 5000 mg Cd kg^{-1} showed higher similarities to the unpolluted samples before incubation. Despite that, several bands were exclusively observed at the end of the incubation period of 5000 mg Cd kg^{-1} (Cd20, Cd31, Cd32 and Cd33), although their low intensities probably prevented the clustering analysis from differentiating them. The fingerprints of samples treated with 1000 mg Cd kg^{-1} slightly differed from those included in group I and II, but were separated by cluster analysis probably due to the presence of bands Cd16, Cd19, Cd21, Cd22 and Cd23.

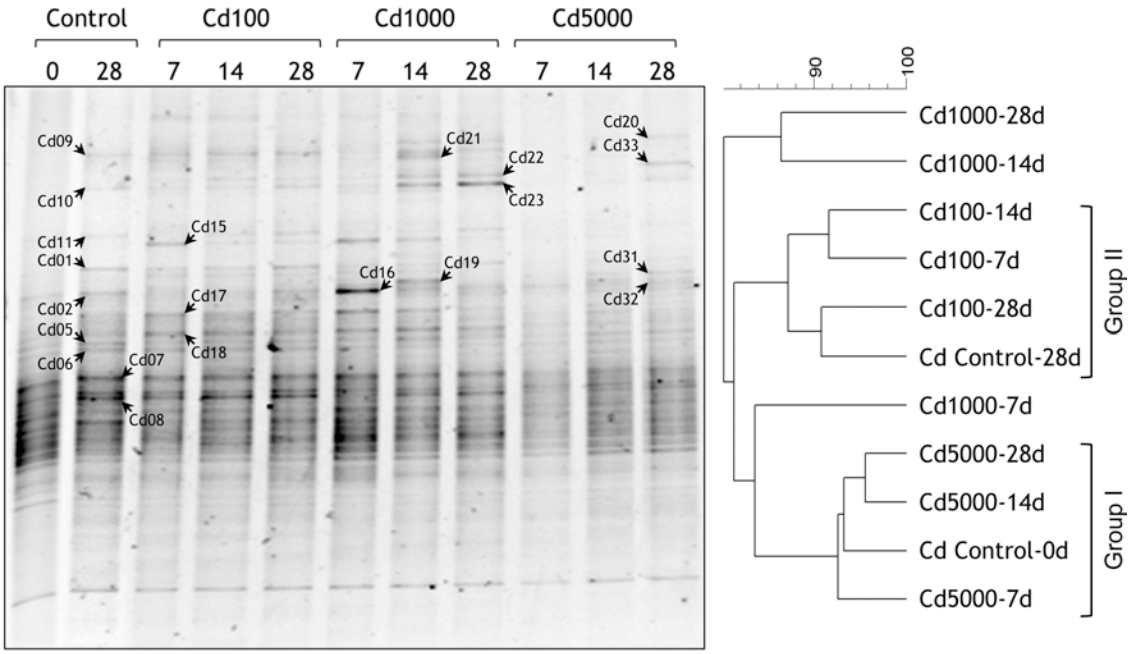


Figure 3. DGGE fingerprints of partial 16S rRNA gene fragments obtained by PCR amplification from Cd-treated soil samples using *Bacteria* universal primers. The concentration of Cd (mg kg^{-1}) and the period of incubation (in days) are indicated for each fingerprint. Arrowheads point out the bands that were excised and sequenced. Fingerprints have been grouped by cluster analysis based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method.

3. Phylogenetic identification of sensitive and resistant bacterial populations

Forty-three relevant bands were excised from DGGE gels and treated for further PCR reamplification and sequencing (Figure 2 and 3). From them, thirty-four bands produced useful sequences, without ambiguous positions, for identification purposes (Table 1).

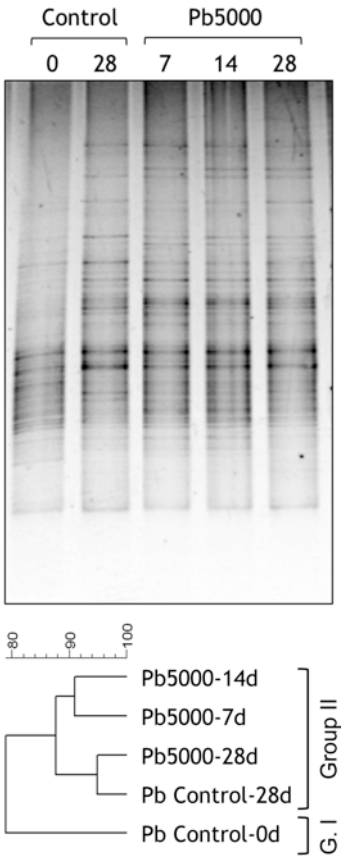


Figure 4. DGGE fingerprints of partial 16S rRNA gene fragments obtained by PCR amplification from Pb-treated soil samples using *Bacteria* universal primers. The concentration of Pb (mg kg^{-1}) and the period of incubation (in days) are indicated for each fingerprint. Fingerprints have been grouped by cluster analysis based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method.

Several bands, although present in the fingerprints of incubated control samples, were absent in all of the treatments with Cr or at high concentrations of Cd, suggesting they probably corresponded to sensitive bacteria. Sequences retrieved from these bands appeared to be closely related to *Flavisolibacter ginsengisoli* (Cr28) and uncultured members of *Cytophagaceae* (Cr26 and Cr27), *Nitrosomonadaceae* (Cr19, Cr20, Cd01 and Cd02) and *Gemmatimonadaceae* (Cr02). In all the cases, homologies with previously published sequences were higher than 98.4%.

In contrast, some bands were exclusively detected in the fingerprints of polluted soil samples and, therefore, they probably were related to resistant bacteria. In Cr-treated soils, retrieved sequences from these bands were affiliated to *Agrococcus jenensis* (Cr09, Cr12 and Cr25), *Mesorhizobium thiogangeticum* (Cr10), *Ramlibacter*

henchirensis (Cr08) and *Stenotrophomonas maltophilia* (Cr07 and Cr24). Homologies with previously published sequences were higher than 99.3%. All of them were detected at 100 mg Cr kg^{-1}. A larger intensity of the bands related to *Stenotrophomonas maltophilia* was clearly evidenced. *Agrococcus jenensis* was also detected at 1000 mg Cr kg^{-1}, corresponding to the most intense band, and even at the end of the incubation period with 5000 mg Cr kg^{-1}. In Cd-treated soils, the presence of bands that probably corresponded to resistant bacteria was less clear than in Cr treatments, due to their relatively low intensity. However, several bands were detected at 1000 and 5000 mg Cd kg^{-1}, and retrieved sequences showed high homologies (> 97.8%) to an uncultured member of the *Burkholderiaceae* (Cd19), and several members of the *Cytophagaceae* (Cd20 and Cd33), as well as *Paucimonas lemoignei* (Cd31) and *Herbaspirillum seropedicae* (Cd16 and Cd32). Moreover, several bands already present in the controls remained or even became more intense at 1000 mg Cd kg^{-1}, suggesting that they corresponded to bacteria that could also resist the moderate toxic effects of intermediate Cd concentrations: *Flavisolibacter ginsengisoli* (Cd21) and an uncultured member of *Cytophagaceae* (Cd22 and Cd23).

4. Isolation and identification of Cr-resistant bacteria

Several isolates were obtained from soil microcosms supplied with 100 and 1000 mg Cr kg^{-1}, which DGGE fingerprints provided the clearest evidences of presumable resistant bacterial populations among the tested pollutants. They were selected for identification according to the observable differences in colony morphology. Most of them showed above 98.3% similarity to sequences of cultured bacteria, according to the 16S rRNA gene sequence homology (Table 2), and distributed in three divisions: *Firmicutes* (68%), *Actinobacteria* (23%) and *Gammaproteobacteria* (9%). The broad diversity of *Firmicutes*, as well as *Actinobacteria* and *Gammaproteobacteria*, was deeply analysed in order to allow a more accurate identification of the isolates (Figure 5). Despite that most of the different isolates were affiliated into *Firmicutes*, the colonies (unequivocally detected by their singular morphological features) related to *Stenotrophomonas maltophilia* (ITCr01) and *Agrococcus jenensis* (ITCr06) had clearly a larger occurrence in the culture plates from the treatments with 100 and 1000 mg Cr kg^{-1}, respectively. Thus, the populations of these bacteria were probably the most abundant in the microcosms after these treatments, as already suggested DGGE results.

Table 1. Closest matches of 16S rRNA gene sequences obtained from DGGE gels. Band codes indicate whether the bands were recovered from Cr or Cd treatments. Closest bacterial species, sequence similarities (%) and affiliations were retrieved according to ARB phylogenetic analysis.

Bands[a]	Acc. Num.	Closest bacterial species	%[b]	bp[c]	Affiliation
		Actinobacteria			
Cr09/**12**/25	FR666796	*Agrococcus jenensis* (AM410679)	100		*Microbacteriaceae*
Cr13, Cd08	FR823419	Uncultured *Rubrobacter* sp. (EU341239)	98.4	551	*Rubrobacteraceae*
		Alphaproteobacteria			
Cr10	FR666808	*Mesorhizobium thiogangeticum* (AJ864462)	98.9		*Phyllobacteriaceae*
		Bacteroidetes			
Cr28, **Cd09**/21	FR823421	*Flavisolibacter ginsengisoli* (AB267477)	98.9	539	*Chitinophagaceae*
Cd17	FR666804	*Pontibacter akesuensis* (DQ672723)	96.7		*Cytophagaceae*
Cd20	FR666806	Uncultured bacterium (EU133668)	98.9		*Cytophagaceae*
Cd33	FR666816	Uncultured soil bacterium (AY493953)	97.8		*Cytophagaceae*
Cr26/27, Cd10/22/**23**	FR823422	Uncultured *Bacteroidetes* (EU979040)	99.6	540	*Cytophagaceae*
		Betaproteobacteria			
Cd19	FR823423	Uncultured bacterium (AB488171)	99.1	548	*Burkholderiaceae*
Cd31	FR666817	*Paucimonas lemoignei* (X92555)	100		*Burkholderiaceae*
Cr08, Cd18	FR666807	*Ramlibacter henchirensis* (AF439400)	99.3		*Comamonadaceae*
Cr19/20, **Cd01**/02	FR823420	Uncultured *Nitrosomonadaceae* (EF018627)	98.4	550	*Nitrosomonadaceae*
Cd15	FR666802	*Naxibacter varians* (AM774587)	98.7		*Oxalobacteraceae*
Cd16/32	FR666803	*Herbaspirillum seropedicae* (EU977754)	99.5		*Oxalobacteraceae*
		Firmicutes			
Cr11/22/23	FR823424	*Bacillus asahii* (AB109209)	99.5	550	*Bacillaceae*
		Gammaproteobacteria			
Cr07/24	FR666795	*Stenotrophomonas maltophilia* (AJ131907)	100		*Xanthomonadaceae*
		Gemmatimonadetes			
Cr02	FR823418	Uncultured *Gemmatimonadetes* (EF612383)	99.6	541	*Gemmatimonadaceae*

[a]A single representative band from each group, including those with identical 16S rRNA gene sequences, was used for phylogenetic identification (codes in bold).
[b]Similarity percentage. [c]Base pare length

Table 2. Phylogenetic affiliation of the bacterial isolates based on 16S rRNA gene analysis and characterization of their resistance to different heavy metals and their capacity to reduce the concentration of Cr(VI). Closest cultured bacterial species and sequence similarities (%) were retrieved according to ARB phylogenetic analysis. (nd): not determined.

Isolate[a]	Sequence acc. num.	Closest cultured bacterial species	%[b]	Soil microcosms procedence of isolates		Resistance (mM)[c]										Cr(VI) remaining (mM)[d]
						Cr(VI)				Cd(II)		Pb(II)		As(V)		
				100 mg Cr kg^{-1}	1000 mg Cr kg^{-1}	0.5	1	2	4	0.5	2	0.2	1	3	5	
		Actinobacteria														
ITCr52	FR823411	*Georgenia ferrireducens* (EU095256)	98.3	ITCr52	-	++	++	-	-	-	-	-	-	-	-	nd
ITCr06	FR823398	*Agrococcus jenensis* (AM410679)	98.3	ITCr15/24	ITCr06/17/18/21 (etc)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ITCr31	FR823406	*Microbacterium esteraromaticum* (Y17231)	98.8	-	ITCr31	+	+	-	-	-	-	-	-	-	-	nd
ITCr03	FR823397	*Arthrobacter crystallopoietes* (X80738)	99.7	ITCr03	-	++	++	++	-	++	++	++	++	+	+	0.14
ITCr08	FR823400	*Micrococcus luteus* (AF542073)	99.2	-	ITCr08	+	-	-	-	-	-	-	-	+	+	nd
		Firmicutes														
ITCr07	FR823399	*Bacillus amyloliquefaciens* (AB255669)	99.7	ITCr04/Cr16	ITCr07/09	++	++	-	-	-	-	-	-	-	-	nd
ITCr10	FR823401	*Bacillus humi* (AJ627210)	97.0	-	ITCr10	++	-	-	-	-	-	-	-	+	+	nd
ITCr14	FR823403	*Bacillus firmus* (D16268)	98.9	ITCr14/33	-	++	++	-	-	-	-	-	-	+	-	nd
ITCr27	FR823404	*Bacillus simplex* (AJ628743)	99.8	-	ITCr20/27	++	++	+	-	-	-	++	++	++	++	0.37
ITCr30	FR823405	*Bacillus licheniformis* (CP000002)	99.5	-	ITCr30/38	++	++	++	-	-	-	+	+	++	++	0.08
ITCr36	FR823407	*Bacillus thuringiensis* (D16281)	100	ITCr36	ITCr53	++	++	+	-	++	++	++	++	++	++	0.24
ITCr39	FR823408	*Bacillus mycoides* (AF155956)	99.1	-	ITCr39/42	++	++	+	-	+	+	+	+	+	+	0.33
ITCr40	FR823409	*Bacillus licheniformis* (CP000002)	99.4	ITCr51	ITCr40	++	++	++	-	-	-	++	++	++	++	0.19
ITCr43	FR823410	*Bacillus mycoides* (AF155956)	100	-	ITCr43	++	++	++	-	-	-	++	++	++	++	nd
ITCr54	FR823412	*Virgibacillus pantothenticus* (D16275)	99.5	-	ITCr54	-	-	-	-	-	-	-	-	-	-	nd
ITCr56	FR823414	*Bacillus cibi* (AY550276)	99.9	-	ITCr56	++	++	-	-	-	-	-	-	+	+	nd
ITCr61	FR823416	*Virgibacillus proomii* (AJ012667)	100	ITCr61	-	-	-	-	-	-	-	-	-	-	-	nd
ITCr63	FR823417	*Bacillus foraminis* (AJ717382)	98.4	-	ITCr63	-	-	-	-	-	-	-	-	-	-	nd
ITCr55	FR823413	*Brevibacillus laterosporus* (AB112720)	100	-	ITCr55	++	++	-	-	-	-	-	-	-	-	nd
ITCr59	FR823415	*Paenibacillus apiarius* (AB073201)	100	-	ITCr59	++	++	-	-	-	-	-	-	+	+	nd
		Gammaproteobacteria														
ITCr12	FR823402	*Enhydrobacter aerosaccus* (AJ550856)	99.8	ITCr12	ITCr22	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ITCr01	FR823396	*Stenotrophomonas maltophilia* (AJ131907)	100	ITCr01/02/11/13 (etc)	-	++	++	-	-	++	++	++	++	++	++	0.17

