UPV EHU
CSIC
INSTITUTO BIOFISIKA INSTITUTUA

ZTF-FCT
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

SOILS UNDER LONG-TERM AIDED PHYTOSTABILIZATION ARE A RESERVOIR FOR TRANSMISSIBLE ANTIBIOTIC RESISTANCE PLASMIDS

Olatz Garaiyurrebaso Rodriguez
Doctoral Thesis
Leioa, 2016

Doctoral thesis

SOILS UNDER LONG-TERM AIDED PHYTOSTABILIZATION ARE A RESERVOIR FOR TRANSMISSIBLE ANTIBIOTIC RESISTANCE PLASMIDS

Olatz Garaiyurrebaso Rodriguez

Supervisor: Dr. Miren Itziar Alkorta Calvo

Co-Supervisor: Dr. Elisabeth Grohmann

Leioa, 2016

Acknowledgements

The present study was carried out at the Department of Biochemistry and Molecular Biology of the University of the Basque Country and the Biofisika Institute (UPV/EHU, CSIC). The author received a research fellowship from the Government of the Basque Country (January 2012-December 2015) and a research contract granted by Fundación Biofisika Bizkaia (January 2016-April 2016).

First, and most especially, I thank my supervisor, Dr. Itziar Alkorta, and my co-supervisor Dr. Elisabeth Grohmann for their guidance during these years. Secondly, I am grateful to Prof. Kornelia Smalla and her group, in particular to Dr. Sven Jechalke, for hosting me in the Julius Kühn Institute and for training me on how to work with DNA from environmental samples.

The realization of this thesis has been possible thanks to the collaborations we had. My most sincere gratitude for their invaluable help goes to Dr. Carlos Garbisu and his team at Neiker, especially Dr. Fernando Blanco, Dr. Lur Epelde, Dr. Anders Lanzén and Dr. Iker Martin, for their unconditional availability and for their contribution on the field of soil phytoremediation, many thanks go to Prof. Jose Maria Becerril and his team, especially Unai Artexte, for helping us sampling the soil in the Lanestosa mine and many thanks go to Dr. Lucia Gallego and her group, especially Dr. Elena Sevillano, for teaching us everything they know about MIC tests and detection of carbapenemase production.

Thanks to everyone at the Department of Biochemistry and Molecular Biology and at the Biofisika Institute for their constant support, especially to my labmates Itxaso Álvarez, Dr. Sandra Águila, Dr. Lide Arana, Eneritz Bilbao and Raúl González. You have helped every step of the way.

And last but not least thanks to my family and Marco for always being there for me.

INDEX

Index of figures

Index of tables

Abbreviations and acronyms

AMP	ampicillin
APS	ammonium persulfate solution
ATP	adenosine triphosphate
BHR	broad host range
BSA	bovine serum albumin
CHL	chloramphenicol
CDC	Centre for Disease Control and Prevention
cDNA	complementary deoxynucleic acid
CEC	cation-exchange capacity
CFU	colony-forming unit
GEN	gentamicin
COW	cow slurry
C_q	quantification cycle
C_t	threshold cycle
DGGE	denaturing gradient gel electrophoresis
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	desoxynucleic acid
dNTPs	desoxynucleoside triphosphates
dsDNA	double-stranded desoxynucleic acid

dtr	DNA transfer and replication
ERY	erythromycin
EDTA	ethylenediaminetetraacetic acid
FDA	fluorescein diacetate
H'	Shannon entropy
HGT	horizontal gene transfer
ichip	isolation chip
ICP-AES	inductively coupled plasma atomic emission spectroscopy
Inc	incompatibility group
J'	Pielou evenness
KAN	kanamycin
LB	Lysogeny Broth
MDR	multidrug-resistant
MGE	mobile genetic element
MGR	maximum growth rate
MHT	modified Hodge test
MIC	minimum inhibitory concentration
mob	mobility genes
mpf	mating pair formation
MRSA	methicillin-resistant *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standards

NMDS	non-metric multidimensional scaling
OD	optical density
OEA	overall enzyme activity
OM	organic matter
oriT	origin of transfer
oriV	origin of replication
OTU	operational taxonomic unit
PBP	penicillin- binding protein
PCR	polymerase chain reaction
POULTRY	poultry manure
PMN	potentially mineralizable nitrogen
PAPER	paper mill sludge mixed with poultry manure (2:1, v/v)
qPCR	quantitative polymerase chain reaction
QAC	quaternary ammonium compounds
R	recipient strain
RIF	rifampicin
rep	replication
RNA	ribonucleic acid
ROS	reactive oxygen species
RR	rarefied richness
rRNA	ribosomal RNA

RT-qPCR	quantitative reverse transcription polymerase chain reaction
STR	streptomycin
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHEEP	sheep manure
SOC	soil organic carbon
SQI	soil quality index
SSC	saline and sodium citrate
T4CP	type IV coupling protein
T4SS	type IV secretion system
TAE	tris-acetate-EDTA buffer
TBE	tris-boric-acid-EDTA buffer
TC-DNA	total community DNA
TET	tetracycline
TEMED	tetramethylethylenediamine
trfA	trans-acting factor A
TSA	Tryptic Soy agar
TSB	Tryptic Soy broth
UPGMA	unweighted pair group method with arithmetic mean
UV	ultraviolet
Vmax	maximum velocity of growth

Abstract

The spread of antibiotic resistance among bacteria has become one of the most important health problems nowadays. Many antibacterial compounds have their roots in natural environments and as a consequence, bacteria have coevolved and shared resistance mechanisms and genes along evolution. Nevertheless, increasing antibiotic concentrations in clinical and natural environments have enhanced this problem during the last 50 years. Due to the frequent administration of antibiotics to livestock, soils fertilized with animal manure often present a wide variety of mobile genetic elements (MGEs) that contain antibiotic resistance genes. Heavy metal resistance has been found associated to mobilizable and/or conjugative plasmids and antibiotic resistance genes. Hence, mine soils represent another ideal source for the dissemination of antibiotic resistance genes by conjugative/mobilizable plasmids.

The long-term effectiveness of different organic amendments was examined as part as an aided phytostabilization field trial on an abandoned Pb/Zn mine with different levels of strong heavy metal contamination. The heavy metal bioavailability along with biological indicators of soil health and microbial biodiversity (16S and 18S rRNA sequencing) were analysed. Paper mill sludge mixed with poultry manure (2:1, v/v) treatment resulted in the highest reduction of Cd, Pb and Zn extractability over cow, sheep and poultry manure treatments. Beneficial effects of this amendment were also observed in terms of recovering the microbial biodiversity and activity, especially in the less contaminated site, while cow slurry was the less successful of all of them.

This work also focused on the search of transmissible plasmids from the bacterial communities present in these unique soils (under both antibiotic and metal pressure). The detection of conjugative/mobilizable IncP and IncQ plasmids and *intI1* sequences indicates that bacteria from these soils have gene-mobilizing capacity with implications for potential dissemination. We obtained a total of 17 *E. coli* transconjugants from different soils selected with ampicillin, erythromycin, chloramphenicol or streptomycin by the exogenous plasmid isolation technique. Almost all transconjugants displayed a multi-resistant phenotype against a broad spectrum of antibiotics including one transconjugant harbouring imipenem resistance. Additionally, the effect of the plasmids on the different aspects of host fitness was discussed. We concluded that the acquisition of plasmids affected the host in varying degrees depending, primarily, on the characteristics of the genes encoded on them and their interaction with the host genome.

Chapter 1.
General introduction

Chapter 1. General introduction

1.1. History of antibiotics

Penicillin, discovered in 1929 by Alexander Fleming (Fleming, 1929), was the first antibiotic used in medical practice. World War II made the use of this antibiotic to be rapidly spread all over the world due to its mass production. The therapeutic antibiotic dosing was calculated simply taking into consideration only two factors: i) it had to be a dose high enough to clean the infected site but ii) without causing a severe toxic effect to the patient. Since their introduction, antibiotics have reduced infectious diseases in humans due to pathogen sensitivity and have been categorized as one of the most revolutionary discoveries of the 20th century. However, this fact had a severe consequence: in 1945 more than 20% of *Staphylococcus aureus* hospital isolates were penicillin-resistant. Consequently, the gene encoding the enzyme conferring penicillin resistance (penicillinase) began to spread world wide among bacteria (Taubes, 2008). Penicillin belongs to the β-lactam group of antibiotics that contain a β-lactam ring in their molecular structure. Penicillinase is the enzyme that breaks the ring structure of the antibiotic and abolishes the effect of penicillin (Barber and Rozwadowska-Dowzenko, 1948).

A decade after the penicillin crisis, the pharmaceutical industry came up with a new semisynthetic penicillin drug called methicillin. Like other β-lactams, methicillin reacts inhibiting the synthesis of bacterial cell walls. However, methicillin-resistant *Staphylococcus aureus* (MRSA) was reported within a year after the introduction of this antibiotic (Jevons *et al.*, 1963). In this case, instead of generating improved β-lactamases, bacteria used a gene called *mecA* to face the methicillin pressure.

The golden age of antibiotics lasted from the 1940s to the 1990s when new antibiotics were continuously introduced into the health care market as it can be seen in Figure 1.1. Nevertheless, outbreaks by antibiotic resistant pathogens became more and more frequent due to a rapid genetic adaptation of the bacteria. Antibiotic resistance is an advantageous trait usually consequence of a secondary adaptation to adverse environmental conditions where competition for resources is intense.

According to the World Health Organization (WHO), antibiotic resistance can be defined as the ability of bacteria to resist the effects of an antibiotic whether by an inherent attribute that protects the cell (intrinsic resistance) or by the acquisition (non-inherited

resistance) of a new mechanism that protects the bacteria against the bactericidal or bacteriostatic effect of the antibiotic.

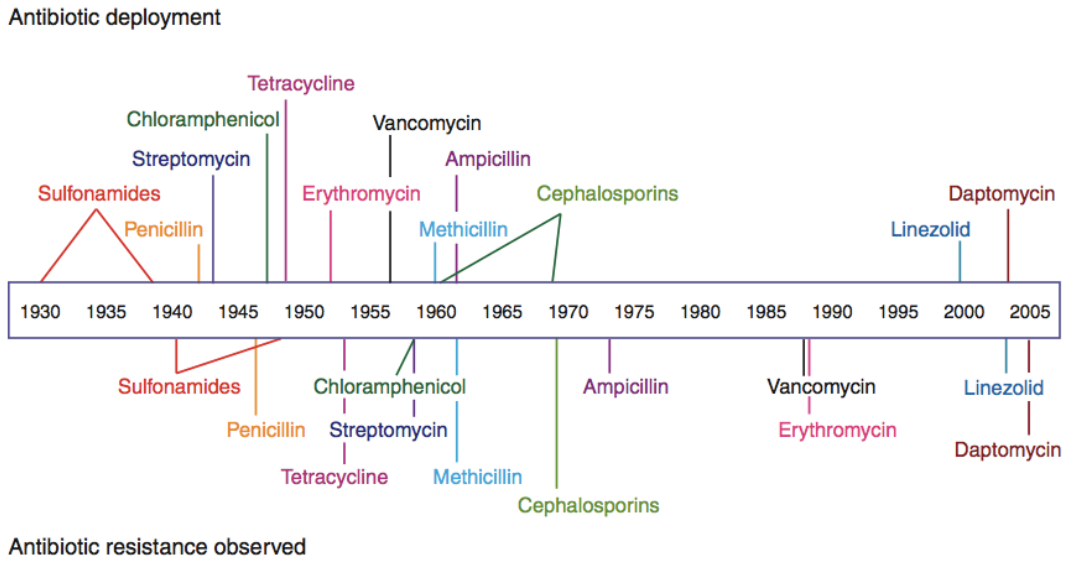


Figure 1.1. Timeline of antibiotic deployment (top) and the appearance of antibiotic resistance in pathogens (bottom). The year each antibiotic was introduced into the market is detailed above the timeline and the year when the corresponding resistance to each antibiotic appeared is shown below the timeline (Clatworthy *et al.*, 2007).

Even though there are many evidences of the relationship between antibiotic use and the increase of resistant pathogens, antibiotics still continue to be the most used therapy against bacterial infections nowadays. Hence, the consequent emergence of antibiotic resistance is considered as one of the few evolutionary facts that can be studied in real time (Baquero *et al*., 2009; Martinez *et al*., 2009). In 1990s, multidrug-resistant bacteria (MDR) were identified and they were called superbugs (Nordmann *et al*., 2007).

As shown in Figure 1.1, plenty of antibiotics have been developed since penicillin and they are commonly classified according to their mechanisms of action or their target as represented in Figure 1.2 (Neu, 1992). Antibiotics can be divided into the following families: i) inhibition of protein synthesis, such as aminoglycosides, chloramphenicol, macrolides, streptothricin and tetracyclines, ii) inhibition of DNA and RNA synthesis, such as quinolones and rifampicin, iii) inhibition or damage of cell wall synthesis like β-lactams and glycopeptides, and iv) modification of folic acid metabolism resulting in the inhibition of cell division in last instance, such as sulphonamides and trimethoprim.

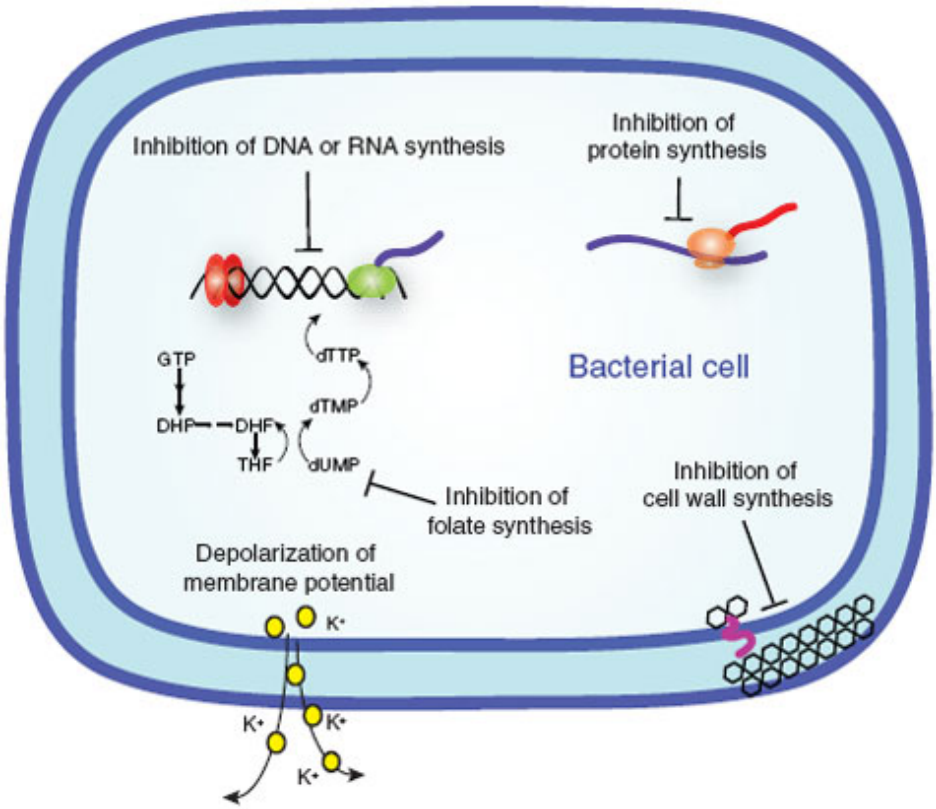


Figure 1.2. Mechanisms of action according to which antibiotics are normally classified. Antibiotics are classically classified depending on whether they inhibit the cell wall synthesis or depolarize the membrane potential, they target DNA synthesis, replication or transcription machinery, they inhibit protein synthesis machinery or metabolic pathways such as folate synthesis (Clatworthy *et al.*, 2007).

Nevertheless, in an attempt to stop the crisis the pharmaceutical industry came with a new powerful antibiotic called vancomycin, which can attack bacteria from different fronts (e.g. inhibition of cell wall cross-linking, alteration of cell-membrane permeability and RNA synthesis). After its introduction into the clinic it took 30 years for vancomycin resistance to appear. But since then, many vancomycin resistant enterococcal strains have appeared. Even so, at the moment, a small number of antimicrobials including imipenem and vancomycin are still considered drugs of last resort (a pharmaceutical agent used after all other treatment options have failed to produce a beneficial response in the patient) for treating antibiotic resistant pathogens. Imipenem is a broad-spectrum carbapenem antibiotic that was designed semi synthetically in 1980 and it is still considered a drug of last resort due to the slow emergence of resistant strains since then (Papp-Wallace *et al.*, 2011). Nowadays, new antibiotics such as linezolid with activity against many vancomycin resistant enterococcal strains have improved the situation, although resistance to linezolid has already been described (Deshpande *et al.*, 2007; Arias *et al.*, 2010).

1.2. The origin of antibiotics and antibiotic resistance

Almost all classes of antibacterial compounds have their origin in natural products obtained from microbial communities in the environment, mainly from soil. Soil represents one of the largest reservoirs of microbial diversity and the bulk of antimicrobials used today in health care have been isolated from soil microorganisms. Soil is a heterogeneous ecosystem and harbours a broad diversity of niches, which are rich habitats with a high genetic diversity at a very small scale. As a consequence, bacteria from these niches had to co-evolve and share resistance mechanisms and their corresponding genes along evolution to avoid suicide and/or to survive in this competitive habitat. Therefore, antibiotic biosynthesis is linked to the occurrence of genes encoding one or more potential resistance proteins that are either specific for the compound produced or more generic, for example efflux pumps. It has been suggested that resistance determinants in antibiotic producer bacteria could be also responsible for the regulation of the biosynthesis pathway (Tahlan *et al*., 2007), besides the self-resistance function. But the situation seems to be more complex: the corresponding resistance proteins evolved long-before the therapeutic use of antibiotics, to play different roles as diverse as detoxification, signal trafficking or metabolic functions.

In Figure 1.3, the main mechanisms responsible for the resistance to antibiotics are shown and they can be classified as follows: i) impermeable barriers, which give bacteria an inherent resistance to certain antibiotics, ii) multidrug resistance efflux pumps, which can secrete antibiotics and/or other molecules outside the cell, iii) resistance mutations that modify the target of the antibiotic, for example by disabling the antibiotic-binding site but leaving the cellular activity of the protein intact, and iv) inactivation of the antibiotic itself that can occur by covalent modification of the antibiotic (Allen *et al*., 2010).

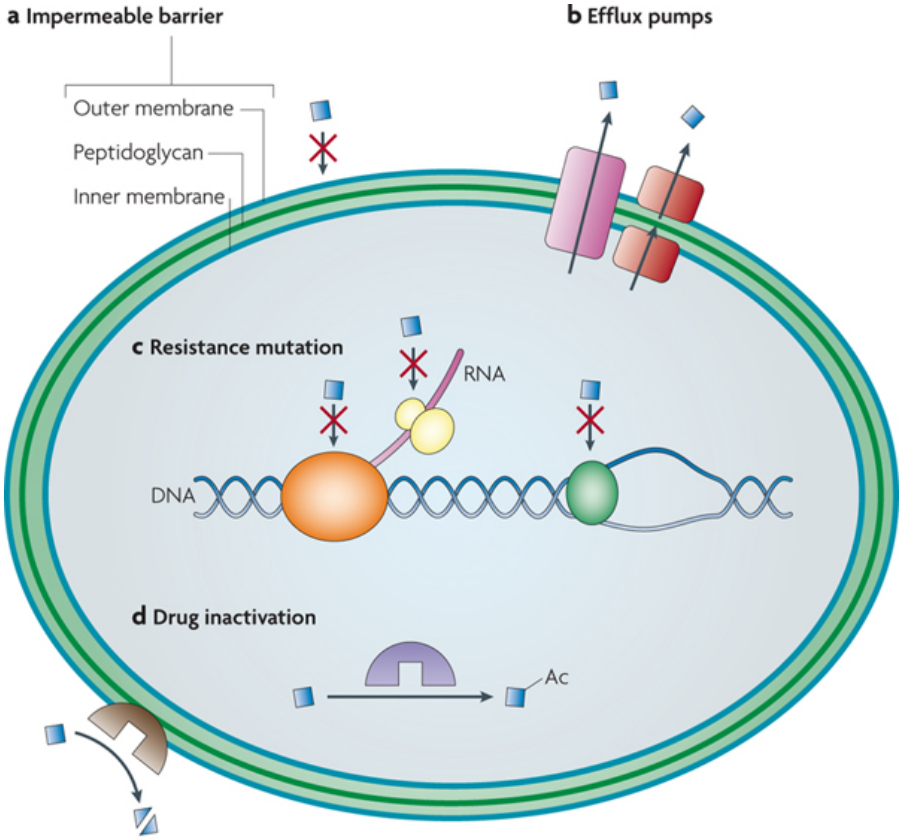


Figure 1.3. Mechanisms of antibiotic resistance in Gram-negative bacteria. **a)** Impermeable barriers. Some bacteria are simply resistant to certain antibiotics due to an impermeable membrane. **b)** Multidrug resistance efflux pumps. They secrete antibiotics outside the cell directly or through the periplasm. **c)** Resistance mutations. These mutations modify the target protein for example by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. **d)** Inactivation of the antibiotic. Inactivation can occur by covalent modification of the antibiotic (Allen *et al*., 2010).

Recent studies support the idea that antibiotics at low concentrations may play a beneficial role serving as signalling molecules in bacterial populations influencing various physiological activities. These activities include bacterial virulence, biofilm formation, signal trafficking, induction of gene expression and triggering specific transcriptional changes. In this way the rate of adaptive evolution and selection for transfer of resistant mutants are enhanced (Davies *et al*., 2006 and 2013; Yim *et al*., 2007; Wang *et al*., 2010; Gullberg *et al*., 2011).

In particular, sub-inhibitory concentrations of antibiotics have the ability to contribute to the generation of an ideal environment where bacteria could thrive by promoting biofilm formation and gene transfer. It was also suggested that bacterial adaptation along with these

two mechanisms might have been selected in the course of evolution with the purpose of exploring new niches (Aminov *et al*., 2009).

The effects of sub-inhibitory concentrations of antibiotics are not only limited to genetic modifications. The low concentrations also influence the bacterial phenotype, for example, by increasing the frequency of emergence of persisters. Persister cells are in a non-growing or extremely slow-growing physiological state and exhibit an elevated multidrug tolerance. The slow growth is a result of a general arrest in metabolic activity, which is also responsible for their intrinsic ability to survive when exposed to antibiotics (Allison *et al*., 2011). Persisters have a high occurrence in surface attached microbial colonies or biofilms. Biofilms are the most common way of living for bacteria in clinic or soil environments and they seem to be responsible for chronic infections. Nevertheless, at high or lethal concentrations of antibiotics these effects are less important as susceptible bacteria are normally killed before any phenotypic change can occur. Furthermore, antibiotics at sub-inhibitory concentrations allow susceptible strains to continue growing, although sometimes at a reduced growth rate. Even so, this will promote an enrichment of pre-existing resistant bacteria, which not necessarily present an affected growth rate (Gullberg *et al*., 2011; Liu *et al*., 2011).

Together, these effects have the ability to accelerate the emergence and spread of antibiotic resistance. This situation is of great importance for the global antibiotic resistance problem as sub-inhibitory concentrations are found in many natural environments such as sewage water and sludge, rivers, lakes, drinking water (Baquero *et al*., 2008; Jiang *et al*., 2013) and they even occur in humans or animals under therapy.

Bacterial physiology and metabolism are considerably plastic, which means that a single gene may express a broad range of different functions depending on the cell context. For instance, a gene responsible for homeostasis regulation or heavy metal detoxification may become useful in the clinical context for antibiotic expulsion and be selected rather for antibiotic resistance. One example of this are the multidrug resistance (MDR) efflux pumps, a membrane-anchored protein family that is ubiquitous and essential in all organisms and provides functional roles other than providing antibiotic resistance (Alonso *et al*., 1999; Lubelski *et al*., 2007) such as virulence, maintaining homeostasis and detoxification of intracellular metabolites among other functions.

Another good example of antibiotic resistance that initiated as a secondary adaptation is the β-lactamase family that inhibits the β-lactam antibiotics such as penicillin (Figure 1.4). As

it was mentioned before, these antibiotics work by inhibiting the cell wall formation or damaging the wall itself. More precisely, they inactivate the transpeptidase or penicillin-binding proteins (PBPs) involved in the synthesis and cross-linking of peptidoglycan and cell wall formation. β-lactamases present a structure similar to PBPs, which are in fact the targets of β-lactam antibiotics. Studies suggest that β-lactamases might have originally been PBPs themselves. Their antibiotic resistance activity may have been a side effect of their original function as a result of diversification (Meroueh *et al.*, 2003). β-lactamases may have been selected initially for other suitable cell wall related functions, such as peptidoglycan recycling, cell division or cell wall assembly.

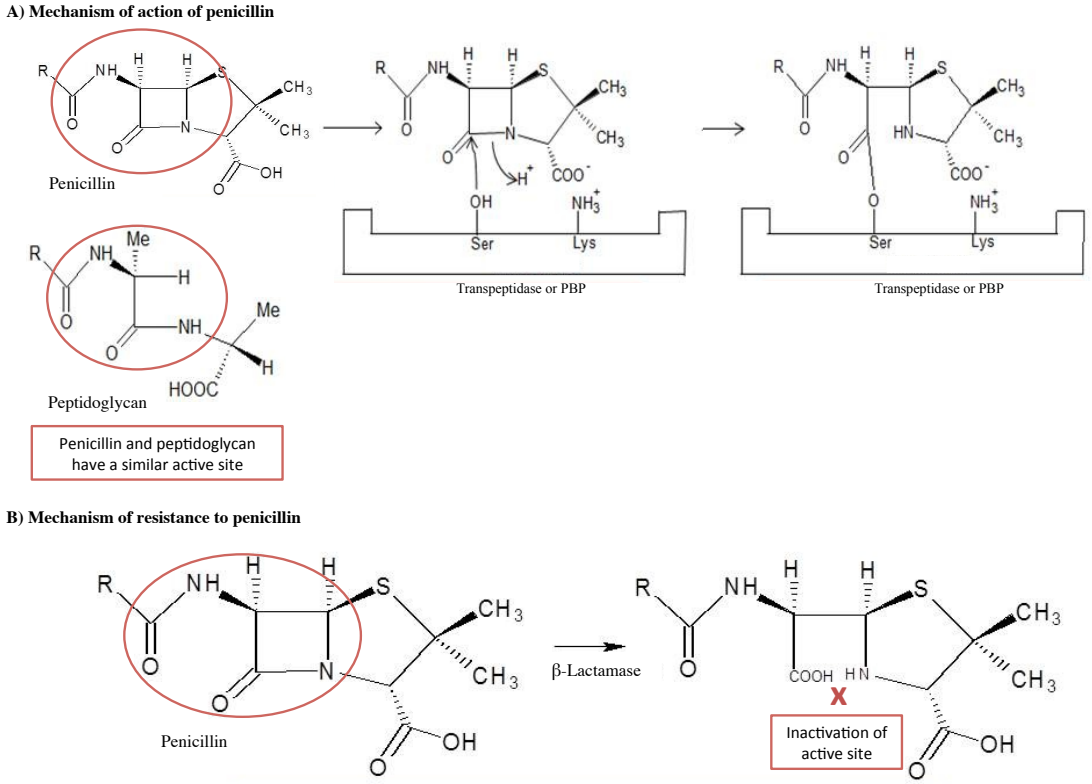


Figure 1.4. Mechanisms of action of penicillin and resistance to penicillin. **A)** Penicillin mimics the active structure of the peptidoglycan. Therefore, it can bind covalently to the transpeptidase or PBP and inhibit the cross-linking action of the peptidoglycan. Peptidoglycan subunits are rendered unable to link to each other, the cell wall is weakened or it cannot be formed. **B)** Bacteria release β-lactamase, which is similar in structure to the transpeptidase or PBP. These enzymes open up the β-lactam ring of penicillin inactivating the active site of the antibiotic. In this manner, penicillin is inactivated before reaching the cell membrane (modification from Yusof *et al.*, 2011).

Due to the fact that the β-lactam group of antibiotics was developed on basis of the natural antibiotics produced by microorganisms, it is easy to understand why resistance genes against these antibiotics are very abundant in soil microorganisms (Allen *et al.*, 2009). The high efficacy and low toxicity of β-lactams are the reasons why these antibiotics are so frequently used in medicine and in agriculture. The common use of β-lactams generates a strong pressure for the acquisition of genes encoding resistance mechanisms against β-lactams. However, a diversity of genes encoding β-lactamases was also found in a remote soil environment with a minimal human-induced selective pressure. Some phylogenetic analyses revealed that the β-lactamases found in those remote places were more related to ancestral enzymes of the same type than to those found in clinical environments (Allen *et al.*, 2009) but still they presented an important nucleotide homology to antibiotic resistance genes from clinical environment (D'Costa *et al.,* 2011). These results indicate that there is a natural selective pressure among bacteria in soil even without the pressure generated by human activities (Giedraitienė *et al.*, 2011).

Similarly, antibiotic resistant bacteria were found at the remote Lechuguilla Cave (USA), a place isolated from the outside world and, especially, from human activities since millions years ago. Interestingly, a significant quantity of the bacterial strains identified from this deserted environment resisted as many as 14 different antibiotics (Bhullar *et al.*, 2012). Even more, some of the resistance genes identified in the isolated bacteria showed a similar structure to those found in the clinical environment, while plenty of them were unknown before this study.

All these data support the idea that soil is the largest reservoir of antibiotic resistance genes and a source of resistance genes of clinical interest, harbouring 30% of all known antibiotic resistance genes found in sequence databases (D'Costa *et al.*, 2006; Aminov and Mackie, 2007; Martínez, 2008; Wright, 2010). It is clear now that there is a large diversity of antibiotic resistance genes in bacterial populations from soil as a consequence of a common production of antibiotics in the same populations. This fact suggest that antibiotic production genes may be essential for many bacteria, highlighting the importance of the antibiotic production and resistance duo for bacterial ecophysisology. Recent studies confirmed the wide spread of antibiotic resistance genes in the environment showing that almost, if not all, environments contain genes with significant similarities to antibiotic resistance genes found in the clinic environment (Nesme *et al.*, 2014).

Nevertheless, the abrupt increase of resistance against antimicrobials is still thought to be caused by a relatively modern development, more precisely, consequence of the selective pressure applied to bacteria by the use and misuse of antimicrobial drugs. The problem with the clinical use of antibiotics during the recent decades is that it has accelerated the natural selection and the adaptation of bacteria to environments with an extremely high antibiotic pressure (Davies and Davies, 2010).

1.3. Problem nowadays

Antibiotic use has been one of the greatest achievements of modern medicine. Nevertheless, despite advances in antibacterial therapy over the last 20 years, today the occurrence of antibiotic resistant bacteria is one of the main medical concerns regarding the treatment of infectious diseases (Volkmann *et al*., 2004; Davies and Davies, 2010). For example, in 2007 the Centre for Disease Control and Prevention (CDC, USA) estimated that the number of deaths due to serious infections caused by MRSA overcame for the first time deaths attributed to HIV/AIDS in the United States for the same year (Taubes, 2008).

Antibiotics represent the largest drug class used in the treatment of infectious diseases caused by bacteria. But the successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. In clinical environments, pathogenic and commensal bacteria are challenged with high concentrations of antibiotics, and bacteria have become resistant to most of the antibiotics developed. Within the hospital setting, resistant pathogens often emerge within a few years after a new antibiotic is introduced (Clatworthy *et al.*, 2007; Schmieder and Edwards, 2012). Nevertheless, it is also remarkable that the emerging of antibiotic resistance does not necessarily imply that the suitability of antibiotics in the therapy against bacterial infections is lost. In fact, some of them still constitute the most powerful weapons when fighting against bacterial infections.

Problems due to antibiotic resistant bacteria are the most common cause of hospital-acquired infections (also known as nosocomial infections) that can range from mild to severe consequences. The emergence of a resistance phenotype towards antimicrobial agents depends on various factors of the host: i) degree of resistance expression, ii) capability of a microorganism to tolerate the resistance mechanism, iii) initial colonization site, and iv) other

factors. Most nosocomial infections are caused by Gram-positive bacteria such as *Enterococcus faecalis*, coagulase-negative staphylococci (CoNS)*, Staphylococcus epidermidis* and *Staphylococcus aureus*, and by Gram-negative bacteria like *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Cleven *et al*., 2006).

The numbers of infections caused by staphylococci have increased in recent years, particularly in intensive care patients, immunosuppressed patients and in patients with prosthetic devices. Almost 50% of all infections in intensive care units in USA can be ascribed to *S. aureus* or to CoNS (Jones *et al*., 2001). Besides, these pathogens frequently colonize the skin of patients and of hospital staff. For this reason, the increase in antibiotic resistance of human pathogens affects not only the treatment of infectious diseases, but also many other medical practices, such as immunosuppression in transplants, anticancer chemotherapy and advanced surgery.

Nosocomial infections result in approximately 100,000 deaths and cost more than 25 billion US$ per year only in the USA (Schmieder and Edwards, 2012). Worldwide, it is estimated that 5 – 10% of patients entering hospitals develop an infection as a result of their stay. These numbers are a result of the lack of knowledge about the processes responsible for the emergence and rapid dissemination of antibiotic resistant pathogens, especially multi-drug resistant bacteria during the last decades. Therefore, it is of great importance to avoid contributions towards the evolutionary success of resistant bacteria over sensitive bacteria before new drugs lose their effectiveness to cure bacterial infections that were treatable before.

The global health problem caused by the emergence of multi-drug resistant bacteria has become now so alarming that a number of international, national, and local surveillance networks have been established to monitor antibiotic resistance trends. Unfortunately, almost all these organizations focus only on monitoring resistance in human clinical environments. The Reservoirs of Antibiotic Resistance Networks (ROAR) is one of the few that goes beyond the clinical isolates and focuses on monitoring antibiotic resistance in bacterial populations found in animals and in natural environments such as soil and water (Marshall *et al*., 2009).

The risk of bacteria developing resistance increases every time bacteria get exposed to antibiotics. It is known that low antibiotic concentrations are not only able to select low-level antibiotic resistant variants, but may produce a substantial stress in bacterial populations, that eventually influences the rate of genetic variation and the diversity of adaptive responses

(Baquero, 2001). Two processes seem to cause the problem. First, antibiotic resistance gene transfer among bacteria is considered as one of the major reasons for the emergence of multi-drug resistant pathogens. Secondly, this spread occurs more easily within surface-attached microbial communities or biofilms, which are commonly found in clinical and soil environments and in immunosuppressed patients. Previous studies suggested that bacteria embedded in biofilms could tolerate levels of antimicrobial agents one order of magnitude higher than planktonic bacteria (Teitzel and Parsek, 2003; Jefferson *et al*., 2005). Thus, work has to be done to elucidate the processes involved in antibiotic resistance and to prevent the dissemination of resistance genes from the environment into the clinical environment.

1.4. Environmental cycle and contamination of ecosystems

Bacteria are ubiquitous organisms that move easily between ecosystems. As most pathogens were susceptible to antibiotics before their introduction for therapy, they must have acquired those antibiotic resistance genes mainly from non-pathogenic microorganisms in the environment. This supports the idea that resistance genes acquired by organisms in one ecosystem can easily be transferred to organisms from different ecosystems, from humans to animals to soil and water and vice versa. Over the last decades, resistance to antibiotics has crossed the limits of the patient and hospital settings to become a global environmental phenomenon. In addition to this, there is nowadays a greater global mobility of population, facilitating the spread of microorganisms and their genes around the world.

In particular, soil, the great reservoir of antibiotics and antibiotic resistance genes, can easily get into contact with antibiotics and antibiotic resistance genes containing microorganisms from diverse sources, for instance, wastewater irrigation, sewage and agricultural and livestock activities. Soil can be both the donor and recipient for antibiotic molecules and/or antibiotic resistance genes as shown in Figure 1.5.

A large proportion of the antibiotics that are released into the environment via the excretion of drugs in urine and faeces, up to 70% of the antibiotics in the market, are not completely metabolised during medical treatment (Kümmerer and Henninger, 2003). Moreover, the discharge of drugs, mainly by industry or hospitals, presents an active form of the antibiotic (Thiele-Bruhn, 2003; Martinez, 2009; Andersson and Hughes, 2012) and when they reach soil or water antibiotics may become mobile and generate not only a local impact

but also disturb the surrounding environments. Its mobility depends mainly on the properties of the antibiotic itself and the hydrological characteristic of the environment (Wegst-Uhrich *et al*., 2014). Although many antibiotics can create a long-term effect on the environment, it could be reduced along time in the absence of new releases. Antibiotics can be degraded as a result of microorganisms' action, hydrolysis or adsorption to particles reducing in this manner the bioavailability (Kümmerer, 2009). Many antibiotics have relatively short effect (days to weeks), but some can persist for months or even years (Massé *et al*., 2014), especially when the concentrations are high. Therefore, highly contaminated environments such as fresh manure, wastewater sewage plants and agricultural soils may exert selective pressure on sensitive bacteria leading to the selection of resistant strains (Heuer and Smalla, 2007; Byrme-Bailey *et al*., 2009; Chang *et al*., 2010; Hu *et al*., 2010).

The same situation occurs when microorganisms that harbour antibiotic resistance genes are introduced into the environment (Allen *et al*, 2010) and become broadly disseminated by different physical and biological forces among environments. Additionally, bacteria have the potential to spread the resistance genes through horizontal gene transfer (HGT) and replication (vertical transfer) generating a bigger effect in the environment. HGT is one of the most important facts that make the spread of antibiotic resistance genes and antibiotics into the environment so threatening.

The coexistence of antibiotics, resistant and non-resistant bacteria, and resistance determinants in the environment raises the frequency of sensible strains becoming resistant due to the low pressure exerted by the sub-inhibitory levels of antibiotics. This involves a great risk for human health as the dissemination of antibiotic resistance can potentially reach pathogenic bacteria as demonstrated before (Baquero *et al*., 2009; Wright, 2010).

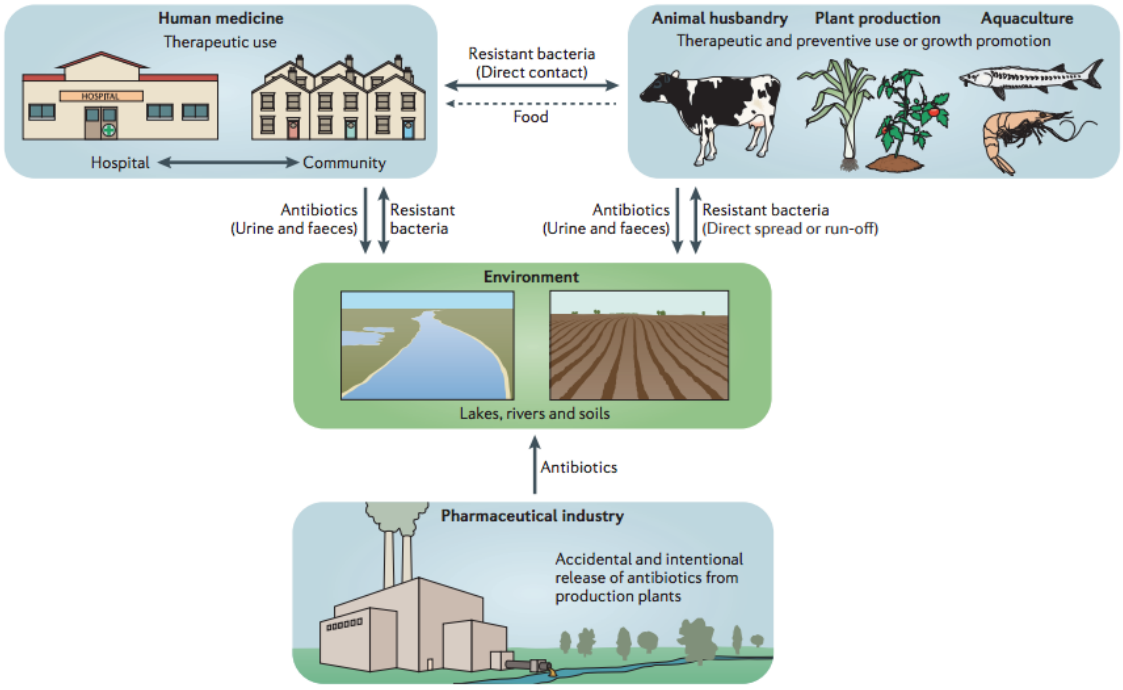


Figure 1.5. Ecology of antibiotics and antibiotic resistance. An overview of the ecology of antibiotics, showing how drugs are cycled between different environments: medical environment, agricultural settings, aquaculture environment, pharmaceutical industry and wider environments (Andersson and Hughes, 2014).

Since the beginning of the twentieth century, livestock production in farms from First World countries felt the necessity to produce larger quantities of animals over a short period of time to meet the new consumer demands. The need for superior (healthier, better quality of meat) and larger livestock, without increasing significantly production costs, led to a use and misuse of antibiotics at sub-inhibitory concentrations in farms for growth promotion, feed efficacy and disease prevention. As a result, both microbiota and pathogens present in the animals experience long-term exposure to sub-inhibitory levels of antibiotics that generally last for the whole production period.

On 1 January of 2006, the European Union banned the non-medicinal use of antibiotics in livestock production as all these techniques seemed to contribute to the spread of antibiotic resistance genes towards bacteria in crops, food production (such as milk, meat and vegetables) and consequently in humans (Ghosh and LaPara, 2007). But in other countries like USA any proposed legislation in this direction has failed to be adopted although the CDC in the USA estimates that approximately 20% of the antibiotic resistant bacteria infections each year originated from foodborne pathogens (Scallan *et al*., 2011).

Animal manure generated in farms is typically applied as fertilizer onto crops. If those animals were previously treated with sub-inhibitory concentrations of antibiotics, there is a high chance that their manure would carry a substantial quantity of bacteria containing antibiotic resistance genes (Heuer and Smalla, 2007) as it is shown in Figure 1.6. Besides, as seen before, the presence of manure in soil increases the HGT rate (Götz and Smalla, 1997) among bacterial populations in those soils. After the fertilization procedure manure bacteria may transfer the antibiotic resistance genes to bacteria present in soil and plants. These observations call for further studies of the abundance of antibiotic resistant bacteria on vegetables, which are often raised in manure-fertilized soil and eaten raw, thereby providing a potential route for antibiotic resistance genes to migrate from the environment to human ecosystems. Currently, there are several groups that study actively the use of manure and its effect on the dissemination of antibiotic resistance among soil bacteria (Heuer and Smalla, 2007; Byrne-Bailey *et al*., 2009; Chee-Sanford *et al*., 2009; Wang *et al*., 2012; Zhu *et al*., 2013).

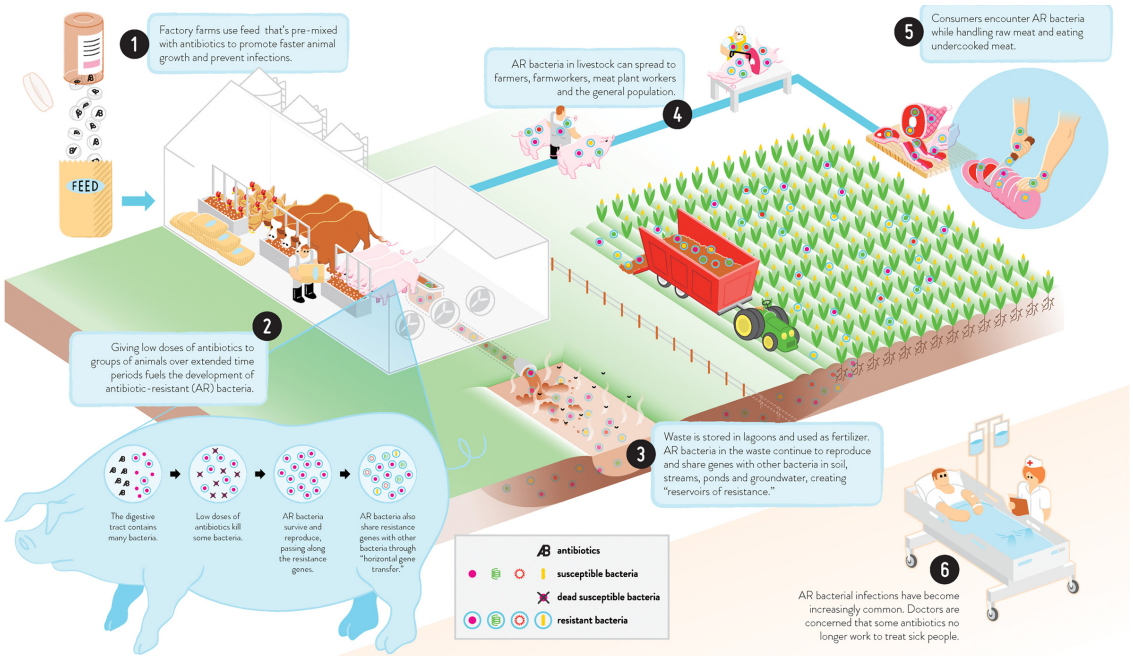


Figure 1.6. The path from the misuse of antibiotics in livestock feed to human infections. 1) Farmers feed with antibiotics to promote growth and prevent infections. **2)** Long-term exposure to sub-inhibitory levels of antibiotics leads to the emergence of resistant strains. **3)** Reservoirs of resistant strains in manure get into contact with environmental sensitive bacteria. **4)** Spread of resistance to soil, crops and farm workers. **5)** Exposure to resistance via food, such as meat, milk and vegetables particularly raw. **6)** Occurrence of antibiotic resistant bacterial infections in humans with difficult treatment (Image source: Food and Water Watch.org).

Sewage water treatment plants also play an important role in the contamination of ecosystems as they receive regular inputs of antibiotics, bacteria, and resistance genes similar to soils where manure was applied (Heuer and Smalla, 2012). Therefore, these wastewater treatment plants represent an important reservoir for antibiotics and antibiotic resistance genes containing bacteria. Likewise, disposal of antibiotics directly from the pharmaceutical industry to the aquatic environment represents a dangerous contribution to the resistome in the environment. These risks might be particularly high for agricultural fields and crops that are irrigated, normally without knowledge, with wastewater. In addition, population growth, as well as urbanization and development of industry in the last century, increased the pressure on water resources in many regions of the world. Thus, instead of decreasing the use of wastewater for irrigation and food production and/or to recharge groundwater for drinking water supply, there is nowadays a notable increase in the re-utilization of wastewater causing long-term health risk (Jimenez and Chavez, 2004; Siemens *et al.*, 2008). Due to this kind of paths, natural ecosystems experience also the pollution by resistance genes selected in clinical environments. As it was mentioned before, soil has been always considered the most probable source of the introduction of antibiotic resistance in the clinical environment through pathogenic bacteria (Fleming, 1932; Martinez, 2008). Now, the route of dissemination of resistance from soil to other environments is not longer considered as a unidirectional path rather a cycle where antibiotics and resistance genes once emerged from the soil come back to the original environment due to pollution caused by anthropogenic activities.

Although natural environments with high levels of metals exist, usually the average occurrence of toxic metals in the environment is low and, besides, most of them are immobilized in sediments and minerals, which make them biologically unavailable (Gadd, 2009). Among these, cadmium (Cd), cobalt (Co), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni), selenium (Se), and zinc (Zn) are the most frequently found metals in contaminated sites (Kavamura and Espósito, 2010). However, modern anthropogenic activities such as mining, agriculture, metallurgy, urbanization, combustion of fossil fuels, waste disposal, and others, have altered the natural biogeochemical cycles, producing an increase in the amount of toxic metals released into the atmosphere and deposited in aquatic and soil environments. Several approaches have been widely used for remediating heavy metal contaminated soils to restore the soil quality and reduce the risk of the toxic effects of the contaminants (e.g. bioremediation, aided phytostabilization). Nevertheless, as a result of the long-term contamination a great diversity of microorganisms successfully shaped to live in such extreme conditions (Galende *et al*., 2014a). Bacteria have developed a broad range of metal resistance

mechanisms that seem to be strongly correlated to antibiotic resistance mechanisms (Baker-Austin, 2006). Therefore metal contamination can function as good as antibiotics as selective agent in the proliferation of antibiotic resistance.

Studying the environmental pool of antibiotic resistance genes and its links to the clinic is of crucial importance as well as tracking down the most probable route of dissemination from the environmental reservoir to pathogens and vice versa and posterior dissemination among pathogens. In this context, a major challenge is to limit the dissemination of antibiotic resistance genes to pathogens where their expression can lead to dangerous infections in humans. Understanding the factors that drive the selection and dissemination of environmental antibiotic resistance genes among different ecosystems would be a valuable starting point to anticipate and prevent dissemination of antibiotic resistance genes within clinical environments. HGT is considered a mechanism of great relevance for bacterial adaptation to the characteristic high levels of antibiotics in clinical environments. This is even more evident since most relevant antibiotic resistance genes in pathogens are usually encountered on MGEs (Schlüter *et al.*, 2007; Stokes and Gilling, 2011; Djordjevic *et al.*, 2013).

1.5. Horizontal gene transfer

Bacteria can acquire antibiotic resistance either through spontaneous mutations or by acquiring resistance genes from other bacteria. However, mutation rates in bacterial populations are generally low as it helps to preserve their genomes and avoid cell death due to unbearable mutations (Martinez *et al*., 2009). HGT, contrarily, allows the exchange of genetic information among bacteria that are not from the same progeny and are even very distantly related. There are three main mechanisms of HGT in bacteria: transformation, transduction and conjugation as explained in Figure 1.7.

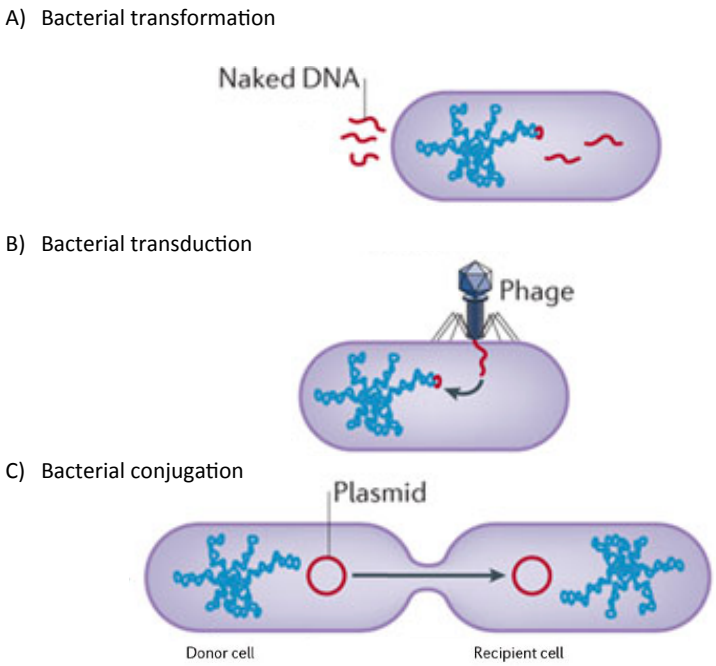


Figure 1.7. Three different ways of horizontal gene transfer. A) Bacterial transformation represents the uptake of exogenous DNA from the surroundings through the cell membrane. **B)** Bacterial transduction refers to the process of DNA transfer from a bacterium to another by a bacteriophage. **C)** Bacterial conjugation is the transfer of genetic information directly between bacteria and requires direct cell-to-cell contact or a bridge-like connection (modification from Stecher *et al*., 2013).

Bacterial transformation is based on the uptake of extracellular DNA from viral particles or more importante from bacterial cell lysis. But for this to happen, the potential recipient cell must have developed a specific state called competence (Thomas and Nielsen, 2005). Also, the success of this process depends on the stability of extracellular DNA in the environment, as there may be substances such as DNases or reactive chemicals that may degrade DNA.

Bacterial transduction, on the other hand, is the process by which bacteria acquire foreign DNA via a bacteriophage. It implies that some host DNA during bacteriophage infection gets into the new virus during viral self-assembly. When this phage then infects another cell, the new host may incorporate the donated DNA into its chromosome by recombination (Thomas and Nielsen, 2005). However, not always complete genes are removed from the bacterial cell, so transferred information may be fragmented and useless.

Bacterial conjugation is a highly efficient process in which genetic information encoded in MGEs such as plasmids is transferred from a donor to a recipient bacterium by direct cell-to-cell contact or a bridge-like connection (Furuya and Lowy, 2006). Resistance determinants are often associated with MGEs such as transposons, integrons, integrative conjugative elements or plasmids. Since broad host range plasmids can mobilize their genes into taxonomically distant species (Thomas, 2000), when resistance determinants are encoded on plasmids they will spread quickly within the genus and even unrelated bacterial genera. On the contrary, when resistance is associated with genes on chromosomes resistant microorganisms will spread more slowly (Giedraitienė *et al*., 2011).

The rate of HGT seems to be high in nutrient-rich environments, which support a great biodiversity of microorganisms and the ecosystem is fertile and healthy. Thereby, in soil, higher rates tend to be found in the rhizosphere in comparison to bulk soils (Van Elsas *et al*., 1998).

1.6. Bacterial conjugation

Among the three mechanisms of HGT, bacterial conjugation is the most significant one for the spread of antibiotic resistance genes among both pathogenic and environmental bacteria. For this reason, the increasing knowledge in the biochemical and molecular bases of bacterial conjugation may help to develop new strategies to fight the increasing number of infections due to multi-resistant pathogens (Thomas and Nielsen, 2005). Conjugation allows bacteria to share genetic information for their survival in adverse environments, contributing in this way to the spread of resistance genes (Christie *et al*., 2014) (Figure 1.8). Nevertheless, the contribution of bacterial conjugation to HGT among soil bacterial populations and the factors driving the transfer and proliferation of plasmid-containing bacteria in the soil environment are yet not fully understood.

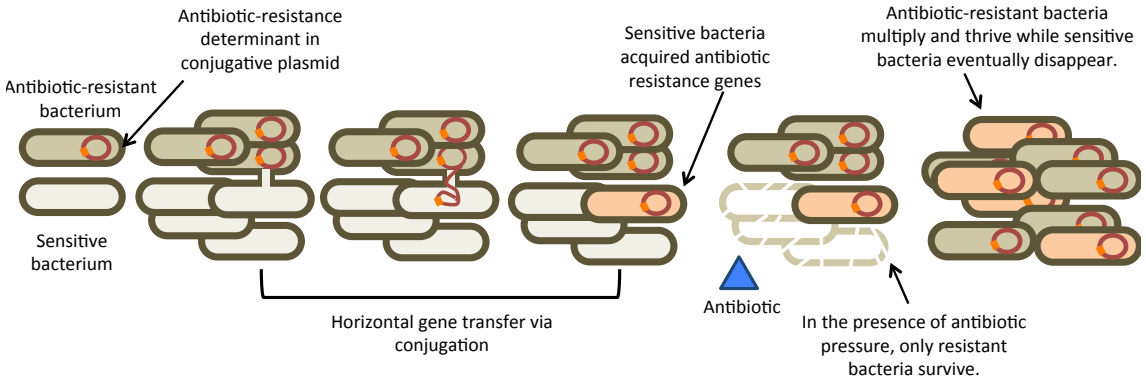


Figure 1.8. How horizontal gene transfer facilitates the spread of drug resistance. Antibiotic resistant bacteria and not resistant ones may co-exist in the same environment. Due to a close contact and other conditions that promote the HGT, resistant bacteria may transfer a copy of the transmissible plasmid harbouring antibiotic resistance determinants to other bacteria. As a consequence, previously sensitive bacteria become now resistant. In the presence of antibiotics, bacteria without the resistance genes will perish, contrarily to resistant bacteria that will multiply and thrive. Eventually all non-resistant bacteria will disappear.

From the molecular point of view, the bacterial transfer of resistance genes via conjugation consists of the transfer of the DNA (plasmid) as a nucleoprotein complex from a donor to a physically attached recipient cell via a multiprotein membrane complex (Christie *et al*., 2005). The system that mediates the process is called Type IV Secretion System (T4SS) and it is composed of two sets of genes that are necessary for plasmid mobilization and transfer.

The first of these two modules is the one composed of the mobility genes (*mob* or *dtr*, for DNA transfer and replication) that encode the proteins required to process the plasmid to be transferred. The key protein in this group is the relaxase that binds the origin of transfer (*oriT*) sequence at the beginning of the process, forming the relaxosome (the relaxase bound to the 5' end of the single-stranded DNA). The relaxase is the only protein that is common to all transmissible plasmids along with the *oriT* sequence. The interaction of the relaxase with the *oriT* sequence catalyses the first and last steps of the conjugation process. The other protein of this group is the type IV coupling protein (T4CP), which connects the relaxosome with the T4SS. The second set of genes (*mpf,* for mating pair formation) encodes the proteins that form the transfer channel. This module presents a variable number of MPF proteins depending on the system.

Plasmids that code for their own sets of *mob* and *mpf* genes are called self-transmissible or conjugative plasmids. In contrast, plasmids that only encode their own set of

mob genes and use the secretion channel of another plasmid are called mobilizable plasmids (Figure 1.9). Therefore, to be transferred mobilizable plasmids require the help of a conjugative plasmid that may be harboured in the same donor cell. Finally, non-mobilizable plasmids are those that are neither conjugative nor mobilizable (Smillie *et al*., 2010).

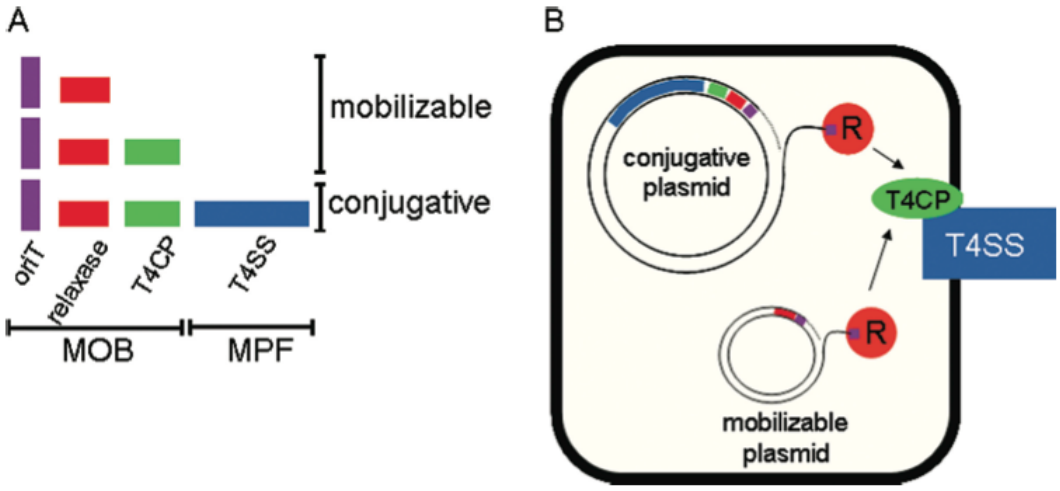


Figure 1.9. Schematic representation of the genetic and protein composition of transmissible plasmids. Self-transmissible or conjugative plasmids code for the four components of a conjugative apparatus: an origin of transfer (*oriT*) (violet), a relaxase (red), a type IV coupling protein (T4CP) (green), and a T4SS (blue). Mobilizable plasmids contain just a MOB module (with or without the T4CP) and need the MPF module of another conjugative plasmid to become transmissible by conjugation (Smillie *et al*., 2010).

1.7. Mobile genetic elements

Bacterial adaptation along evolution has primarily been shaped by the high plasticity of bacterial genomes that allows them to exchange and rearrange genomic sequences and consequently to gain new and advantageous traits. This has been extensively demonstrated with the bacterial resistance to antibiotics. Even though mutation events contribute substantially to the bacterial adaptation, HGT seems to be the main cause of the rapid proliferation of antibiotic resistance genes across a wide diversity of bacteria and environments. Nevertheless, beyond HGT, the loss, rearrangement and acquisition of functional modules have a large impact in the processes of rapid bacterial adaptation and development of resistance (Wozniak and Waldor, 2010; Bertels and Rainey, 2011). MGEs are

the main players in the process of mobilizing and reorganizing genes within genomes (intracellular mobility) or between bacterial cells (intercellular mobility).

Plasmids that can spread among bacterial populations by conjugation have common backbones that can incorporate different modules. Antibiotic resistance can be transferred by plasmids that acquire transposons, which in turn can contain integrons that recruit antibiotic resistance genes (Thomas, 2000; Baquero *et al*., 2004; Martinez *et al*., 2009) as it is shown in Figure 1.10.

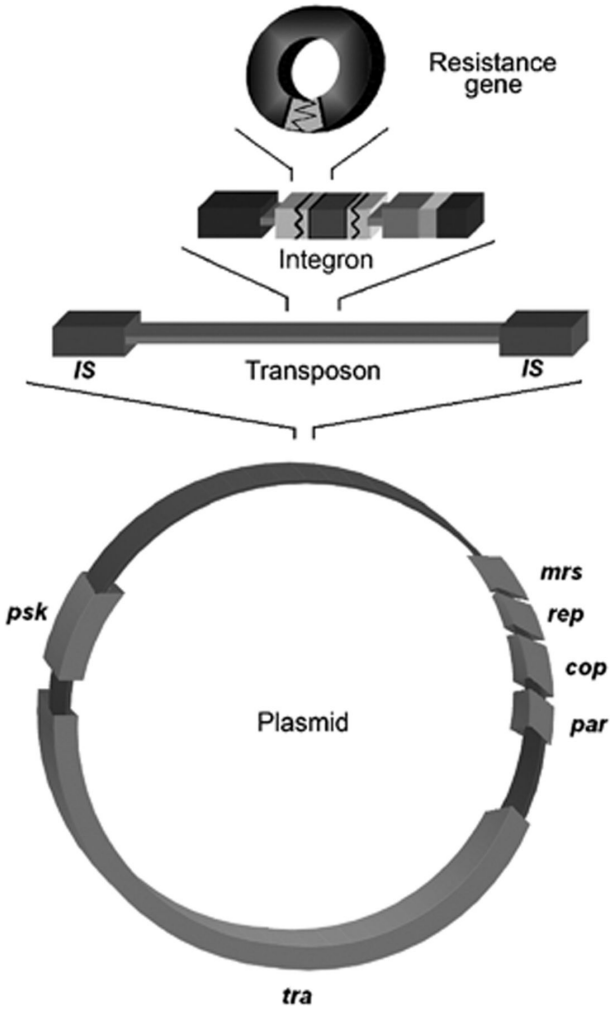


Figure 1.10. The hierarchical scheme of the elements involved in resistance dissemination. The elements involved in HGT have common backbones that can incorporate different modules. The simplest element would be the resistance gene itself, which can be nested, along with others, inside an integron. The integron may insert in a transposon and finally this one into a plasmid. IS, insertion sequences; *tra*, transfer genes; *psk*, postsegregational killing; *mrs*, multimer resolution; *rep*, replication; *cop*, copy number control; *par*, partitioning (Martinez *et al*., 2009).

Integrons can acquire, exchange, and express genes embedded within gene cassettes. Therefore, they have an important role in clustering antibiotic resistance genes with a major role in the development of antibiotic resistance and consequent adaptation of bacteria. They are composed of three main elements: the integrase gene, *intI*; a specific recombination site, *attI*; and a promoter (Boucher *et al*., 2007). Gene cassettes lack a promoter as a general rule; thus, they use the integron promoter for their expression. The last integrated cassette (usually there are no more than 10 gene cassettes within the same integron) is located the closest to the promoter (Collis and Hall, 2004) and as a consequence, it shows the highest level of expression in the integron. Accordingly, rearranging the order of cassettes can modulate changes in the relative expression of individual gene cassettes.

Integrons depend, as mentioned before, on MGEs to promote their dissemination among bacteria. Most integrons have been described in a wide range of Gram-negative bacteria, while being only sporadically seen in Gram-positive bacteria (Martin *et al*., 1990; Nesvera *et al*., 1998; Shi *et al*., 2006; Xu *et al*., 2010; Barraud *et al*., 2011). Integrons have usually been associated with multidrug-resistant pathogens (Bass *et al*., 1999; Nijssen *et al*., 2005; Laroche *et al*., 2009) as more than one resistance gene may be linked in the same cassette. In this case, processes of second-order selection can happen and eventually promote the spread of resistance genes even in the absence of their respective antibiotics.

Class 1 integrons are the best-studied integron group since they are very well adapted in a broad range of environments, widely distributed among Gram-negative bacteria of clinical interest and are the most frequently reported in human and animals (Walsh, 2006). The *intI* gene sequence of the class 1 integrons, also called *intI1*, is highly conserved among clinical isolates, while it shows variability in environmental isolates (Gillings *et al*., 2008a).