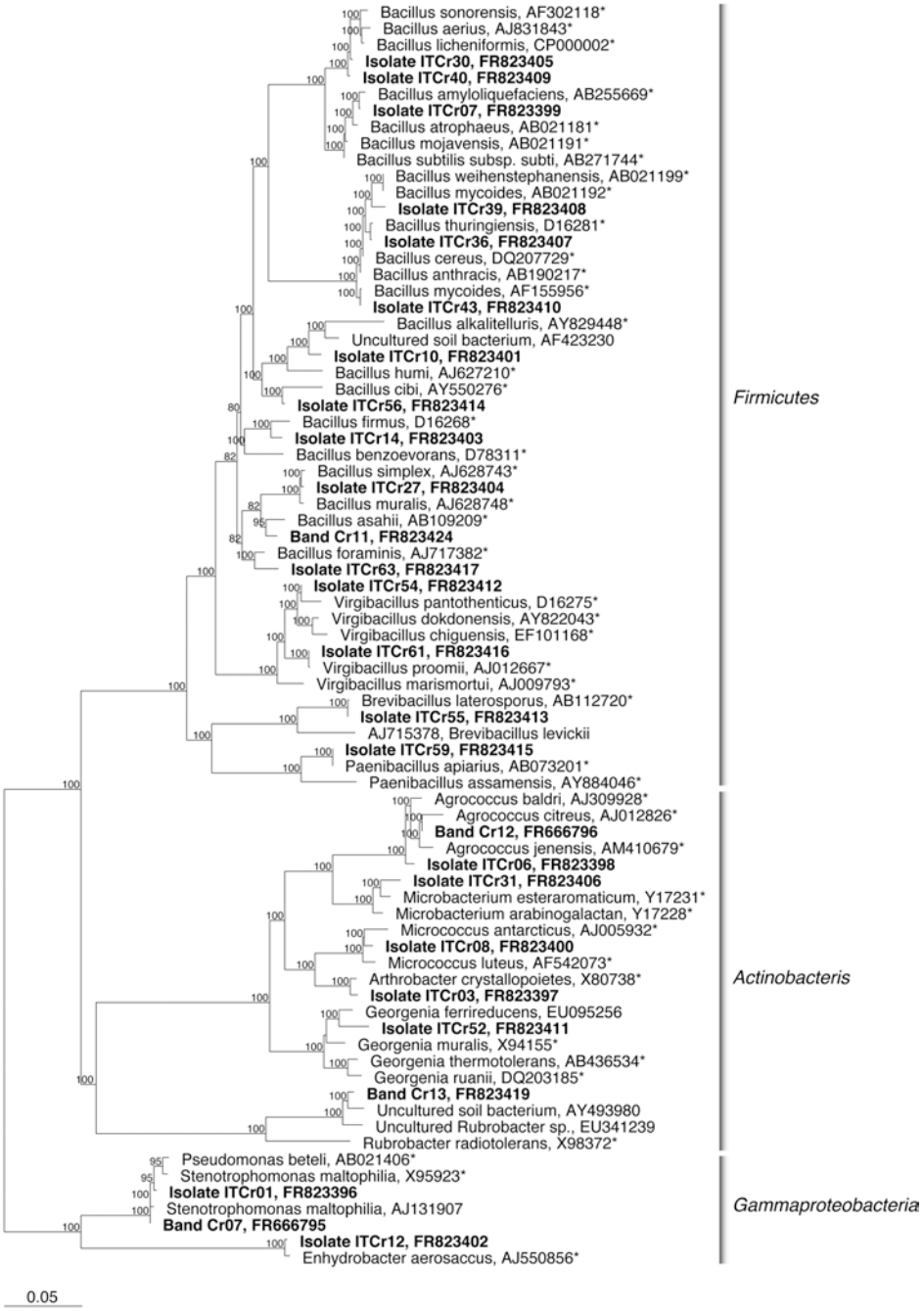
[a]A single representative isolate from each group, including those with identical 16S rRNA gene sequences, used for phylogenetic identification and characterization

[b]Similarity percentage (%). All compared sequences are longer than 1400 bp

[c]Qualitative determination according to the development of the colonies in comparison to the controls: similar (++), lower (+) and null (-)

[d]Cr(VI) remaining in the liquid cultures with minimal media from an initial concentration of 0.5 mM Cr(VI). Control experiments without inocula showed no variation in respect to the initial Cr(VI) concentration

Figure 5. Maximum likelihood phylogenetic tree calculated for 16S rRNA gene sequences of *Firmicutes*, *Actinobacteria* and *Gammaproteobacteria* obtained in this study from both DGGE bands and isolates (in boldface) and those of their closest relatives. Bootstrap values >60% are indicated at branch nodes. Representative sequences retrieved from DGGE bands are indicated as "Band", and those obtained from the isolated resistant bacteria are indicated as "Isolate". Type strain sequences included in SILVA database are marked with an asterisk (*). The scale bar indicates 5% estimated sequence divergence.



5. Characterization of metal resistance and Cr (VI) reduction by the isolates

Twenty-two different isolates, obtained from highly Cr-polluted soil microcosms, were tested in minimal media supplied with different amounts of Cr, Cd, Pb and As to estimate their resistance to these toxic metals (Table 2). A single representative for each group of isolates with identical 16S rRNA gene sequences was chosen. However, strains related to *Agrococcus jenensis* (ITCr06) and *Enhydrobacter aerosaccus* (ITCr12) could not be tested properly since they did not grow in the medium used for this test (neither in other tested minimal media). Most of the isolates grew in the presence of 0.5 and 1 mM Cr(VI), but only seven strains, mostly related to species of *Bacillus*, were able to resist up to 2 mM Cr(VI). Resistance to other heavy metals, mainly Pb and As, was found to be also achieved by these isolates. Moreover, the strain related to *Stenotrophomonas maltophilia* (ITCr01) was able to grow in the presence of all heavy metals at the tested concentrations, despite it was found to resist only up to 1 mM Cr(VI).

The closest relatives of the most resistant strains to Cr were found to be *Arthrobacter crystallopoietes* (ITCr03) and several species of *Bacillus* (ITCr27, ITCr30, ITCr36, ITCr39, ITCr40 and ITCr43). All of them, as well as the strain ITCr01 (*Stenotrophomonas maltophilia*), were tested for their capacity to reduce the concentration of Cr(VI) in liquid cultures with minimal media. However, strain ITCr43 (tentatively identified as *Bacillus mycoides*) could not be properly assessed since its growth in liquid medium was not achieved. From 0.5 mM Cr(VI), concentrations decreased to values ranging from 16% to 74% in a 48 h period (Table 2). At the end of the incubations, growth led strains ITCr03 (*Arthrobacter crystallopoietes*) and ITCr01 (*Stenotrophomonas maltophilia*) to reach higher OD values (2.0 and 0.77, respectively) than those related to *Bacillus* (around 0.3). The two former strains, as well as ITCr30 and ITCr40 (both related to *Bacillus licheniformis*) showed the highest decreasing rates of Cr(VI) concentration during the incubations.

Discussion

1. Avaluation of the effects of the metals added to the soil microcosms by both respirometric analyses and molecular methods

Since soil microorganisms constitute the main part of the biomass and regulate all nutrient cycles, they are good ecological receptors for the assessment of metal toxicity (Lazzaro et al., 2006a). In this agricultural soil, a wide overview of the influence of the different heavy metals (Cr, Cd and Pb) on the soil system has been

revealed by assessing activity and composition of the microbial community. Metal-treated soil microcosms showed significant inhibition in the respirometric activities and variations in the presence of different bacterial groups or species as a consequence of the addition of pollutants, even at low concentrations in the case of Cr.

Cr and Cd lead to significant changes in the bacterial composition especially in amended soil samples that exhibited more noticeable effects on their respirometric activity. Few variations in the DGGE fingerprints between controls and treatments at 5000 mg kg^{-1} of Cr and Cd could apparently indicate the absence of changes in the community composition. Nevertheless, GELCompar clustering analysis of band patterns (Figure 2 and 3) evidenced higher similarities of the treatments with the non-incubated controls than with the controls after incubation, and respirometric results indicated a strong inhibition in these treatments. Therefore, a lack of bacterial development throughout the incubation, probably due to a strong impairment of the microbial community by the toxic effects of metals, is suggested to have happened in these highly polluted soil microcosms, rather than the preservation of the active dominant bacterial populations (Cáliz et al., 2011a). The fingerprints of the treatment with only 10 mg Cr kg^{-1} showed higher similarities with the non-incubated control as well. Despite this observation, the microbial community of these less polluted soil microcosms can not be considered as strongly affected as in the treatment with 5000 mg Cr kg^{-1}, since respirometric activity clearly showed lower inhibition levels.

The bacterial community composition at the treatment of 5000 mg Pb kg^{-1} showed no variations respect to unpolluted soil microcosms, despite respirometric values decreased 37% in comparison to the control samples. Also treatments at 10 mg Cr kg^{-1} and 1000 mg Cd kg^{-1} showed only few significant changes on the bacterial community, despite of inhibition of their respirometric activity. This statement suggests that clear changes in microbial composition were mainly detected in soil microcosms which respirometric values were reduced more than 40% respect to the controls. However, other experiments performed with the same soil (Cáliz et al., 2011b) showed clear changes in the composition of the bacterial community when respirometric activity was reduced only 10%, as a consequence of artificial pollution with chlorophenols. In addition, respirometric activity was even stimulated in other treatments with lower chlorophenol concentrations, suggesting that the development of resistant bacteria, that were able to grow on chlorophenols, probably caused an overestimation of the activity status of the community in most treatments. Therefore, although DGGE could be more useful than respirometric

assays to detect ecotoxicological effects of organic pollutants that can be used as C sources, the latter seem to be even more sensitive than molecular techniques to show the effects of other toxic compounds, such as heavy metals.

2. The bioavailability of heavy metals in the soil as a key factor to determine their toxic effects

Quantification of the bioavailable fraction of metals in the soil microcosms was performed to further investigate on the differential behavior of the microbiota depending on the pollutant. These analyses showed that almost all the added Cr was still bioavailable in the soil, since Cr(VI) compounds are highly water-soluble and adsorb poorly to soil and organic matter, making them mobile in soil and groundwater (James and Bartlett, 1983). However, lower values of soluble Cd and Pb concentrations were detected when compared to the total added, in agreement with several studies (Echeverría et al., 1998; Covelo et al., 2007; Lazzaro et al., 2008). Shortly after addition of Cd and Pb, these cations could have been sorbed by the soil matrix, in association to organic matter (Roane et al., 2001; Broos et al., 2007), or converted to less soluble forms and remained in soil as hydroxy or carbonate precipitates (Bataillard et al., 2003), thus reducing the available fraction exposed to the microbiota. These results evidenced the capacity of soil physicochemical properties to reduce the negative impact of Cd and Pb, as already pointed out by other authors (Konopka et al., 1999; Shi et al., 2002; Lazzaro et al., 2006b). Actually, this calcareous soil must have a great potential to retain high amounts of these metals, because Pb and Cd were found to be almost unavailable, even in the treatments with their highest concentrations.

Since large metal fractions can be present in biologically unavailable forms, total concentrations of heavy metals are poor indicators of their presumable toxicity in soil environment (Lazzaro et al., 2006b). Therefore, only the soluble fraction of the added metals has been considered as an indicator of the biologically available forms (Blaser et al., 2000; Turpeinen et al., 2004; Lazzaro et al., 2006b), to analyse the effects of the different metals on the microbial community. According to this statement, it can be considered that a similar inhibition level of the respirometric activity was achieved at lower concentrations of bioavailable pollutants in Cd treatments than in Cr ones, which pointed out the former as the most damaging of both metals in this soil. In addition, Pb was able to produce a decrease in the respirometric values at even lower bioavailable concentrations than the other metals. Consequently, these results suggest that Pb is potentially the most toxic of the assayed pollutants, although the comparison among the different treatments

indicated a lower negative effect of this metal at the same amounts of added pollutants, due to its lowest bioavailability. Some studies conducted by Microtox® also revealed that the EC_{50} value for Pb is lower than the corresponding to Cr and Cd (Tarkpea et al., 1986; Sillanpää and Oikari, 1996; Villaescusa et al., 1997).

The agricultural Mediterranean soil surveyed in our study is thus considered as low sensitive to the addition of high amounts of Cd and Pb by means of keeping both metals as biologically unavailable forms. This behavior on natural soils could even be useful to avoid the widespread of pollutants through different environments by acting as a filter itself. However, the chemical equilibrium of unavailable forms of the metals could be altered by many factors, such as acid rain (Shaoping et al., 2008; Stevens et al., 2009), flooding (Hoang et al., 2008; Presley et al., 2010), fertilizer addition (Perilli et al., 2009; Santos et al., 2010), landfill wastes (Ginocchio et al., 2009), etc., enabling the pollutants to be available. Many chemical processes are involved in the transformation of trace elements in soil, but precipitation–dissolution, adsorption–desorption, and complexation are the most important processes controlling bioavailability and mobility of trace elements in soils (He et al., 2005). Variations in the available concentrations of the pollutants could lead to dramatic consequences, as suggested especially by the results derived from Pb experiments that showed significant effects in the microbiota at very low concentrations of soluble metal (less than 1 mg kg^{-1}). This statement also suggests that the toxic effects of pollutants may be unnoticed in a real contamination event due to the protection by the chemical properties of the soil itself. However, this soil should be controlled to avoid that changes in the environment could increase toxicity by the sorbed metals.

3. Resistance of bacteria to high concentrations of heavy metals

Several resistant bacteria were clearly detected by PCR-DGGE in soil microcosms polluted with intermediate to high amounts of Cr, even after only a few days of incubation, showing a fast adaptation of the microbial community to the new adverse situation by the selection and development of resistant populations. Since changes in the community composition depend on the pollutant concentration of the treatment, it seems that different tolerances to Cr of the resistant bacteria can be also key factors determining their selection during microcosms incubations. In contrast, presumable resistant bacterial populations to Cd were mainly detected in soil samples amended with the highest concentration. However, the low intensity of their respective bands in the fingerprints could indicate they had difficulties to grow in an incubation period of 28 days due to the strong toxic effects of this pollutant.

Considering the treatments with Pb, development of resistant bacteria could not be detected by PCR-DGGE, although the resistance tests confirmed their presence in the studied soil, because of the low influence that this pollutant caused in the soil microcosms.

Resistant bacteria detected by PCR-DGGE in Cr-treated soil microcosms were more widespread through different phylogenetic groups than the resistant strains isolated from the same treatments, which belonged mainly to the groups of *Actinobacteria* and *Firmicutes*. Most of the isolated bacteria could not be detected by molecular analysis, as was also found in the experiments with chlorophenols (Cáliz et al., 2011b), despite that several of them were able to grow in the presence of high concentrations of the respective pollutants in the resistance tests. The preferential identification of gram-positive bacteria by culture methods in comparison to molecular techniques has been often described (Ellis et al., 2003; Branco et al., 2005), and several reasons have been put forward to explain this phenomenon, such as PCR bias, the difficulty of lysing spores and the easiness of these bacteria to grow on plate cultures (Ellis et al., 2003). Therefore, culture-dependent and culture-independent methods must be considered as complementary approaches to detect resistant bacteria in this soil. Only the most abundant isolates in the culture plates from the treatments of 100 and 1000 mg Cr kg^{-1}, related to *Stenotrophomonas maltophilia* and *Agrococcus jenensis*, respectively, could be clearly detected by molecular approaches in soil microcosms as well. These results are in agreement with Campbell *et al.* (2009), who reported that PCR-DGGE analyses are especially useful to detect the most representative bacteria of the soil community.