Previous studies suggested that the prevalence of the class 1 integrons in bacterial communities is clearly subjected to direct or indirect antibiotic pressure in clinical, agricultural, and environmental ecosystems (Skurnik *et al*., 2005; Daikos *et al*., 2007; Barlow *et al*., 2009; Luo *et al*., 2010; Kristiansson *et al*., 2011). With regard to the last two ecosystems, manure amendments proved to have the ability to significantly increase the pool of MGEs encoding antibiotic resistance (Heuer *et al*., 2011). Even after a few months or a couple of years after the application of manure, the prevalence of class 1 integrons, although significantly lower in comparison to the one seen right after the application, was still higher than in control soils without any amendment treatment (Byrne-Bailey *et al*., 2010; Gaze *et al*., 2011). Other factors that can contribute to the relative abundance of class 1 integrons in soils are quaternary

ammonium compounds (QACs) or heavy metals; thus, probably playing a role in their spread before the antibiotic era. In fact, many class 1 integrons present a *qacEΔ1* gene attached to the 3' end, which is a functional deletion of the *qacE* gene that still confers resistance to QACs. Moreover, industrial activities that normally result in heavy metal contamination such as mining, contribute as well to the enrichment of class 1 integrons in the environment (Wright *et al*., 2008; Rosewarne *et al*., 2010). And more importantly, co-selection of antibiotic and heavy metal resistance genes has been previously described (Aminov and Mackie, 2007).

However, it is of great importance to mention that MGEs can affect bacterial populations in different ways as a consequence of the different nature of bacterial species and plasmids. Another factor that is crucial is the incompatibility of same or similar plasmids to coexist in the same cell (Novick, 1987). According to this, plasmids have been classically classified in incompatibility (Inc) groups meaning that only plasmids with different replication systems can coexist in the same cell. Some of the best-characterized groups are the IncP, IncN and IncW type plasmids, which are self-transmissible or conjugative plasmids, and IncQ type plasmids, which are mobilizable plasmids.

1.8. Cultivation-dependent and independent approaches

Due to its chemical and physical heterogeneity, soil is a particularly challenging system to work with. Besides, a vast biodiversity of bacteria still remains unknown in soil due to the incapability to grow them in laboratory conditions. In like manner, molecules isolated from soil microorganisms during the golden ages of the antibiotic production may likely come from those easy-to-cultivate bacteria. This suggests an even more diverse *in situ* production of antibiotics and their corresponding antibiotic resistance genes, which probably remain still unknown. Therefore, studies based on cultivable bacteria are considered unrepresentative and biased.

Another reason for this concern is that cultivation with specific antibiotics in the laboratory may activate or amplify some antibiotic resistance genes that may be present only in trace-levels in their natural habitats. Therefore, the results may not reflect the actual abundance of bacterial species, MGEs or antibiotic resistance genes. To circumvent this and evaluate the entire microbiome of this complex ecosystem, cultivation-independent

techniques, investigation of the extracted total-community DNA or exogenous plasmid isolation can be used (Figure 1.11).

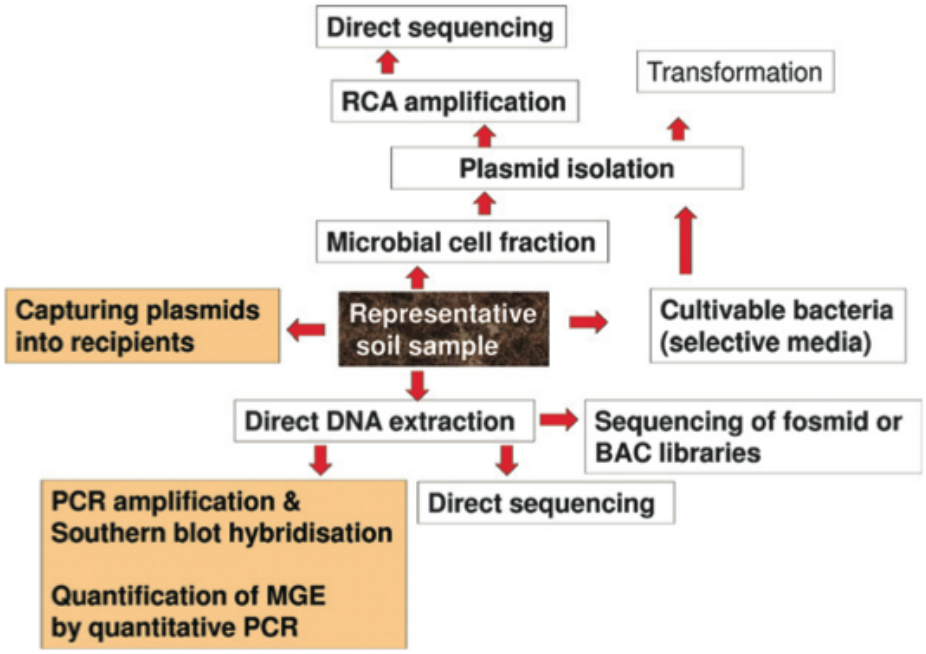


Figure 1.11. Cultivation-dependent and independent approaches to detect and analyse MGEs from soil bacteria. RCA: rolling circle amplification; BAC: bacterial artificial chromosome; MGE: Mobile genetic element (Heuer and Smalla, 2012).

Cultivation-independent detection and capture of plasmids from soil bacteria, and complete sequencing have provided new insights into the ecology of plasmids. A large number of sequenced plasmid genes have no similarity to previously sequenced genes and a high proportion are of unknown function (Tett *et al*., 2007; Sentchilo *et al*., 2013; Xiong *et al*., 2013). In this respect, new concepts such as global metagenomics approaches and development of high-throughput sequencing technologies can now try to circumvent this well-admitted cultivation bias for the study of antibiotic resistance in the environment (Martinez, 2008; Nesme *et al*., 2014).

Information on the entire microbial community present in soil can be obtained by combination of the direct extraction of total community DNA with next-generation sequencing, mitigating in this way cultivation biases. This is what it is referred now as metagenomics (Walsh, 2000; Handelsman, 2004; Monier *et al*., 2011). For instance, screening for antibiotic molecules by functional metagenomics allowed the isolation of a DNA fragment

from soil that was used to synthesize a new antibiotic molecule such as Tetarimycin A (Kallifidas *et al.,* 2012), which is notably active against MRSA.

Analogously, the capture of transferable plasmids directly into bacteria that grow easily under laboratory conditions, termed exogenous plasmid isolation, enables plasmid isolation independently of the cultivability of their original host. There are several limitations of this technique: i) it gives no information on the species from which the plasmid was transferred, ii) it depends on plasmid replication in the recipient, and iii) it works mainly with Gram-negative bacteria. Furthermore, the type of plasmids captured depends on the accessibility and abundance of these in soil (Heuer and Smalla, 2012).

Although plasmids are broadly spread among bacterial communities in soil, particular plasmids may be only harboured in a small proportion of the bacterial population. Therefore, the detection of a particular plasmid may represent also quite a challenge as its abundance might often be below the detection limit. However, under the conditions of selective pressure (such as antibiotic or heavy metal) they might become detectable (Heuer and Smalla, 2012). But as antibiotics are used as selective markers, the outcome may be a result of this pressure rather than the reality. However, combining the information obtained from the total community DNA and the information collected from the captured plasmids it may be possible to obtain a more in depth insight in the bacterial community in soil, the genetic diversity and the transferability of the antibiotic resistance genes (Heuer and Smalla, 2012).

To detect MGEs as well as antibiotic resistance genes, PCR techniques are frequently used in combination with Southern blot hybridization using probes generated from reference plasmids. This combined technique increases the sensitivity of detection and also confirms sequence specificity of the amplicons, although the detection is still limited to only already known sequences.

An important feature of many conjugative broad host range (BHR) plasmids is that they efficiently mobilize plasmids, which results in the acquisition of more plasmids to study. In fact, many conjugative BHR plasmids have been isolated based on their ability to mobilize IncQ plasmids. BHR plasmids most often reported from soil bacteria belong to the IncP-1 group (Van Elsas *et al.,* 1998; Adamczyk and Jagura-Burdzy, 2003; Norberg *et al*., 2011). The first IncP-1 plasmids were isolated from hospital specimens and thus IncP-1 plasmids were originally categorized as clinically important plasmids. Nowadays, this plasmid group is considered ubiquitous and several studies using cultivation-independent methods have detected and

isolated IncP-1 plasmids from soils and related ecosystems such as sewage and manure (Heuer *et al.,* 2002; Smalla *et al.,* 2006). Studies on the effect of the introduction of veterinary medicines into soil via manure on the abundance of transferable antibiotic resistance recently led to the discovery of a novel plasmid group: low GC-type plasmids. Plasmids belonging to this group were the plasmids most frequently captured in *E. coli* directly from manure-treated soils (Bihn *et al*., 2008; Heuer *et al*., 2009).

1.9. Current trends

Antibiotics were first introduced into the market to fight against infectious diseases caused by pathogenic bacteria. However, bacteria rapidly acquired resistance to these antibiotics and the development of new and better antibiotics was soon required. During the golden age of antibiotic therapy, the antibiotic pool was substantially expanded while new applications were also established, such as growth promotion in agricultural practices. But in the last couple of decades, the situation has changed. Lately, the number of new antibiotics launched is lower and in most cases, they are structurally similar to or have the origin in antibiotics already in use or in antibiotics that lost effectiveness due to bacterial resistance (Baquero, 2009). So, antibiotic resistance is spreading faster than the introduction of new compounds into clinical practice. We have already jumped into an uncertain but crucial age in which the future of the bacterial infection control may be determined by our success in developing novel antibiotics or alternative strategies to push back the outbreaks of multi-drug resistant pathogens.

A global analysis of the phenomenon of antibiotic resistance needs to be addressed starting from the soil, the source of most antibiotics and resistance determinants, and all the way to the bacterial adaptation to stressful conditions in the clinical ecosystem, where most of the outbreaks emerged. As it is now confirmed, findings on the ecology of antibiotic resistance genes in natural ecosystems could have considerable impact on clinical microbiology as well as being an ideal system for studying adaptive traits in bacterial populations. These studies would also be a great contribution to fundamental microbiology and ecology. The different features of this process include the physiological role of resistance determinants, the global networks that mediate the contact among bacterial populations and pressure determinants such as antibiotics and heavy metals, the regulatory networks that modulate genetic events leading to the acquisition of resistance such as mutation, recombination and HGT and the effect on the

bacterial physiology of the acquisition of MGEs harbouring resistance determinants (Hamilton-Miller, 2004). Studies on the mobile gene pool exchanged by bacteria will hopefully help to tackle the problem of antibiotic resistance dissemination in the clinic.

Despite the increasing health problem due to antibiotic resistance in pathogenic bacteria, little is still known regarding the diversity, dissemination and origins of resistance genes; especially for the uncultureable majority of environmental bacteria (Schmieder and Edwards, 2012). It is estimated that the number of species successfully cultured from soil under standard laboratory conditions represent less than one percent of the total population and, thus, the few cultured ones do not accurately represent the overall biodiversity (Head *et al*., 1998; Whitman *et al*., 1998; Béjà *et al*., 2002).

The uncultivable microbial population represents the largest unexplored pool of biological and chemical novelty. Recently, a few studies showed that species from this pool could be actually grown successfully inside diffusion chambers incubated *in situ*. Incubation *in situ* provides microorganisms with either naturally occurring nutrients or growth factors. With this approach, a novel high-throughput platform for parallel cultivation and isolation of previously uncultivated microbial species from a variety of environments was developed (Nichols *et al*., 2010). This platform, also called isolation chip (ichip) is composed of several hundreds of miniature diffusion chambers, each inoculated with (on average) a single environment cell (Figure 1.12). The growth recovery by this method approaches 50%, as compared to 1% of cells from soil that will grow on a nutrient Petri dish. Once a colony is produced, a substantial number of uncultured isolates are able to grow *in vitro*. This cultivation approach is an innovative way to tap into the rich biodiversity, which is also hiding a lot of chemical diversity that may include other new antibiotics.

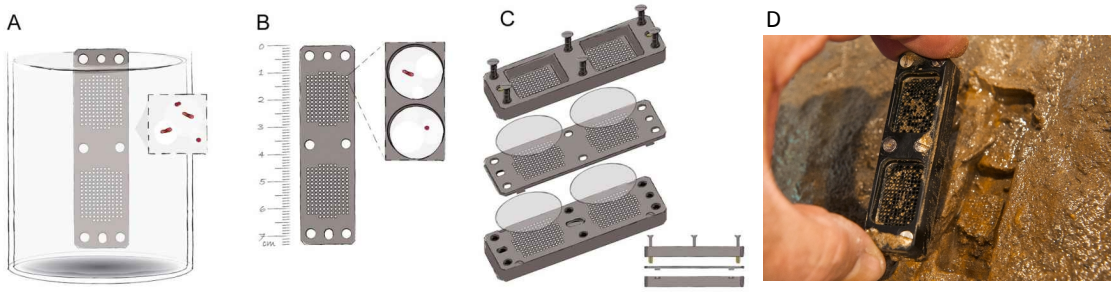


Figure 1.12. Isolation chip or ichip design and application for high-throughput microbial cultivation *in situ*. A) Dipping a plate with multiple through-holes into an agar suspension with mixed environmental cells leads to capturing (on average) a single cell. **B)** Cells get immobilized inside small agar plugs, preventing cell migration. **C)** Ichip assembly: membranes cover arrays of through-holes from each side;

upper and bottom plates with matching holes press the membrane against the central (loaded) plate. Each of the through-holes actually serves as a miniature diffusion chamber. **D)** For incubation, ichips return to the environment that served as the source of cells for *in situ* incubation with their naturally occurring nutrients and growth factors (modification from Ling *et al*., 2015).

Extracts from 10,000 bacterial isolates obtained by growth in ichips were screened for antimicrobial activity on plates overlaid with *S. aureus*. A previously unknown Gram-negative β-protebacteria (provisionally called *Eleftheria terrae*) showed activity against MRSA (Ling *et al*., 2015). These bacteria produce teixobactin, a bacterial cell wall inhibitor, which has become a promising antibiotic against Gram-positive bacteria. The teixobactin shows excellent activity against bacteria causing diseases such as colitis, tuberculosis, conjunctivitis, meningitis and pneumonia. Moreover, studies have not yet found any bacteria that are resistant to this antibiotic (Ling *et al*., 2015).

Rather than targeting a protein whose gene is mutable, facilitating resistance, teixobactin has two non-protein cell wall targets on Gram-positive bacteria. Teixobactin binds to highly conserved portions of two precursor polymers of peptidoglycan and cell wall teichoic acid. Therefore, teixobactin has no effect on Gram-negative bacteria, which are in fact the producers of the teixobactin compound. Taking in-to account these considerations, it is estimated that resistance to teixobactin may take longer to emerge, as there is no need for the producer species to have self-protecting resistance mechanisms. Still, researchers postulated that teixobactin resistance is likely to emerge. The rate of evolution of large-scale resistance will depend on the dosage and frequency of the antibiotic's use. Assuming that the antibiotic is efficacious and well tolerated in humans, teixobactin should be reserved only for serious disease cases.

A more sensible use of antibiotics for infection control could help to move in the right direction, as this would reduce the levels of antibiotics that are released into the environment. Similarly, prohibiting the use of antibiotics for non-therapeutic activities, such as for growth promotion, and reducing the contamination of natural ecosystems with antibiotics or heavy metals, could drastically reduce the exposure of bacteria to sub-inhibitory antibiotic concentrations, thereby reducing the overall selective pressure. However, subsequent restrictive measures to control the use of several key antibiotics were not as successful as expected, suggesting that there are components guiding the evolution and dissemination of these resistance processes that have yet to be understood (Martinez and Baquero, 2002; Martinez *et al*., 2007).

Therefore, to find a promising solution, it is necessary to understand the evolutionary process behind resistance; in particular the role of these genes and how they disseminate from the source: natural environments. Knowledge of the sequences of resistance genes, function and expression patterns may elucidate a way to selectively target the resistance mechanisms with specific drugs. Likewise, finding a specific inhibitor against the key protein/s involved in the transfer of resistance genes among bacteria may represent a good starting point for the inhibition or control of the transfer process. The future may not rely solely on finding or designing new antibiotics, given the rapidity with which bacteria acquire and transfer resistance genes to a wide variety of pathogens, but on the search of specific inhibitors against either resistance determinants or against the transfer of resistance genes among bacteria.

Uncovering the details of the molecular mechanisms, the regulatory networks and a more in depth view of the functional roles played by antibiotics and antibiotic resistance in different natural ecosystems (both affected and not affected by human activities) may help to understand the processes leading to the emergence of antibiotic resistant pathogens. Finally, these findings would bring the possibility to predict the way the resistance genes will spread and potentially reduce their dissemination.

Chapter 2.
Objectives

Chapter 2. Objectives

Nowadays infection outbreaks to which almost all antibiotics are ineffective are more and more frequent. In this regard, dissemination of antibiotic resistance genes among pathogenic bacteria represents an increasing global health problem. To try to tackle the different aspects of this problem, it is necessary to understand the role of the dissemination and the role of the antibiotic resistance genes in clinical and natural environments. Soil is the main reservoir of antibiotics and antibiotic resistance genes. Nevertheless, there is still limited knowledge regarding the diversity, dissemination and origins of resistance genes from bacteria in soil and, in particular, from the nonculturable fraction. As seen before, anthropologic activities, such as animal husbandry and mining, can enhance the proliferation and diversity of such mechanisms and the eventual link to the clinic.

In this thesis, we aimed to study the environmental mobile and antibiotic resistance gene pool from soils with both heavy metal and antibiotic contamination as a result of mining activities and an aided phytostabilization field trial with organic amendments, respectively. In this regard, this study contributes to broaden the knowledge of the different aspects of the dissemination of antibiotic resistance genes by conjugative/mobilizable plasmids in natural environments.

Objective 1. Evaluate the long-term effectiveness of the aided phytostabilization using different organic amendments at the surroundings of an abandoned Pb/Zn mine.

Objective 1.1. Estimate the reduction of heavy metal availability.

Objective 1.2. Study the soil quality recovery.

Objective 1.3. Analyse the presence of mobile genetic elements that could be related to the spread of antibiotic resistance.

Objective 2. Capture conjugative plasmids from the bacterial communities present in these unique soils.

Objective 2.1. Examine the antibiotic and metal resistance phenotype of transconjugants.

Objective 2.2. Evaluate the effect of the acquisition of plasmids on the host fitness.

Chapter 3.
Effect of long-term aided phytostabilization on soils

Chapter 3. Effect of long-term aided phytostabilization on soils

3.1. Introduction

3.1.1. Soil is a valuable resource threatened by anthropogenic contamination

In 400 B.C., the father of medicine Hippocrates considered the "health of the soil" as a relevant factor to human health (Krupenikov *et al*., 2011). From then onwards, a number of researchers have evidenced the relationship between the soils' status and human health (Brevik and Sauer, 2015). The term "soil health" refers to the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality, and maintain plant and animal health (Doran and Zeiss, 2000). Soil is the original habitat of most currently known antibiotic-producing bacteria and it represents an exceptionally rich ecosystem regarding both microbial abundance and species diversity. Soil also harbours a large genetic diversity at small spatial scale. In the rhizosphere of agricultural soils (the top 10 cm of fertile soil) there may be present more than 10^6 bacterial cells only in one gram of soil. But, of course, bacterial abundance and diversity depend among other factors, on the soil matrix (e.g. water percentage, soil pH, temperature, redox potential, nutrient sources, area, climate) and most importantly on pollutant concentrations and human practices (Young and Crawford, 2004).

As a consequence of modern activities, soil contamination has turned into an important environmental issue as it has been explained in chapter 1. Interestingly, the relative abundance of the class 1 integrase gene (*intI1*) has been reported as a proxy for anthropogenic contamination (i.e. a universal marker of selective pressures imposed by anthropogenic contamination) (Gillings *et al*., 2015). The *intI1* gene is linked to genes conferring resistance to antibiotics, disinfectants and heavy metals, which means that it might be an excellent measure of the general level of resistance to selective agents that are most likely to be present in contaminated areas (Gillings *et al*., 2015). Therefore, the relative abundance of *intI1* is a priori a good candidate for a biological screening of the presence of contaminants in soil, prior to a chemical characterization of specific contaminants.

Agricultural contamination

Due to the frequent administration of antibiotics to livestock, soils fertilized with animal manure present a wide variety of MGEs that contain antibiotic resistance genes. Nevertheless, this overuse of antibiotics in food animal production is still an underestimated problem. Animals are fed with antibiotics for growth promotion, feed efficiency and disease prevention thereby reducing the production costs. The problem here is that in contrast with human medicine, this antibacterial administration to food animals is rarely under supervision. And most critical, some antibiotics used in human and animal medicine are closely related if not the same. So, if resistance to antibiotics arises in early stages of the food chain, sooner or later those antibiotics will be useless in human therapy against bacterial infections (Ghosh and LaPara, 2007).

Due to this concern, actions have been taken globally to promote the prudent use of antibiotics in agriculture. Currently, the European Union authorities have banned the utilization of some antimicrobials for growth promotion in agriculture. Nevertheless, countries like the United States have still little regulation in this field (Scallan *et al*., 2011).

There are different ways in which the use of antibiotics in agriculture contributes to the appearance of resistances in bacteria that cause nosocomial infections. For example, the use of antibiotics itself exerts a selective pressure on the gut bacteria of farm animals (Baquero *et al*., 1993) that could acquire antibiotic resistance and potentially spread antibiotic resistance genes to the environment; more precisely to soil, plants and water by the use of manure. It has been shown that there is a significant share of antibiotic resistance genes between soil bacteria and gut microbiota (D'Costa *et al*., 2011; Forsberg *et al*., 2012) and even pathogenic bacteria (Allen *et al*., 2009; Nesme *et al.,* 2014). One of the main routes by which resistance genes enter the food chain is through amendment of crop soils with manure from antibiotic-treated animals, which are considered a reservoir of such genes.

And even more important, dairy cow manure amendment was found to enhance the proliferation of resident antibiotic resistant bacteria and genes encoding β-lactamases in soil even though the cows from which the manure was derived had not been treated with antibiotics (Udikovic-Kolic *et al*., 2014). These unexpected findings indicate the importance of understanding the behaviour of antibiotic resistance genes in the environment, including their response to agricultural practices and spread into the food chain.

Mining contamination

During the last century, modern anthropologic practices such as fossil fuel combustion, incineration, agricultural practices, industrial and metal extraction (mining) activities have contributed significantly to the heavy metal toxicity of ecosystems (Besser *et al*., 2009; Epelde *et al.*, 2015). Metals are not easy to degrade or destroy and can subsequently represent a long-term disturbance in soils. However, metal bioavailability (the fraction of a metal that might be absorbed by plants or soil microorganisms) depends on many factors including its chemical behaviour, soil properties (pH, cation exchange capacity), plant species, fertilization and irrigation characteristics. The reduction of metal bioavailability is mainly an effect of their frequent adsorption to soil particles and/or precipitation in less toxic forms and it represents a key factor in the risk assessment of contaminated sites.

The prevalence of heavy metal resistance in soil or sediment bacteria has often been correlated with heavy metal contamination. Studies also suggested that IncP-1 plasmids might play an important role in the adaptation of microbial communities in surface and subsurface soils exposed to heavy metal pollution such as mercury pollution (Smalla and Heuer, 2006; de Lipthay *et al*., 2008). Mine soils are characterized by high heavy metal concentrations as a consequence of the anthropogenic activities performed in the last centuries. Regarding soil microbial communities, when exposed to chronic metal pollution characteristic of mine soils, the most tolerant microorganisms can also be negatively affected and result in reduced activity, biomass and diversity (Epelde *et al*., 2009a).

The association of antibiotic- and metal-resistant phenotypes in environmental bacteria was hypothesized as early as 1974, when the most prevalent *E. coli* strain in sludge-contaminated estuarine sediment sites showed multiple antibiotic and metal resistance (Koditschek and Guyre, 1974). Therefore, a combination of these two selective contaminations in soil could represent an ideal source to find plasmids of interest to study the selective and dissemination mechanisms along with their resistance genes. Here, unique soils in which both high pressure of antibiotics and/or antibiotic resistance genes and heavy metals are present were chosen as sites of study.

3.1.2. Soil remediation

Historically, in the Basque Country metal-mining activities represented a fundamental component of its industrial development, which has resulted in an environmental problem of great concern in the region due to the metal pollution of soils. Mining areas are classically characterized by high concentrations of metals (Cd, Pb and Zn are the most common contaminants) and poor soil fertility (low organic matter and nutrient content). Among mine wastes, tailings are known to have the largest environmental impact, as they have the highest concentrations of toxic elements

Traditionally, physicochemical methods were used to remediate metal contaminated soils. However, this classic approach is frequently expensive and often results in irreversible damage of soil (Alkorta *et al*., 2010). Consequently, nowadays, a variety of biological methods of soil remediation (e.g. bioremediation, phytoremediation) are receiving much attention, mainly owing to their lower cost and environmentally friendly character (Juwarkar *et al*., 2014).

Aided phytostabilization is a more environmentally friendly *in situ* phytoremediation process, based on the combination of utilization of metal tolerant plants (phytostabilization) with organic and/or inorganic amendments (chemical stabilization) aimed to reduce metal bioavailability in soil (Bolan *et al*., 2011) but not the total heavy metal concentrations in soil.

The use of plants native to metal enriched soils for phytostabilization of metal contaminated sites is highly recommended, since they are adapted not only to tolerate metal contamination but also to the other adverse environmental factors (lack of organic matter, nutrients and/or topsoil) characteristic of many metal contaminated sites (Ernst, 2005). In this respect, *Festuca rubra* (red fescue) has been previously described as a pseudometallophyte excluder. It presents an extensive root system, a relatively large biomass and a low root-to-shoot metal translocation. *F. rubra* is the predominant plant species in the study area, which is proof of its capacity to tolerate high metal concentrations and shortage of soil nutrients. In this manner, *F. rubra* is considered a promising metal tolerant plant to use for soil pollution remediation (Epelde *et al*., 2010; Barrutia *et al*., 2011).

Chemical stabilization refers to the use of organic and/or inorganic amendments, which can improve soil physicochemical properties, such as water- and nutrient-holding capacity, reduce toxic metal bioavailability by immobilizing metals in excess, supply OM and nutrients to the soil ecosystem (stimulating soil biological activity) (Arienzo *et al*., 2004; Guo *et*

al., 2006) and provide essential nutrients for plant growth (Alvarenga *et al*., 2009). In addition, phytostabilization allows the reuse of wastes from farming and livestock activities and industrial by-products (Kabas *et al*., 2012; Galende *et al*., 2014b). Previous studies on chemical immobilization with organic amendments, such as cow manure (Meeinkuirt *et al*., 2012), cow slurry (Epelde *et al*., 2009a), paper mill sludge (Battaglia *et al*., 2007), sheep and horse manure (Pérez-Esteban *et al*., 2013), and chicken manure (Wei *et al*., 2010), have reported the capacity of these amendments to reduce Cd, Pb, and Zn bioavailability in contaminated soils.

For all these reasons, aided phytostabilization is currently being considered as the most promising technology for the remediation of contaminated sites (Garbisu *et al*., 2002); contrarily to phytoextraction which is being relegated to a second place due to the extremely long period of time required to reduce soil metal concentration (Salt *et al*., 1998).

3.1.3. Ecosystem services

It is widely accepted that it is not possible to assess the impact of soil contaminants by simply measuring the levels of those contaminants (Ludwig and Iannuzzi, 2005). Such measurements provide information about "contamination" (presence of a substance where it should not be or at concentrations above the natural background level for the area), but they do not provide information about "pollution" (contamination that causes adverse biological effects on resident organisms) (Chapman, 2007).

In the last years, it has been frequently highlighted that the ultimate goal of all soil remediation techniques (physicochemical and biological) must be not only to reduce the concentration of contaminants in the soil (or the bioavailable fraction) but, most importantly, to restore soil quality. After all, some physicochemical remediation technologies reduce the concentration of soil contaminants at the expense of negatively affecting the integrity of the soil ecosystem (Epelde *et al*., 2009b). As a result, it has been proposed the assessment of the effectiveness of biological remediation methods in terms of the recovery of ecosystem services or ecological attributes (e.g. vigor, organization, stability, suppressiveness, redundancy, carbon sequestration, primary productivity, raw materials habitats, biodiversity or erosion control) (Garbisu *et al*., 2011; Epelde *et al*., 2014a).

As indicators of soil quality recovery, microbial and physicochemical soil properties might be of great value for long-term monitoring programs (Epelde *et al*., 2014b). Along with

this, ecological stability (resistance and resilience) has a great significance in the remediated soil monitoring program, given the crucial importance of maintaining soil function after the treatment (Creamer *et al*., 2010; Griffiths and Philippot, 2013). In this respect, microbial parameters that provide information on the biomass, activity and diversity of soil microbial communities (Gómez-Sagasti *et al*., 2012) are valuable indicators of soil quality, owing to their rapid response, sensitivity and capacity to provide information that integrates many environmental factors (Garbisu *et al*., 2011). Therefore, biological parameters in combination with physicochemical indicators have been proposed to use when assessing the potential ecological risks of metal contaminated soils and long-term monitoring of aided phytostabilization (Alvarenga *et al*., 2008).

Bacteria are not spread homogeneously over the soil. Bacteria in the rhizosphere live in biofilms, which form niches where the competition for carbon resources is strong. Niches are packed in small pores formed between mineral particles, soil organic matter, roots and under the influence of plant root exudates where nutrients are significantly more abundant. Inversely, large pores between mineral particles are mostly devoid of bacteria (Bais *et al*., 2006; Demanche *et al*., 2008; Forsberg *et al*., 2012). The different ecological niches promote the development of different microorganisms, including bacteria and fungi, and their individual characteristics. In this challenging environment, where resources are scant and living conditions fluctuant, an extensive genetic and phenotypic diversity, especially MGEs, is imperative to thrive. The close proximity of diverse microbial species within micro-niches certainly favours the exchange of genetic determinants via HGT, the consequent dissemination of antibiotic resistance among bacteria and eventually the acquisition by pathogens (Forsberg *et al*., 2012; Yang *et al*., 2013). This suggests that environmental heterogeneity has a role in the maintenance of MGEs diversity (Hall *et al*., 2015). The unique diversity of MGEs can also be altered by the presence of contaminants shaping the microbial profile in different ways. Therefore, the recovery of soil quality should be also aimed to restore this diversity of MGEs characteristic of each bacterial community.

3.2. Materials and methods

3.2.1. Selection of soils

The studied area is located in the surroundings of an abandoned Pb-Zn mine in the province of Biscay (Basque Country, Spain) (43° 13' N; 3° 26' W). This Atlantic region of Spain displays a temperate climate with a mean annual rainfall of about 1,400 mm and a mean annual temperature of 11-15 °C. The mining area includes open pits, waste rocks, tailing dams and parts affected by mining subsidence. The mine area has been partly colonized by plants in the last approximately 30 years, after mining activities ceased in the late 1970s. Nowadays, the mine shows a wide range of metallicolous plant populations, including the hyperaccumulator *Noccaea caerulescens* and several metal excluders such as *Ulex europaeus*, *Agrostis capillaris* and *Festuca rubra* (Barrutia *et al.*, 2011). *F. rubra*, the dominant plant species in this area, has been recommended for the revegetation and phytostabilization of mine tailings (Epelde *et al.*, 2010; Becerra-Castro *et al.*, 2012; Galende *et al.*, 2014a).

For this study, two vegetated sites with a strong heavy metal contamination, mostly lead (Pb) and zinc (Zn), were selected. Both sites (site 1 and site 2) present Pb and Zn contamination but differ in the concentration of those contaminants. The spatial coordinates for each of the two sites are TM SigPac Basque Country 30T 0464952, 786206 and 30T 0464782, 4786572, respectively. In order to carry out an aided phytostabilization experiment in the two selected sites, in 2010, different organic amendments (an amount equivalent to 150 kg nitrogen ha^{-1} for each amendment) were superficially applied in triplicate in 1 m^2 soils as shown in Galende *et al.* (2014a). Organic amendments were incorporated into the soil due to their ability to reduce heavy metal bioavailability and improve soil physicochemical characteristics while supplying nutrients for plant growth and increasing soil biological activity (Alkorta *et al*., 2010; Bolan *et al.*, 2011).

Four different organic amendments were added: cow slurry (COW), sheep manure (SHEEP), paper mill sludge mixed with poultry manure (2:1, v/v) (PAPER) and poultry manure (POULTRY). A control with no amendment was included in each site (CONTROL) (Galende *et al.*, 2014a). These four amendments were chosen due to their wide availability, low cost and their easy application in agriculture. Fresh cow slurry was directly applied to the experimental soil. Poultry and sheep manure was air-dried for 6 months in 1 m piles. Paper mill sludge and poultry manure were mixed (2:1, v/v), aerated regularly and stored at ambient temperature for one month (Galende *et al*., 2014a).

3.2.2. Collection of samples

Triplicate samples (composite samples) of rhizosphere soil (10 cm depth) were collected from each treated or control soil. Each of these three samples consisted of about 150 g, coming from a composite sample of 10 different sub-samples of about 15 g each, taken randomly at different places within the soils. The samples were kept at 4 °C in sterile bags during transportation.