Several isolates obtained from polluted soil microcosms showed low or null resistance to Cr in the performed tests. The presence of these bacteria in the polluted soils can probably be explained by the existence of microenvironments with low Cr(VI) concentrations, which can be a result of the activity of the Cr(VI)-reducing strains, enabling bacteria to stabilize according to their metal resistance ability (Branco et al., 2005) or the possibility of avoiding high heavy metal exposure within microsites of bacterial colonies or soil microaggregates (Lakzian et al., 2007). In contrast, seven isolates, distributed in different domains such as *Actinobacteria, Firmicutes* and *Gammaproteobacteria,* were able to resist high concentrations of Cr in the minimal medium supplied with glucose and also showed the capacity to reduce the concentration of Cr(VI). These results evidenced that these abilities are not exclusive of a single bacterial group, although the mechanisms they use may differ (Bruins et al., 2000), possibly reflecting horizontal genetic transfer as reported in several studies (Francisco et al., 2002; Coombs and Barkay, 2004; Becker et al., 2006;

Lakzian et al., 2007). These results also indicated that several retrieved bacteria from a previously unpolluted soil already had or acquired the capacity to reduce the concentration of Cr(VI) from the environment, thus suggesting the potential of native populations for soil restoration. In this sense, wild microbial strains, able to resist high concentrations of different potential toxic elements without previous contact with them, have already been reported (Konopka et al., 1999; Roane and Pepper, 1999; Jackson et al., 2005; Viti et al., 2006; Garavaglia et al., 2010).

The most resistant isolates to Cr were phylogenetically related to *Arthrobacter crystallopoietes* (Camargo et al., 2004) and several species of *Bacillus*, which have been widely detected in Cr-polluted environments (Camargo et al., 2005; Viti and Giovannetti, 2005) and even described to transform Cr(VI) to Cr(III) through enzymatic reduction (Camargo et al., 2003; Camargo et al., 2004; Horton et al., 2006; Abou-Shanab et al., 2007; Verma et al., 2009). Furthermore, these strains with the greatest resistance to Cr were also resistant to the other tested inorganic pollutants, in agreement to the coexistence in plasmids of the genes related to multiple resistances to heavy metals in bacteria. In this sense, Verma *et al.* (2009) recently described that the resistance to a wide array of metals and antibiotics, as well as the reduction of Cr(VI), by four different *Bacillus* species is plasmid-mediated. Strain ITCr01 (*Stenotrophomonas maltophilia*) also showed moderate resistance to Cr, as well as to high concentrations of Cd, Pb and As. This species is well known to be resistant to heavy metals (Alonso et al., 2000; Kozdrój and van Elsas, 2000; Antonioli et al., 2007; Chien et al., 2007; Pages et al., 2008), by means of different detoxification and protecting strategies such as efflux systems (Alonso et al., 2000), production of extracellular polysaccharide or Cr-reducing capacity (Morel et al., 2009). In contrast, *Agrococcus jenensis*, *Mesorhizobium thiogangeticum* and *Ramlibacter henchirensis*, which strains were found by PCR-DGGE analysis to develop at high Cr-polluted soil microcosms, have never been described as metal-resistant species.

Several bacterial strains resistant to chlorophenols were also isolated from the same soil (Cáliz et al., 2011b), and some of them were found to have 2,4,6-trichlorophenol and/or pentachlorophenol degrading abilities. Some of these strains show identical phylogenetic affiliation with isolates recovered in the present work, such as those related to *Georgenia ferrireducens* and *Bacillus mycoides,* suggesting that these isolates could resist both inorganic and organic tested pollutants. Other closely related but not identical strains, with pairwise homologies among them above 99.5%, were also obtained from treatments with the different kinds of pollutants. These isolates were related to several species of *Bacillus* and *Arthrobacter crystallopoietes*. *Stenotrophomonas maltophilia* (ITCr01), which has been only retrieved from Cr-polluted

soil microcosms, was also able to grow in the presence of high concentrations of chlorophenols (Cáliz et al., 2008). Therefore, our results pointed out the presence of multiresistant bacteria in a Mediterranean soil without previous adaptation or contact with all these pollutants, suggesting the capacity of this soil to cope with pollution of both heavy metals and chlorophenols, and thus to presume the potential of the soil and some of the isolated strains to face up to co-contamination events.

Concluding remarks

The combination of respirometric assays with molecular methods is useful to assess the effects of any disruption in the microbial community of a soil, as respirometry shows changes in a brief period of time and molecular analyses allow an accurate detection and identification of the community components subjected to alterations.

Bioavailability is a key factor controlling the effects of metal pollutants in soil, and can strongly change the effective concentrations of pollutants that have an impact on the microbial community. In this study, the intrinsic toxicity was found to be higher in Pb than Cd and Cr, but actually, Cr became the most damaging metal, followed by Cd and finally Pb, due to differential soil bioavailability. However, any change in the soil characteristics could modify it and lead to different toxic effects for each metal.

A natural soil may contain resistant bacterial strains to different pollutants, even without previous contact with them, which populations could play an important role protecting the soil itself or the surrounding environment. These microorganisms can be useful for bioremediation processes, since some strains have shown to be able to reduce the concentrations of these pollutants, and they could be especially suitable for the restoration of polluted environments with similar characteristics to this calcareous Mediterranean soil, even in multiple contamination events.

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Chapter IV

Emerging resistant microbiota from an acidic soil exposed to toxicity of Cr, Cd and Pb is influenced by the bioavailability of these metals

Abstract

The involvement of the microbial community of an acidic forest soil in relation to several heavy metals has been studied in microcosms under controlled laboratory conditions. Soil samples were artificially polluted with Cr(VI), Cd(II) or Pb(II) at concentrations ranging from 0.1 to 5000 mg kg^{-1} and incubated along 28 days. A decrease in the soil respirometric activity and changes in the microbial community composition were detected from 10/100 mg kg^{-1} Cr and 1000 mg kg^{-1} Cd and Pb. Presumable resistant bacterial and fungal populations might have developed in most of these polluted microcosms, but the microbiota was severely impaired at the experiments with the highest additions of Cr, although the acidic condition of the soil reduced the bioavailable concentrations of this metal. Results showed that bioavailability is an important factor determining the impact of the heavy metals assayed, and even a higher toxicity of Pb can be achieved if their soluble fractions are considered instead of the total added amounts. Moreover, multiresistant microorganisms were isolated from metal-polluted soil microcosms, some of them showing the capacity to reduce Cr(VI) concentrations between 54-70% of the initial value. These potentially useful strains for bioremediation were related to several species of *Streptomyces* and *Bacillus*.

Introduction

Soil environments contain diverse and complex microbial communities, with microorganisms capable to develop a huge array of environmental processes. Soil bacteria can be able to resist toxicity and perform biodegradation or transformation of most chemical pollutants, most of them from anthropogenic processes, which include inputs of trace elements through use of fertilizers, organic manures, and industrial and municipal wastes, irrigation and wet and/or dry deposits (He et al., 2005). These inputs clearly contribute to the presence of heavy metals in soil environment.

However, soil pollution may also impact soil microbial communities, affecting microbial diversity and functions, such as nutrient transformations and organic

matter mineralization. The effect of toxic compounds on soil microbiota is related to their inherent toxicity and their availability, which also depends on the physicochemical properties of the soil. Therefore, tests with microbial populations are usually included in ecotoxicity assessment of soil pollution (Eisentraeger et al., 2000; Hollender et al., 2003).

Among potentially toxic elements, Cr and Cd are widely present in polluted soils. The widespread use of Cr in the metal industries and subsequent contamination problems has led to a strong interest on this metal. Although trace quantities are required for some metabolic activities, e.g. glucose and lipid metabolism, Cr is considered to be toxic, and is designated a priority pollutant in many countries (Lloyd, 2003). Indeed, Cr(III) is considered 1000 times less mutagenic than Cr(VI) (Lloyd, 2003; and references within). Interestingly, both biotic and abiotic reduction of Cr (VI) to Cr (III) are possible (Kozuh et al., 2000). Cd is a non-essential metal, toxic at low concentrations. Cd (II) adsorption to microorganisms and organic or inorganic particles is frequent. The most important Pb source has been the addition of this metal to gasoline to improve the combustion, as well as the white paint and lead arsenate insecticides, and it is related with reduced IQ and behavioral abnormalities (Carpenter, 2001). Pb has no known beneficial effects to bacterial cells and is toxic even at low concentrations (Trajanovska et al., 1997).

The main objectives of this research were to use artificially polluted soil microcosms to assess the impact of different heavy metals (Cr, Cd and Pb) on the indigenous microbial community of a previously unpolluted Mediterranean soil. Soil respirometric assays allowed to assess the effect of pollutants on the soil microbial activity, showing the global effect on the carbon cycle. Molecular techniques were also used for analyzing the variations on the soil microbial community composition and for the detection of resistant microbiota. Isolation of metal resistant bacteria and fungi were performed from these experimental incubation procedures. Finally, several strains were tested to consider their usefulness in soil bioremediation processes from their resistance and degradation capabilities.

This research is a part of a wider study including the assessment of chlorophenolic pollutants in relation to this soil and even to another agricultural calcareous soil (Cáliz et al., 2011a; Cáliz et al., 2011b; Marti et al., 2011). This global focus also allows to analyze these results in the frame of the influence of a wider array of pollutants on different kinds of soil environments, as well as to consider the potential of the microbiota to face up to co-contamination events

*M*aterial and methods

1. Soil samplings and microcosms incubations

Experiments were performed with a pine woodland soil without any prior history of exposure to the organic pollutants assayed. The soil was collected in July 2006 from the superficial layer (A horizon) of a site located in the Mediterranean area of Vilassar de Dalt, Barcelona (UTM: 31 T 444376E 4596459N). After sampling, the soil was sieved (<2 mm) and stored by air-drying conservation method until the forthcoming experiments. This soil was classified as haplic arenosol (FAO-UNESCO, 1998). It was an acidic (pH 5.6, measured in water soil extract, proportion 1:2.5) and coarse-textured (87% sand) soil of granitic origin, with low concentrations of organic carbon (0.71%) and carbonates (0.1%). This soil type has adequate C/N ratio (10.14) to perform respirometric assays, which corresponds to the properties of standard soil proposed by the OECD for ecotoxicity testing in terrestrial environments.

After a pre-incubation at the field soil humidity (5.1% dry weight), microcosms with 50 g of soil were treated separately with 0.1, 1, 10, 100, 1000 and 5000 mg kg^{-1} Cr(VI), Cd(II) or Pb(II), and without pollutants (control). The contamination of soil microcosms was performed with aqueous solutions of dichromate (K_2CrO_4), $CdSO_4$ or $PbCl_2$. In order to perform respirometric analysis, soil samples were placed in closed reactors (500 mL) with a water content equivalent to the 60% of the soil water holding capacity and incubated in the dark at 25 °C during 28 days. All experiments were performed in triplicate. Control and polluted soil microcosms destined to molecular analysis (PCR-DGGE) were incubated likewise, but harvested and sampled destructively after 7, 14 and 28 days. Non-incubated soil samples without pollutant were used as initial controls. After each harvest, soil samples were stored at -80 °C until extraction of nucleic acids.

2. Analyses of metals in the soil solution

Soluble fractions of Cr, Cd and Pb in the soil samples were determined in triplicate from aqueous extracts (DIN, 1984), with subsequent quantification by inductively coupled plasma using optical detection (ICP-OES) for Cr and mass spectrophotometry (ICP-MS) for Cd and Pb.

3. Respirometric analysis of microbial activity

The heterotrophic aerobic microbial activity was analyzed by respirometry. Microcosms processed with this purpose were incubated in manometric respirometers as described above, which allow the determination of sample oxygen consumption (Oxitop®, WTW). The cumulative oxygen consumption (Cumulative Respiration, CR) was periodically registered throughout the incubation period. To determine the statistical significance of the differences between treated samples and controls, an ANOVA followed by Duncan's *post-hoc* test ($p<0.05$) (SPSS 16) was done.

Data-derived from respirometric assays were considered to select treatments for a throughout analysis of the changes in the microbial community composition by PCR-DGGE. All the treatments with concentrations that resulted in a significant inhibitory effect on respirometry tests and those with the highest concentration of pollutants that showed low or no inhibitory effect were analyzed by molecular methods.

4. DNA extraction

DNA was extracted from 0.5 g of soil samples using the FastDNA® SPIN Kit for Soil (Bio101, Carlsbad, USA), as described in (Cáliz et al., 2011b). Previously, the optimal amount of soil sample was experimentally determined from the relationship between the weight of processed soil samples and the concentration of extracted DNA (data not shown) to accomplish a high nucleic acid yield while avoiding saturation of the extraction procedure. The integrity of DNA was checked by agarose gel electrophoresis and the amount and purity of nucleic acid were determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop, Technologies, Inc., Wilmington, DE). DNA extracts were stored at -80 °C until processed.

5. PCR amplification

All chemicals and *Taq* polymerase used for the PCR-amplification of DNA extracts were provided by Applied Biosystems (Foster City, CA). Amplifications were done in a 9700 GeneAmp thermal cycler (Applied Biosystems, Perkin-Elmer, CA). Partial 16S rRNA gene fragments covering the variable V3 to V5 regions were obtained by PCR amplification using the *Bacteria* universal primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-

3') (Muyzer et al., 1993; Muyzer et al., 1995). Partial sequences of the internal transcriber gene spacer ITS from fungi were obtained by PCR amplification using primers ITS1-F (5'- CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS2 (5'- GCTGCGTTCTTCATCGATGC-3') (White et al., 1990). A 44-bp GC-rich clamp sequence was added to the 5' ends of primers 357F and ITS1-F to allow further separation of PCR products by DGGE (Muyzer and Smalla, 1998). Reaction mixtures and PCR amplification conditions, specific for both primers pairs, were applied as previously described (Cáliz et al., 2011a). PCR amplification products were checked by agarose gel electrophoresis, and those with the correct size and similar yields (~100 ng μL^{-1}) were used for DGGE analysis.

6. DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) was performed using an INGENY PhorU system (Ingeny International BV, Goes, The Netherlands). PCR products were separated in 6% (w/v) polyacrylamide gels prepared with a 35 to 75% and 20 to 80% urea-formamide vertical gradient for bacteria and fungi, respectively, according to the instructions of the manufacturer [100% denaturant agent contains 7M urea and 40% deionized formamide (McCaig et al., 2001)]. Electrophoresis was performed for 12 h with 1x TAE buffer at 60 °C, at a constant voltage of 160 V. Gels were stained for 30 min with 1x SybrGold (Invitrogen Molecular Probes, Eugene, OR) and visualized under UV excitation. Bands of interest were chosen after detailed analysis of fingerprint images and excised from gels for further reamplification and sequencing.

Digital images were analysed by using the GELCompar II v.6.1 software package (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Comparison between samples loaded on different DGGE gels was completed using normalized values derived from control samples common in all of them. Calculation of the pair-wise similarities of densitometric profiles was based on Pearson's correlation coefficients with an optimization of 2%. Cluster analysis based on this similarity matrix was done by UPGMA (unweighted pair-group method with arithmetic averages).

7. Isolation of metal resistant bacteria

Soil samples contaminated with 100 or 1000 mg kg^{-1} Cr, or 5000 mg kg^{-1} Cd or Pb, respectively, were chosen for the isolation of resistant bacteria after 28 days of incubation under the same conditions described above for respirometric and molecular analysis. Samples consisting of 1 g of fresh soil from each microcosm

were suspended in 100 mL of sterile Ringer's solution (Scharlab, Barcelona, Spain) in 250 mL conical flasks and dispersed by stirring for 20 min at 200 rpm. The resultant suspensions were serially diluted and plated in triplicate on solid 10-fold diluted Luria-Bertani medium (Sigma-Aldrich GmbH, Steinheim, Germany) supplemented with K_2CrO_4, $CdSO_4$ or $PbCl_2$ to final concentrations of 50, 100, 500 and 1000 mg L^{-1}. Plates were incubated up to a week at 25 °C in the dark. Several colonies were selected from each soil microcosm according to their different morphology. Most of them belonged to bacteria, and a few fungi could be isolated in culture plates, as well. Pure cultures were obtained after repetitive inoculation in fresh medium and saved for further identification and characterization.

8. Sequencing DNA from DGGE bands and isolates

DNA from excised DGGE bands was rehydrated in 50 µL of sterile ddH_2O, eluted after incubation at 65 °C for 30 min and reamplified using 2 µL of the eluate with the PCR conditions and the corresponding primers (without GC clamp) described above for bacteria and fungi. PCR products were stored at -80 °C until sequencing.

Nucleic acids were also extracted from colonies picked up directly from agar plates using Wizard™ Genomic DNA Isolation Kit (Promega, Madison, WI, USA). The isolation of DNA from bacteria and fungi was performed following the manufacturer's indications. The amount of DNA was quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop, Technologies, Inc., Wilmington, DE) and stored at -80 °C until processed. Partial 16S rRNA gene fragments were amplified by PCR using the *Bacteria* universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') (Lane, 1991). Reaction mixtures and PCR amplification conditions were applied as previously described (Cáliz et al., 2011a). Partial sequences of the internal transcriber gene spacer ITS were amplified as described above for fungi. PCR amplification products were checked by agarose gel electrophoresis. Products of the correct size were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and stored at -80 °C until sequencing.

Finally, the PCR-amplified DNA products, obtained from the DGGE bands and the isolates, were sequenced by Macrogen Inc. (Seoul, South Korea). Primers 357F and 907R were used in order to sequence the PCR-products from the DGGE bands of bacteria. Primers 27F and 1492R were used to sequence the 16S rRNA gene of the isolates, in combination with the universal primer Eb787F (Baker et al., 2003) as

reverse and forward, respectively. Both fungi-derived DGGE bands and isolates were sequenced using primers ITS1-F and ITS2.

9. Phylogenetic identification of bacteria and fungi

All bacterial- and fungal-retrieved sequences were compared for the closest relatives in NCBI database (http://www.ncbi.nlm.nih.gov/blast/) using the BLASTN algorithm tool (Altschul et al., 1990). The presence of chimera was checked using the Bellerophon tool (Huber et al., 2004). Bacterial sequences were properly aligned using the online automated aligner SINA (SILVA Incremental Aligner) available at SILVA website (http://www.arb-silva.de/; (Pruesse et al., 2007). Alignments were imported into the ARB software package (http://www.arb-home.de/; (Ludwig et al., 2004) and loaded with the SILVA 16SrRNA-ARB-compatible database (SSURef-102, February 2010). The phylogenetic tree was constructed by maximum likelihood (RAxML) analyses using reference sequences and sequences of the isolates longer than 1200 bp. Subsequently, the shorter sequences obtained from DGGE bands were added by applying the parsimony tool implemented in ARB, thereby maintaining the overall tree topology. Closest relative bacteria were identified based on the phylogenetic tree affiliations, and sequence similarities were calculated using the ARB distance matrix tool.

Sequences of the bacterial 16S rRNA and fungal ITS regions derived either from DGGE bands or from isolates were deposited in GenBank under accession numbers HE577948 to HE578011.

10. Characterization of resistant bacteria

Isolates were incubated at 25 °C during one week in the medium described by Francisco *et al.* (2010) with slight modifications (Cáliz et al., 2011b), in order to test for their resistance to different heavy metals and metalloid: Cr(VI), Cd(II), Pb(II) and As(V). The pH was adjusted to 7.5 using NaOH. To prepare solid plates, agar was added to a final concentration of 15 g L^{-1}. After autoclaving, each pollutant were added to the media by using stock solutions of K_2CrO_4, $CdSO_4$, $PbCl_2$ or KH_2AsO_4 to obtain plates with different concentrations: 0.5, 1, 2 or 4 mM Cr(VI); 0.5 or 2 mM Cd(II); 0,2 or 1 mM Pb(II); 3 or 5 mM As(V). Resistance of the isolates was checked by qualitative observation of the colony development in polluted plates in comparison to the controls without pollutants.

The most resistant isolates were checked for their capacity to reduce the concentration from 0.5 mM Cr(VI) in 100 mL cultures with the medium described above. Experiments were conducted in Erlenmeyer flasks (500 mL) on an orbital shaker (150 rpm) at 25 °C for 48 h. Controls without inocula were set up likewise. Growth was measured at the end of the incubations by registering optical density (OD) at 600 nm. Cr(VI) concentration was analyzed from culture supernatant after the centrifugation of cells using diphenylcarbazide method (American-Public-Health-Association, 1998).

Results

1. Respirometric estimation of microbial activity inhibition

The overall state of the soil microbiota was assessed by cumulative respiration analyses. No inhibitory effects were detected at the microcosms incubated with up to 1 mg Cr kg^{-1} (Figure 1), but oxygen consumption decreased at treatments with higher amounts of added Cr(VI). The highest amounts of Cr added as Cr(VI) to soil microcosms (1000 and 5000 mg kg^{-1}) resulted in strong inhibitory effects of the respirometric activity values, more than 80% inhibition in respect to the control soil values. Considering treatments with Cd and Pb, the absence of inhibitory effects was detected at higher concentrations than Cr, producing a significant reduction of the oxygen consumption only above 1000 mg kg^{-1} of both pollutants. Moreover, at the same added amount of each pollutant, a slightly lower inhibition of the respirometric activity was obtained with Pb than with Cd.

Analyses of soluble metal concentrations in soil microcosms indicated that they were considerably lower than the initially spiked amounts for all tested metals (Figure 1). In most of the treatments, bioavailable Cr decreased up to values below 20% in comparison to the amounts actually added of this metal and ranged from 60 to 80% in the treatments of 1000 and 5000 mg kg^{-1}. Moreover, almost all the added Cd and Pb was unavailable to the microbiota, recovering up to 32% and only 4.2%, respectively, in the treatments of 1000 and 5000 mg Cd kg^{-1} and 5000 mg Pb kg^{-1}. According to these data, the measured concentrations of 1.318 mg Cr kg^{-1} and 1.580 mg Pb kg^{-1} were the lowest soluble amounts of metals producing significant decreases of the respirometric values, while no effects were detected at a similar concentration of Cd (1.420 mg kg^{-1}). Moreover, considering the highest polluted treatments, a higher reduction of oxygen consumption was detected at lower soluble amounts of Cr (776.8 mg kg^{-1}) than Cd (1598.4 mg kg^{-1}).

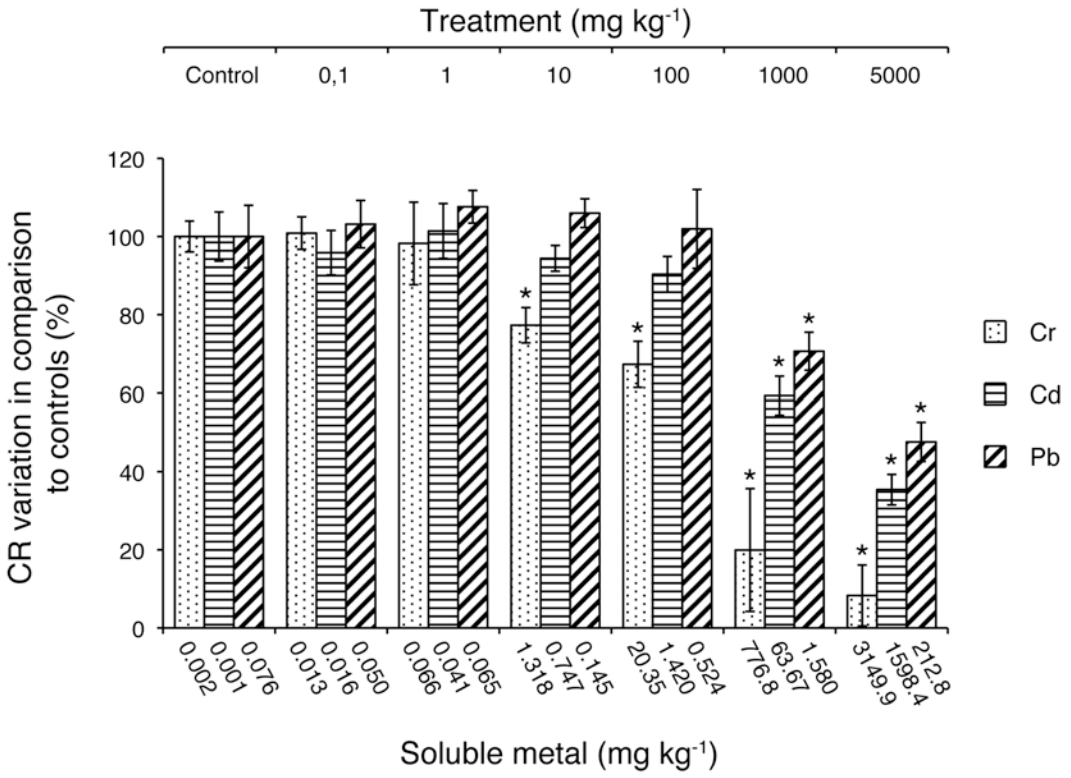

Figure 1. Cumulative oxygen consumption (expressed as a percentage in relation to the respective controls) obtained from the CR assays of the polluted microcosms after 28 days of incubation. Variation coefficient is indicated around the mean values. Soluble metal concentrations determined for each treatment are also indicated in the X-axis; standard deviations show no relevant differences among these values. (*): Significant inhibition compared to controls ($p<0.05$).

2. DGGE fingerprints of soil samples

Changes in the microbial community composition of soil microcosms were analyzed from PCR-DGGE fingerprints of 16S rRNA (*Bacteria*) and ITS (*Fungi*) gene fragments to assess the effects of the pollutants and to determine the concentrations that produced different degrees of alteration.

Taking into account bacterial fingerprints, soil samples clustered in several groups according to similarities of the densitometric DGGE profiles (Figure 2). The fingerprints of the non-incubated and incubated controls were separated in different groups (group I and group V, respectively) with more than 70% similarity within each one, thus indicating that several changes appeared on soil bacterial community

as an effect of the incubation itself. The fingerprints of treatments with low concentrations of pollutants (10 mg kg^{-1} Cr and 100 mg kg^{-1} Cd and Pb) also clustered together with the incubated control samples in group V. Soil samples amended with the highest concentrations of Cr(VI), which revealed a strong inhibition in respirometric activity, were included within group I, closely related to the non-incubated control samples.

Three additional groups (II, III and IV) could be distinguished, with changes involving the disappearance of certain bands present in the control samples, probably related to pollutant sensitive bacteria, and the appearance of a few new bands that could be related to the development, as dominant populations, of several presumably resistant bacteria. Most of these changes were detected after only 7 days of incubation and persisted throughout all the incubation period. Groups II and III, represented by samples amended with 5000 mg kg^{-1} Pb and Cd, respectively, showed the most different DGGE fingerprints.

The analysis of the molecular fingerprints of fungi led to similar results: Community composition changed along with the increased concentrations of the pollutants (Figure 3). Soil samples of non-incubated and incubated controls also clustered separately, showing the effects of the incubation. Group III included the treatments with low concentrations of all pollutants, as well as the incubated controls, with more than 80% similarity, indicating that no effects were produced at these concentrations. On the opposite, highly polluted samples showed significant differences with the controls, according to the clear appearance and disappearance of several bands. Group II, which included the Pb-treated samples, showed the highest similarities with the group containing the incubated control, while samples amended with the highest concentrations of Cr and Cd were included in group I and exhibited the most different fingerprints. No PCR products were obtained at 5000 mg Cr kg^{-1}.

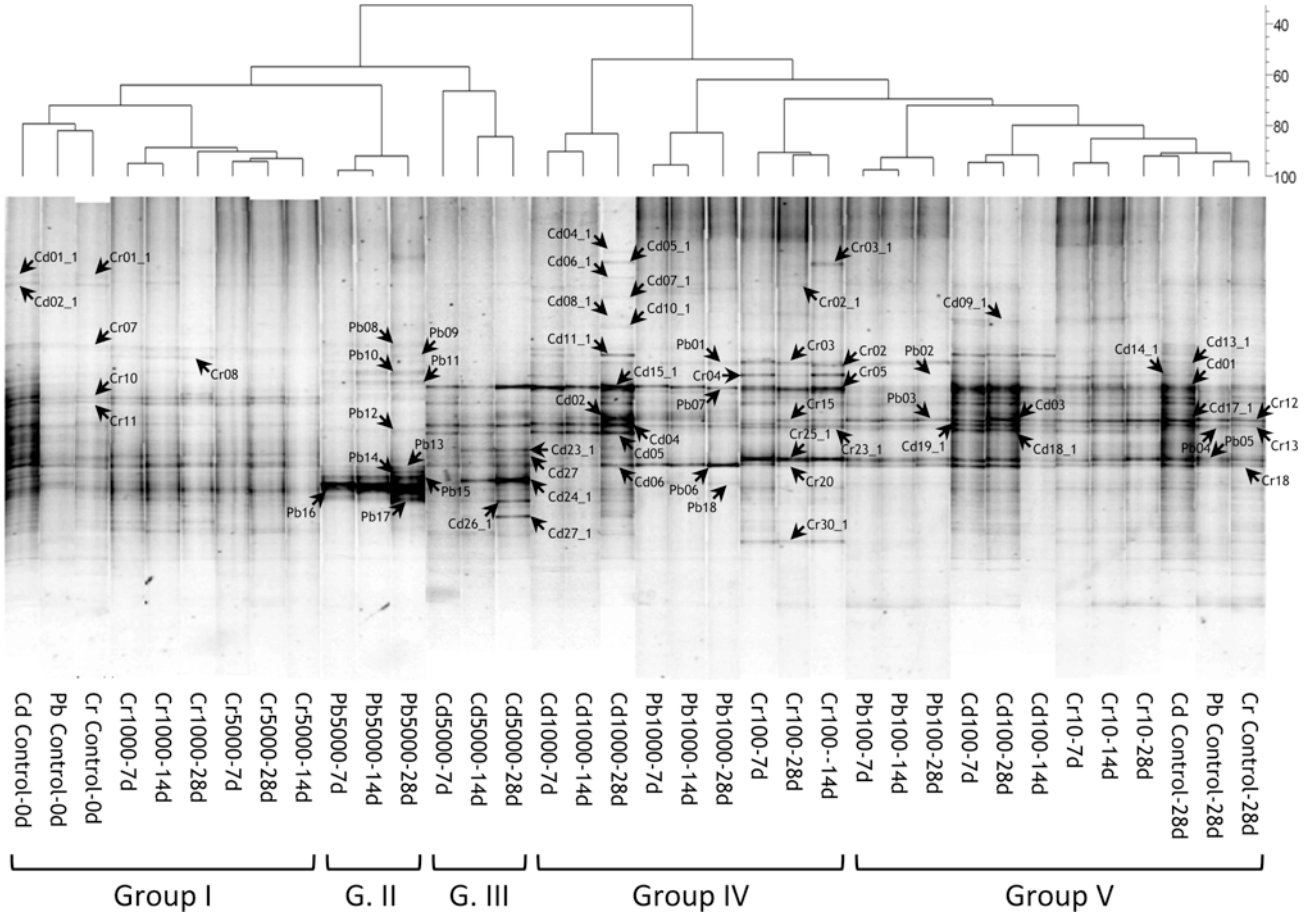

Figure 2. DGGE fingerprints of partial 16S rRNA gene fragments obtained with *Bacteria* universal primers, as they have been grouped in a dendogram by GelCompar clustering analysis. Arrowheads indicate the bands that were excised and sequenced. Bands codes indicate whether the bands were recovered from Cr, Cd or Pb treatments. The dendogram is based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method. Codes for soil samples indicate consecutively the pollutant used in the treatment, its concentration (mg kg^{-1}) and the period of incubation (in days).