Upon arrival, soil samples were placed on a tray covered with sterile foil and allowed to dry between 24 h and 48 h at room temperature, until the soil fragments were easy to break apart. To achieve uniform drying, aggregates were crumbled in small pieces. Once soil samples were dry, they were sieved with a sterile 2 mm stainless steel sieve (CISA, Barcelona, Spain). One third of each sieved sample was stored at -20 °C for molecular analyses and the remaining fraction at 4 °C for inmediate studies.

3.2.3. Physicochemical characterization of soils

For the determination of soil physicochemical parameters, each CONTROL soil from site 1 and site 2 were analysed in triplicate. Soil physicochemical properties were measured following standard methods (MAPA, 1994). Briefly, soil texture was determined by laser diffractometry, soil pH was measured in water (1:2:5, w/v), total and oxidable OM by dichromate wet digestion, total N by the Kjeldhal method, carbonate content by neutralization with HCl, and available phosphorous by the Olsen method. Cation exchange capacity and exchangeable potassium, calcium and magnesium were determined by atomic absorption spectroscopy after saturation with 1 N sodium acetate and posterior treatment with 1 N ammonium acetate.

3.2.4. Heavy metal characterization of soils

Pseudo-total heavy metal concentrations

Pseudo-total concentrations of heavy metals were determined by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) after microwave digestion with nitric acid and hydrochloric acid (McGrath and Cunliffe, 1985).

$CaCl_2$-extractable concentrations of heavy metals

$CaCl_2$-extractable concentrations of Cd, Pb and Zn were measured by ICP-AES after extraction with 0.01 M $CaCl_2$ (Houba *et al.*, 2000). The $CaCl_2$-extractable concentrations are used as an estimation of the bioavailable fraction of heavy metals in soil.

3.2.5. Soil microbial parameters

Enzyme activities

Regarding soil enzyme activities, β-glucosidase (EC 3.2.1.21), arylsulphatase (EC 3.1.6.1) and acid phosphatase (EC 3.1.3.2) activities were determined spectrophotometrically (Dick *et al*., 1996; Taylor *et al*., 2002). Briefly, 1 g of dry weight soil was mixed with 1.6 ml of buffer (20 mM modified universal buffer (MUB), pH 6.0, for β-glucosidase; 500 mM acetate buffer, pH 5.8, for arylsulphatase; 20 mM MUB, pH 6.5, for acid phosphatase), 0.4 ml of substrate (4-nitro-phenyl-β-D-glucopyranoside (1.5%, w/v), potassium 4-nitrophenyl sulphate (1.3%, w/v) and 4-nitrophenyl phosphate disodium salt (1.85%, w/v) for β-glucosidase, arylsulphatase and acid phosphatase, respectively). After incubation at 37 °C for 45 min, the reaction was stopped with 0.4 ml of 500 mM $CaCl_2$ and 1.6 ml of 500 mM NaOH. The mixture was then centrifuged at 3,500 × g for 3 min and the absorbance value of the samples was read at 410 nm.

For urease (EC 3.5.1.5) activity (Kandeler and Gerber, 1988), 1.75 ml of 100 mM borate buffer (pH 10) and 0.25 ml of 820 mM urea were added to 1 g dry weight soil. The mixture was then incubated at 37 °C for 1 h and the reaction stopped with 6 ml of acidified 2 M KCl. After centrifugation at 3,500 × g for 3 min, 0.25 ml of the supernatant fraction were mixed with 3.75 ml of distilled water, 2 ml of sodium salicylate/sodium nitroprusiate mixture (17%, w/v and 0.12%, w/v, respectively) and 0.3 M NaOH. Finally, 0.8 ml of sodium dichloroisocyanurate were added and allowed to react for 30 min. The absorbance value of the samples was finally read at 670 nm.

Fluorescein diacetate (FDA) hydrolysis was estimated according to Galende *et al*. (2014a): 1 dry weight soil, 4 ml of 0.1 M Tris (pH 7.6) and 50 µl of FDA solution (0.2% in acetone, w/v) were mixed and incubated for 12 min at 25 °C. The reaction was stopped with 4 ml of acetone and, after centrifugation at 3,500 × g for 5 min, the absorbance value of the samples was spectrophotometrically determined at 490 nm.

For the dehydrogenase activity (EC 1.1) determination (Taylor *et al*., 2002), 1 g dry weight soil was mixed with 0.4 ml of 100 mM of trometamol, tris-hydroxymethyl aminomethane buffer (pH 7) and 0.4 ml of substrate (iodonitrotetrazolium chloride (0.5%, w/v)). After incubation at 25 °C for 3 h, the reaction was stopped with 8 ml of methanol and centrifuged at 3,500 × g for 3 min. The absorbance value of the samples was read at 490 nm.

Soil organic carbon

The soil organic carbon (SOC) is the main source of energy and nutrients for soil microorganisms. It is one of the most important constituents of the soil due to its capacity to affect the cation-exchange capacity (CEC), the structural stability and the nutrient and water-holding capacity. Briefly, 1.5 g of each soil sample were mixed with 7.5 ml of distilled water during 15 min and centrifuged at 5,500 rpm for 5 min. Then, 2 ml of supernatant were digested at 150 °C for 90 min with 3.5 ml of 0.18% chromic oxide and 65% sulphuric acid (Wei *et al*., 2008). The SOC values were calculated by absorbance at 445 nm.

Potentially mineralizable nitrogen

Potentially mineralizable nitrogen (PMN) refers to the amount of nitrogen that is mineralized from an organic form to a plant-available inorganic form by the soil microbial community. Briefly, 1.6 g of each soil sample in 3 ml of distilled water were incubated over seven days at 40 °C in an anaerobic environment (Powers, 1980; Mulvaney, 1996; Canali and Benedetti, 2005). After this incubation, 5 ml of 3.2 M KCl were added, mixed during 15 min and centrifuged at 5,500 rpm for 5 min. The ammonium concentrations were calculated by absorbance at 670 nm compared to samples without the 7 days incubation.

Antibiotic resilience

The response of soil microorganisms to disturbance can be expressed as resistance and resilience, whose effects determine the ecosystem stability. Resistance is the inherent capacity of the system to withstand the disturbance, whereas resilience is the capacity to recover after the disturbance (McNaughton, 1994; Seybold *et al*., 1999).

The resilience against penicillin of soil microbial communities was tested here. For each soil, 400 g of fresh sample were stirred vigorously during 10 min with 35 ml of 100 mM phosphate buffer (pH 7). Immediately after that, 100 µl of the soil suspension were mixed with 100 µl of sterile 1/10 LB medium (Sigma Aldrich, St. Louis, USA) and supplemented with 75 µg ml^{-1} of penicillin. Plates were incubated under constant shaking at 20 °C for 24 h. Approximately every 1.5 h the absorbance at 450 nm was recorded.

Microbial biomass

Microbial biomass was measured by the fumigation-extraction method (Vance *et al*., 1987). First, 5 g dry weight soil were fumigated for 24 h with amylene stabilized $CHCl_3$ and extracted with 20 ml of 0.5 M K_2SO_4. Then, 3.5 ml of chromium reagent (chromium (VI) oxide (0.06%, w/v); sulfuric acid (65%, v/v)) were added to 2 ml of extract and incubated at 150 °C for 60 min. After incubation, organic C concentration was determined spectrophotometrically at 445 nm. Microbial biomass was calculated as the difference between C concentration of the fumigated and unfumigated extracts (an extractability of 0.38 was assumed) (Wu *et al*., 1990).

3.2.6. Extraction and purification of total community DNA (TC-DNA) from soils

Extraction of TC-DNA from 0.5 g soil (wet weight) was carried out according to the manufacturer's protocol, FastDNA™ SPIN kit for Soil, using the FastPrep FP24 bead-beating system (MP Biomedicals, Santa Ana, USA). DNA solutions were further purified by Geneclean Spin Kit (MP Biomedicals, Santa Ana, USA).

To determine the DNA yield, TC-DNA samples extracted from soil were analysed by standard agarose electrophoresis to confirm their successful extraction and purification. One µl of TC-DNA was mixed with 0.2 µl of 6× TBE loading buffer (5 ml 5× TBE, 5 ml 87% glycerol, 30 mg bromophenol blue and 30 mg xylencyanol). As DNA molecular weight marker, 5 µl of 1 Kb Plus DNA Ladder (Thermo Scientific, Waltham, USA) were used. Next, samples were loaded onto an agarose gel (0.8%, w/v) in 0.5× TBE buffer (45 mM Tris (pH 8), 45 mM boric acid and 1 mM EDTA) and run at a voltage of 50 V for 45 min. Once the electrophoresis was completed, the gels were stained with a 1:10,000 ethidium bromide solution for 30 min under continuous shaking and then visualized under UV light.

3.2.7. Quantification of 16S rRNA gene copy numbers by quantitative Polymerase Chain Reaction (qPCR) assays

In order to quantify the bacterial population in each soil, bacterial 16S rRNA gene copy numbers were determined by qPCR in TC-DNA extracted from soil. Quantification of the 16S rRNA gene was performed in a CFX96 Real-Time System (BioRad Life Technologies, Hercules, USA). The primers and the Tm1389F TaqMan® probe are listed in Table 3.1 (Suzuki *et al.*, 2000). The TaqMan® probe is labelled with both a fluorophore (5'-FAM) and a quencher dye (3'-TAMRA) that will quench the fluorescence of FAM until the reaction takes place.

Concentrations of reaction components and the cycle program of the qPCR assay are listed in Tables 3.2 and Table 3.3, respectively. Taq polymerase and buffer used were the TrueStart Taq buffer and Taq Polymerase (Fermentas, Waltham, USA). To determine the copy number of the 16S rRNA in the soil samples, serially diluted gel purified *E. coli* 16S rRNA gene cloned into pGEM-T vector served as template for the generation of standard curves (Heuer *et al*., 2008; Vogel *et al*., 2014).

Table 3.1. Primers and TaqMan® probe used for determination of 16S rRNA gene copy numbers.

Gene	Primer/Probe*	Primer Sequence (5'-3')**	Product Size (bp)	Reference
Bacterial 16S rRNA	BACT1369 - F	CGGTGAATACGTTCYCGG	460	Suzuki *et al.*, 2000
	PROK1492 - R	GGWTACCTTGTTACGACTT		
	Tm1389F TaqMan® probe	CTTGTACACACCGCCCGTC with 5'-FAM and 3'-TAMRA		

*F: forward primer, R: reverse primer.

**W: A or T; Y: C or T.

Table 3.2. Reagents and respective concentrations for quantification of 16S rRNA gene copy numbers (50 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
12.75	Aqua ultrapure	
5	TrueStart Taq buffer (10×)	1×
5	dNTPs (2 mM)	0.2 mM
6	$MgCl_2$ (25 mM)	3 mM
2.5	BSA (2 mg ml^{-1})	0.1 mg ml^{-1}
6	Forward primer	1.2 µM
5	Reverse primer	1 µM
2.5	TaqMan probe	0.5 µM
0.25	TrueStart Taq polymerase (5 U μl^{-1})	1.25 U/sample
5	DNA template	

Table 3.3. qPCR program for determination of 16S rRNA gene copy number.

Cycles	Temperature (°C)	Time (s)	Step
1	95	300	Denaturation
40	95	15	Annealing
	56	15	Elongation

3.2.8. Denaturing Gradient Gel Electrophoresis (DGGE)

The diversity profile of the dominant species of the bacterial community from the soils was studied by DGGE (Denaturing Gradient Gel Electrophoresis) (Weinert *et al.*, 2009). This method is based on the separation of different DNA bands with the same molecular weight by its content of GC. Since GC content is different for each species, this technique allows discerning among samples of great complexity (Myers *et al*., 1985).

16S rRNA genes of each TC-DNA sample extracted from soil samples were amplified by GC-PCR (Heuer *et al.*, 1997). This amplification adds a short GC sequence of approximately 40 nucleotides to one end of the DNA product, facilitating the separation of the products in the

subsequent electrophoresis, as the GC-rich sequence acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands.

PCR amplification of 16S rRNA gene fragments in bacteria (GC-PCR)

Oligonucleotides used to amplify these highly conserved sequences in bacteria were F984GC forward and R1378 reverse primer (Nübel *et al.*, 1996; Heuer *et al.*, 1997). *F984GC* and *R1378* primers have the following sequences 5´-AACGCGAAGAACCTTAC-3´ and 5´-CGGTGTGTACAAGGCCCGGGAACG-3', respectively. A GC-rich sequence (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG) is attached to primer F984GC, in order to add a GC clamp for subsequent DGGE analysis.

Concentrations of reagents and the qPCR program are listed in Table 3.4 and Table 3.5, respectively. Acetamide was added to the reaction mixture to facilitate the denaturation of dsDNA and to reduce non-specific annealing of primers (Reysenbach *et al*., 1992). GoTaq Flexi Buffer and Taq polymerase were used (Promega, Madison, USA).

Table 3.4. Reagents and respective concentrations for amplification of the 16S rRNA gene fragment in bacteria (25 µl PCR reaction each)**.**

Volume (µl)	**Reagent**	**Final Concentration**
9.62	Aqua ultrapure	
5.0	GoTaq Flexi Taq buffer (5×)	1×
2.5	dNTPs (2 mM)	0.2 mM
3.75	$MgCl_2$ (25 mM)	3.75 mM
2	Acetamide (50%) (w/v)	4% (v/v)
0.5	Primer F984GC (10 µM)	0.2 µM
0.5	Primer R1378 (10 µM)	0.2 µM
0.125	GoTaq Flexi polymerase (5 U μl^{-1})	0.625 U/sample
1	DNA template	Approx. 20 ng

Table 3.5. GC-PCR program for amplification of the 16S rRNA gene fragment in bacteria.

Cycles	Temperature (°C)	Time (s)	Step
1	94	300	Initial denaturation
35	94	60	Denaturation
	53	60	Annealing
	72	120	Elongation
1	72	600	Final elongation

PCR products were analysed by agarose gel (1%, w/v) electrophoresis in 0.5× TBE buffer. 10 µl of PCR products were mixed with 2 µl of 6× TBE loading buffer prior to loading on the gel. 5 µl of GeneRuler 1 Kb Plus DNA Ladder (Thermo Scientific, Waltham, USA) was used as marker. Samples were loaded onto the gel and run at 50 V for 45 min.

Once the electrophoresis was completed, the gel was stained with ethidium bromide solution for 30 min with shaking and finally visualized under UV light.

Denaturing Gradient Gel Electrophoresis

This technique was performed in an Ingeny PhorU system (Ingeny, Goes, The Netherlands). The electrophoresis cassette was assembled as shown in Figure 3.1.

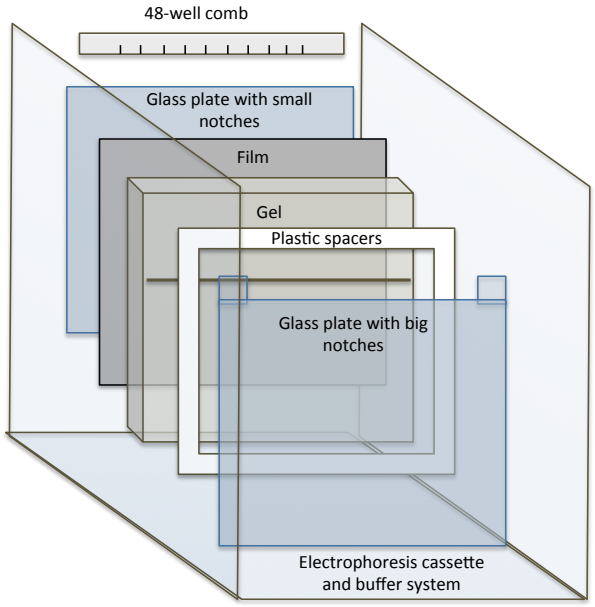


Figure 3.1. Electrophoresis cassette assembly. The main elements of the cassette assembling are depicted. The GelBond PAG Film (Lonza, Rockland, USA) was used to handle the gel after the electrophoresis more easily.

To cast the gel with a denaturing gradient two solutions with different concentrations of urea and formamide were used. One of the solutions consisted of a low concentration of urea and formamide (urea and formamide (46.5%, w/v), acrylamide (6.2%, w/v) in 1× TAE buffer). The second solution consisted of a high concentration of urea and formamide (65% urea and formamide, acrylamide (9%, w/v) in 1× TAE buffer). In addition, 46.4 µl of tetramethylethylenediamine (TEMED) and 129 µl of ammonium persulfate solution (APS) (50%, w/v) were added to both solutions. To create this gradient, the cassette was connected to a peristaltic pump with a workflow of 5 ml min^{-1}.

To prepare the stacking gel 80 µl of APS (50%, w/v) and 29 µl of TEMED were added to a solution of acrylamide (15%, w/v) in 1× TAE buffer (40 mM Tris (pH 7.4), 20 mM $NaCH_3COO$ and 1 mM Na_2EDTA) and allowed to polymerise completely for 30 min inside the cassette.

The cassette was then pre-run for 2 h at 140 V in 1× TAE buffer at 58 °C. After the pre-run, 6 µl of each PCR product with 2 µl of DGGE loading buffer (0.5% bromphenolblue, 0.5% xylencyanol, 0.2 ml 0.5M EDTA (pH 8) and 68.97 ml 87% glycerol in 100 ml) were loaded into the gel as well as 6 µl of the bacterial DGGE standard along with 6 µl of loading buffer. The bacterial DGGE standard was composed of 16S rRNA gene amplicons (GC-PCR) of 11 different bacterial strains (with different electrophoretic mobility). The strains in the mix were *Clostridium pasteurianum* DSM 525, *Erwinia carotovora* (Pectobacterium) DSM 30168, *Agrobacterium tumefaciens* DSM 30205, *Pseudomonas fluorescens* R2f, *Pantoea agglomerans*,

Nocardia asteriodes N3, *Rhizobium leguminosarum* DSM 30132, *Actinomadura viridis* DSM 43462, *Kineosporia aurantiaca* JCM 3230, *Nocardiopsis atra* ATCC 31511 and *Actinoplanes philippinensis* JCM 3001 (Smalla *et al.*, 2001). The electrophoresis was run at 140 V for 17 h.

After completing the electrophoresis, the gel was carefully detached from the cassette to process it. First, the DNA was fixed to the gel by soaking it in fixation solution (acetic acid (0.5%, v/v), ethanol (10%, v/v) and ultrapure distilled water) for 2 × 30 min. Next, the gel was kept in $AgNO_3$ solution (0.2%, w/v) for 15 min. The gel was rinsed twice with ultrapure distilled water for 1 min. Then, the gel was submerged in NaOH buffer (1.5%, w/v) with 800 µl of formaldehyde (37%, v/v) until appearance of strong bands.

To stop the reaction, the gel was submerged into stop solution (sodium carbonate (0.75%, w/v) in ultrapure distilled water) for at least 10 min. Finally, the gel was submerged into conservation solution (ethanol (25%, v/v) and glycerol (10%, v/v) in ultrapure distilled water) for 7 min. The gel was air-dried for at least 24 h and further analysed.

Statistical analysis

DGGE profiles were compared pairwise using the software GelCompar II 6.5 (Applied Maths, Gent, Belgium) according to the provider's instructions. This software facilitates scanning of the electrophoresis gels and correction of background and other disturbances. Pairwise Pearson similarity coefficients were used for construction of dendrograms (Rademaker and De Bruijn, 2004). The dendrograms represent the similarity in structure of the samples in hierarchical clusters (distance between individual biological samples). In this way, instead of assigning bands and comparing their intensities or presence/absence, which can cause a severe bias for complex patterns, the lanes were normalized and compared based on the unweighted pair group method with arithmetic mean (UPGMA) cluster algorithm (Kropf *et al.*, 2004).

The Pearson's similarity matrix was used to make a permutation test based on the significant difference between within-group similarities and between-group similarities, which should be larger for the actual samples in most cases than for random permutations, if there are significant treatment effects. In all tests, significant differences were set at $p < 0.05$. Using the same starting point (the correlation coefficients) as the cluster analysis, the results of this test do not depend on the cluster algorithm used before.

3.2.9. Bacterial 16S rRNA and eukaryotic 18S rRNA sequencing

Bacterial 16S rRNA and eukaryotic 18S rRNA sequence amplification and indexing by PCR

This technique allows the amplification of the 16S rRNA from environmental microbial communities by dual nested Illumina sequencing (MiSeq v2) using a dual indexing approach (modified from Caporaso *et al*., 2012). To do so, 5 random nucleotides are inserted between the linker and the forward primer as described in Schirmer *et al.* (2015). The amplification of the sequences consists of two PCR reactions, or nested PCR, to first amplify the 16S rRNA V4 region and 18S rRNA V7 region and then to include barcode sequences to label the samples for the subsequent massive sequencing.

The primers used for the first PCR are listed in Table 3.6. The primers used for the second PCR are listed in Table 3.9 along with the barcode sequences used for each of the soil samples. Reagents and their corresponding concentrations and the thermal program for the first and second PCR reactions can be found in Tables 3.7 and 3.8 and 3.10 and 3.11, respectively. The Taq polymerase buffer was the Qiagen HotStart Master Mix (Qiagen, Hilden, Germany), which already contained the Qiagen HotStart polymerase.

Table 3.6. Primers for the amplification of the 16S rRNA and 18S rRNA highly conserved sequences.

Gene	Primer/ Probe*	Primer Sequence (5'-3')**	Product Size (bp)	Reference
Bacterial 16S rRNA	FAdapter-N5-519F	CTACACTCTTTCCCTACGACGACGCTCTTCCG ATCT-NNNNN-CAGCMGCCGCGGTAA	253	Adaptation from Øvreås *et al.*, 1997
	RAdapter-806R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCT-GGACTACHVGGGTWTCTAAT		Modification from Caporaso *et al*., 2011
Eukaryotic 18S rRNA	Illumina-1183Fmod	ACACTCTTTCCCTACACGACGCTCTTCCGATC TNNNNNAATTTGACTCAACRCGGG	226	Jessica Rey, unpublished
	Illumina-1443Rmod	GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTGRGCATCACAGACCTG		

*F: forward primer, R: reverse primer.

**M: A or C; H: A, C or T, V: A, C or G, W: A or T and N: any nucleotide.

Table 3.7. Reagents and respective concentrations for the amplification of the 16S rRNA and 18S rRNA highly conserved sequences (20 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
8.6	Aqua ultrapure	
10	Qiagen HotStart Master Mix 2×	1×
0.2	Forward primer 100 µM	1 µM
0.2	Reverse primer 100 µM	1 µM
1	DNA template	Approx. 20 ng

Table 3.8. qPCR program for the amplification of the 16S rRNA and 18S rRNA highly conserved sequences.

Cycles	Temperature (°C)	Time (s)	Step
1	95	900	Initial denaturation
20	95	20	Denaturation
	55	30	Annealing
	72	30	Elongation
1	72	420	Final elongation

Table 3.9. Primers for the addition of the index to the amplified 16S rRNA and 18S rRNA highly conserved sequences.

Gene	Primer/Probe *	Primer Sequence (5'-3')**	Reference
Bacterial 16S rRNA and eukaryotic 18S rRNA	FAdapter-barcode-FLinker	AATGATACGGCGACCACCGAGATCTACAC-X-ACACTCTTTCCCTACACGACG	Caporaso *et al.*, 2011
	RAdapter-barcode-RLinker	CAAGCAGAAGACGGCATACGACAT-X-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	

*F: forward primer, R: reverse primer.

** X are replaced by a unique barcode sequence for each sample. See table below.

Site	Soil	Sequence	FAdapter-barcode-FLinker	RAdapter-barcode-RLinker
1	COW	Bacterial 16S rRNA	F5-F8	R1
	SHEEP		F1-F4	R1
	PAPER		F5-F8	R2
	POULTRY		F1-F4	R2
	CONTROL		F5-F8	R3
2	COW		F1-F4	R3
	SHEEP		F5-F8	R4
	PAPER		F1-F4	R4
	POULTRY		F5-F8	R5
	CONTROL		F1-F4	R5
1	COW	18S rRNA	F5-F8	R6
	SHEEP		F1-F4	R6
	PAPER		F5-F8	R7
	POULTRY		F1-F4	R7
	CONTROL		F5-F8	R8
2	COW		F1-F4	R8
	SHEEP		F5-F8	R9
	PAPER		F1-F4	R9
	POULTRY		F5-F8	R10
	CONTROL		F1-F4	R10

Barcode	Sequence
F1	A**A**TGATACGGCGACCACCGAGATCTACAC**TAGATCGC**ACACTCTTTCCCTACACGACG
F2	A**A**TGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**ACACTCTTTCCCTACACGACG
F3	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**ACACTCTTTCCCTACACGACG
F4	AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**ACACTCTTTCCCTACACGACG
F5	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**ACACTCTTTCCCTACACGACG
F6	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**ACACTCTTTCCCTACACGACG
F7	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**ACACTCTTTCCCTACACGACG
F8	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**ACACTCTTTCCCTACACGACG

Barcode	Sequence
R1	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R2	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R3	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R4	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R5	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R6	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R7	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R8	CAAGCAGAAGACGGCATACGAGAT**CCTCTCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R9	CAAGCAGAAGACGGCATACGAGAT**AGCGTAGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
R10	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Table 3.10. Reagents and respective concentrations for the addition of the index to the amplified 16S rRNA and 18S rRNA highly conserved sequences (50 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
10	Aqua ultrapure	
25	Qiagen HotStart Master Mix 2×	1×
5	FAdapter-barcode-FLinker 10 µM	1 µM
5	RAdapter-barcode-RLinker 10 µM	1 µM
5	5-10 ng/µl DNA template (purified first PCR amplicon)	0.5-1 ng/µl

Table 3.11. qPCR program for the addition of the index to the amplified 16S rRNA and 18S rRNA highly conserved sequences.

Cycles	Temperature (°C)	Time (s)	Step
1	95	900	Initial denaturation
10	95	20	Denaturation
	61	30	Annealing
	72	30	Elongation
1	72	420	Final elongation

Bacterial 16S rRNA and 18S rRNA amplicon sequence purification

The purification of the PCR amplicons was carried out with the Agencourt AMPure XP PCR purification system and the Agencourt SPRIStand (Beckman Coulter, Pasadena, USA) according to the manufacturer's instructions. The Agencourt AMPure XP PCR purification system is based on a solid-phase reversible immobilization paramagnetic bead technology for high-throughput purification of PCR amplicons.

Illumina Miseq sequencing

Sequencing was carried out using an Illumina MiSeq with the V2 kit (approximately pair-ended 2 × 250 nt length) at Tecnalia, Miñano, Spain.

Sequence data analysis

Amplicon sequence read-pairs were quality-filtered and overlapped using usearch (Edgar, 2013) (options fastq_maxdiff=5, fastq_maxee=0.5). Resulting 16S rRNA sequences were truncated from both ends in order to remove N5 and primer sequences (to a length of 253 nt). 18S rRNA amplicon sequences were instead trimmed using cutadapt (Martin, 2011) to remove adapters and primers, because of their variable length. All quality-filtered overlapped sequences from 16S and 18S rRNA amplicons were merged across datasets and clustered into operational taxonomic unit (OTU) sequences at 97% and 98% sequence similarity within a data set to each other, respectively, using vsearch (Rognes *et al*., 2015) as described in Edgar (2013). Briefly, sequences were de-replicated, sorted by abundance, singletons (abundance=1) discarded, remaining sequences clustered into OTUs, and finally chimera filtered using the uchime de novo method. 16S rRNA OTU sequences were also de novo chimera filtered using the ChimeraSlayer reference database rdp_gold.fa (for 16S rRNA). OTU abundances were obtained by mapping reads back to the representative OTU sequences.

OTU sequences were aligned to the SilvaMod v106 reference database using *blastn* (v.2.2.25+ task megablast) and classified taxonomically using CREST (default parameters) (Lanzén *et al*., 2012). Taxon distributions were studied at order rank and 18S rRNA taxon data was further divided into fungi, metazoa and protists (unicellular eukaryotic organisms including mostly unicellular algae, protozoa and slime molds). Abundance data for eukaryotic subsets

was re-normalised, excluding taxa without order rank classifications.

Statistical analyses

OTU and taxon abundance data was imported and analysed using R. Relative taxon abundances were plotted using the package *ggplot2* (Wickham, 2009). Other visualisation, multivariate statistics and calculation of diversity indices were performed using the R base or Vegan (Oksanen *et al*., 2013) packages. The diversity indices considered in this study were rarefied richness (RR) (expected richness at a comparable number of reads, representing the lowest read depth), Shannon entropy (H') and Pielou evenness (J'). Bray-Curtis dissimilarity between samples based on relative OTU distributions (obtained using *decostand*) was used for multivariate statistics, namely Non-metric multidimensional scaling (NMDS; function *metaMDS*) and ANalysis Of SIMilarities (ANOSIM, function *anosim*). Function ordiellipse was used to calculate elliptic areas representing the distribution of groups of samples (or replicates) in NMDS coordinate space, based on standard deviations and 95% confidence.

Group-wise ANOVA (analysis of variance) was used to compare diversity indices or taxon abundances between samples with different contamination levels and treatments. Where more than two groups were compared, Tukey's Range Test was used to refine p-values. Obtained p-values from ANOVA comparisons were subjected to Bonferroni correction for multiple hypotheses testing when comparing taxon abundance data (and not reported unless significant after correction). When assessing the effect of a treatment, only significant difference with respect to the corresponding CONTROL soil was considered, except for ANOSIM.

3.2.10. Quantitative Polymerase Chain Reaction (qPCR) assays

qPCR assays were used to assess quantitatively the presence of class 1 integrons by the detection of class 1 integrase gene (*intl1*), the presence of quaternary ammonium compound resistance gene (*qacE+qacEΔ1*), streptomycin (*aadA)*, tetracycline (*tetM)* and sulphonamide (*sul1*) resistance genes. Quantification of these genes was performed in a CFX96 Real-Time System (BioRad Life Technologies, Hercules, USA). Primers and TaqMan® probes used for each gene are listed in Table 3.12. The TaqMan® probe is labelled with both a fluorophore (5'-FAM)

and a quencher dye (3'-TAMRA) that quenches the fluorescence of FAM until the reaction takes place.

Concentrations of reagents and the qPCR program are given in Tables 3.13 and 3.14, respectively. TrueStart Taq buffer and Taq Polymerase (Fermentas, Waltham, USA) were used. To determine the copy number of each gene in the soil samples serially diluted gel purified PCR products from pKJK5 *intl1* (196 bp), pB10 *qacE* + *qacEΔ1* (69 bp), pKJK5 *aadA* (635 bp), pAT101 *tetM* (88 bp) and R388 *sul1* (67 bp) were used as template for the generation of standard curves (Heuer *et al*., 2008).

Table 3.12. Primers and Taqman® probes for quantification of *intl1, qacE + qacEΔ1, aadA, tetM* and *sul1*.

Gene	Plasmid	Primer/Probe*	Primer Sequence (5'-3')**	Product Size (bp)	Reference
intl1	pKJK5	*intl1 - F*	GCCTTGATGTTACCCGAGAG	196	Barraud *et al*., 2010
		intl1 - R	GATCGGTCGAATGCGTGT		
		intl1 - Taqman® probe	ATTCCTGGCCGTGGTTCTGGGTTTT		
qacE + qacEΔ1	pB10	*qacE + qacEΔ1 - F*	CGCATTTTATTTTCTTTCTCTGGTT	69	Jechalke, 2014
		qacE + qacEΔ1 - R	CCCGACCAGACTGCATAAGC		
		qacE + qacEΔ1 - Taqman® probe	TGAAATCCATCCCTGTCGGTGT		
aadA	pKJK5	*aadA - F*	TTGATTTGCTGGTTACTGTG	635	Walsh, 2011
		aadA - R	CTTAGTGATCTCGCCTTT		
		aadA - Taqman® probe	TGGTAGGTCCAGCGGCGGAG		
tetM	pAT101	*tetM - F*	GGTTTCTCTTGGATACTTAAATCAA TCR	88	Peak *et al*., 2007
		tetM - R	CCAACCATAYAATCCTTGTTCRC		
		tetM - Taqman® probe	ATGCAGTTATGGARGGGATACGCT ATGGY		
sul1	R388	*sul1 - F*	CCGTTGGCCTTCCTGTAAAG	67	Heuer and Smalla, 2007
		sul1- R	TTGCCGATCGCGTGAAGT		
		sul1 - Taqman® probe	CAGCGAGCCTTGCGGCGG		

*F: forward primer, R: reverse primer.

**R: A or G; Y: C or T.

Table 3.13. Reagents and respective concentrations for amplification of *intl1, qacE + qacEΔ1, aadA, tetM* and *sul1* (50 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
22.75	Aqua ultrapure	
5	TrueStart Taq buffer (10×)	1×
5	dNTPs (2 mM)	0.2 mM
5	$MgCl_2$ (25 mM)	2.5 mM
2.5	BSA (2 mg ml^{-1})	0.1 mg ml^{-1}
1.5	Forward primer (10 pmol μl^{-1})	0.3 µM
1.5	Reverse primer (10 pmol μl^{-1})	0.3 µM
1.5	Taqman probe	0.3 µM
0.25	TrueStart Taq polymerase (5 U μl^{-1})	1.25 U/sample
5	DNA template	Approx. 20 ng

Table 3.14. PCR program for detection of *intl1, qacE + qacEΔ1, aadA, tetM* and *sul1*.

Cycles	Temperature (°C)	Time (s)	Step
1	95	600	Initial denaturation
40	95	30	Denaturation
	60	60	Annealing

3.2.11. Polymerase Chain Reaction (PCR) assays

PCR combined with Southern Blot DNA hybridization were used to assess the presence of different genes in the bacterial communities from the different soil samples. In particular, the presence of class 1 integrase gene (*intl1*) and the presence of quaternary ammonium compound resistance gene (*qacEΔ1*) were tested. In addition, plasmid backbone regions related to replication (IncP-1 subgroups *trfA*, IncN *rep* and V216 *rep*) and origin of replication regions (IncQ *oriV*) were studied. As positive control plasmid pKJK5 for *intl1*, pB10 for *qacEΔ1* (Sandvang *et al.,* 1997), pTH10 for IncP-1 (all subgroups) *trfA,* pTH10 for IncP-1α *trfA,* R751 for IncP-1β *trfA,* (Bahl *et al.,* 2009)*,* RSF1010 for IncQ *oriV,* RN3 for IncN *rep* (Götz *et al*., 1996) and pHHV216 for pV216 *rep* (Heuer *et al.*, 2009) were used. Oligonucleotide sequences, amplicon

sizes and plasmids used as positive controls are listed in Table 3.15. Concentrations of reagents are given in Tables 3.16 to 3.20 while PCR programs are given in Tables 3.21 to 3.25.

Table 3.15. Primers to detect *intl1, qacEΔ1,* IncP-1α *trfA,* IncP-1β *trfA,* IncP-1ε *trfA,* IncP-1γ *trfA,* IncP-1γ like *trfA,* IncP-1δ *trfA,* IncP-1ζ *trfA,* IncQ *oriV,* IncN *rep* and pV216 *rep*.

Gene	Plasmid	Primer Name*	Primer Sequence (5'-3')**	Product Size (bp)	Reference
intl1	pKJK5	*intl1 – F*	CCTCCCGCACGATGATC	280	Sandvang *et al.,* 1997
		intl1 - R	TCCACGCATCGTCAGGC		
qacEΔ1	pB10	*qacEΔ1 - F*	ATCGCAATAGTTGGCGAAGT	226	
		qacEΔ1 - R	CAAGCTTTTGCCCATGAAGC		
IncP-1α *trfA*	RP4 or pTH10	*trfA* α, β, ε - *F*	TTCACSTTCTACGAGMTKTGCCAGGAC	281	Bahl *et al.,* 2009
IncP-1β *trfA*	R751				
IncP-1ε *trfA*	pKJK5	*trfA α, β, ε - R*	GWCAGCTTGCGGTACTTCTCCCA		
IncP-1γ *trfA*	pQKH54	*trfA* γ - *F*	TTCACTTTTTACGAGCTTTGCAGCGAC		
		trfA γ - *R*	GTCAGCTCGCGGTACTTCTCCCA		
IncP-1γ like *trfA*	pKS208	*trfA* γ like - *F*	TTCACCTTCTACGAACTGTGTAATGAC		
		trfA γ like - *R*	GTCAAGGCCCGATACTTCTCCCA		
IncP-1δ *trfA*	pEST4011	*trfA* δ - *F*	TTCACGTTCTACGAGCTTTGCACAGAC		
		trfA δ - *R*	GACAGCTCGCGGTACTTTTCCCA		
IncP-1ζ *trfA*	pMCBF1	*trfA* ζ - *F*	TTCACTTTCTACGAAATCTGCAAAGAC		
		trfA ζ - *R*	GATAGCTTCCGATACTTTTCCCA		
IncQ *oriV*	RSF1010	IncQ *oriV* - *F*	CTCCCGTACTAACTGTCACG	436	Götz *et al*., 1996
		oriV - *R*	ATCGACCGAGACAGGCCCTGC		
IncN *rep*	RN3	IncN *rep* - *F*	AGTTCACCACCTACTCGCTCCG	165	
		IncN *rep* - *R*	CAAGTTCTTCTGTTGGGATTCCG		

pV216 *rep*	pHHV216	pV216 *rep - F*	AATTGACCGATTTAGTTGTGACC	912	Heuer *et al.*, 2009
		pV216 *rep - R*	TGCTGATTTGYTTTGGAGATAC		

*F: forward primer, R: reverse primer.

**K: G or T; M: A or C; S: G or C; W: A or T; Y: C or T.

Table 3.16. Reagents and respective concentrations for amplification of *intI1* (25 µl PCR reaction each)**.**

Volume (µl)	**Reagent**	**Final Concentration**
15.87	Aqua ultrapure	
2.5	TrueStart Taq buffer (10×)	1×
1.5	dNTPs (2 mM)	0.2 mM
2.5	$MgCl_2$ (25 mM)	2.5 mM
0.75	Forward primer (10 pmol μl^{-1})	0.3 µM
0.75	Reverse primer (10 pmol μl^{-1})	0.3 µM
0.125	TrueStart Taq polymerase (5 U μl^{-1})	1 U/sample
1	DNA template	Approx. 20 ng

Table 3.17. Reagents and respective concentrations for amplification of *qacEΔ1* (25 µl PCR reaction each)**.**

Volume (µl)	**Reagent**	**Final Concentration**
15.37	Aqua ultrapure	
2.5	EP-Taq buffer* (10×)	1×
2.5	dNTPs (2 mM)	0.2 mM
2.5	$MgCl_2$ (25 mM)	2.5 mM
0.5	Forward primer	0.2 µM
0.5	Reverse primer	0.2 µM
0.125	EP-Taq polymerase* (5 U μl^{-1})	1 U/sample
1	DNA template	Approx. 20 ng

*EP-Taq Buffer and Polymerase (JKI, Quedlinburg) (Dealtry *et al.*, 2014).

Table 3.18. Reagents and respective concentrations for amplification of *trfA* sequence from all IncP-1 subgroups (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
13.37	Aqua ultrapure	
2.5	TrueStart Taq buffer (10×)	1×
2.5	dNTPs (2 mM)	0.2 mM
2.5	$MgCl_2$ (25 mM)	2.5 mM
1.5	Primer mix forward	0.2 µM
1.5	Primer mix reverse	0.2 µM
0.125	TrueStart Taq polymerase (5 U μl^{-1})	0.625 U/sample
1	DNA template	Approx. 20 ng

Table 3.19. Reagents and respective concentrations for amplification of IncQ *oriV* and IncN *rep* (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
15.75	Aqua ultrapure	
2.5	EP-Taq buffer* (10×)	1×
2.5	dNTPs (2 mM)	0.2 mM
2.5	$MgCl_2$ (25 mM)	2.5 mM
0.25	Forward primer	0.1 µM
0.25	Reverse primer	0.1 µM
0.25	EP-Taq polymerase* (5 U μl^{-1})	0.125 U/sample
1	DNA template	Approx. 20 ng

*EP-Taq Buffer and Polymerase (JKI, Quedlinburg) (Dealtry *et al.*, 2014).

Table 3.20. Reagents and respective concentrations for amplification of pV216 *rep* (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
14.75	Aqua ultrapure	
2.5	EP-Taq buffer* (10×)	1×
2.5	dNTPs (2 mM)	0.2 mM
2.5	$MgCl_2$ (25 mM)	2.5 mM
0.75	Forward primer	0.3 µM
0.75	Reverse primer	0.3 µM
0.25	EP-Taq polymerase* (5 U μl^{-1})	1.25 U/sample
1	DNA template	Approx. 20 ng

*EP-Taq Buffer and Polymerase (JKI, Quedlinburg) (Dealtry *et al.*, 2014).

Table 3.21. PCR program for detection of *intl1*.

Cycles	Temperature (°C)	Time (s)	Step
1	94	300	Initial denaturation
35	94	30	Denaturation
	55	30	Annealing
	72	30	Elongation
1	72	300	Final elongation

Table 3.22. PCR program for detection of *qacEΔ1.*

Cycles	Temperature (°C)	Time (s)	Step
1	95	900	Initial denaturation
35	94	60	Denaturation
	50	60	Annealing
	72	30	Elongation
1	72	600	Final elongation

Table 3.23. PCR program for detection of the *trfA* sequence from all IncP-1 subgroups.

Cycles	Temperature (°C)	Time (s)	Step
1	94	300	Initial denaturation
35	94	30	Denaturation
	60	20	Annealing
	72	20	Elongation
1	72	300	Final elongation

Table 3.24. PCR program for detection of IncQ *oriV* and IncN *rep*.

Cycles	Temperature (°C)	Time (s)	Step
1	94	300	Initial denaturation
35	94	60	Denaturation
	x*	60	Annealing
	72	60	Elongation
1	72	600	Final elongation

*IncQ *oriV* 57 °C; IncN *rep* 55 °C.

Table 3.25. PCR program for detection of pV216 *rep*.

Cycles	Temperature (°C)	Time (s)	Step
1	95	300	Initial denaturation
35	94	30	Denaturation
	56	30	Annealing
	72	60	Elongation
1	72	300	Final elongation

3.2.12. Southern blotting

Southern blot consists of the transfer of the amplified DNA separated by agarose gel electrophoresis to a membrane and subsequent hybridization with a specific probe. This

technique was carried out following the instructions in the DIG System User's Guide for Filter Hybridization (Roche, Mannheim, Germany) with some modifications.

Labelling of DNA probes by random primer labelling

The DIG System is a simple, effective system for nonradioactive labelling and detection of nucleic acids. Digoxigenin (DIG) labelled probes were generated from PCR amplified fragments obtained with reference plasmids: pKJK5 for *intI1*, pB10 for *qacEΔ1* (Sandvang *et al.,* 1997), pTH10 for IncP-1 (all subgroups) *trfA,* pTH10 for IncP-1α *trfA,* R751 for IncP-1β *trfA,* (Bahl *et al.,* 2009), RSF1010 for IncQ *oriV,* RN3 for IncN *rep* (Götz *et al*., 1996) and pHHV216 for pV216 *rep* (Heuer *et al.*, 2009) (Table 3.9).

To purify the PCR products from reference plasmids, 75 µl of the PCR products of *intI1*, *qacEΔ1,* IncP-1 (all subgroups) *trfA,* IncP-1α *trfA,* IncP-1β *trfA,* IncQ *oriV* and pV216 *rep* were separated by agarose gels (1%, w/v) electrophoresis in 1× TBE buffer and 75 µl of the PCR product of IncN *rep* was separated by agarose gel (1.5%, w/v) electrophoresis in 1× TBE buffer. Then, the band of the correct size of the PCR product was extracted from the gel with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). Approximately 300-500 ng of the previously-denatured gel-purified PCR product were mixed with the labelling reagents: 2 µl of 10× Hexanucleotide Mix (Roche, Mannheim, Germany), 2 µl of 10× DIG DNA Labelling Mix (Roche, Mannheim, Germany), 1 µl (2 U μl^{-1}) of Klenow enzyme, and DNA Polymerase I (Roche, Mannheim, Germany). The mix was incubated overnight at 37 °C. To stop the reaction, 2 µl of 0.2 M EDTA buffer (pH 8.0) were added to the mix. The DNA probes were stored at -20 °C for further use.

DNA transfer to nylon membrane through capillary transfer

PCR products (75 µl) of *intI1*, *qacEΔ1,* IncP-1 (all subgroups) *trfA,* IncP-1α *trfA,* IncP-1β *trfA,* IncQ *oriV* and pV216 *rep* were separated by agarose gel (1%, w/v) electrophoresis in 1× TBE buffer and 75 µl of the PCR product of IncN *rep* was separated by agarose gel (1.5%, w/v) electrophoresis in 1× TBE buffer. As DNA molecular weight markers, 5 µl of 1 Kb Plus DNA Ladder (Thermo Scientific, Waltham, USA) and 5 µl of the DNA molecular weight marker VI, DIG-labeled (Roche, Mannheim, Germany), were used. The gels were run at 50 V for 4-5 h.

Denaturation of the gel was carried out by soaking it in denaturation solution (0.5 M NaOH and 1.5 M NaCl) for 2 × 15 min at room temperature under constant shaking. After

rinsing, gels were soaked in neutralization solution (1 M Tris-HCl (pH 7.5) and 1.5 M NaCl) for 2 × 15 min at room temperature under constant shaking. Finally, the gel was equilibrated for at least 10 min in 20× SSC buffer (3 M NaCl and 300 mM sodium citrate (pH 7)).

Simultaneously, the capillarity transfer set was prepared as shown in Figure 3.2. Samples were allowed to transfer overnight at room temperature.

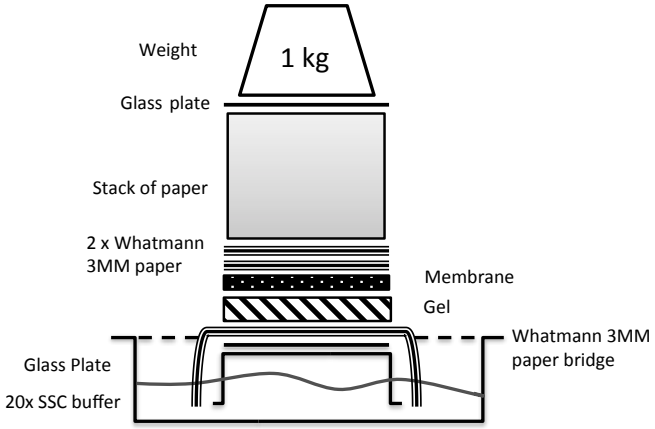


Figure 3.2. Southern Blot capillarity transfer set. A reservoir of approximately 300 ml of 20× SSC buffer was placed on the bottom of the transfer set. Then, a piece of Whatman 3MM paper (Sigma Aldrich, St. Louis, USA), previously soaked in 20× SSC buffer was laid on top of the glass plate and the sides of the 3MM paper hanged into the reservoir of 20× SSC buffer. The gel was placed upside down on top of the soaked Whatman 3MM paper and subsequently, a piece of Hybond-N uncharged Nylon membrane (Amersham Pharmacia Biotech, Amersham, UK) was put on top of the gel. To complete the blot assembly, two pieces of Whatman 3MM paper previously soaked in 20× SSC buffer and an approximately 7.5 cm high stack of filter paper, a glass plate, and 1 kg of weight were added on top.

Fixation of the membrane

After the overnight transfer, the membrane was washed for 5 min in 2× SSC to reduce the unspecific background. Then, the DNA was fixed to the membrane by baking the membrane for 2 h at 80 °C.

Hybridization of the DNA

Pre-hybridization step was carried out at 62 °C for 2 h with 30 ml of previously warmed pre-hybridization buffer (5× SSC, formamide (20%, v/v), blocking-reagent (2%, w/v), Na-

lauroylsarcosine (0.1%, v/v) and SDS (0.02%, v/v)) per 100 cm^2 of membrane surface area. After the incubation, the pre-hybridization buffer was discarded and 30 µl of previously denatured DIG-labelled probe was added to fresh 10 ml of pre-hybridization buffer (hybridization buffer). The membrane was incubated with the hybridization buffer overnight at 62 °C at constant rotation.

Washing at different stringencies

On the next day, the membrane underwent two-stage stringent washes to disrupt undesired hybrids. The first wash was made with low stringency (high salt concentration and low temperature) to remove non-specifically bound probe which otherwise could lead to high background. The membrane was incubated 2 × 5 min at room temperature with 50 ml low stringency buffer (2× SSC containing SDS (0.1%, v/v)) per 100 cm^2 of membrane.

The second wash was a high stringency wash (low salt concentration and high temperature) to remove undesired hybrids of low homology. The membrane was incubated 2 × 15 min at 68 °C with 50 ml of pre-warmed high stringency buffer (0.5× SSC containing SDS (0.1%, v/v)) per 100 cm^2 of membrane.

Detection of probe-target hybrids

Hybrids were detected with an enzyme-linked immunoassay. All the following steps were carried out at room temperature at constant rotation. Volumes were calculated for 100 cm^2 of membrane. The membrane was washed with 50 ml of washing buffer (100 mM maleic acid, 150 mM NaCl (pH 7.5) and Tween 20 (0.3%, v/v)) for 5 minutes. To prevent non-specific interaction of the antibody with the membrane, the membrane was blocked by 30 min incubation with 100 ml blocking buffer (blocking-reagent (1%, w/v), 100 mM maleic acid and 150 mM NaCl (pH 7.5)).

Next, the membrane was incubated for 30 min with 20 ml of blocking buffer supplemented with 2 µl of Anti-DIG-alkaline phosphatase (1:10,000) (Roche, Mannheim, Germany). Then, the membrane was washed 2 × 15 min with 100 ml of washing buffer to eliminate excess of antibody. Finally, it was incubated for 2 × 5 min with 20 ml of sterile alkaline solution (0.1 M Tris-HCl and 0.1 M NaCl (pH 9.5)) in order to activate the phosphatase.

To start the enzymatic reaction, 1,000 µl of chemiluminescent substrate CDP star® ready to use solution (Roche, Mannheim, Germany) were added onto the membrane and incubated for 5 min. After incubation, excess of CDP-star solution was removed, and the membrane was exposed for 20 minutes to a High Performance Chemiluminescence Film (GE Healthcare Life Science, Little Chalfont, UK) and developed.

3.2.13. Statistical analysis

ANOVA were applied to the following tests: $CaCl_2$-extractable concentrations of heavy metals, soil microbial properties and determination of 16S rRNA gene copy numbers by qPCR. Where more than two groups were compared, post-hoc pairwise comparisons were performed with Tukey-Kramer adjustment ($p < 0.05$). All statistical analyses were carried out with the Statview software (SAS Institute Inc. Software Informer).

In order to determine the overall effect of aided phytostabilization on soil quality here, from the following 13 soil microbial parameters, a quality value for each soil was calculated, based on the treated-soil quality index (T-SQI) proposed by Mijangos *et al*. (2010).

$$SQI = 10^{\log m + \frac{\sum_{i=1}^{n} (\log n_i - \log m)}{n}}$$

Where m is the control value for the CONTROL soil (set to 100%) and n corresponds to the measured values for each parameter as a percentage of the control value. This index is appropriate for the assessment of soil quality in those cases where the soil has been intentionally treated. When evaluating the effects of treatments, the T-SQI takes into account not only the magnitude of the change but also the direction (increase/decrease) of such change (Epelde *et al*., 2014a).

The following parameters were selected for the studied soil quality: (1) β-glucosidase activity; (2) urease activity; (3) acid phosphatase activity; (4) dehydrogenase activity; (5) FDA; (6) arylsulphatase activity; (7) potentially mineralizable nitrogen (PMN); (8) microbial biomass; (9) total bacteria from 16S rRNA; (10) RR-16S rRNA; (11) H'-16S rRNA; (12) RR-18S rRNA; and (13) H'-18S rRNA.

3.3. Results

3.3.1. Physicochemical characterization and heavy metal concentrations of soils

Physicochemical characterization of soils

The soil physicochemical parameters for site 1 and site 2 (CONTROL soils) are listed in Table 3.26. Site 1 was classified as sandy-loamy soil, while site 2 showed a loamy texture. Both sites presented slightly acid pH (6.5 and 6.7, respectively). Cation exchange capacity (CEC) was high for both sites (>10 cmol kg^{-1}) and clearly dominated by calcium. However, when comparing site 1 *versus* site 2, the CEC was slightly higher for site 1 than for site 2 (19.980 cmol kg^{-1} and 15.117 cmol kg^{-1} for site 1 and site 2, respectively). The same relation was observed for the oxidable and total organic matter (OM). Site 1 harboured a higher oxidable and total OM (86.1 g kg^{-1} and 199.8 g kg^{-1}, respectively) levels compared to site 2 (72.3 g kg^{-1} and 151.2 g kg^{-1}, respectively).

Table 3.26. Soil characterization. Values are expressed on a dry weight basis (mean ± SE, n = 3).

	Site 1	**Site 2**
Coarse sand (%)	39.110 ± 0.887	11.690 ± 1.645
Fine sand (%)	43.843 ± 1.061	39.873 ± 1.249
Total sand (%)	82.953 ± 1.923	51.563 ± 1.249
Loam (%)	14.103 ± 1.393	38.820 ± 0.320
Clay (%)	2.943 ± 0.542	9.616 ± 0.175
Classification	Sandy-loam	Loam
pH (1:2.5) water	6.486 ± 0.111	6.713 ± 0.029
Oxidable organic matter (g kg^{-1})	86.133 ± 5.279	72.300 ± 7.304
Total organic matter (g kg^{-1})	199.800 ± 28.315	151.166 ± 9.012
Nitrogen total (g kg^{-1})	7.133 ± 0.865	4.266 ± 0.536
C/N ratio	7.286± 1.198	9.926 ± 0.312
Phosphorus (Olsen) (g kg^{-1})	0.007 ± 0.001	0.002 ± 0.000
Calcium (g kg^{-1})	1.343 ± 0.091	0.760 ± 0.082
Magnesium (g kg^{-1})	0.287 ± 0.029	0.177 ± 0.009
Potassium (g kg^{-1})	0.156 ± 0.033	0.084 ± 0.003
Cation exchange capacity (cmol kg^{-1})	19.980 ± 2.832	15.117 ± 0.901

Heavy metal concentration of soils

The majority of the heavy metals detected in both sites of study, but especially Pb and Zn pseudo-total concentrations greatly surpassed the Reference Critical Values reported for the ecosystem protection in the Basque Country (120 mg kg^{-1}, 106 mg kg^{-1} for Pb and Zn, respectively) (IHOBE, 1998; BOPV, 2005). Nevertheless, site 2 showed a significantly higher contamination for all of the heavy metals tested than site 1 (Table 3.27).

Table 3.27. Pseudo-total heavy metal concentrations. Values are expressed on a dry weight basis (mean ± SE, n = 3).

	Site 1	**Site 2**
Aluminium (Al) (mg kg^{-1})	3,771 ± 43.544	13,527 ± 156.196
Arsenic (As) (mg kg^{-1})	18.5 ± 0.748	134 ± 0.774
Cadmium (Cd) (mg kg^{-1})	6 ± 0.312	13.6 ± 0.157
Chromium (Cr) (mg kg^{-1})	10.4 ± 0.360	31.7 ± 0.183
Copper (Cu) (mg kg^{-1})	27.9 ± 1.289	50.2 ± 0.290
Iron (Fe) (mg kg^{-1})	14,954 ± 86.337	94,119 ± 1086.793
Manganese (Mn) (mg kg^{-1})	243 ± 4.209	745 ± 17.205
Nickel (Ni) (mg kg^{-1})	8.3 ± 0.144	52.6 ± 0.607
Lead (Pb) (mg kg^{-1})	16,285 ± 188.043	28,587 ± 330.094
Selenium (Se) (mg kg^{-1})	<3	<3
Zinc (Zn) (mg kg^{-1})	15,529 ± 179.313	61,007 ± 352.224
Mercury (Hg) (mg kg^{-1})	10.3 ± 0.476	14.3 ± 0.330

3.3.2. PAPER treatment was the most effective to reduce the long-term bioavailability of Cd, Pb and Zn

The main goal of any aided phytostabilization is to reduce the bioavailability of heavy metals in soil. To measure the long-term effects of the aided-phytostabilization experiment started in 2010 with the application of cow manure (COW), sheep manure (SHEEP) and paper mill sludge mixed with poultry manure (2:1, v/v) (PAPER) on both site 1 and site 2, the $CaCl_2$-extractable Cd, Pb and Zn concentrations in soil were determined in 2014 and compared to the control soils where no amendment was applied (CONTROL). Soil treated with poultry manure (POULTRY) in 2010 was not included in this study as it received a further application of manure in 2014 prior to our study.

Among the three heavy metals tested, values of $CaCl_2$-extractable heavy metal concentrations were much higher for Zn than for Pb or Cd (Figure 3.3). PAPER was the amendment that produced a more effective reduction ($p < 0.05$) in bioavailable Cd for both sites 1 and 2, in comparison to the effect caused by the application of COW or SHEEP treatment (Figure 3.3A).

Regarding Pb bioavailability, site 2 showed a significant higher concentration ($p < 0.05$) of $CaCl_2$-extractable Pb than site 1 due to probably an also higher pseudo-total concentration of Pb on site 2 (16,285 ± 188.0 and 28,587 ± 330.1 mg kg^{-1} for site 1 and 2, respectively) (Figure 3.3B). Amendment application did not show any long-term reduction in bioavailable Pb in site 1. In site 2, COW treated soil showed the highest concentration of bioavailable Pb indicating that it was the least effective in reducing Pb bioavailability. Although the differences were not statistically significant, SHEEP and PAPER treated soils showed lower levels of bioavailable Pb in comparison to CONTROL soil.

Again, site 2 showed a significant higher concentration ($p < 0.05$) of $CaCl_2$-extractable Zn (Figure 3.3C) than site 1 due to probably an also higher pseudo-total concentration of Zn on site 2 (15,529 ± 179.3 and 61,007 ± 352.2 mg kg^{-1} for site 1 and 2, respectively). PAPER treatment reduced significantly ($p < 0.05$) the concentration of bioavailable Zn in both sites. In addition, SHEEP treatment caused a similar reduction of bioavailable Zn in site 2.

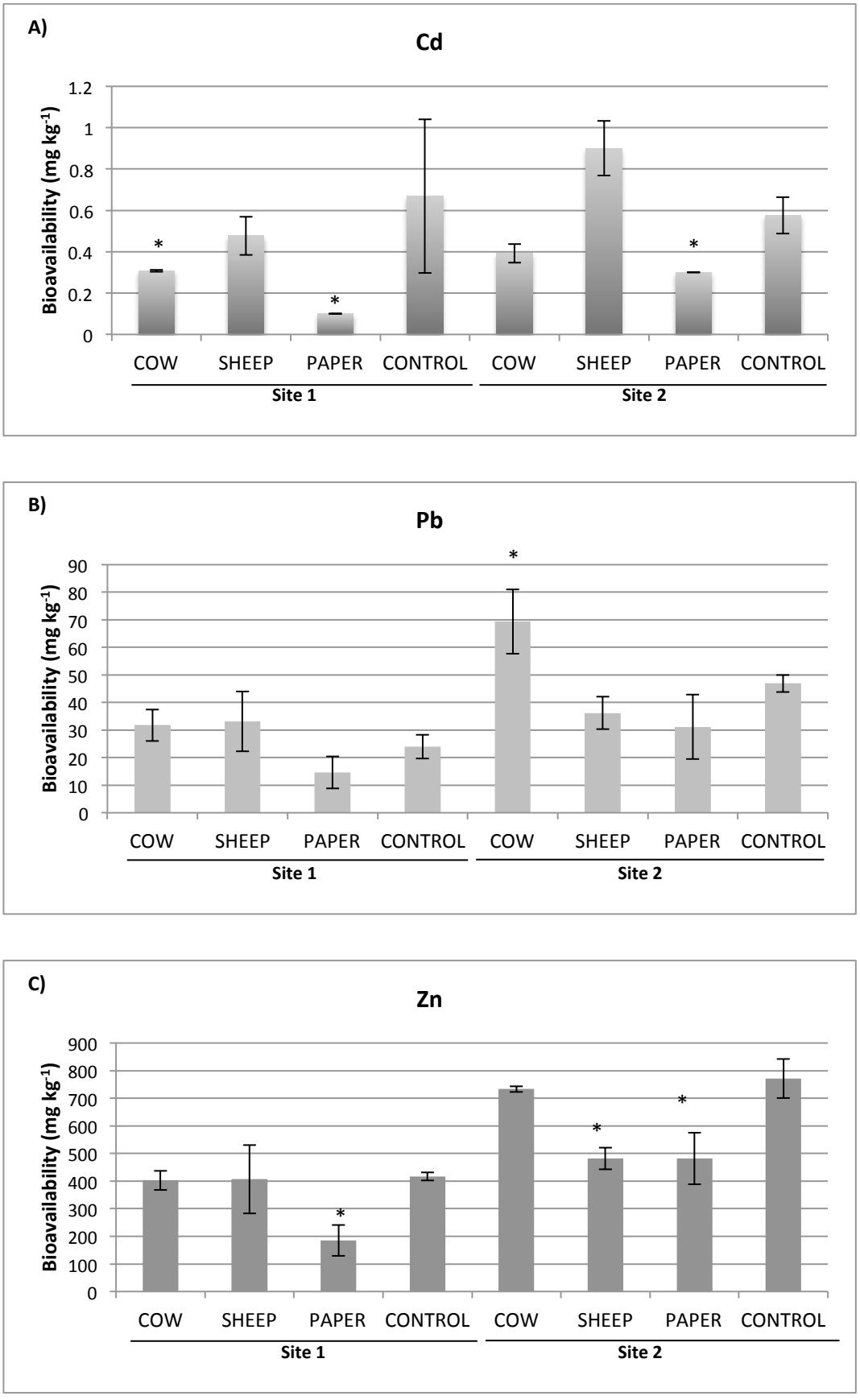


Figure 3.3. Cd (A), Pb (B) and Zn (C) bioavailable concentrations (mg kg^{-1}) of each of soil studied. (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

Taking into consideration the observed changes for Cd, Pb and Zn bioavailability fractions, PAPER was the most effective amendment in terms of long-term reduction of heavy metal bioavailability in the study sites.

3.3.3. Biological indicators of soil quality

Apart from reducing heavy metal bioavailability, the other main objective of any aided-phytostabilization is to restore, or at least improve, soil quality. To evaluate the long-term effect of COW, SHEEP and PAPER amendment application on soil quality, different soil microbial properties were selected as indicators of such quality. Once again, POULTRY treated soil was not included in this study (see above).

First, the three main nutrient cycles (C, N and S) in soil were studied as indicators of the functional status of the microbial community in each soil.

In relation to the carbon cycle, β-glucosidase enzyme activity and soil organic carbon concentration (SOC) were analyzed. When comparing both sites, site 1 presented a significantly higher ($p < 0.05$) β-glucosidase enzyme activity and SOC concentration than site 2 (Figure 3.4 and Table 3.28). In particular, SHEEP significantly reduced the β-glucosidase enzyme activity in soil when compared to CONTROL, COW and PAPER.

Table 3.28. Soil organic carbon concentration (SOC). SOC concentration was expressed for each soil sample as mg kg^{-1} of soil (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

Site	Soil	SOC (mg kg^{-1})
1	COW	<40*
	SHEEP	113.257 ± 32.606
	PAPER	79.219 ± 2.935
	CONTROL	94.267 ± 13.855
2	COW	<40
	SHEEP	<40
	PAPER	<40
	CONTROL	<40

Regarding SOC values in site 1, COW showed a significantly lower SOC concentration in soil in comparison to the CONTROL, SHEEP and PAPER (Table 3.28) treated soils. Contrarily, in

site 2, the β-glucosidase enzyme activity and SOC concentration displayed equivalent values in all soil samples independently of the applied treatment.

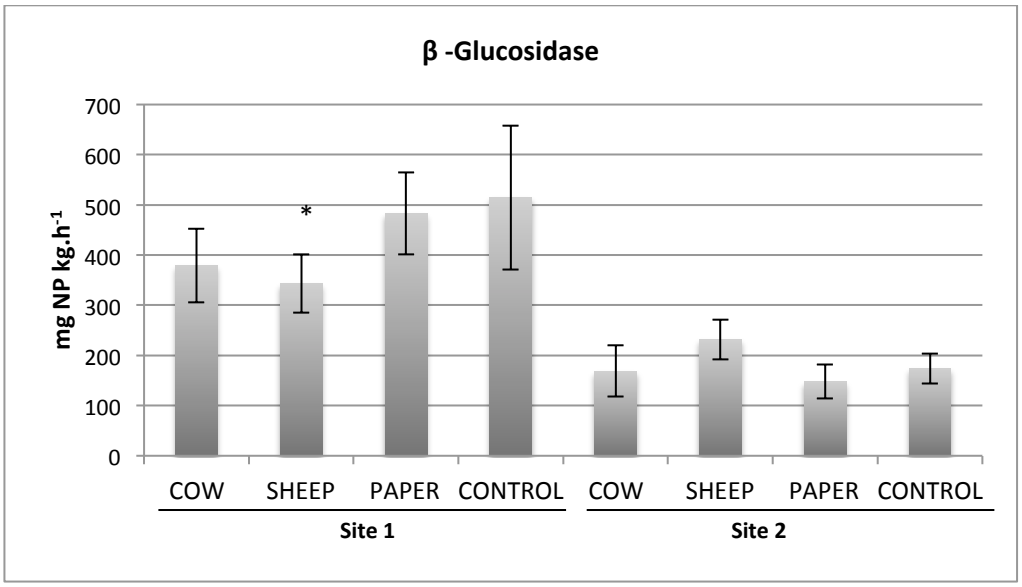


Figure 3.4. β-glucosidase enzyme activity. NP: *p*-nitrophenol (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

In relation to the nitrogen cycle, urease enzyme activity and potentially mineralizable nitrogen (PNM) were studied. When comparing both sites, site 1 presented a significantly higher ($p < 0.05$) urease enzyme activity than site 2 along with a higher PNM value (Figure 3.5 and Table 3.29).