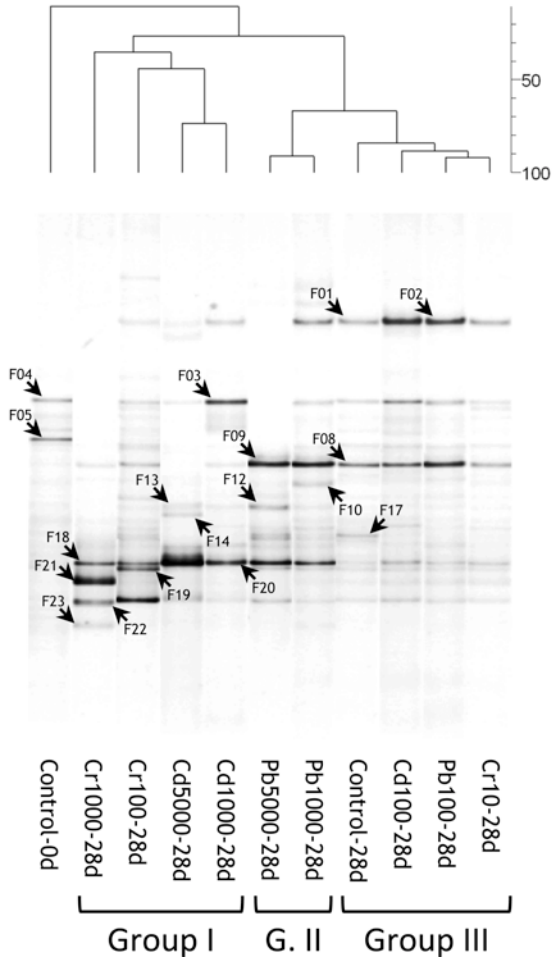

Figure 3. DGGE fingerprints of the internal transcriber gene spacer fragments obtained with ITS fungal primers, as they have been grouped in a dendogram by GelCompar clustering analysis. Arrowheads indicate the bands that were excised and sequenced. The dendogram is based on calculated pair-wise similarities of densitometric profiles (Pearson's correlation coefficients with an optimization of 2%). Grouping has been made by using a UPGMA method. Codes for soil samples indicate consecutively the pollutant used in the treatment, its concentration (mg kg^{-1}) and the period of incubation (in days).

3. Phylogenetic identification of sensitive and resistant bacterial populations

Up to 82 relevant bands were excised from bacteria- and fungi-derived DGGE gels and treated for further PCR reamplification and sequencing (Figure 2 and 3, respectively). From them, 71 bands produced useful sequences, without ambiguous positions, and were used for identification purposes (Table 1). Most sequences yielded very high similarity values with previously published sequences. Some of the DGGE bands resulted in identical sequences despite appearing in different

positions of the gel (i.e., bands Pb08/Pb15, Pb11/Pb12/Pb13/Pb14/Pb16 and Cd01/Cd09_1/Cd14_1, in Figure 2), probably due to variable melting behaviours or the presence of multiple ribosomal gene copies in a single organism (Prat et al., 2009). On the other hand, some bands appearing in different samples at the same gel position resulted in different sequences (e.g. band Cd01, Cd15.1 and Cr05, in Figure 2).

Several bands, present in the fingerprints of incubated controls, were absent in metal-treated samples that showed inhibition of the respirometric activity, suggesting they probably corresponded to pollutant sensitive bacteria. Sequences retrieved from these bands belonged to *Sphingomonas jaspsi* (Cd01, Cd09_1 and Cd14_1), *Thermomonas* sp. (Cd18_1), *Ramlibacter* sp. (Cd03, Cd17_1 and Pb03) and *Tumebacillus permanentifrigori* (Pb05). These last two phylotypes could only be considered Cd and Pb sensitive bacteria, because their related bands were still detected at 100 mg Cr kg^{-1} (bands Cr15 and Cr25_1, respectively). In all cases, homologies with previously published sequences were higher than 97.2%.

In contrast, other bands were exclusively detected in the fingerprints of polluted soil samples, probably belonging to resistant bacteria. In Cr(VI)-treated soils, retrieved sequences from these bands were related to *Geodermatophilus obscurus* (Cr30_1), *Phenylobacterium mobile* (Cr23_1), *Rhizobium leguminosarum* (Cr05), *Niastella koreensis* (Cr02_1 and Cr03_1), *Cupriavidus campinensis* (Cr02), *Massilia aerilata* (Cr04) and *Paenibacillus contaminans* (Cr08). Most of these phylotypes were only detected at 100 mg kg^{-1}, and homologies with previously published sequences were higher than 98.5%. Retrieved sequences from bands that were exclusively detected at the highest concentration of Cd were closely related to *Leifsonia kribbensis* (Cd24_1), *Actinoallomurus coprocola* (Cd27_1) and *Methylobacterium radiotolerans* (Cd23_1). Other bands that were detected at lower concentration of Cd showed high homologies (most of them above 97.5%) to several members of the *Bacteroidetes* (Cd07_1, Cd04_1, Cd05_1, Cd06_1, Cd08_1 and Cd10_1), as well as to *Burkholderia graminis* (Cd04), *Cupriavidus metallidurans* (Cd11_1) and *Frateuria aurantia* (Cd02 and Cd05). Concerning Pb, up to eleven identified bands (from Pb08 to Pb18), most of them exclusively detected at 5000 mg Pb kg^{-1}, showed high homologies (>98.7%) with species of *Streptomyces*. Phylogenetic relationships of the retrieved sequences within this group have been deeply analysed (Figure 4). Other bands, common in the fingerprints of different metal-treated samples, could be related to presumable resistant bacteria to several of the assayed pollutants. Retrieved sequences from these bands belonged to *Burkholderia caledonica* (Cd15_1 and Pb07), *Bacillus acidiceler*

(Cr03 and Pb01) and an unidentified member of the *Bacillales*, strain Gsoil 1105 (Cr20, Cd06 and Pb06).

Considering the fungal sensitive populations, sequences affiliated to *Cryptococcus terreus* (F08) and an unclassified fungus (F01) were retrieved from bands disappearing at high concentrations of Cr and Cd. In contrast, these fungi were probably resistant to Pb, since related bands (F09 and F02, respectively) were present at high concentrations of this pollutant. Other bands related to a sensitive *Zygomycete* sp. (F03 and F04) were absent at high concentrations of Cr and Pb, but this species was probably stimulated by the addition of Cd, as indicated by the intense band detected at 1000 mg Cd kg^{-1}. In all cases, homologies with previously published sequences were higher than 99.1%. Several members of the *Ascomycota* were exclusively detected at the highest concentrations of the pollutants, probably related to resistant fungi. Sequences with high homology (>99.1%) with different strains of *Trichoderma atroviride*, DAOM 233966 (F18 and F20) and NG 13 (F19), were found at these treatments with Cr, and the former was also detected in Cd- and Pb-treated samples. Two additiontal fungi, related to *Engyodontium album* (F21) and *Penicillium corylophilum* (F22), were exclusively detected at the highest concentration of Cr.

4. Isolation and identification of resistant bacteria and fungi

Several isolates were obtained from soil microcosms supplied with 100 and 1000 mg kg^{-1} of Cr, and 5000 mg kg^{-1} of Cd or Pb, which DGGE fingerprints provided evidences of presumable resistant microbial populations. They were selected according to the observable differences in colony morphology. 16S rRNA or ITS gene sequence analysis indicated that most of the isolates showed more than 98.1% similarity to sequences of cultured bacteria and fungi (Table 2). Bacterial isolates distributed mainly in two divisions, *Actinobacteria* and *Firmicutes*, but several strains obtained only from the Cd-polluted microcosm were also related to *Methylobacterium radiotolerans*. Three species of fungi, all of them clustered into the group of *Ascomycota*, were isolated mainly from Pb treatments.

Table 1. Closest matches of 16S rRNA and ITS gene sequences obtained from bacterial and fungal DGGE analyses, respectively. Band codes indicate whether the bands were recovered from Cr, Cd or Pb treatments; F indicates the bands derived from fungal analysis. Closest bacterial species, sequence similarities (%) and affiliations were retrieved according to ARB phylogenetic analysis. Identification of fungal species was performed by comparing sequences for the closest relative in NCBI database using the BLASTN search tool.

Bands[a]	Closest bacterial species	%[b]	Affiliation
	Bacteria		
	Actinobacteria		
Cr30_1	*Geodermatophilus obscurus* (X92355)	98.7	*Geodermatophilaceae*
Cd24_1	*Leifsonia kribbensis* (EF466129)	99.8	*Microbacteriaceae*
Pb10	*Streptomyces psammoticus* (AB184554)	99.8	*Streptomycetaceae*
Pb08/**15**	*Streptomyces tubercidicus* (AB184304)	98.7	*Streptomycetaceae*
Pb09	*Streptomyces tubercidicus* (AB184304)	99.4	*Streptomycetaceae*
Pb11/12/13/**14**/16	*Streptomyces tubercidicus* (AB184304)	98.8	*Streptomycetaceae*
Pb17	*Streptomyces tubercidicus* (AB184304)	98.9	*Streptomycetaceae*
Pb18	*Streptomyces ciscaucasicus* (AB184208)	99.6	*Streptomycetaceae*
Cd27_1	*Actinoallomurus coprocola* (AB364579)	99.4	*Thermomonosporaceae*
	Alphaproteobacteria		
Cr10	*Bradyrhizobium elkanii (U35000)*	100	*Bradyrhizobiaceae*
Cr11	*Bradyrhizobium elkanii (U35000)*	99.8	*Bradyrhizobiaceae*
Cr23_1	*Phenylobacterium mobile* (AY035307)	99.4	*Caulobacteraceae*
Cd23_1	*Methylobacterium radiotolerans (*AB175640)	100	*Methylobacteriaceae*
Cr05	*Rhizobium leguminosarum* (AM181757)	99.2	*Rhizobiaceae*
Cd01/09_1/14_1	*Sphingomonas jaspsi (*AB264131)	97.2	*Sphingomonadaceae*
	Bacteroidetes		
Cr01_1	*Niastella koreensis (*DQ244077)	97.2	*Chitinophagaceae*
Cr02_1	*Niastella koreensis (*DQ244077)	99.1	*Chitinophagaceae*
Cr03_1	*Niastella koreensis (*DQ244077)	96.9	*Chitinophagaceae*
Cd01_1	*Niastella koreensis (*DQ244077)	96.3	*Chitinophagaceae*
Cd07_1	*Niastella koreensis (*DQ244077)	99.6	*Chitinophagaceae*
Cd04_1	Uncultured *bacterium (*EF516948)	95.7	*Chitinophagaceae*
Cd05_1	*Segetibacter* sp. 6424S-61 (GQ421847)	98.0	*Chitinophagaceae*
Cd06_1	*Chitinophaga pinensis* DSM 2588 (CP001699)	100	*Chitinophagaceae*
Cd08_1	*Mucilaginibacter oryzae* (EU109722)	97.6	*Sphingobacteriaceae*
Cd10_1	*Sphingoterrabacterium koreensis* (AB267721)	98.3	*Sphingobacteriaceae*
	Betaproteobacteria		
Cd04	*Burkholderia graminis* (U96939)	99.3	*Burkholderiaceae*
Cd11_1	*Cupriavidus metallidurans* (Y10824)	99.2	*Burkholderiaceae*
Cd15_1, Pb07	*Burkholderia caledonica* (AF215704)	100	*Burkholderiaceae*
Cr02	*Cupriavidus campinensis* (AF312020)	99.3	*Burkholderiaceae*
Cr12/15, Cd03/17_1, Pb03	*Ramlibacter* sp. HTCC332 (AY429716)	98.5	*Comamonadaceae*
Cr07	Uncultured *Nitrosomonadaceae* (EF018627)	97.6	*Nitrosomonadaceae*
Cr04	*Massilia aerilata* (EF688526)	99.6	*Oxalobacteraceae*
	Firmicutes		
Cr25_1, **Pb05**	*Tumebacillus permanentifrigori* (DQ444975)	99.3	*Bacillaceae*
Cr03, Pb01	*Bacillus acidiceler* (DQ374637)	99.8	*Bacillaceae*
Cr18/20, Cd06, **Pb06**	*Bacillales bacterium Gsoil 1105* (AB245375)	100	*Bacillaceae*
Cr08	*Paenibacillus contaminans* (EF626690)	97.8	*Paenibacillaceae*
	Gammaproteobacteria		
Cd02	*Frateuria aurantia* (AB091194)	99.3	*Xanthomonadaceae*
Cd05	*Frateuria aurantia* (AB091194)	97.5	*Xanthomonadaceae*
Cd18_1	Uncultured *Thermomonas* sp. (EU202907)	97.4	*Xanthomonadaceae*
	Fungi		
	Ascomycota		
F17	Uncultured *Ascomycota* C31 H07 (EU490091)	97.6	*Ascomycota*
F21	*Engyodontium album* MC A31 (HQ115665)	97.1	*Mitosporic Ascomycota*
F22	*Penicillium corylophilum* G9 (GU566277)	98.3	*Eurotiomycetes*
F18, **F20**	*Trichoderma atroviride* DAOM 233966 (EU280133)	99.1	*Sordariomycetes*
F19	*Trichoderma atroviride* NG 13 (HQ115671)	100	*Sordariomycetes*
F12	*Fusarium oxysporum* Fo15 (GU724514)	100	*Sordariomycetes*
	Basidiomycota		
F05	*Clavulina cf. cinerea* BIO 10296 (EU862214)	99.6	*Agaricomycetes*
F08, **F09**	*Cryptococcus terreus* CBS 1895 (AF444319)	100	*Tremellomycetes*
F10	*Cryptococcus aerius* CBS 4192 (AF444376)	100	*Tremellomycetes*
	Unclassified		
F03, F04	*Zygomycete* sp. AM-2008a (EU428773)	100	*Fungi incertae sedis*
F01, **F02**	Uncultured fungus F66N0BQ01AGX0G (HQ125130)	99.1	Unclassified

[a] A single representative band from each group, including those with identical 16S rRNA or ITS gene sequences, used for identification (codes in bold).
[b] Similarity percentage.
[c] Base pair length.

Figure 4. Maximum likelihood phylogenetic tree calculated for 16S rRNA gene sequences of *Actinobacteria* obtained in this study from both DGGE bands and isolates (in boldface) and those of their closest relatives. Bootstrap values >60% are indicated at branch nodes. Representative sequences retrieved from DGGE bands are indicated as "Band", and those obtained from the isolated resistant bacteria are indicated as "Isolate". Type strain sequences included in SILVA database are marked with an asterisk (*). The scale bar indicates 5% estimated sequence divergence.