In particular, in site 1, COW and SHEEP showed a significantly lower urease enzyme activity in comparison to CONTROL and PAPER treated soils. Contrarily, in site 2, the urease enzyme activity was equivalent in all soils independently of the applied treatment. Alongside, PNM values presented no significant differences as a result of the different amendment application neither in site 1 nor site 2.

Table 3.29. Potentially mineralizable nitrogen (PMN). PMN was expressed for each soil as mg kg^{-1} of soil (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

Site	Soil	PMN (mg kg^{-1})
1	COW	79.725 ± 1.598
	SHEEP	93.660 ± 12.856
	PAPER	124.019 ± 12.019
	CONTROL	108.903 ± 8.523
2	COW	41.061 ± 4.154
	SHEEP	54.239 ± 5.449
	PAPER	44.646 ± 4.426
	CONTROL	46.412 ± 1.372

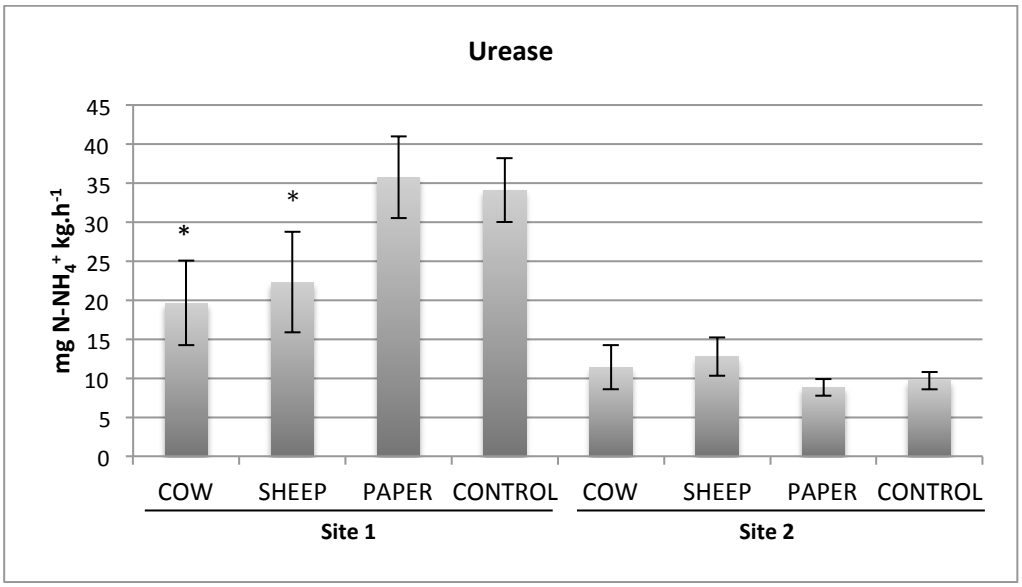


Figure 3.5. Urease enzyme activity. (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

Additionally, acid phosphatase, dehydrogenase, and fluorescein diacetate (FDA) hydrolysis activities were measured and arylsulphatase enzyme activity (Figure 3.6) was quantified to evaluate the sulphur cycle in soil.

When comparing both sites, site 1 and site 2 did not show any significant difference on the level of acid phosphatase enzyme (Figure 3.6A) or arylsulphatase enzyme activities (Figure 3.6B). Regarding amendment application, PAPER treated soil presented a significantly higher arylsulphatase enzyme activity in comparison to the rest of the soils (Figure 3.6B), but not to the CONTROL soil. Apart from this, no other significant differences were observed among the different soils for the acid phosphatase enzyme or arylsulphatase enzyme activity.

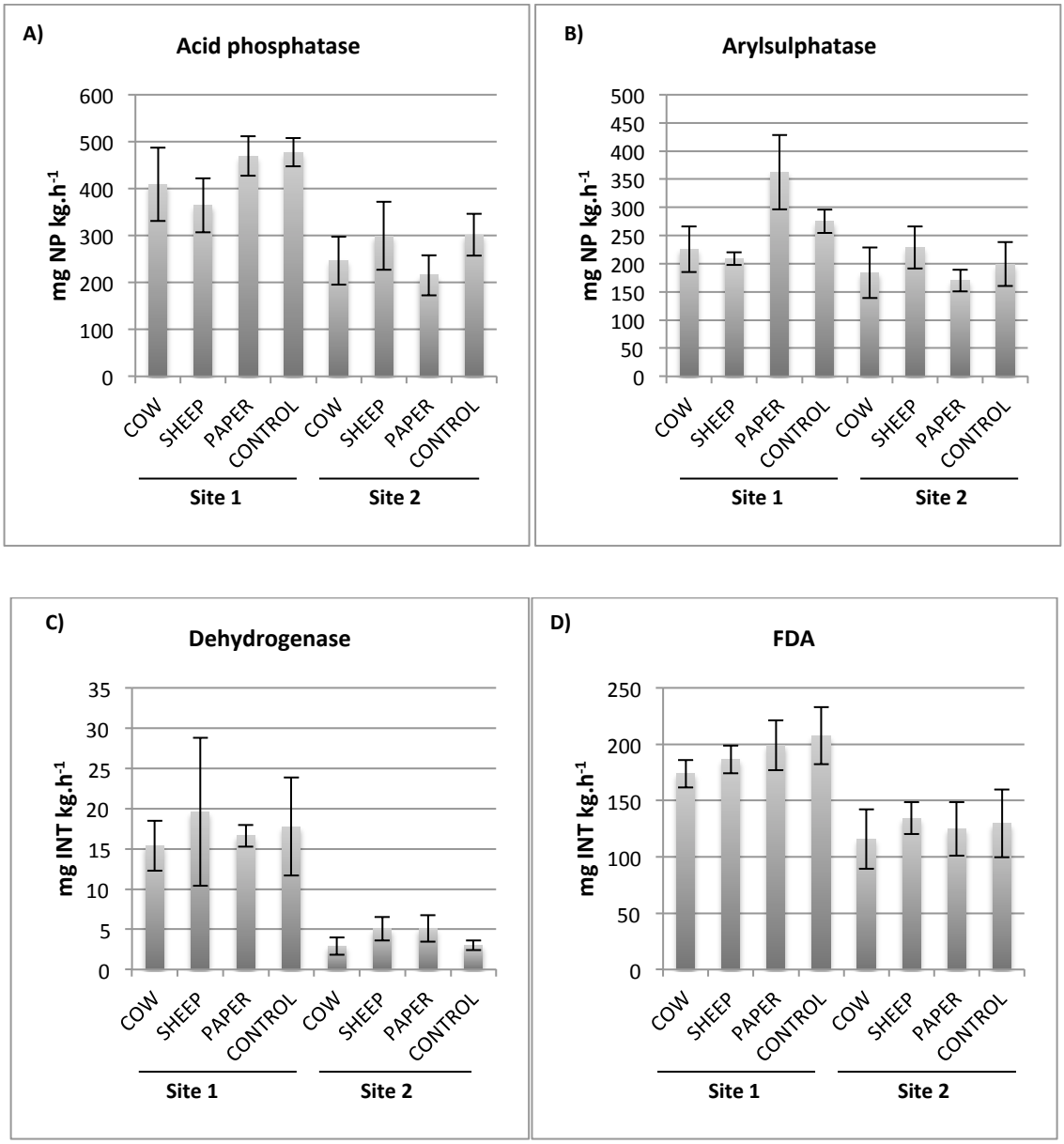


Figure 3.6. Acid phosphatase (A), arylsulphatase (B) and dehydrogenase (C) enzyme activities, fluorescein diacetate (FDA) hydrolysis (D). (mean ± SE, n=4).

In relation to dehydrogenase and FDA (Figure 3.6C and D, respectively) activities, site 1 presented a significantly higher ($p < 0.05$) activity than site 2 for both of them. Nevertheless, regarding amendment application, there was no significant effect on dehydrogenase enzyme activity or FDA for any of the two sites.

Finally, the geometric mean of the values of arylsulphatase, β-glucosidase, acid phosphatase and urease enzyme activities was also expressed as an indicator of overall enzyme activity (OEA) (Figure 3.7).

$$OEA = (\text{arylsulphatase} \cdot \beta\text{-glucosidase} \cdot \text{acid phosphatase} \cdot \text{urease})^{1/4}$$

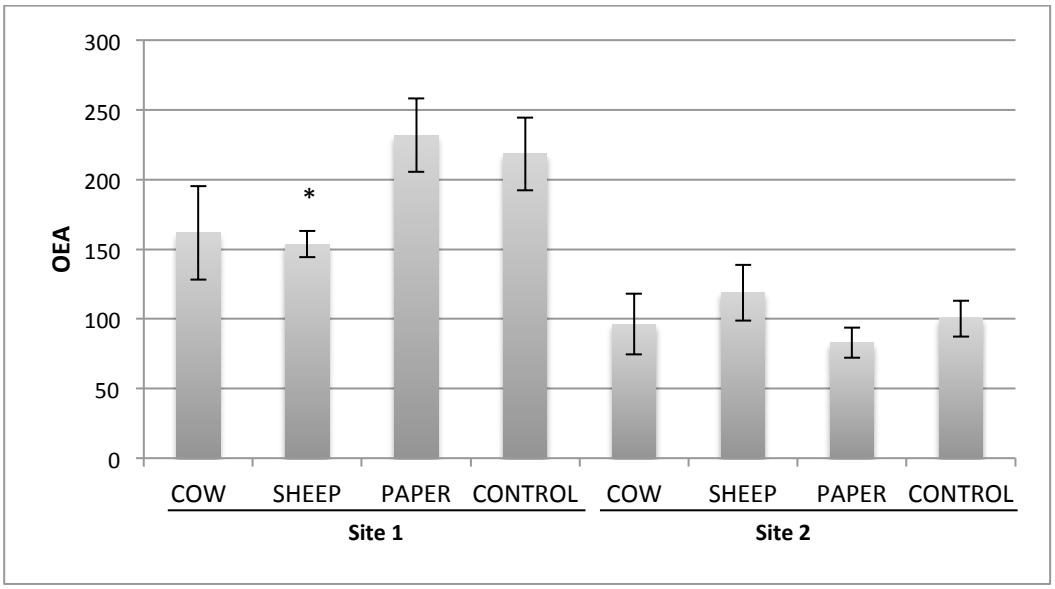


Figure 3.7. Overall enzyme activity (OEA). (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

In relation to OEA, site 1 presented a significantly higher ($p < 0.05$) value than site 2 (Figure 3.7). In site 1, SHEEP produced a significantly lower reduction ($p < 0.05$) on the OEA in soil. However, in site 2, no significant differences were observed for OEA values.

Penicillin resilience

Resilience was measured for each soil after penicillin application. In particular, resilience was expressed as the (i) maximum velocity of growth (Vmax) of the soil bacterial community and (ii) time to reach that maximum velocity (t for Vmax), after penicillin application. In general terms, a positive correlation between both parameters was observed (Table 3.30). As it can be seen in Table 3.30, all soil bacterial communities were disturbed by the addition of penicillin as Vmax was lower in the presence *versus* the absence of penicillin (negative); in addition, the time required to reach Vmax increased in the presence *versus* the absence of penicillin.

Table 3.30. Maximum velocity (Vmax) of growth curve and time for maximum velocity (t for Vmax) when incubated with or without penicillin for 24 h. (mean ± SE, n=4).

Site	Soil	Vmax negative	Vmax penicillin	t for Vmax negative(d)	t for Vmax penicillin (d)
1	COW	2.290 ± 0.163	0.917 ± 0.129	0.881 ± 0.021	1.262 ± 0.146
	SHEEP	1.688 ± 0.175	1.122 ± 0.107	0.892 ± 0.024	1.147 ± 0.175
	PAPER	2.203 ± 0.233	1.032 ± 0.059	0.859 ± 0.023	0.889 ± 0.018
	CONTROL	1.428 ± 0.134	1.027 ± 0.126	0.933 ± 0.020	0.952 ± 0.051
2	COW	0.948 ± 0.394	0.362 ± 0.140	1.032 ± 0.058	1.878 ± 0.070
	SHEEP	0.995 ± 0.124	0.551 ± 0.039	0.926 ± 0.044	1.451 ± 0.207
	PAPER	0.969 ± 0.085	0.530 ± 0.103	0.986 ± 0.043	1.327 ± 0.178
	CONTROL	0.896 ± 0.085	0.723 ± 0.117	1.054 ± 0.079	1.643 ± 0.127

To compare the results among the different soils, both Vmax and time for Vmax where normalized with the negatives grown without penicillin (Table 3.31). Both CONTROL soils from site 1 and 2 presented the highest Vmax values. Moreover, CONTROL soil from site 1 required the shortest time to reach the Vmax point. By contrast, COW treated soils exhibited the lowest Vmax and required the longer time to recover after the penicillin perturbation in both sites.

Table 3.31. Normalized maximum velocity (Vmax) of the growth curve and time for maximum velocity (t for Vmax) when incubated with penicillin for 24 h. The values were normalized with the results obtained for the same samples incubated without penicillin (mean ± SE, n=4).

Site	Soil	Vmax	t for Vmax (d)
1	COW	0.400 ± 0.131	1.432 ± 0.394
	SHEEP	0.665 ± 0.143	1.285 ± 0.326
	PAPER	0.468 ± 0.077	1.035 ± 0.044
	CONTROL	0.720 ± 0.217	1.020 ± 0.118
2	COW	0.382 ± 0.367	1.820 ± 0.264
	SHEEP	0.554 ± 0.224	1.568 ± 0.335
	PAPER	0.547 ± 0.311	1.346 ± 0.408
	CONTROL	0.806 ± 0.382	1.560 ± 0.264

These results, although not statistically significant ($p > 0.05$), suggest a stronger resilience of the soil bacterial community towards penicillin in the CONTROL soils versus treated soils.

3.3.4. Heavy metal contamination affects soil microbial biomass

The microbial biomass of each of the soil was determined as an indicator of the effect of the aided-phytostabilization on the soil microbial communities and, hence, on soil quality. Microbial biomass refers to the living fraction of the soil OM. The soil microbial biomass rapidly responds to changing environmental conditions, such as, for instance, an addition of carbon substrates or an increase in heavy metal concentration. In this respect, the microbial biomass can be a more reliable indicator than the level of OM.

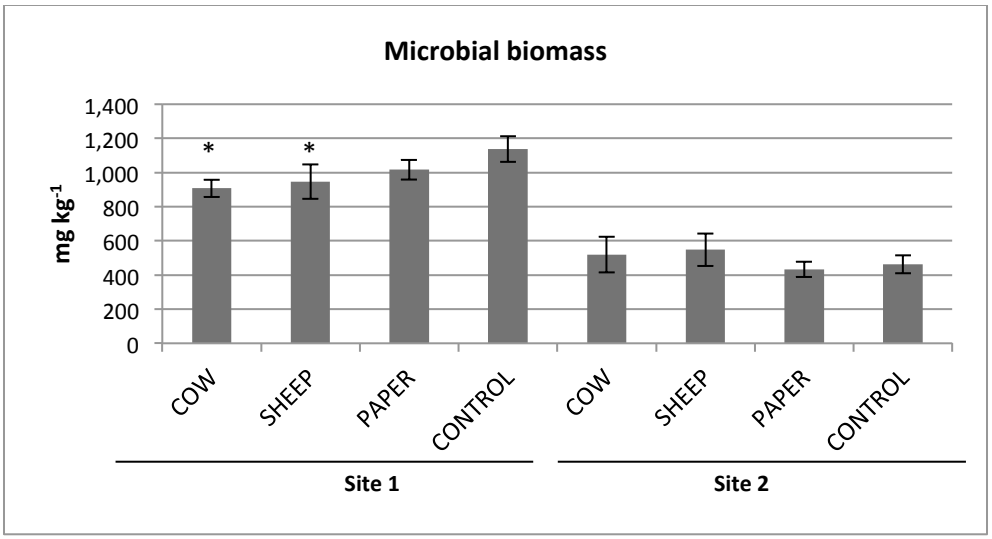


Figure 3.8. Quantification of the microbial biomass. (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

In general terms, higher values of microbial biomass were detected in site 1 than in site 2, which implies that soil in site 1 harbour a larger microbial community than soil in site 2 (Figure 3.8). More specifically, in site 1 the highest values of microbial biomass were found for the CONTROL soil where no amendment was applied, while COW and SHEEP treated soils showed significantly lower ($p < 0.05$) values. In site 2, no significant differences among treatments were observed.

Total community DNA (TC-DNA) from soil

After the extraction of TC-DNA from soil carried out with the FastDNA™ SPIN kit for Soil and the posterior purification with the Geneclean Spin Kit, TC-DNA was visualized by agarose gel (0.8%, w/v) electrophoresis in 0.5× TBE buffer as shown in Figure 3.10. This DNA

extraction procedure delivered high molecular weight DNA with a slightly different quantity between replicates. The highest amount of DNA was obtained in soil samples taken from site 1 (Figure 3.9A) in comparison to site 2 (Figure 3.9B).

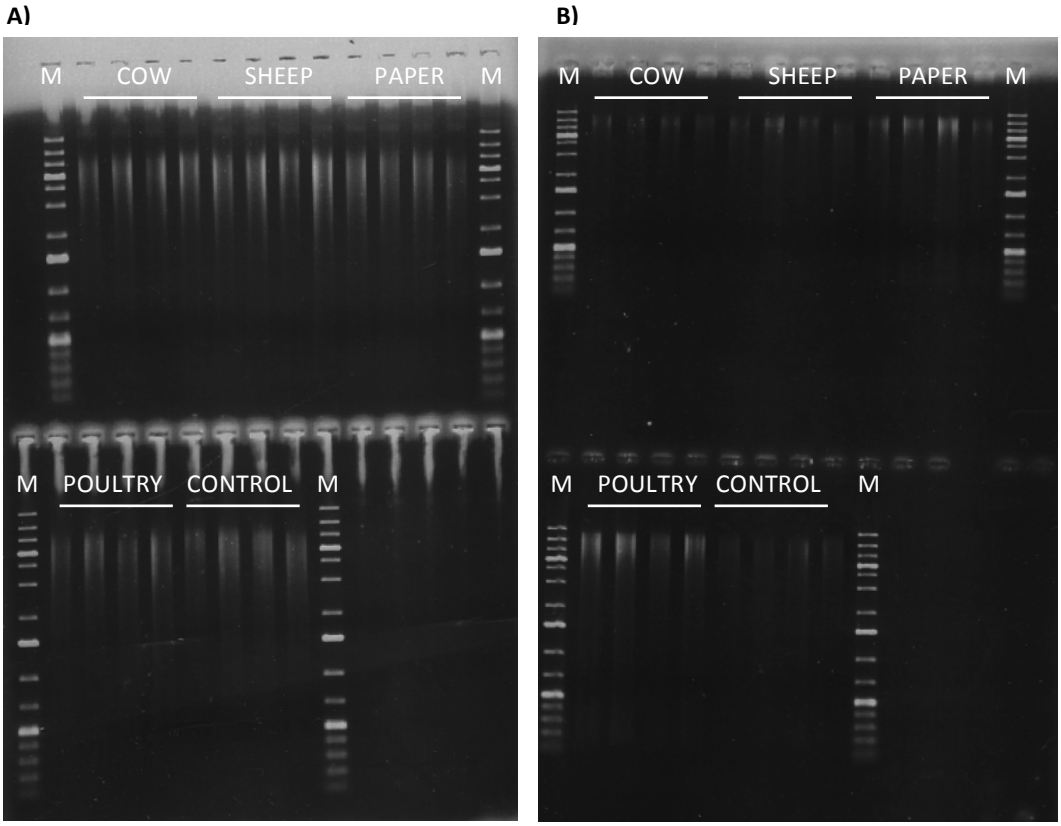


Figure 3.9. Agarose gel electrophoresis of TC-DNA from the different soils in site 1 (A) and site 2 (B). Lanes M: GeneRuler 1 Kb Plus DNA Ladder DNA molecular weight marker (Thermo Scientific). Quadruplicates of each sample were analysed.

16S rRNA gene copy numbers by qPCR

Quantification of the bacterial population present in soil was carried out by 16S rRNA-qPCR. In Figure 3.10 the amplification curve (A) and the standard curve (B) for the qPCR assay are represented. Successful amplifications for all of the samples studied can be seen in both graphs. The threshold cycle (C_q) refers to the first cycle at which the fluorescence of the amplification surpasses the fluorescence due to background noise. This value is represented in Figure 3.10A as the point where the amplification curve meets/crosses the threshold line. In Figure 3.10B, a standard curve (R^2: 0.999) was built with serial dilutions of of the *E. coli* 16S rRNA gene cloned into pGEM-T vector. In this manner, the absolute values of the initial concentration of the 16S rRNA gene copy of each sample were determined.

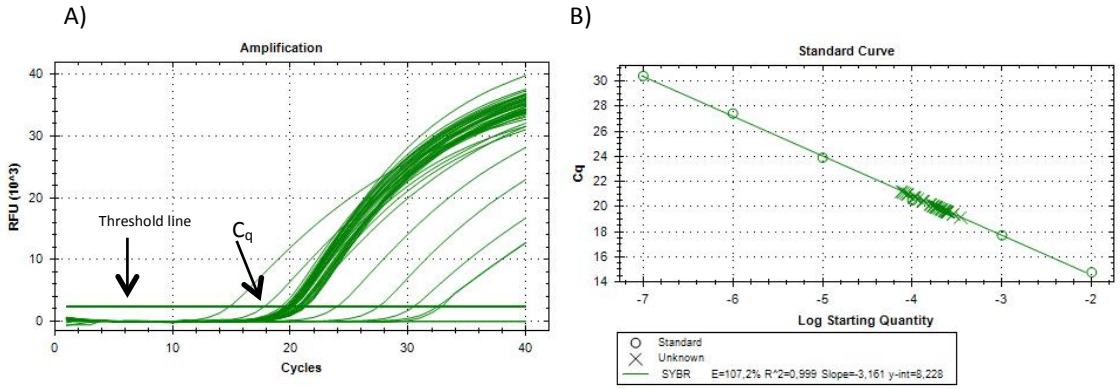


Figure 3.10. Quantification of 16S rRNA gene copy numbers by qPCR. A) Amplification curves. Each soil was studied in quadruplicate. The C_t (threshold cycle) value for each sample is the point where the amplification curve meets the threshold line. **B) Standard curve.** The standard curve was constructed by plotting the log of the starting quantity of the serial dilutions of the *E. coli* 16S rRNA gene standard against the C_q (quantification cycle) values obtained.

Statistically significant differences were observed among the soil when comparing the 16S rRNA gene copy numbers ($p < 0.05$). In general terms, a higher value of the 16S rRNA gene copy number was detected in site 1 in comparison with site 2 (Figure 3.11), which implies that in site 1 the bacterial community in soil is larger that in site 2. More specifically, the highest number of copies was found for the COW treated soil in site 1 and the lowest number was obtained for the CONTROL soil in site 2.

In site 2, COW and SHEEP treated soils and CONTROL soil were significantly different ($p < 0.05$) from the other soil samples but they did not differ significantly among each other. Furthermore, COW treated soil in site 1 also significantly differed from the PAPER treated soil in site 1 and the POULTRY treated soil in site 2.

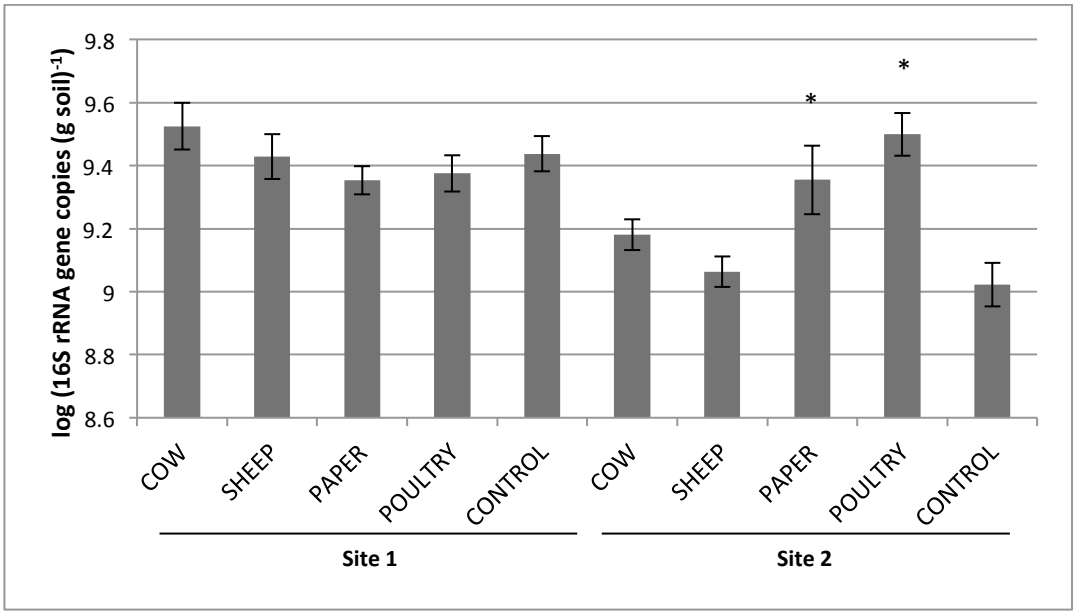


Figure 3.11. Quantification of 16S rRNA gene copy numbers of each of the soils studied. (mean ± SE, n=4). *Asterisks indicate significance ($p \leq 0.05$).

3.3.5. Effect of heavy metals and aided phytostabilization on the diversity of soil microbial communities

Denaturing Gradient Gel Electrophoresis (DGGE)

The profile of dominant bacterial species present in the soils was studied by DGGE of the amplified 16S rRNA genes. An amplification product size of approximately 473 bp corresponding to the amplified 16S rRNA fragment with a GC-clamp attached was detected for all samples (Figure 3.12).

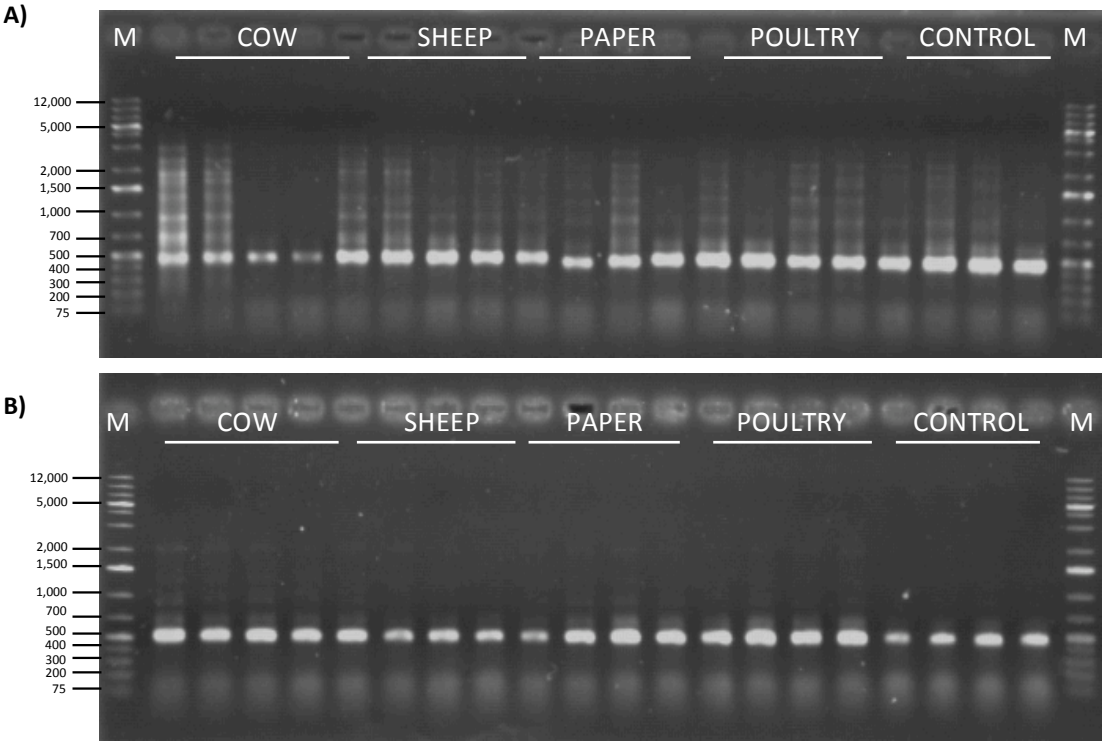


Figure 3.12. Detection of amplified 16S rRNA genes from bacterial communities in the different soils in site 1 (A) and site 2 (B). Lanes M: GeneRuler 1 Kb Plus DNA Ladder DNA molecular weight marker (Thermo Scientific).

DGGE analysis of PCR-amplified 16S rRNA gene fragments displayed a high number of bands indicating the presence of a large number of different ribotypes for all soil types (Figure 3.13, 3.15A and 3.16A). UPGMA cluster analysis of DGGE fingerprints revealed a clustering according to the soil site as seen in Figure 3.14. The green cluster, which corresponds to soils from site 1, was clearly separated from the soils from site 2-cluster shown in magenta. Also, UPGMA cluster analysis of DGGE fingerprints showed distinct sub-clusters in each site in accordance with the type of amendment that each soil received (Figure 3.15B and 3.16B). In both figures, the red cluster represented the quadruplicates from the COW treated soil, the green cluster corresponded to the SHEEP treated soil, the blue cluster to the PAPER treated soil, the yellow cluster to the POULTRY treated soil and the pink cluster to the CONTROL soil (no amendment). These analyses confirmed the high similarity of bacterial communities that existed between the four independent replicates of each soil.

Permutation tests based on Pearson similarity coefficients showed that bacterial communities from soils in site 1 and soils in site 2 formed separated clusters that had a 30.5% of difference. When studying the amendment effect among the soils in site 1, a significant

difference of 37.7% was found among the different soils. For site 2, the treatment effect was significantly lower, with a difference of only 25.9%.

When comparing the CONTROL soil with the treated soils within the same site, the highest similarity was found for COW treated soil with a difference of only 6.5 and 7.1% for site 1 and site 2, respectively. On the contrary, the highest difference between any treated soils in comparison to the CONTROL soil was found for PAPER treated soil with a difference of 74.3 and 54.9%, for site 1 and site 2, respectively.

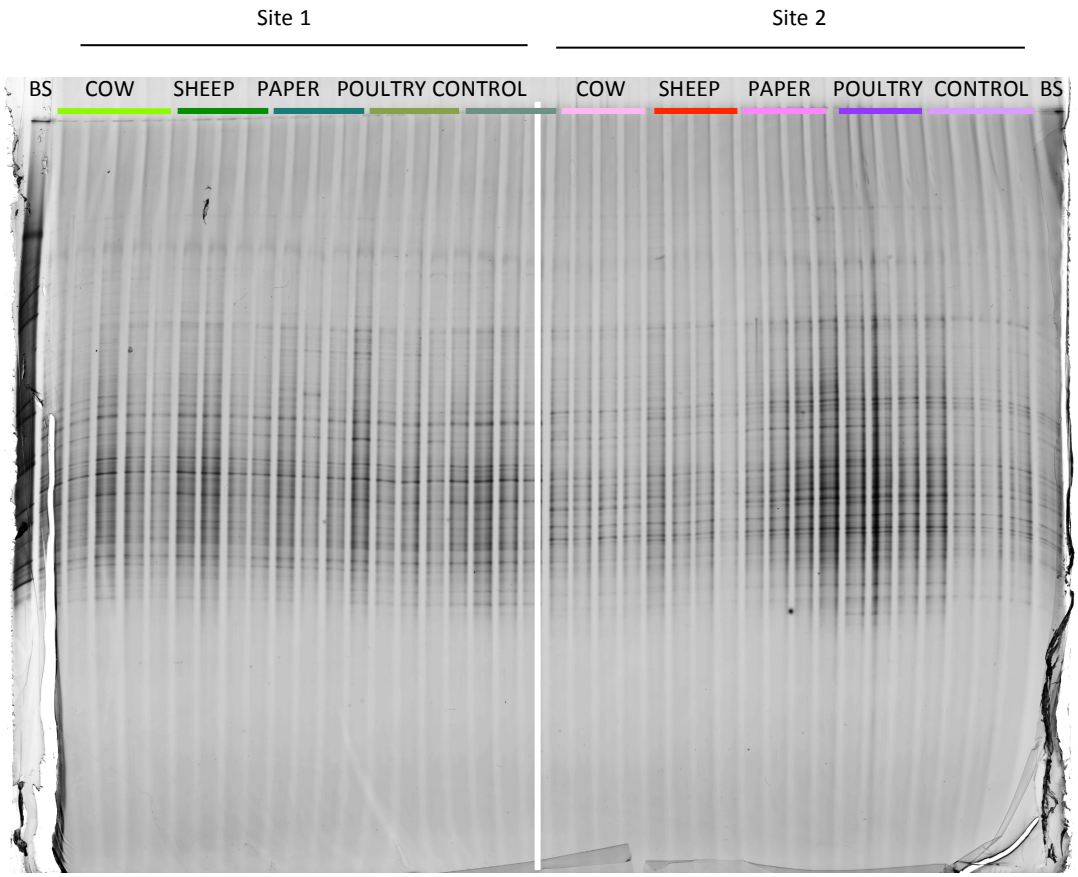


Figure 3.13. DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from the different soils. BS: bacterial DGGE standard designed from 11 different bacterial strains.

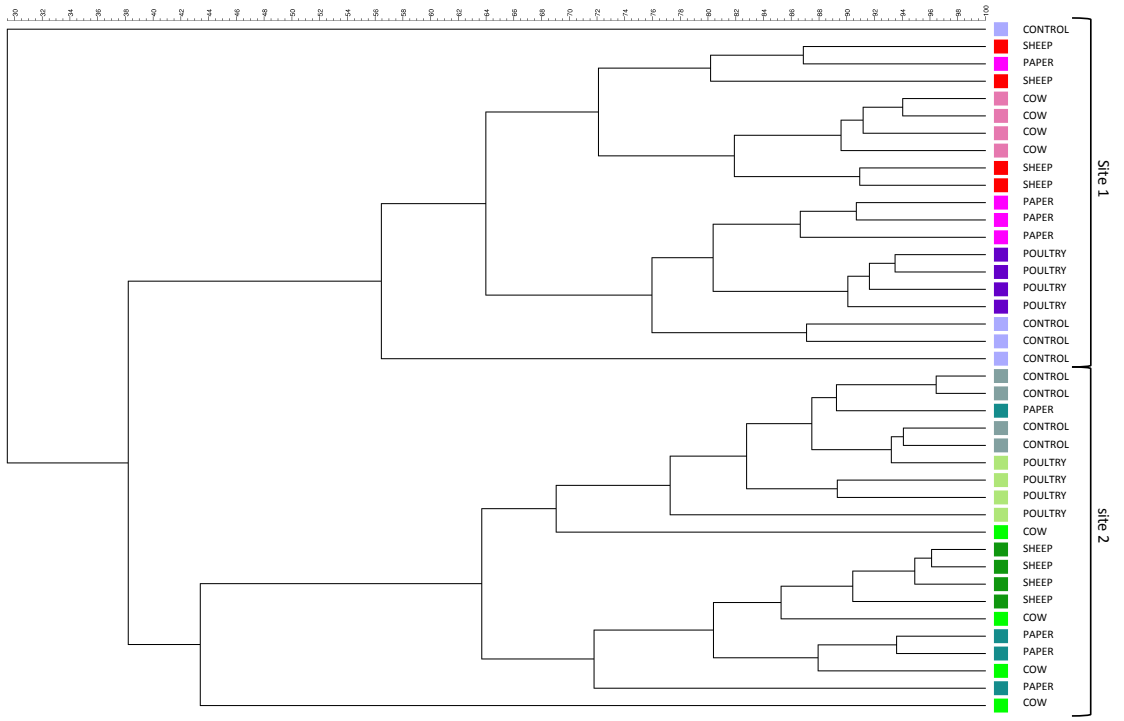


Figure 3.14. Dendrogram of the DGGE profiles of bacterial communities from the different soils. The cluster analysis was based on Pearson correlation coefficient as similarity measure and the unweighted pair-group method with arithmetic averages (UPGMA).

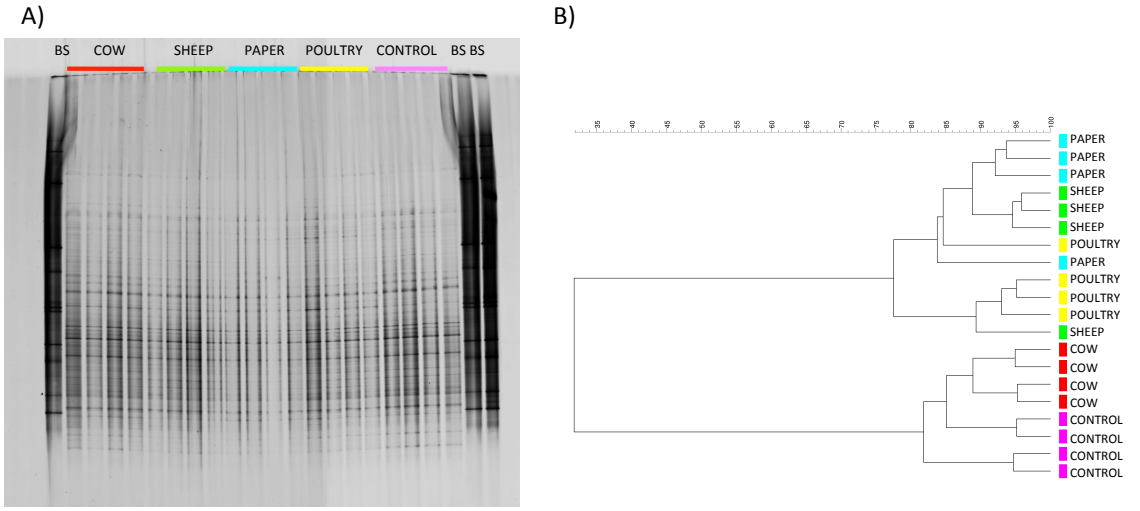


Figure 3.15. A) DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from the different soils in site 1. BS: bacterial DGGE standard designed from 11 different bacterial strains. **B) Dendrogram of the DGGE profiles of bacterial communities from the different soils in site 1.** The cluster analysis was based on Pearson correlation coefficient as similarity measure and the unweighted pair-group method with arithmetic averages (UPGMA).

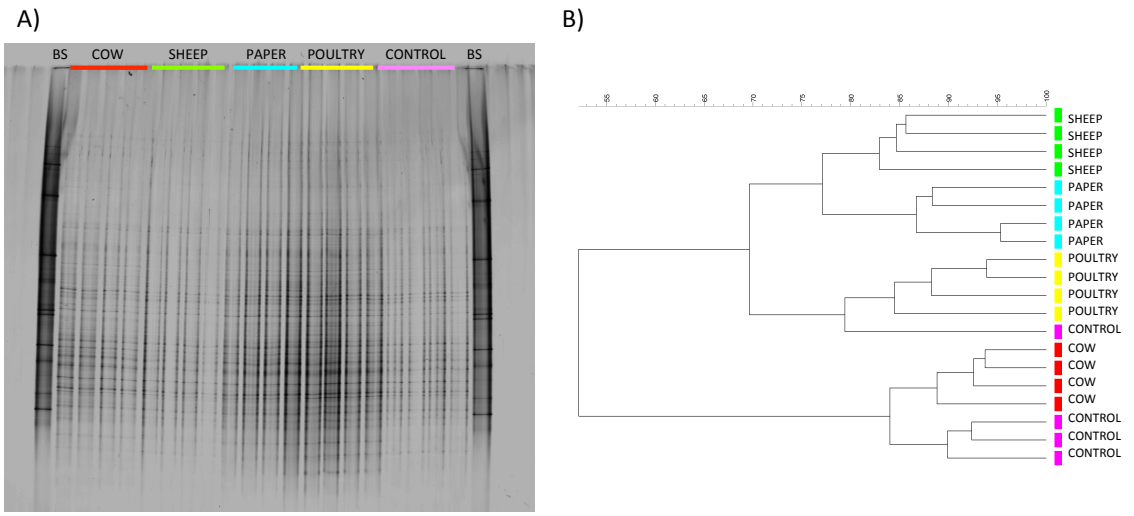


Figure 3.16. A) DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from the different soils in site 2. BS: bacterial DGGE standard designed from 11 different bacterial strains. **B) Dendrogram of the DGGE profiles of bacterial communities from the different soil in site 2.** The cluster analysis was based on Pearson correlation coefficient as similarity measure and the unweighted pair-group method with arithmetic averages (UPGMA).

Sequence datasets, taxonomic and functional diversity

Paired-end Illumina HiSeq sequencing of the total soil transcriptome resulted in 68-132 thousand and 104-191 thousand sequence reads per sample for 16S and 18S, respectively. In total they clustered to 10,120 16S OTUs and 5,798 18S OTUs (Table A3.1).

Prokaryotic (16S) and eukaryotic (18S) RR correlated nicely between samples (Figure 3.17), indicating an interaction between both communities in these soils.

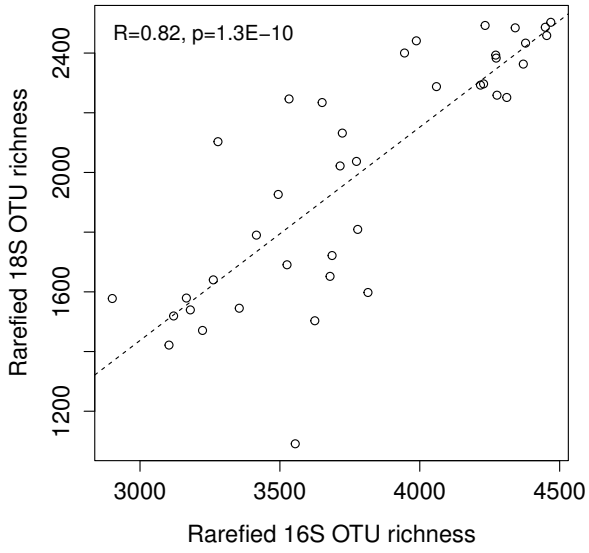


Figure 3.17. Rarefied richness (RR) of 16S rRNA OTU against 18S rRNA for soils in site 1 and site 2.

When analyzing both site 1 and site 2 according to their level of heavy metal contamination, all diversity indices (RR, H' and J') were significantly lower in the highly contaminated site 2 in comparison to the less contaminated site 1 (Figure 3.18).

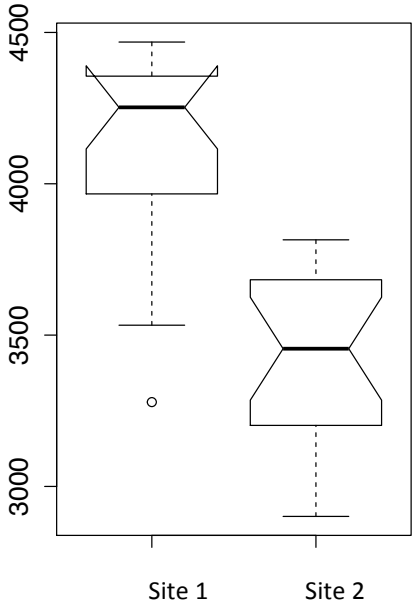


Figure 3.18. Rarefied richness (RR) of 16S rRNA for site 1 and site 2 ($p = 1.6 \cdot 10^{-8}$)**.**

In the less contaminated site, site 1, SHEEP treated soil presented lower values of 16S and 18S diversity indices (RR, H' and J') (Figure 3.19 and Figure 3.20A). However, in site 2 with a stronger heavy metal contamination, SHEEP treated soil, showed also lower 16S RR, H' and J' values (not significant) but higher 18S RR, H' and J' values instead (Figure 3.20B).

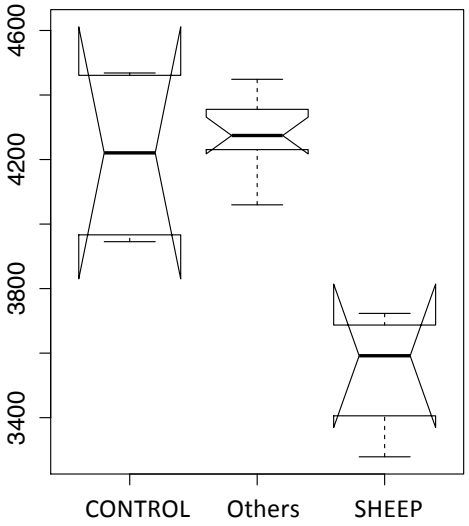


Figure 3.19. Rarefied richness (RR) of 16S rRNA for soils in site 1 (p = 2.43 10^{-6})**.**

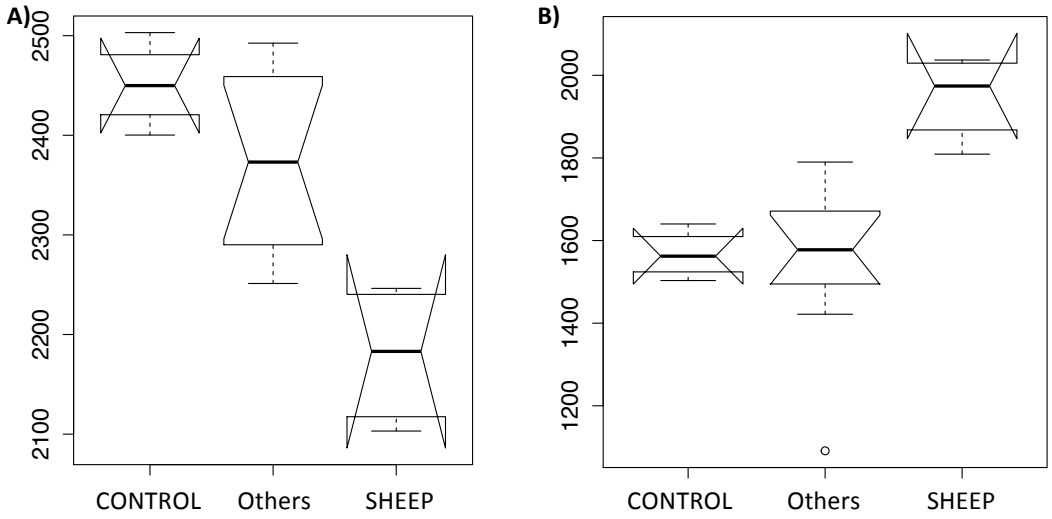


Figure 3.20. Rarefied richness (RR) of 18S rRNA for soils in site 1 (A) (p = 5 · 10^{-4}) **and site 2 (B)** (p = 0.0017)**.**

Taxonomy independent analysis (based on OTU composition)

ANOSIM (analysis of similarities) provides a statistic of the likehood that samples in a defined group are more similar to each other than to other samples. Results (Table 3.32) showed that the biggest difference in community composition among these soils depends on contamination, both for prokaryotes and eukaryotes. Relative to amendment treatment, in the less contaminated site 1, the biggest difference in community composition was found for PAPER treated soil, both for prokaryotes and eukaryotes and for SHEEP treated soil, only for

prokaryotes. Along the same lines, in the more contaminated site 2, the strongest effect on composition was observed for POULTRY treated soil.

Table 3.32. Analysis of similarities or ANOSIM. The first column indicates the factor (contamination or amendment treatment) that was analyzed in each case and the third column specifies the effect from which was analyzed.

Factor	Samples	Effect from	R (16S)	R (18S)
Contamination	All soils	Site 1 vs. site 2	0.97***	0.78***
Treatment	Site 1	Any treatment	0.76***	0.62***
Treatment	Site 1	PAPER	0.68***	0.53**
Treatment	Site 1	SHEEP	0.53***	0.17
Treatment	Site 2	Any treatment	0.72***	0.3**
Treatment	Site 2	POULTRY	0.83***	0.64**

*Asterisks indicate significance (*** ≤ 0.001, ** ≤ 0.01, * ≤ 0.05).

Two-dimensional NMDS (non-metric multidimensional scaling) is a method for visualizing differences between many samples in two dimensions. NMDS results (Figure 3.21) supported the main ones obtained with ANOSIM (Table 3.32): the difference in heavy metal contamination provoked the biggest influence in composition and soil communities clustered by site (site 1 and site 2, less and more contaminated sites, respectively).

Among the different soils within each site, NMDS also supports the difference in composition caused by SHEEP treatment on prokaryotes but only in site 1 (SHEEP cluster was significantly separated from CONTROL soil). Also, the biggest difference in community composition of the PAPER treated soil in site 1 was confirmed and additionally for site 2 but only for prokaryotes. However, the composition effect caused by POULTRY treatment in site 2 (found with ANOSIM) was not supported by NMDS: POULTRY treated soil did form a cluster quite separated from the CONTROL samples but the samples themselves were so separated from each other that it was not significantly according to the NMDS analysis.

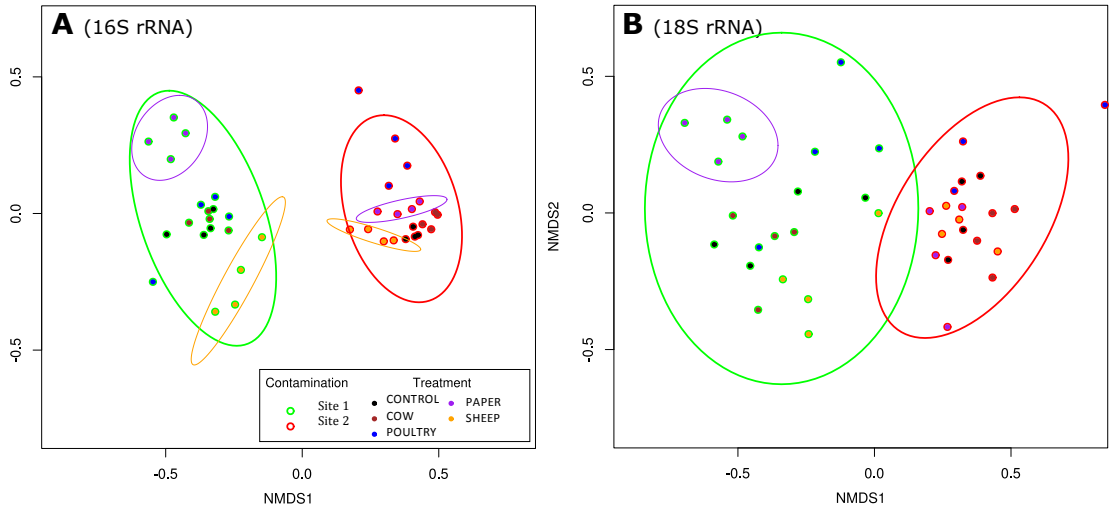


Figure 3.21. Two-dimensional NMDS plots for 16S rRNA (A) and 18S rRNA (B). Ellipses represent distribution of a treatment or contamination group with 95% confidence based on standard deviation in NMDS space.

Taxonomic composition

Bacteria dominated the community in all soils in relation to protist, fungi and metazoan (Table A3.1). The taxa distribution identified (order rank) in both sites appeared consistent with the richness indicator. Although most groups were more abundant in site 1, (e.g. *Rhizobiales*, *Cyanobacteria group ML635J-21*, *Betaproteobacteria group TRA3-20*, *Streptosporangiales*) several taxa also appeared especially well adapted to high heavy metal contamination in site 2 (e.g. *Chloroflexi subdiv. 10 group KD4-96*, *Rhodobacterales*, *Thermoleophilia group AKIW543*, *Acidobacteria group JH-WHS99*) (Figure 3.22 and Table 3.33).

Table 3.33. Relative distribution of prokaryotic, protist fungi and metazoan taxa groups that are either more abundant in site 1 or in site 2 according to the heavy metal contamination level. First column indicates taxa more abundant in site 1 and column two indicates taxa more abundant in site 2.

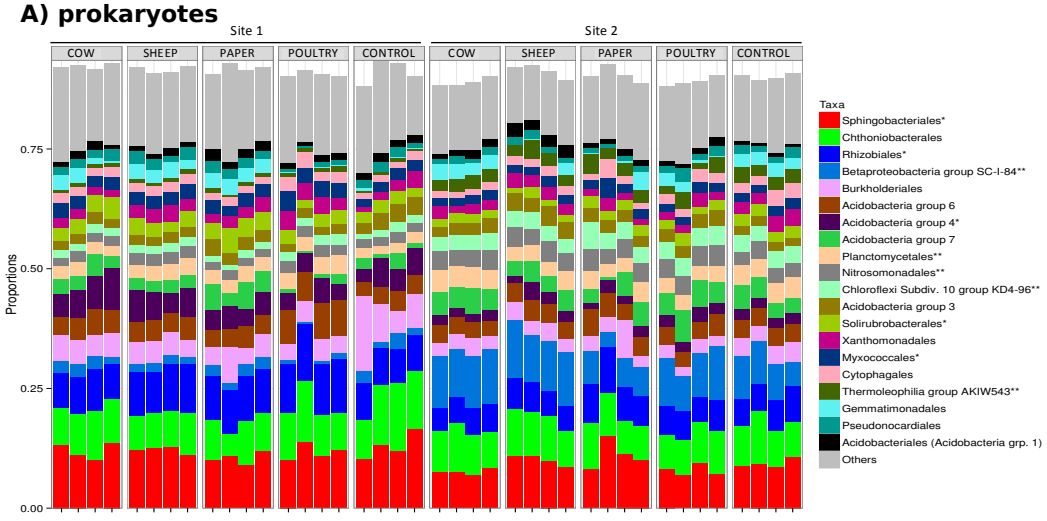
Site 1		**Site 2**	
Prokaryotic orders	***p* value**	**Prokaryotic orders**	***p* value**
Phycisphaerae WD2101 soil group	$8 \cdot 10^{-14}$	Chloroflexi subdiv. 10 group KD4-96	$1 \cdot 10^{-16}$
Bdellovibrionales	$4 \cdot 10^{-12}$	Thermoleophilia group AKIW543	$9 \cdot 10^{-15}$
Betaproteobacteria group TRA3-20	$1 \cdot 10^{-10}$	Betaproteobacteria group SC-I-84	$2 \cdot 10^{-14}$

Acidobacteria group 4	$4 \cdot 10^{-10}$	Nitrosomonadales	$8 \cdot 10^{-14}$
Streptosporangiales	$2 \cdot 10^{-9}$	Thermomicrobia group JG30-KF-CM66	$7 \cdot 10^{-13}$
Rhizobiales	$4 \cdot 10^{-8}$	Acidobacteria group 11	$1 \cdot 10^{-12}$
Corynebacteriales	$1 \cdot 10^{-7}$	vadinHA64	$5 \cdot 10^{-8}$
Elusimicrobia Lineage IIa	1·10-^{7}	Rhodobacterales	$8 \cdot 10^{-8}$
Opitutales	$3 \cdot 10^{-7}$	Acidobacteria group 15	$1 \cdot 10^{-7}$
Cyanobacteria group ML635J-21	$4 \cdot 10^{-7}$	Rhodocyclales	$2 \cdot 10^{-7}$
Caulobacterales	$1 \cdot 10^{-6}$	Rubrobacterales	$3 \cdot 10^{-7}$
Chromatiales	$6 \cdot 10^{-6}$	Ktedonobacterales	$3 \cdot 10^{-6}$
Vampirovibrio	$8 \cdot 10^{-6}$	Acidobacteria group 13	$3 \cdot 10^{-6}$
Cyanobacteria group SHA-109	$1 \cdot 10^{-5}$	Chtomonadetes	$2 \cdot 10^{-5}$
Flavobacteriales	$2 \cdot 10^{-5}$	Acidobacteria group 18	$4 \cdot 10^{-5}$
Solirubrobacterales	$2 \cdot 10^{-5}$	Candidatus Nitrososphaera	$2 \cdot 10^{-4}$
Sphingomonadales	$3 \cdot 10^{-5}$	Acidobacteria group JH-WHS99	$2 \cdot 10^{-4}$
Phycisphaerae mle1-8	$5 \cdot 10^{-5}$	Chloroflexi subdiv. 10 group Gitt-GS-136	$2 \cdot 10^{-4}$
Sphingobacteriales	$6 \cdot 10^{-5}$	Desulfurellales	$2 \cdot 10^{-4}$
Elusimicrobia Lineage IV	$6 \cdot 10^{-5}$	Planctomycetales	$2 \cdot 10^{-4}$
Elusimicrobia Lineage IIb	$7 \cdot 10^{-5}$	Thermotogales	$3 \cdot 10^{-4}$
Gemmatimonadetes BD2-11 terrestrial group	$8 \cdot 10^{-5}$	Gammaproteobacteria BD7-8 marine group	$3 \cdot 10^{-4}$
Micromonosporales	$9 \cdot 10^{-5}$	Actinobacteria group PeM15	$3 \cdot 10^{-4}$
Rickettsiales	$1 \cdot 10^{-4}$		
Myxococcales	$1 \cdot 10^{-4}$		
Fungal orders		**Fungal orders**	
Chaetothyriales	$5 \cdot 10^{-10}$	Mortierellales	$5 \cdot 10^{-9}$
Tremellales	$1 \cdot 10^{-9}$	Rhizophydiales	$9 \cdot 10^{-8}$
Auriculariales	$4 \cdot 10^{-8}$	Cladochytriales	$3 \cdot 10^{-4}$
Pleosporales	$2 \cdot 10^{-7}$		
Mucorales	$5 \cdot 10^{-7}$		

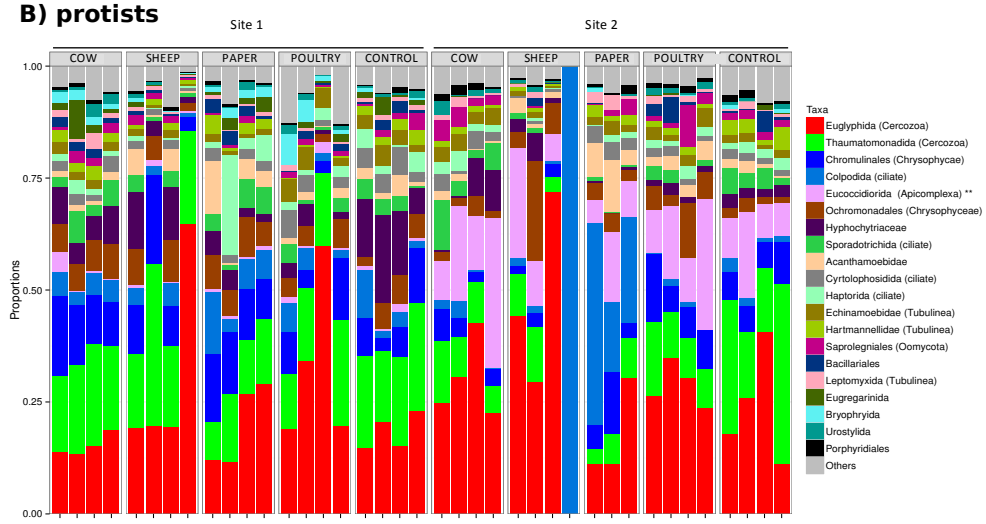
Diaporthales	$2 \cdot 10^{-5}$		
Lichinales	$2 \cdot 10^{-5}$		
Eurotiales	$2 \cdot 10^{-4}$		
Diversisporales	$2 \cdot 10^{-4}$		
Glomerales	$2 \cdot 10^{-4}$		
Monoblepharidales	$2 \cdot 10^{-4}$		
Hypocreales	$4 \cdot 10^{-4}$		
Neocallimastigales	$5 \cdot 10^{-4}$		
Protist orders		**Protist orders**	
Philasterida	$7 \cdot 10^{-4}$	Eucoccidiorida	$2 \cdot 10^{-7}$
		Spumellaria	$6 \cdot 10^{-4}$
Metazoan orders		**Metazoan orders**	
Rhabditida	$1 \cdot 10^{-7}$	Tylenchida	$4 \cdot 10^{-7}$
Parachela	$1 \cdot 10^{-5}$		
Leucosolenida	$5 \cdot 10^{-4}$		

Once again, a trend related to heavy metal contamination was evident at order level. Although these results are based on relative abundances, it could be as well related to the fact that in site 2 the total microbial biomass was lower than in site 1.

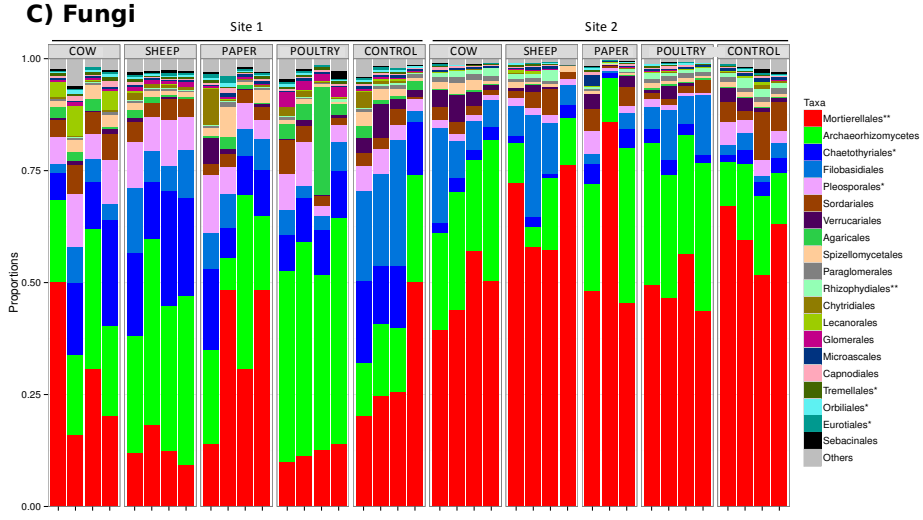
A) prokaryotes



B) protists



C) Fungi



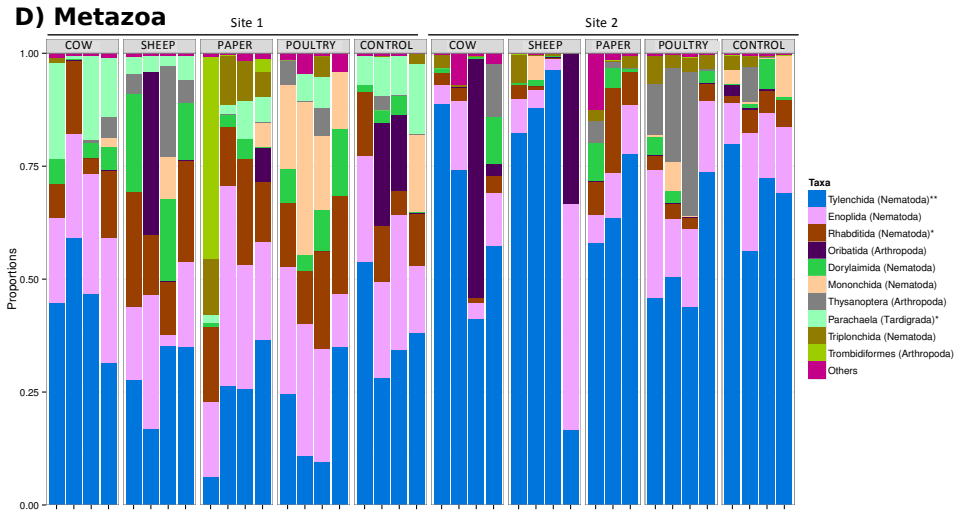


Figure 3.22. Relative abundances and distribution of the 20 most abundant taxa for prokaryotic (A), protist (B), fungi (C) and metazoan (D) at order rank. Groups marked with * were significantly overrepresented in less contaminated samples (site 1) and those marked with ** in more contaminated samples (site 2).

In particular, Erysipelotrichales (a firmicute) was overrepresented in COW treated soils (Figure 3.23) while Triplonchida (a nematode) was more abundant in POULTRY treated soils (Figure 3.24), regardless of the contamination level.