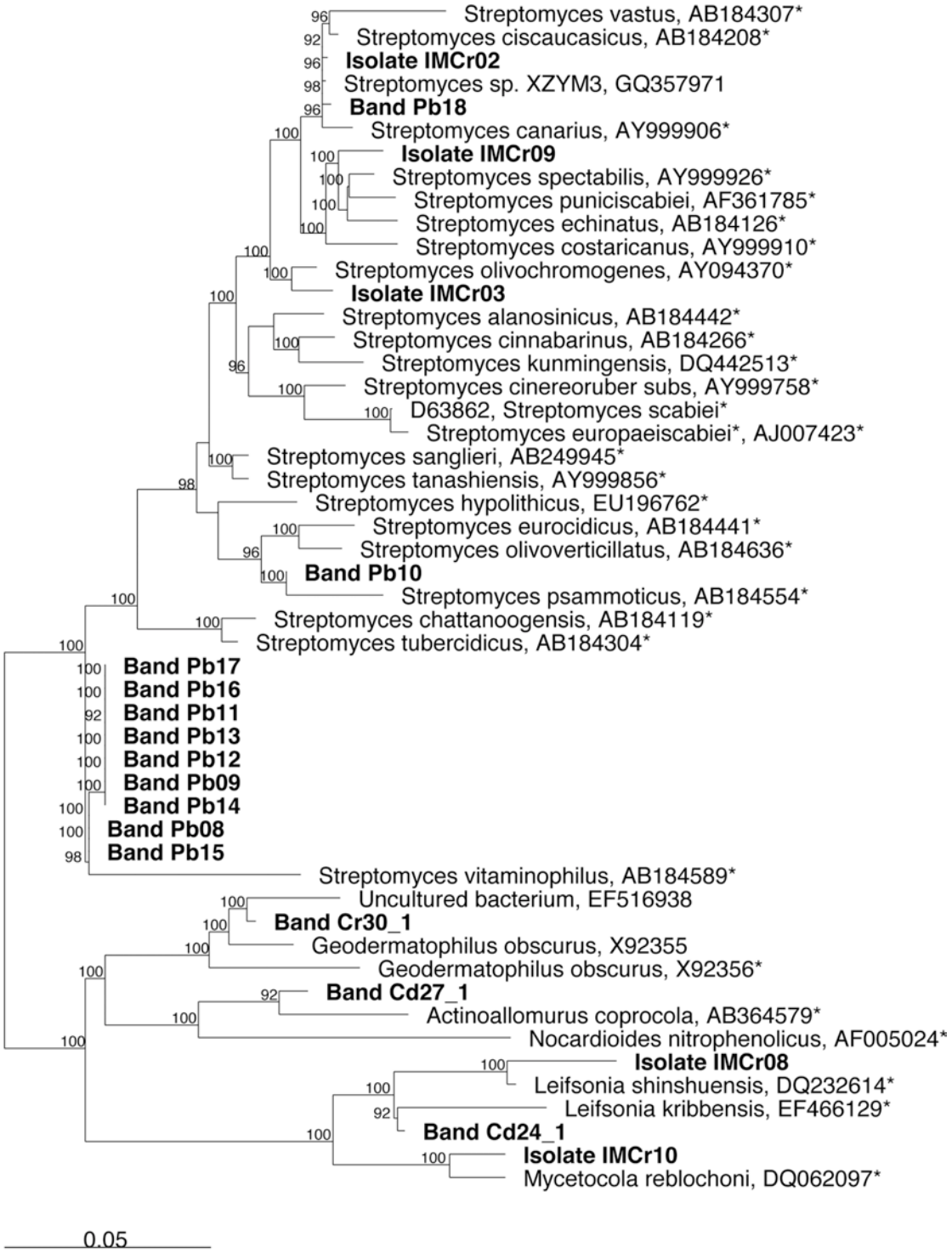

Table 2. Phylogenetic affiliation of bacterial and fungal isolates based on 16S rRNA and ITS gene analysis and characterization of their resistance to the tested metals and metaloids, as well as their capacity to reduce the concentration of Cr(VI). Isolate codes indicate the soil microcosms procedence: ITCr, ITCd or ITPb for microcosms polluted with Cr, Cd or Pb, respectively. Closest cultured bacterial species and sequence similarities (%) were retrieved according to ARB phylogenetic analysis. Identification of fungal species was performed by comparing sequences for the closest relative in NCBI database using the BLASTN search tool. (nd): not determined

Isolate[a]	Closest cultured bacterial species	%[b]	Isolates procedence in soil microcosms[c]				Resistance (mM)[d]										Cr(VI)[e]
							Cr(VI)				Cd(II)		Pb(II)		As(III)		
			Cr-100	Cr-1000	Cd-5000	Pb-5000	0.5	1	2	4	0.5	2	0.2	1	3	5	
	Bacteria																
	Actinobacteria																
IMCr08	*Leifsonia shinshuensis* (DQ232614)	100	IMCr08	-	IMCd02/09	-	++	++	-	-	-	-	++	-	-	-	nd
IMCr10	*Mycetocola reblochoni* (DQ062097)	98.1	-	IMCr10/12	-	-	++	++	-	-	-	-	++	-	-	-	nd
IMCr02	*Streptomyces ciscaucasicus* (AB184208)	99.7	IMCr02	-	-	IMPb02	++	++	++	+	-	-	++	++	-	-	0.23
IMCr03	*Streptomyces olivochromogenes* (AY094370)	99.4	IMCr03/04	-	-	-	++	++	++	+	-	-	++	++	-	-	0.23
IMCr09	*Streptomyces spectabilis* (AY999926)	98.8	-	IMCr09	-	-	++	++	++	-	-	-	++	++	-	-	0.20
	Alphaproteobacteria																
MCd01	*Methylobacterium radiotolerans* (AB175640)	100	-	-	IMCd01/06/07	-	-	-	-	-	++	++	++	++	-	-	nd
	Firmicutes																
IMCr01	*Bacillus thuringiensis* (D16281)	100	IMCr01	-	-	-	++	++	++	-	++	-	++	++	-	-	0.15
IMCr07	*Bacillus weihenstephanensis* (AB021199)	100	IMCr07	-	-	IMPb01	++	++	+	-	+	-	++	++	++	-	nd
IMCr11	*Bacillus acidiceler* (DQ374637)	100	IMCr05/06	IMCr11	-	-	++	++	-	-	-	-	++	++	-	-	nd
IMCd03	*Bacillus simplex* (AJ628743)	99.5	-	-	IMCd03	-	++	++	+	-	-	-	++	++	++	++	0.20
IMCd04	*Brevibacillus brevis* (D78457)	99.4	-	-	IMCd04	IMPb03/05	++	++	-	-	-	-	++	-	-	-	nd
	Fungi																
	Ascomycota																
IMPb04	*Trichoderma atroviride* DAOM 233966 (EU280133)	100	-	-	-	IMPb04	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IMPb06	*Penicillium corylophilum* G9 (GU566277)	98.3	-	-	-	IMPb06	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IMCd05	*Lecythophora hoffmannii* N40 (FJ903377)	100	-	-	IMCd05	IMPb07	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a]A single representative isolate from each group, including those with identical 16S rRNA and ITS gene sequences, used for phylogenetic identification and characterization.

[b]Similarity percentage (%). Compared sequences are longer than 1400 bp for bacterial isolates, and between 200 and 300 bp for fungal isolates.

[c]The corresponding concentrations of the pollutants assayed are expressed as mg kg^{-1}.

[d]Qualitative appreciation for development of the colonies in comparison to the controls: similar (++), lower (+) and null (-).

[e]Cr(VI) remaining in the liquid cultures with minimal media from an initial concentration of 0.5 mM Cr(VI). Control experiments without inocula showed no variation in respect to the initial Cr(VI) concentration

5. Characterization of metal resistance and Cr (VI) reduction by the isolates

Bacterial isolates were tested in minimal media supplied with different amounts of Cr, Cd, Pb and As to assess their resistance to these toxic metals (Table 2). A single representative for each group of isolates with identical 16S rRNA gene sequences was chosen. Most of the isolates grew in the presence of 1-2 mM Cr(VI), but only *Streptomyces* strains (IMCr02 and IMCr03) were able to withstand 4 mM Cr(VI). Resistance to Pb was usually achieved by most of the isolates, but only a few strains could grow in the presence of Cd or As. The strain IMCd01 (*Methylobacterium radiotolerans*) showed the highest resistance to Cd, although it was unable to tolerate any of the assayed concentrations for Cr and As. Only a few strains, all of them related to species of *Bacillus* (IMCr01, IMCr07 and IMCd03), could withstand most of the tested metals.

The most resistant strains to Cr, belonging to species of *Streptomyces* (IMCr02, IMCr03, IMCr09) and *Bacillus* (IMCr01, IMCr07 and IMCd03), were also tested for their capacity to reduce the concentration of added Cr(VI) in liquid cultures with minimal media. However, the strain IMCr07 (*Bacillus weihenstephanensis*) could not be properly assessed, since its culture in liquid medium could not be achieved. All strains were able to reduce the concentration of Cr(VI), in a 48h period, between 54% and 70% from an initial concentration of 0.5 mM Cr(VI) (Table 2). At the end of the incubations, growth led to similar OD values for *Bacillus* strains (around 0.55). OD values for *Streptomyces* strains were not determined since their cells were grouped in aggregates, but their growth was clearly observed in the liquid cultures.

Discussion

1. Evaluation of the effects of the metals added to the soil microcosms by both respirometric analyses and molecular methods

Since soil microorganisms constitute the main part of the biomass and regulate all nutrient cycles, they are good ecological receptors for the assessment of metal toxicity (Lazzaro et al., 2006). Microorganisms assessed by various physiological, biochemical or molecular techniques, have been recommended as biological indicators of metal contamination (He et al., 2005). In the forest soil surveyed in this study, an overview of the influence of the different heavy metals (Cr, Cd and Pb) on the soil system has been revealed by assessing activity and composition of the microbial community. Fungal populations have been considered, besides bacteria,

since the soil assessed is clearly acidic and its climatic origin includes a long dry period, factors favoring the development of these microorganisms (Reith et al., 2002; Hobbie and Gough, 2004; Kimura and Asakawa, 2006). Despite that, DGGE fingerprints of the control samples evidenced a lower diversity of the soil fungal community composition in comparison to bacteria, as previously reported by Fierer *et al.* (2005) and Wang *et al.* (Wang et al., 2010).

Metal-treated soil microcosms showed inhibition in the respirometric activity and variations in the community composition of both bacterial and fungal groups. According to the clustering analysis, significant changes in the bacterial and fungal populations were detected above the same added amounts for each metal (from 100 mg kg^{-1} Cr and 1000 mg kg^{-1} Cd and Pb), thus indicating that sensitive microorganisms of these groups had similar pollutant tolerances. As higher was the inhibition of the activity, as lower the similarities in the fingerprints between polluted soil samples and incubated controls. Thus, detrimental effects on the microbial community increased with the pollutant concentration, linking the level of toxicity with the added amount of each metal (Speir et al., 1995).

The most noticeable and drastic effects on the microbial community were detected at the treatments of 1000 and 5000 mg Cr kg^{-1}, which led to a strong inhibition of the respirometric activity (more than 80% in comparison to unpolluted samples) and even of microbial development, as suggested by the molecular analysis. DGGE fingerprints of these samples clustered with non-incubated controls in group I (Figure 2), indicating the lack of significant changes in bacterial community composition throughout the incubation with these high concentrations of Cr. Similar results were obtained in other experiments with the same soil spiked with other kinds of pollutants (Cáliz et al., 2011a). A strong impairment of the fungal populations in soil samples amended with 5000 mg Cr kg^{-1} was also suggested by the negative PCR amplification of fungal ITS gene fragments, which probably indicated even a damage of the DNA of the dead fungi.

Comparison between both methods shows that a significant decrease in the respirometric activity values corresponded to changes in the DGGE fingerprints, in respect to the incubated controls. The only exception was the treatment with 10 mg Cr kg^{-1}, which showed no changes in the microbial community composition but inhibition in the respirometric activity. Clear variations in the microbial community were detected, however, only at treatments with the highest metal concentrations, in agreement to the most drastic decreases in the activity, as well. In contrast, other experiments performed with the same soil (Cáliz et al., 2011a) showed that changes

in the microbial composition of several chlorophenol-treated samples were detected even in treatments producing unnoticeable alterations in the cumulative respiration. Respirometric assays could be more suitable and specific to detect ecotoxicological effects of heavy metals than organic pollutants, due to the overestimation of the activity status caused by chlorophenol-degrading microorganisms, as previously reported in similar experiments with a calcareous soil (Cáliz et al., 2011b)

2. The bioavailability of heavy metals in the soil as a key factor to determine their toxic effects

The mobility and availability of trace elements in soils are controlled by many chemical and biochemical processes, such as precipitation-dissolution, adsorption-desorption, complexation-dissociation and oxidation-reduction (He et al., 2005), which are affected by pH and biological activity. In general, free cationic metal species are more bioavailable at acidic pH values (Hughes and Poole, 1991; Smith, 1994; Antoniadis et al., 2008), since they may not be sorbed by binding sites of the soil matrix, clay materials or organic matter, and therefore the interactions between metals and potential metal-complexing ligands are limited. Moreover, under basic conditions, cations tend to form unavailable hydroxy- or carbonate-metal complexes (Bataillard et al., 2003). According to these statements, Cd and Pb were sparingly available in our acidic soil microcosms when low amounts of these cations were spiked and high amounts were to be added to significantly increase their presence in the soluble fraction. Despite that, the bioavailability of Pb was largely the lowest in comparison to the other tested pollutants, confirming the difficulty of this metal to remain in the soluble fraction (Echeverría et al., 1998; Covelo et al., 2007). In contrast, the bioavailability of Cr is faintly influenced by the interactions with the soil matrix, since it is an anionic species on its Cr(VI) ionic form, but its mobility is related to the oxidation state. Cr was added to soil microcosms in its oxidized form Cr(VI), which is the most toxic and water-soluble (James and Bartlett, 1983). However, at acidic pH values, the chemical reduction reactions favoured the predominance of Cr(III), whose features limit its bioavailability and mobility (Richard and Bourg, 1991), thus explaining the decrease of soluble Cr detected in comparison to the initial amounts spiked to soil microcosms. These results agree with the capacity of the soil physicochemical properties to reduce the potentially higher negative impact of Cr on the microbiota.

Since large metal fractions can be present in biologically unavailable forms, total concentrations of heavy metals are poor indicators of their presumable toxicity in soil environment (Lazzaro et al., 2006). Therefore, only the soluble fraction of the

added metals can be considered as an indicator of the potentially toxic forms (Blaser et al., 2000; Turpeinen et al., 2004; Lazzaro et al., 2006) to analyse the effects of the different metals on the microbial community. According to this statement, similar inhibition levels of the respirometric activity were achieved at low and similar concentrations of bioavailable Cr and Pb, while no effects were detected considering similar amounts of Cd. Consequently, these results suggest that Pb is potentially the most toxic metal in this soil, together with Cr, although the comparison among the different treatments indicated a lower negative effect of this metal at the same amounts of added pollutants, in agreement with its lowest bioavailability.

3. Resistance of bacteria and fungi to high concentrations of heavy metals

Soil microcosms exposed to Cr, Cd or Pb showed a fast response of the microbial community to the new adverse situations by selection and development of supposedly resistant populations. In addition, most of the isolates obtained from these artificially polluted soil microcosms were also confirmed as resistant to several of the pollutants assayed. Thus, these results prove the survival of several metal resistant microorganisms in the surveyed soil, even without any prior exposure to these toxic compounds, in agreement with similar studies (Diaz-Ravina and Baath, 1996; Lazzaro et al., 2008).

While most of the fungi detected by the molecular approach at highly polluted soil microcosms were restricted to *Ascomycota* lineage, as already expected (Vadkertiová and Sláviková, 2006; Zafar et al., 2007), presumable resistant bacteria were found to be widespread among several phylogenetic groups. Moreover, only a few different species of fungi were detected in these treated-soil samples, in contrast to the diversity of identified bacteria. These differences may be explained by the low complexity of the natural fungal composition of the soil itself. The greater diversity of presumable resistant bacterial community inhabiting this soil may confer a better adaptability and flexibility in comparison to the fungal assemblage, and thus, a higher potential to survive in more diverse events of contamination or perturbation. In the case of Cr-treated soil microcosms, however, some fungi were found to develop in highly polluted treatments (1000 mg Cr kg^{-1}), while no bacteria were detected, as showed by the PCR-DGGE analysis, suggesting a higher efficiency of members of the fungal group to cope with high concentrations of Cr (Khan and Scullion, 2000). In contrast, several isolated bacteria from these polluted microcosms showed high resistance to Cr in the tests performed. Therefore, resistant bacteria were probably present at too low abundances to be detected by the

molecular methods, but longer incubations could evidence their later emergence. Similar studies also suggested a slow down in the development of resistant microorganisms, especially at high concentrations of pollutants, which only became detectable after long incubation periods (Cáliz et al., 2011a; Cáliz et al., 2011b).

Isolation procedures allowed recovering mainly gram-positive bacteria from metal-treated soil microcosms, in agreement to several previous studies (Viti and Giovannetti, 2001; Branco et al., 2005; Viti and Giovannetti, 2005). Some of the isolates showed identical affiliation, or were closely related, to most representative phylotypes identified from DGGE fingerprints. Most of the resistant fungi detected by the molecular analysis were obtained in pure cultures, thus showing a good correlation between culture-dependent and culture-independent approaches.

Some bacterial and fungal microorganisms could be detected either in soil samples amended with any of the pollutants assayed. They were related to *Burkholderia caledonica* (Cd15_1 and Pb07), *Bacillus acidiceler* (Cr03 and Pb01), an unidentified member of the *Bacillales* (Cr20, Cd06 and Pb06) and *Trichoderma atroviride* (F18 and F20). Moreover, most of the isolates tested to assess their resistance to the heavy metals (Cr, Cd and Pb) and a metalloid (As) showed the capacity to grow in the presence of several of these toxic elements, in agreement to the coexistence in plasmids of the genes usually related to multiple resistances.

The most resistant isolates to Cr were found to reduce more than 50% from an initial concentration of 0.5 mM Cr(VI) in 48h period, which indicated their potential for the soil restoration. Different species of *Bacillus* are well known as Cr-resistant, able to transform Cr(VI) to Cr(III) through enzymatic reduction (Camargo et al., 2003; Verma et al., 2009), but this ability is less known in the genus *Streptomyces* (Poopal and Laxman, 2009; Polti et al., 2010). Moreover, a wide diversity of phylotypes related to *Streptomyces* were also detected by the molecular analysis at highly polluted soil microcosms with Pb, pointing out that members of this group successfully coped with different kinds of toxic metals.

Phylotypes affiliated to *Leifsonia kribbensis* (Cd24_1), *Actinoallomurus coprocola* (Cd27_1) and *Methylobacterium radiotolerans* (Cd23_1) were exclusively detected in treated soil microcosms at the highest added amounts of Cd. The later could also be isolated in pure cultures and showed the highest resistance to Cd in the tests performed, in agreement with the presence of genes coding for proteins of heavy metal efflux pumps that can be detected in genomic sequences of *Methylobacterium radiotolerans* and other species of this genus. Moreover, several identical strains related to *Leifsonia shinshuensis* were isolated from Cr- and Cd-polluted soil

microcosms, and even a member of this genus had already found to reduce Cr(VI) to Cr(III) (Puzon et al., 2008).

Several bacterial strains, which were previously isolated from the same soil, were also found to be resistant to chlorophenols (Cáliz et al., 2011a), and some of them were found to have 2,4,6-trichlorophenol and/or pentachlorophenol degrading abilities. Some of these strains show identical phylogenetic affiliation with isolates retrieved in the present work, such as those related to *B. thuringiensis*, *B. weihenstephanensis* and *B. acidiceler*, suggesting that these isolates could resist both inorganic and organic tested pollutants. Two species of *Burkholderia* (*B. graminis* and *B. caledonica*) were detected by PCR-DGGE analyses in most of the chlorophenol-treated soil microcosms and in the presence of Cd. Moreover, identical fungi were also found to develop in different soil microcosms amended with heavy metals and with chlorophenols, such as *Engyodontium album*, *Penicillium corylophilum* and the isolated strains related to *Trichoderma atroviride*. Therefore, these results pointed out the presence of multiresistant bacteria in this Mediterranean soil, without previous adaptation or contact with all these pollutants. This finding suggests the capacity of this soil to cope with pollution of both heavy metals and chlorophenols, and thus presumes the potential of the soil and some of the isolated strains to face up to co-contamination events.

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General discussion

1. Evaluation of soil microcosms by both respirometric analyses and molecular methods

Two methodologies were used to assess the influence of the pollutants on the microbial community of previously unpolluted Mediterranean soils. The first approach, based on respirometric measurements, has shown the effects of pollutants on microbial activity. The cumulative soil respiration, commonly used as an estimation of the aerobic activity of the heterotrophic microbiota in toxicity assessments (Strotmann et al., 2004; Plaza et al., 2005; Montserrat et al., 2006; Martí et al., 2007; Coello et al., 2009), is a useful technique to evaluate the overall soil state. The second approach, based on a PCR-DGGE analysis that is widely used in soil microbiology (Edenborn and Sexstone, 2007; Campbell et al., 2009), has revealed its capability to infer changes in the microbial community composition of the experimental soil microcosms. Both, the respirometric techniques and the molecular approach, showed a significant effect of the pollutants on the soil microbial communities, thus indicating that not only the activity but also the abundance of several microbial groups were influenced by the added contaminants.

The presence of pollutants produced a decline in the activity of the heterotrophic microbiota, while showing significant changes in the composition of the soil microbial community. Observable changes involved the disappearance of certain DGGE bands, probably related to pollutant sensitive microbiota, and the appearance of several new bands, pointing out the development of presumably resistant microbiota as new dominant populations. At intermediate pollutant concentrations, these changes were rapidly detected and persisted throughout the incubation period. However, at the highest pollutant concentrations, the new bands appeared later in the incubation period, thus indicating a lag phase for the adaptation of the resistant microbiota due to the toxic effects of the compounds. Moreover, some of the pollutants led to drastic effects in the microbial community at these concentrations, which implied strong inhibition of the respirometric activity and even of microbial development, as suggested by the molecular analysis. DGGE fingerprints of soil samples after incubation with these pollutants presented higher similarities with the non-incubated controls than with the controls after incubation, indicating the lack of significant changes in the microbial community composition throughout the incubation.

The presence of MCP and TCP at an intermediate concentration (100 mg kg^{-1}) in the microcosms of the soil CRe led to an increase on the respirometric activity in

comparison to controls, thus indicating stimulation of the activity (Chapter I). This increase was probably due to the biodegradation of these pollutants, as supported by the presence of strains with biodegrading capabilities that were isolated from the same soil. Chaudri et al. (2000) suggested that resistant microbial populations from PCP-amended soil samples proliferated by metabolizing the organic pollutant, as well as the carbon liberated by the death of the sensitive populations. CP concentrations in other soil microcosms would be too low to evidence the stimulation of the activity due to insuficient amounts of extra C, as had been already observed by Chaudri et al. (2000). As an opposite situation, high concentrations of CP did not show this increase on the activity (Chapters I and II), probably because the concentrations were high enough to produce strong inhibition effects on the microbiota. However, an overestimation of the activity status probably occurred in some of these high-polluted microcosms, as the results suggested. Experiments with CP showed a clear impact on the microbial community composition at significant but low inhibition of the respirometric activity (Chapter I), or even with unnoticeable effects on the activity in comparison to controls (Chapter II). In contrast, in the experiments performed with heavy metals, clear changes in the microbial community were only detected when the respirometric activity was highly inhibited, and some treatments did not show any variation in the composition of the microbiota despite of significant decreases in the activity values (Chapters III and IV). These results indicate that DGGE could be more useful than respirometric assays to detect ecotoxicological effects of organic pollutants that can be used as C sources, but the latter seem to be more sensitive than molecular techniques to show the effects of other toxic compounds, such as heavy metals. Therefore, an overestimation of the activity status should be taken into account in ecotoxicity assessments of organic pollutants, which is concerning for the establishment of pollutant concentrations producing toxic effects. Both methodologies are thus helpful and complementary approaches for ecotoxicological assessments.

The influence of pollutants on the microbial community composition was analysed by PCR-DGGE of extracted DNA and RNA from soil microcosms. Targeting of DNA may indicate changes in relative abundance of all the microbiota, while RNA is assumed to better reflect the metabolically active microorganisms (Nogales et al., 2001; Girvan et al., 2003). Results from both molecular approaches, however, have only been shown for the experiments with CP in the soil CRe (Chapter I) because, unfortunately, DGGE analysis of extracted RNA from samples of the soil HAr led to incoherent data. Most representative phylotypes detected in the CRe soil microcosms were identified by both DNA- and RNA-derived data, in agreement with the experiments reported by Mahmood et al. (2005) and Nogales et al. (2001),

who also found a good correspondence of both approaches. Nevertheless, a few phylotypes were detected by only one of the molecular approaches. Phylotypes detected only by DNA-DGGE analysis could be indicative of quiescent bacteria present in the soil microcosms, since active cells usually contain higher numbers of ribosomes than quiescent cells (Nogales et al., 1999). The contrary situation, exclusively evidenced when new bacterial populations initiate development, might be explained because targeting RNA specially increases sensitivity (Nogales et al., 2001). A controversial finding was evidenced when considering the phylotype affiliated to *Bacillus asahii*. The bands related to this phylotype disappeared in DNA-DGGE fingerprints of samples with high CP concentrations but were repeatedly detected at most of these samples in RNA analysis. These results could indicate that *B. asahii* was a metabolically active microorganism in CP polluted soil microcosms. However, the isolated strain that was also affiliated to *B. asahii* showed no evidence of CP resistance by performed tests. Unless some kind of artefact arose from the RNA approach, the detection of ribosomal RNA located in spores of *B. asahii* could provide a reasonable explanation for this phenomenon. The high toxicity of added CP to soil microcosms could have favoured the spore formation of *B. asahii*, a naturally occurring bacterium of this soil. While most bacteria died, the proportion of ribosomal RNA targets of *B. asahii* increased respect to those of other non-sporulating bacteria, thus allowing a clear detection of this phylotype at the highest concentration of CP only by RNA analysis.

2. Toxicity of CP and heavy metals depending on soil characteristics

Since soil microorganisms constitute the main part of the biomass and regulate all nutrient cycles, they are good ecological receptors to evaluate the toxicity of pollutants (Lazzaro et al., 2006). In ecotoxicological tests, in which the pollutants are assayed by adding known concentrations, the effective amount of pollutants may be very different from the nominal concentration, thus inducing underestimation of toxicity. The toxicity of pollutants depends on the soil, which has particular physicochemical properties conditioning the bioavailability of pollutants and microbial populations with specific sensitivities. In the Mediterranean soils surveyed in this study, a wide overview of the influence of different CP (MCP, TCP and PCP) and heavy metals (Cr, Cd and Pb) on the soil system have been revealed by assessing activity and composition of the microbial community. Data obtained from the respirometric techniques and the molecular approach, as well as from the analysis of available amounts of pollutants, has been used to determine the effects of the pollutants and the concentrations producing different degrees of alteration.

2.1. Assessment of CP toxicity (Chapters I and II)

Considering both Mediterranean soils and the properties of CP, some considerations have to be done. Both soils have pH values below the pKa of MCP, so the pollutant remains mainly in the non-ionized form. According to the Henderson Hasselbach equation, theoretically about <1% and 20% of the pollutant will be ionized, respectively, in Arenosol and Regosol. This is a volatile form, with high vapor pressure and favorable Henry's Law constant value to be volatilized. It has to be taken into account that only the molecular form of an ionizable compound has the physicochemical properties that are attributed to it, whereas the ionized fraction becomes a different molecule, with ionic behaviour and different physicochemical properties. Volatilization is faster in soil HAr, which has coarse texture, than in soil CRe, allowing the gas diffusion processes. Moreover, the organic matter type and content in soil CRe, as well as the different biological activity (shown by the controls in the respiration test) and the presence of fine mineral fractions, contribute to the sorption processes competing against solubilization and volatilization compared to soil HAr (Fingler et al., 2004; Banks and Schwab, 2006). TCP is less volatile than MCP and has a nearly neutral pKa, thus, most TCP will be non-ionized in soil HAr (<4% ionization approximately), in a quite hydrophobic, volatile and less soluble form (Fingler et al., 2004). However, the same compound will be mostly ionized (more soluble and less volatile) in CRe (theoretical ionization about 90% of the compound). The volatility of PCP was negligible in both soils (Martí et al., 2011), as predicted from its physicochemical properties. The pKa of this compound allows it to be in the ionized and more soluble form (Fingler et al., 2004), especially in the alkaline soil CRe (~90 and 99% in HAr and CRe, respectively). Therefore, the effective concentration of PCP in both soils should be similar to the initially spiked considering its volatility. Regarding the soil sorption processes related with these pollutants, different theoretical possibilities could be found depending on the soil properties (Shimizu et al., 1992; Fröbe et al., 1994; DiVincenzo and Sparks, 2001; He et al., 2006; Pu and Cutright, 2006; Martí et al., 2011). The molecular form of MCP, which is predominant in both soils, can be sorbed by hydrophobic partitioning in the soil organic matter (as in the case of soil CRe with mature humus type) or it can react with the soil organic matter if its maturity degree is low and some reactivity is still present (as in soil HAr occurs). TCP sorption mechanism may be similar to MCP in soil HAr (due to the predominance of the undissociated form), but the sorption by means of ionic exchange processes can be produced in soil CRe, especially Ca-mediated binding to the organomineral complex. PCP sorption may occur by the same mechanisms; in this case, the compound may be almost completely dissociated in soil CRe, so a

reduced partitioning in the organic matter is likely to occur, whereas cationic exchange (mediated by Ca cation bridges) and solubilization may be important points to take into account.

The cumulative respiration showed an initial toxic effect from 1000 mg MCP kg^{-1}, which was stronger in HAr than in CRe. Moreover, molecular analysis showed that resistant bacterial populations appeared later in soil HAr, and even a lack of development at the highest pollutant concentrations, probably indicating the higher toxicity of MCP at this soil as well. The different impact of MCP would be explained because of its higher availability in CRe than in HAr, since volatilization was lower in the former soil and higher extractable amounts were available, especially water-soluble (Martí et al., 2011). Thus, the low intrinsic toxicity of MCP allowed part of the microbial populations of the soil CRe to adapt and to degrade this available compound, as indicated the slight stimulation of the activity at 100 mg MCP kg^{-1}. Biodegradation of MCP in polluted soils has already been reported by Lallai and Mura (2004). In contrast, the higher volatility of MCP in soil HAr could produce an immediate toxic effect throughout the soil matrix, which is favoured by the soil texture, promoting the gas diffusion. Considering the experiments with TCP, this compound produced inhibition of the respirometric activity and changes in the composition of the microbial community from 100 mg kg^{-1} in HAr and 1000 mg kg^{-1} in CRe. The higher toxicity of TCP in soil HAr was probably due to its lower adsorption (low clay, organic matter and calcium content) and, thus, this compound could produce toxic effects at lower concentrations. Moreover, the higher volatility of TCP in HAr could also produce an immediate toxic effect throughout the soil matrix, as mentioned for MCP. The lower volatility and higher bioavailability of TCP in soil CRe allowed the microbiota to easily access the molecule and degrade it in a greater degree, as indicated the stimulation of the activity at 100 mg TCP kg^{-1}. The impact of PCP on the microbiota was evidenced from 100 mg kg^{-1} in both soils, but the inhibition of the respirometric activity was clearly stronger in CRe at higher PCP concentrations. The later development of the microbial populations also evidenced the higher toxic effects of PCP in soil CRe. Because PCP strongly adsorbs to soil matrix, particularly under acidic conditions (Kenaga, 1980; van Gestel and Ma, 1988; Mahmood et al., 2005), its lower bioavailability in soil HAr could be responsible for these results, by limiting the effective concentration of pollutant that can reach the microorganisms. The lower decrease of the respirometric activity values observed in soil HAr at the highest PCP concentrations could be due to the development of presumably resistant bacteria, evidenced by PCR-DGGE results, which could maintain the respiration levels while sensitive microbiota remained inactive. Even though PCP is the least soluble of the

compounds assayed, it was still sufficiently available to generate high toxicity levels, since it is minimally volatile.

2.2. Assessment of heavy metals toxicity (Chapters III and IV)

Many chemical and biochemical processes, such as precipitation-dissolution, adsorption-desorption, complexation-dissociation and oxidation-reduction, control the mobility and availability of trace elements. These processes are affected by soil properties and biological activity (He et al., 2005). This study has analysed two types of pollutants that behave in a different way when they are in soils: cationic (Cd and Pb) and anionic (Cr) species. Cd and Pb may form very insoluble compounds (hydroxides/carbonates) in the soil. Moreover, cations are adsorbable in the soil cation exchange matrix, clay materials, and organic matter. Thus, when added to the soil CRe, Cd and Pb tended to remain sorbed to the soil solid matrix or blocked by different mechanisms, due to its alkaline pH value and calcareous nature, with higher cation exchange capacity and exchangeable calcium compared to the soil HAr. Moreover, metals can be driven into the soil solution not only by solubilisation as separated ions, according to the chemical equilibriums established with the soil components (Bataillard et al., 2003), but also by means of the formation of soluble complexes with the organic matter, which depends on the type of organic matter present in the soil (Shi et al., 2002; Bataillard et al., 2003; Vig et al., 2003). The soil HAr has a high degree of soluble organic matter due to its humus type and calcium and carbonate contents are comparatively low. All these properties, together with its acidic pH value, make it easy to maintain soluble forms of Cd and Pb. According to the Pourbaix diagrams (Takeno, 2005), Pb can be found as Pb(II) when soil pH is lower than 6 (soil HAr) and as hydroxide when pH is over 6 (soil CRe). In contrast, Cd is found as Cd(II) in the pH range of both soils (below pH 9). Therefore, Pb availability is clearly different in both soils, whereas Cd may be available. Considering Cr, this element was added to soils as an anion, which tends to remain as an anionic soluble species in either alkaline, neutral or acidic media at both acidic and alkaline pH values (Zayed and Terry, 2003). Furthermore, Cr undergoes chemical and biological mediated reduction processes. Chemical reduction to Cr(III) and eventual precipitation as Cr_2O_3 can easily occur at acidic pH values, when soluble oxidable organic matter is available (Kozuh et al., 2000; Bolan et al., 2003; Banks et al., 2006). These conditions favour Cr(VI) reduction in HAr, but this process is also possible in CRe. The soluble metal concentrations measured in the samples showed that the proportion of soluble forms of Cr was considerably higher in the soil CRe, in agreement with the considerations above.

Since the reduced forms of Cr are less soluble and also less toxic (Shi et al., 2002), the availability of Cr(VI) conditions its toxicity, which depends on the soil properties.

According to the added amounts of heavy metals to soil microcosms, Cr impacted on the microbial populations of both soils at lower initial concentrations than Cd and Pb: 10 mg kg^{-1} Cr and 1000 mg kg^{-1} Cd and Pb. These effects were probably due to the highest bioavailability of the anionic species that occurred in these soils. However, the concentration of pollutants that produced ecotoxicological effects changed when considering the soluble metal concentrations. According to the bioavailable amounts of metals, Pb showed effects at lower concentrations than the other metals in CRe, while Cr and Pb did in HAr. Therefore, these results suggest that Pb is potentially the most toxic of the assayed pollutants when considering effective concentrations, in agreement with several studies conducted by Microtox® which revealed lower EC_{50} values for Pb than the for Cr and Cd (Tarkpea et al., 1986; Sillanpää and Oikari, 1996; Villaescusa et al., 1997).

3. Resistant microbiota to high concentrations of CP and heavy metals

The development of presumably resistant microbial populations was evidenced by PCR-DGGE in both Mediterranean soils amended with high concentrations of CP (Chapters I and II) or heavy metals (Chapters III and IV). Bacteria were affiliated to several phylogenetic groups such as *Firmicutes*, *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Betaproteobacteria*, while fungi mainly belonged to *Ascomycota*. The molecular approach also indicated the development of some of these microorganisms in treatments with different pollutants. It is the case of bacteria related to *Pseudomonas putida* and *Lysobacter gummosus*, or uncultured *Sphingomonadales* and *Burkholderiaceae*, which were able to develop at highly polluted microcosms with TCP and PCP in the soil CRe. In HAr, bacteria related to *Bacillus acidiceler* and an unidentified member of the *Bacillales* (strain Gsoil 1105) were found in the presence of all the heavy metals assayed, whereas different bacteria and fungi (related to *Burkholderia caledonica*, *Burkholderia graminis*, *Engyodontium album* and two different strains of *Trichoderma atroviride*) could resist most of both inorganic and organic tested pollutants.

Culture techniques allowed to recover most representative and presumably resistant microorganisms that were detected by PCR-DGGE analysis in highly polluted soil microcosms. However, other isolated microorganisms that were not detected by the molecular approach, mainly belonging to *Actinobacteria* and *Firmicutes*, were also

obtained from contaminated soils. Although culture methods usually favoured the identification of gram-positive bacteria, in comparison to molecular techniques (Viti and Giovannetti, 2001; Ellis et al., 2003; Branco et al., 2005; Viti and Giovannetti, 2005), both methodologies have appeared as complementary approaches to detect the resistant microbiota of both Mediterranean soils. Several isolates were confirmed as especially resistant to high pollutant concentrations by the performed tests. Some of these strains (related to *Georgenia ferrireducens*, *Stenotrophomonas maltophilia* and several species of *Bacillus*) were found to resist both organic and inorganic pollutants.

These results prove the survival of several microorganisms in the presence of different pollutants without a previous adaptation or contact with them, in agreement with previous studies (Andreoni et al., 1998; Konopka et al., 1999; Roane and Pepper, 1999; McGrath and Singleton, 2000; Jackson et al., 2005; Mahmood et al., 2005; Viti et al., 2006). Moreover, they point out the presence of multiresistant microbiota in two different Mediterranean soils, suggesting the capacity of previously unpolluted soils to cope with both CP and heavy metals, and thus to presume the potential of these soils and some of the isolated strains to face up to co-contamination events.

Resistant fungi were detected in the acidic soil HAr (Chapters II and IV), but no evidences of its development were found in the calcareous soil CRe (DGGE fingerprints from fungal analysis of this soil are not included because of the lack of relevant bands). Moreover, resistant bacteria were specifically detected for each soil environment since no common species were evidenced between both soils. For example, members of the genus *Pseudomonas* or *Burkholderia*, which are widely known as pollutant resistant bacteria (Kiyohara et al., 1992; Radehaus and Schmidt, 1992; Männistö et al., 1999; Kharoune et al., 2002; Yang et al., 2006; Webb et al., 2010), were exclusively developed in CRe or in HAr, respectively. Therefore, the emerging resistant microbiota was shown to be the best suited to each environment, probably in agreement with the soil characteristics and the growing requirements that favoured the development of such microorganisms. These findings point out the importance to consider both the microbial features and the soil characteristics before designing bioremediation strategies that use bioaugmentation, in order to achieve a good performance of the process.

4. Potentially useful strains for bioremediation

The use of microorganisms for bioaugmentation requires culturable specific strains that must be able to perform high pollutant degradation activity in particular contaminated sites (Mera and Iwasaki, 2007). However, these easily culturable microorganisms may have a low adaptability to soils with different characteristics than the environments from where they were isolated (Pu and Cutright, 2007). Therefore, a wide array of degraders, expressing different features in distinct environments, may be useful to develop bioaugmentation and bioremediation strategies for different polluted sites. For this purpose, the most resistant bacteria that were isolated from both kinds of Mediterranean soils were tested to assess their usefulness in bioremediation processes.

The isolates most resistant to Cr showed the capacity to reduce Cr(VI) concentrations up to 84% of the initial value (Chapters III and IV). Several of them were affiliated to genera (or species) that had already been described to have this capacity, such as *Bacillus* (Camargo et al., 2003; Verma et al., 2009), *Arthrobacter* (Camargo et al., 2004; Horton et al., 2006) and *Stenotrophomonas* (Morel et al., 2009). However, the strains with the highest resistance to Cr were related to different species of *Streptomyces*, a genus less known by this capacity. *Streptomyces* is the largest genus of *Actinobacteria*, which are predominantly found in soil environments, and thus they could be useful microorganisms in soil bioremediation processes.

The isolates most resistant to CP were found to reduce the concentrations of TCP or PCP in a different degree, several of them up to 30-35% of the initial value (Chapters I and II). Some of them were affiliated to known genera of CP degraders such as *Pseudomonas* (Radehaus and Schmidt, 1992; Resnick and Chapman, 1994), *Burkholderia* (Kharoune et al., 2002; Webb et al., 2010) and *Kocuria* (Karn et al., 2010). The strains related to *Lysobacter gummosus* and *Luteibacter rhizovicinus* belonged to species that had never been reported as CP degraders, although other members of these genera showed the capacity to degrade a broad diversity of organic pollutants (Leigh et al., 2006; Li et al., 2008; Wang et al., 2010a; Wang et al., 2010b) and other *Xanthomonadaceae* are also well known to perform CP degradation (Goswami et al., 2007). Finally, the most interesting and novel finding was provided by strains related to *Paenibacillus taichungensis* and two species of *Bacillus*, since CP degraders belonging to *Firmicutes* had never been described so far (Field and Sierra-Alvarez, 2008). Moreover, the *Bacillus*-related strain that was isolated from the soil CRe showed the highest CP degradation rates to both TCP and PCP and it is also a candidate to be a new species, based on the 16S rRNA gene sequence criterion. *Bacillus* and

Paenibacillus are gram-positive spore-forming bacteria, very commonly distributed in soils; these features confer on them a strong interest as soil autochthonous bacterial species with potential for bioremediation application.

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Conclusions and future prospects

Conclusions

Both respirometric techniques and molecular (PCR-DGGE) approach have shown a **significant impact of the pollutants** on the soil microbial communities, thus indicating that not only the activity but also the relative abundance of several microbial groups are influenced by the contact with the contaminants added to the microcosms.

While respirometry shows changes on the activity status in a brief period of time, molecular analysis allows an accurate detection and identification of the microbial populations subjected to alterations, either sensitive or resistant to the pollutants. In general, **both methodologies have revealed as helpful and complementary approaches** for ecotoxicological assessments. However, DGGE could be more useful than respirometric assays to detect ecotoxicological effects of organic pollutants that can be used as C sources, leading to an overestimation of the activity status. In contrast, respirometric techniques seem to be more sensitive than the molecular approach to show the effects of heavy metals.

Despite some exceptions, **DNA- and RNA-derived data have shown a good correspondence**. RNA-DGGE analysis provides valuable information about the metabolically active microorganisms, but some pitfalls in comparison to DNA-DGGE analysis have also been evidenced.

The **toxicity of CP generally increases with the increasing degree of chlorination**. However, the toxic effects of MCP and TCP were stronger in the soil HAr than in the soil CRe, while the impact of PCP was weaker in the soil HAr even when compared with MCP and TCP (regarding the highest pollutant concentrations), because of the **different effects of the particular properties of each soil on the fate of CP**.

Bioavailability has strong effects on the impact of the heavy metals, and even a different potential toxicity ranking can be achieved if their soluble fractions are considered instead of the total concentration. Cr showed the most toxic effects regarding the added amounts of metal, but Pb should be considered the most toxic tested metal according to their soluble fractions.

Culture-dependent and culture-independent methods have shown as useful and complementary approaches to detect resistant microbiota in both Mediterranean soils.

Several of the **isolated microorganisms have been found to resist high concentrations of the tested pollutants**, and even to be able to **degrade TCP and/or PCP**, or to **reduce the concentration of Cr(VI)**.

Some of the most resistant strains have been phylogenetically related to *Paenibacillus taichungensis* and to different species of *Bacillus* and *Streptomyces*, all of them gram-positive spore-forming bacteria and commonly distributed in soils. Their singular features confer them a strong interest as **soil autochthonous microbiota with potential for bioremediation applications**. Some of the microorganisms are able to cope with both kinds of pollutants, thus indicating the potential of native soil populations to face up even to **situations of co-contamination**.

Although these Mediterranean soils were not influenced by any previous adaptation or contact with these pollutants, both of them hold resistant microorganisms to CP and heavy metals. This finding suggests that **the own autochthonous microbiota could also play an important role protecting the soil system** in eventual contamination episodes of these soils.

Suggestions for future prospects, ecotoxicological assessments and bioremediation applications

Although respirometric techniques and their derived parameters (CR, SIR), DNA- or RNA-based PCR-DGGE analyses and cultivation methods have been shown as useful and complementary approaches and all of them usually led to similar results, their comparisons indicate that some methodologies can be more appropriate to particular kinds of pollutants or soils, as well as to different purposes in the analyses or investigations to carry out. Therefore, a **good selection of the best-fitted methods** to the aims of studies or analyses to be performed or the development of multidisciplinary approaches should be taken in consideration in future works or ecotoxicological assessments. In particular, the molecular approaches based on acid nucleic analyses seem to provide new and interesting insights to the assessment of the role of soil microbiota under toxicity stress.

Bioavailability has revealed as a key factor controlling the effective concentrations of pollutants that impact on the soil microbial community. Although it can be difficult to estimate, **pollutant bioavailability should be undoubtedly considered in ecotoxicological assessments**. It must be also beard in mind that any change in the soil system could modify the bioavailability and therefore the fate of the pollutants, leading to alterations in their toxic effects.

The emerging resistant microbiota has shown to be the best suited to each environment. This statement points out that it is important to consider the microbial features and the soil characteristics before designing bioremediation strategies that use bioaugmentation, in order to achieve a good performance of the process. In this sense, the bacterial strains with potential for bioremediation isolated from these Mediterranean soils can be considered an interesting starting point for future research focused on the development of more effective bioremediation strategies based on the use of native strains for the restoration of polluted soils. The **pollutant biodegradation capabilities of the isolated resistant strains should be accurately tested in axenic cultures and inoculated soil microcosmos and mesocosmos** with contamination and co-contamination assays performed under different conditions. The knowledge of the behaviour and effectiveness of these strains in their native soils and other environments with different degrees of similarity to them should give a more precise estimation of the extent of their capabilities and potential for the development of bioaugmentation or biostimulation strategies to cope with eventual contamination contingencies.