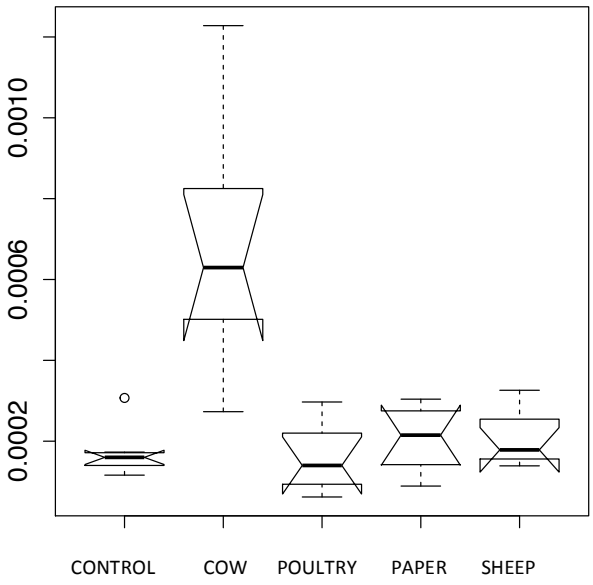


Figure 3.23. Relative abundance of Erysipelotrichales ($p = 3.1 \cdot 10^{-8}$)**.**

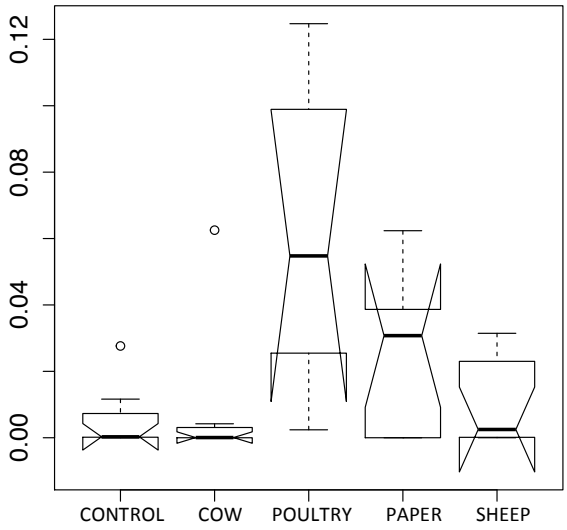


Figure 3.24. Relative abundance of Triplonchida (p = 0.00088)**.**

Any other effect was only significant in either the less or the more contaminated site. In site 1, Chlamydiales was more abundant in the COW treated soil. Rhizobiales, Chloroflexi subdivision 10 group S085, Glomerales and Pleosporomycetidae were more abundant in the PAPER treated soil. However in site 2, Chlorobiales were more abundant in COW treated soil, Rhodospirillales in SHEEP soil while Chloroflexi subdivision 10 groups S085 and Gitt-GS-136, Phycisphaerae and SAR324 were more abundant in POULTRY treated soil.

3.3.6. Overall aided phytostabilization effect

The following parameters were selected for the study of the overall long-term effect of the aided phytostabilization on soil microbial communities: 1) β-glucosidase activity; (2) urease activity; (3) acid phosphatase activity; (4) dehydrogenase activity; (5) FDA; (6) aryIsulphatase activity; (7) potentially mineralizable nitrogen (PMN); (8) microbial biomass; (9) total bacteria from 16S rRNA; (10) RR-16S rRNA; (11) H'-16S rRNA; (12) RR-18S rRNA and (13) H'-18S rRNA.

According to the SQI values, in site 1, COW and SHEEP treated soils showed a significantly (p < 0.0001) lower soil quality after the treatment (Table 3.34). This effect was confirmed by the AMOEBA (a general method of ecosystem description and assessment; Ten Brink *et al*., 1991) plot where each parameter was represented by its relative abundance in the form of a two-dimensional chart of 13 axes. In the AMOEBA plot for site 1, COW and SHEEP treated soils were more disturbed in comparison to the CONTROL soil (Figure 3.25), especially in relation to the microbial activity.

On the contrary, although the SQI value did not show a significant change, PAPER treated soil displayed an improvement in several microbial activity parameters related to the N cycle (urease enzyme activity and PMN) and the sulphur cycle (arylsulphatase enzyme activity).

Table 3.34. Soil quality index (SQI) of the different soils in site 1 when the CONTROL soil was set to 100% as reference. (mean ± SE; n=4). *Asterisks indicate significance ($p \leq 0.05$).

Soils	Treatment	SQI
COW	Cow slurry	80.270 ± 8.861*
SHEEP	Sheep manure	80.115 ± 5.358*
PAPER	Paper mill sludge and poultry manure	95.948 ± 6.379
CONTROL	No amendment	100

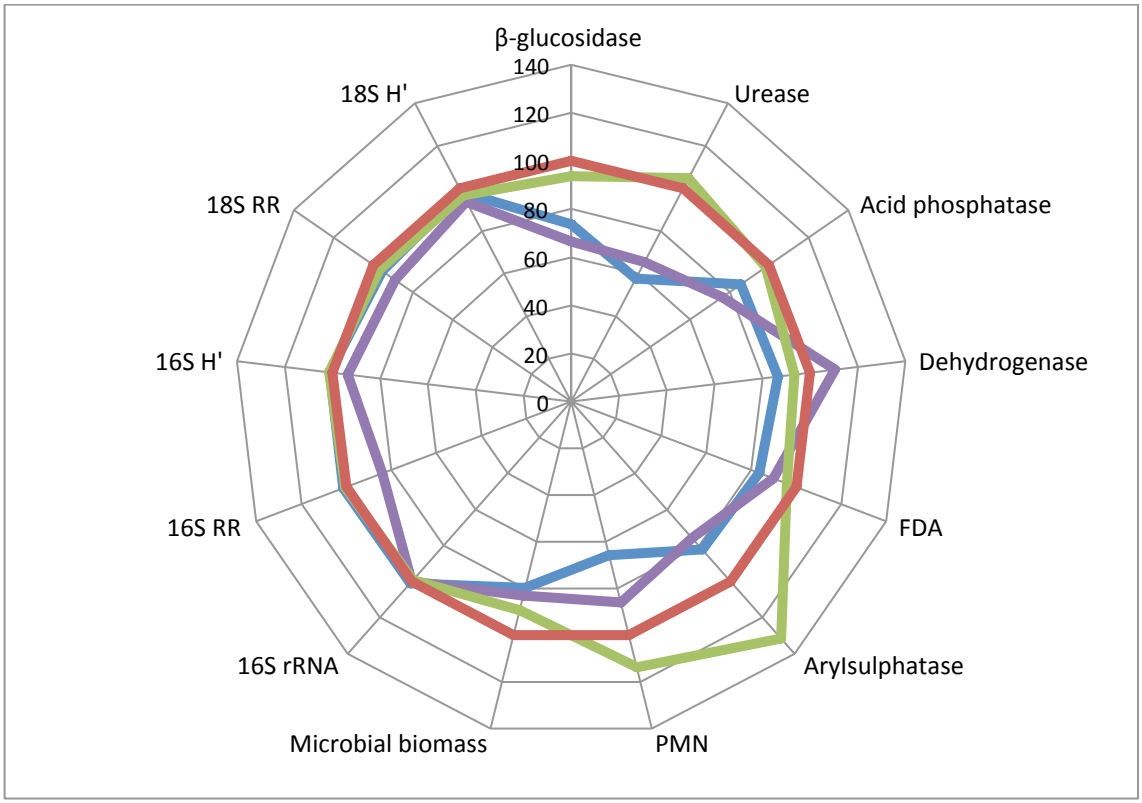


Figure 3.25. AMOEBA plot of the effect of treatments on soil microbial activity, biomass and diversity in site 1. CONTROL soil was set as reference (100%). Red line: CONTROL soil; blue line: COW treated soil; lavanda line: SHEEP treated soil and green line: PAPER treated soil.

Regarding site 2, SHEEP treated soil significantly ($p < 0.05$) improved its soil quality after treatment (Table 3.35). This effect was confirmed once again by the AMOEBA plot for site 2, where most of the parameters for the SHEEP treated soil increased in comparison to the CONTROL soil (Figure 3.26).

On the contrary, although the SQI value did not show any significant change, PAPER treated soil in site 2 displayed an improvement, especially, in dehydrogenase enzyme activity.

Table 3.35. Soil quality index (SQI) of the different soils in site 2 when the CONTROL soil was set to 100% as reference. (mean ± SE; n=4) *Asterisks indicate significance ($p \leq 0.05$).

Soils	Treatment	SQI
COW	Cow slurry	102.839 ± 15.178
SHEEP	Sheep manure	125.197 ± 13.289*
PAPER	Paper mill sludge and poultry manure	105.209 ± 7.747
CONTROL	No amendment	100

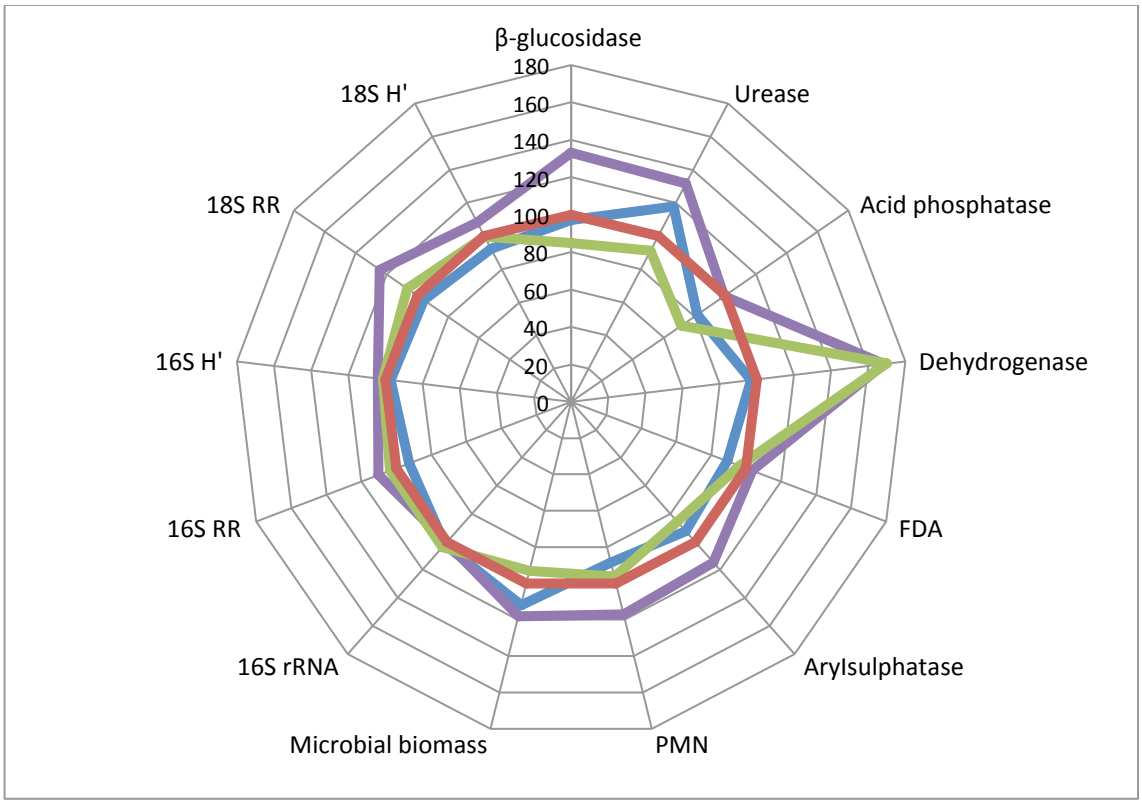


Figure 3.26. AMOEBA plot of the effect of treatments on soil microbial activity, biomass and diversity in site 2. CONTROL soil was set as reference (100%). Red line: CONTROL soil; blue line: COW treated soil; lavanda line: SHEEP treated soil and green line: PAPER treated soil.

3.3.7. Detection of class 1 integrons, quaternary ammonium compound resistance and plasmids in TC-DNA from soils

qPCR assays

The results obtained by qPCR for the presence of class 1 integrase gene (*intI1*), the presence of quaternary ammonium compound resistance gene (*qacE+qacEΔ1*), streptomycin (*aadA)*, tetracycline (*tetM)* and sulfonamide resistance genes (*sul1*) were rated as inconclusive since amplifications of the unknown samples started during the last cycles of the reaction (Figure 3.27A). This fact made them difficult to separate from the background noise or from the amplification of unspecific sequences. Similarly, in Figure 3.27B the amplified samples appeared at concentrations lower than 10^{-9} ng μl^{-1}. These results indicate that either these genes are absent in the samples or that they are present in concentrations below the qPCR detection limit.

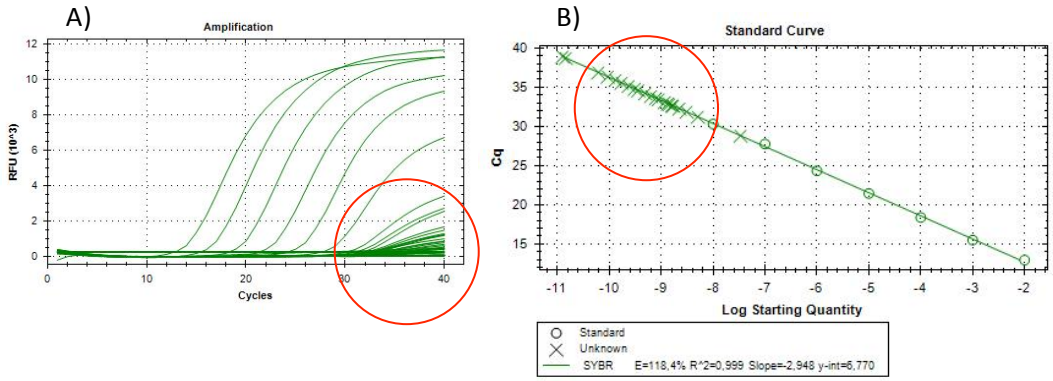


Figure 3.27. A) Amplification curves for *qacE+qacEdelta*. Each soil was studied in quadruplicate. The Ct value for each sample is the point where the amplification curve meets the threshold line. **B) Standard curve for *qacE+qacEdelta*.** The C_t was plotted against the log of the starting quantity for each sample and standard dilution. Marked in red are the amplified samples.

PCR and Southern Blot assays

TC-DNA extracted from the soils was PCR-amplified and analysed by Southern Blot hybridization to screen for the presence of class 1 integrons by the detection of class 1 integrase gene (*intI1*), quaternary ammonium compound resistance gene (*qacEΔ1*) and plasmid backbone regions related to replication (IncP-1 (all subgroups) *trfA*, IncN *rep* and V216 *rep*) and the IncQ origin of replication (*oriV*) (Figures 3.28 to 3.35). The results revealed the presence of class 1 integrons in all soils except for SHEEP treated soil in site 2, for at least one

of the four replicates for each soil. Similarly, *qacEΔ1* was also detected in all soils, except for SHEEP treated soil in site 2.

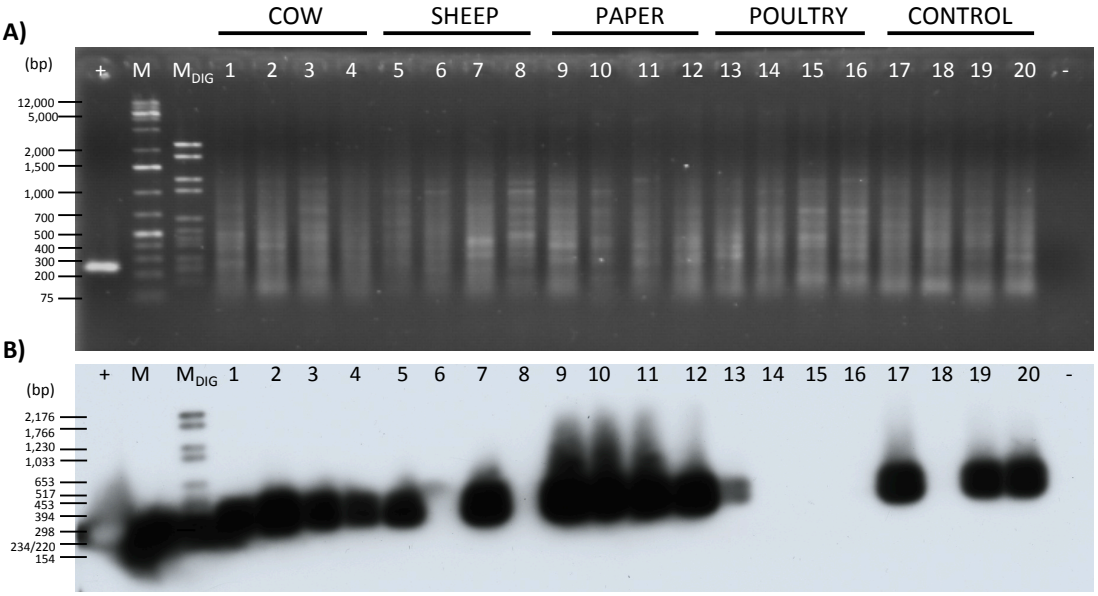


Figure 3.28. Detection of *intl1* sequence in TC-DNA samples of the different soils in site 1. A) *intl1* sequence (280 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 1 was used as template for the PCR reactions. **B)** Amplicons of *intl1* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 1-4: samples of COW treated in quadruplicate. Lanes 5-8: samples of SHEEP treated soil in quadruplicate. Lanes 9-12: samples of PAPER treated soil in quadruplicate. Lanes 13-16: samples of POULTRY treated soil in quadruplicate. Lanes 17-20: samples of CONTROL soil in quadruplicate. Lane +: pKJK5, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

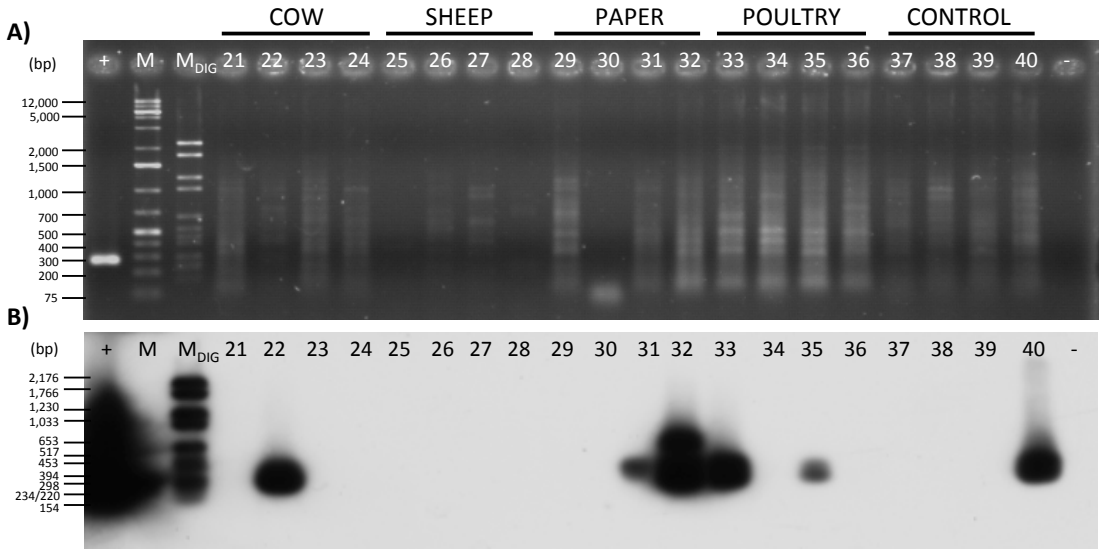


Figure 3.29. Detection of *intl1* sequence in TC-DNA samples of the different soils in site 2. A) *intl1* sequence (280 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 2 was used as template for the PCR reactions. **B)** Amplicons of *intl1* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 21-24: samples of COW treated in quadruplicate. Lanes 25-28: samples of SHEEP treated soil in quadruplicate. Lanes 29-32: samples of PAPER treated soil in quadruplicate. Lanes 33-36: samples of POULTRY treated soil in quadruplicate. Lanes 37-40: samples of CONTROL soil in quadruplicate. Lane +: pKJK5, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

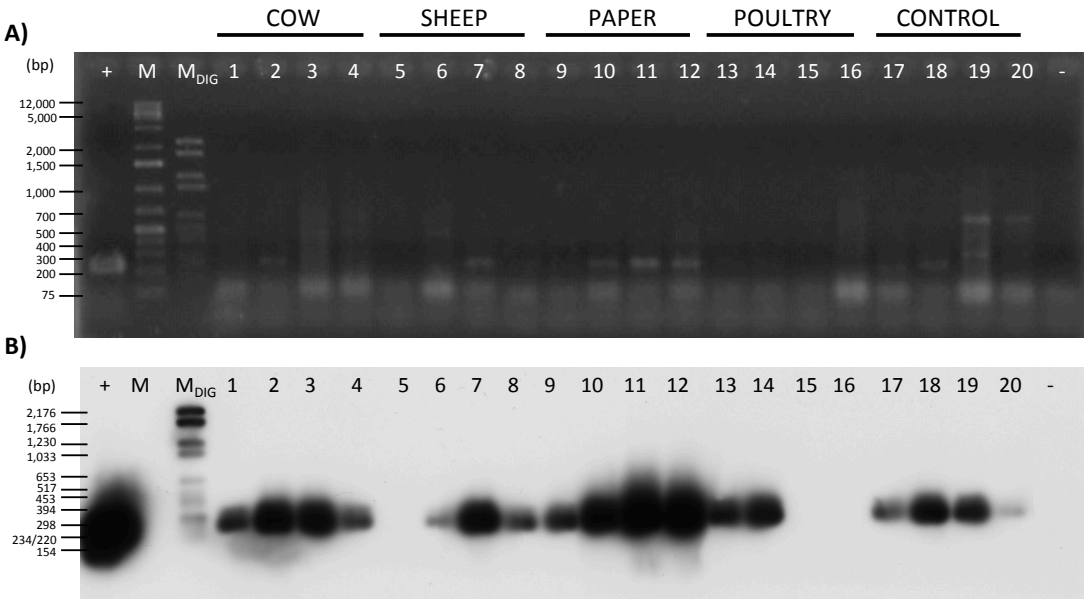


Figure 3.30. Detection of *qacEΔ1* sequence in TC-DNA samples of the different soils in site 1. A) *qacEΔ1* sequence (226 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 1 was used as template for the PCR reactions. **B)** Amplicons of *qacEΔ1* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 1-4: samples of COW treated in quadruplicate. Lanes 5-8: samples of SHEEP treated soil in quadruplicate. Lanes 9-12: samples of PAPER treated soil in quadruplicate. Lanes 13-16: samples of POULTRY treated soil in quadruplicate. Lanes 17-20: samples of CONTROL soil in quadruplicate. Lane +: pB10, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

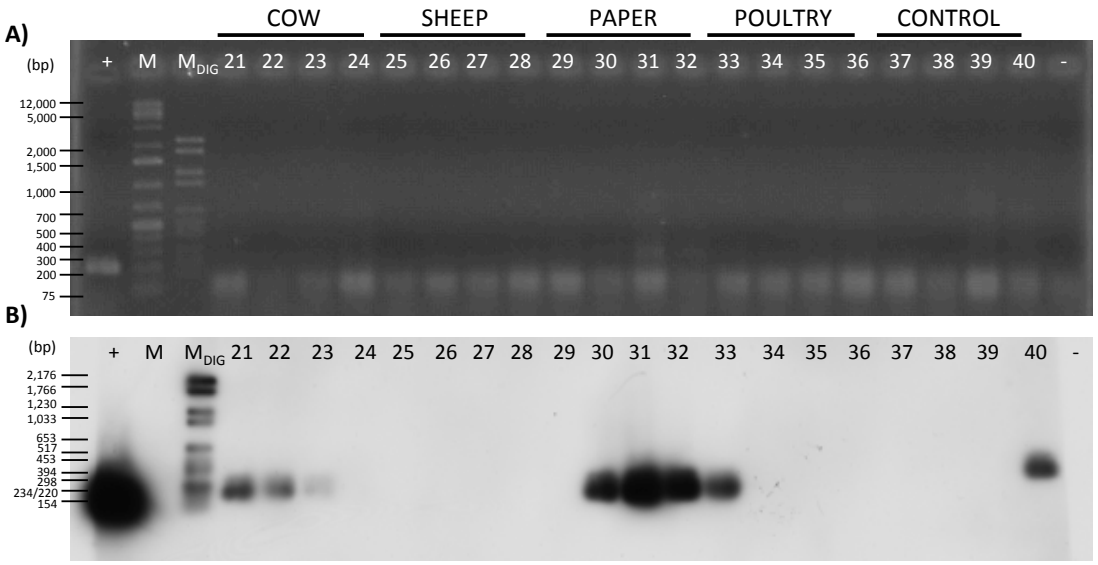


Figure 3.31. Detection of *qacEΔ1* sequence in TC-DNA samples of the different soils in site 2. A) *qacEΔ1* sequence (226 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 2 was used as template for the PCR reactions. **B)** Amplicons of *qacEΔ1* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 21-24: samples of COW treated in quadruplicate. Lanes 25-28: samples of SHEEP treated soil in quadruplicate. Lanes 29-32: samples of PAPER treated soil in quadruplicate. Lanes 33-36: samples of POULTRY treated soil in quadruplicate. Lanes 37-40: samples of CONTROL soil in quadruplicate. Lane +: pB10, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

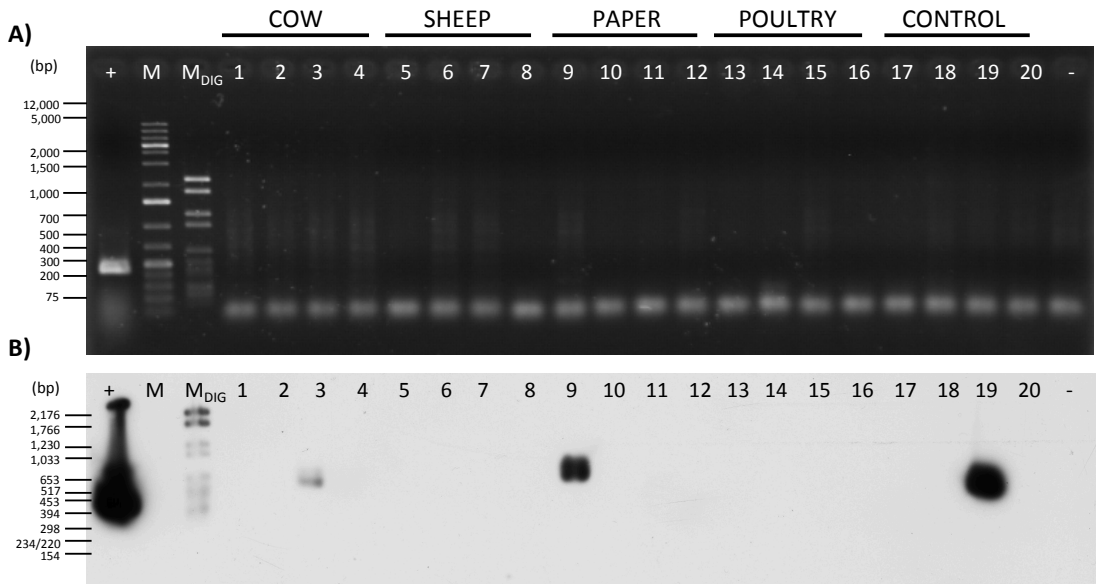


Figure 3.32. Detection of IncQ *oriV* sequence in TC-DNA samples of the different soils in site 1. A) IncQ *oriV* sequence (436 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 1 was used as template for the PCR reactions. **B)** Amplicons of IncQ *oriV* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 1-4: samples of COW treated in quadruplicate. Lanes 5-8: samples of SHEEP treated soil in quadruplicate. Lanes 9-12: samples of PAPER treated soil in quadruplicate. Lanes 13-16: samples of POULTRY treated soil in quadruplicate. Lanes 17-20: samples of CONTROL soil in quadruplicate. Lane +: RSF1010, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

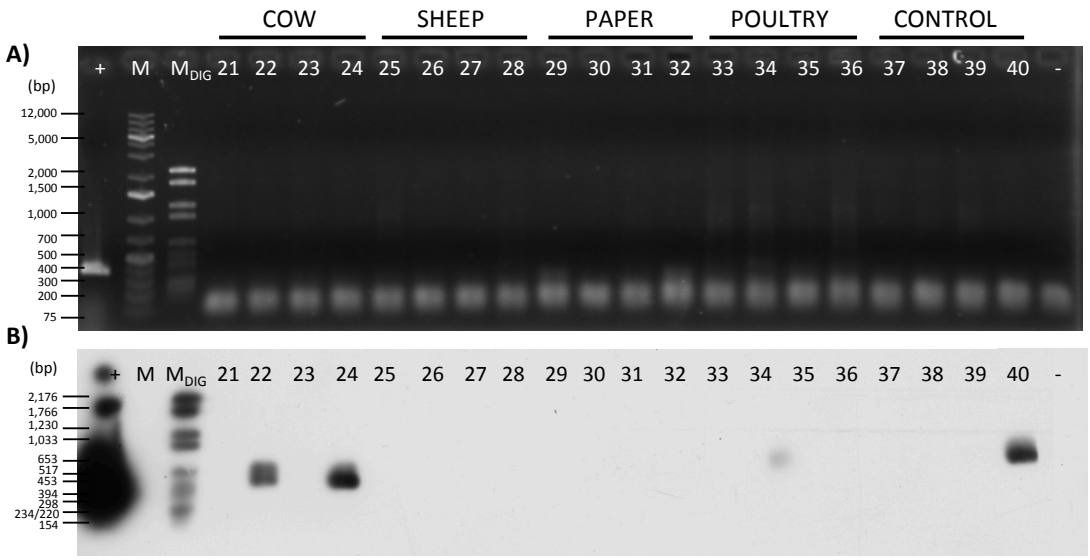


Figure 3.33. Detection of IncQ *oriV* sequence in TC-DNA samples of the different soils in site 2. A) IncQ *oriV* sequence (436 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 2 was used as template for the PCR reactions. **B)** Amplicons of IncQ *oriV* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 21-24: samples of COW treated in quadruplicate. Lanes 25-28: samples of SHEEP treated soil in quadruplicate. Lanes 29-32: samples of PAPER treated soil in quadruplicate. Lanes 33-36: samples of POULTRY treated soil in quadruplicate. Lanes 37-40: samples of CONTROL soil in quadruplicate. Lane +: RSF1010, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

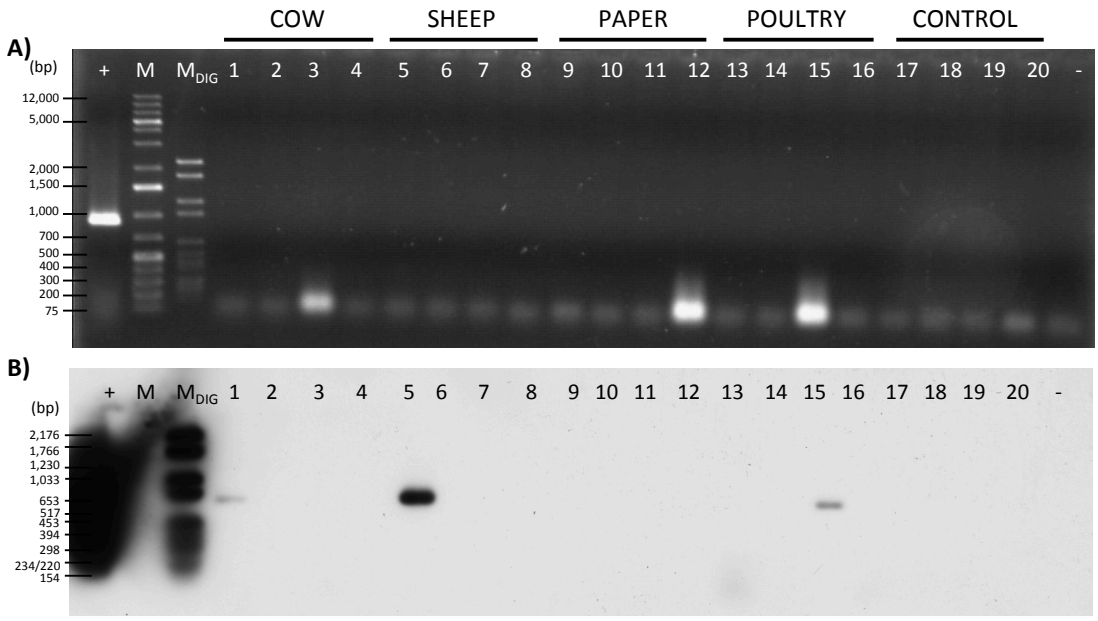


Figure 3.34. Detection of pV216 *rep* sequence in TC-DNA samples of the different soils in site 1. A) p*V216 rep* sequence (912 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 1 was used as template for the PCR reactions. **B)** Amplicons of pV216 *rep* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 1-4: samples of COW treated in quadruplicate. Lanes 5-8: samples of SHEEP treated soil in quadruplicate. Lanes 9-12: samples of PAPER treated soil in quadruplicate. Lanes 13-16: samples of POULTRY treated soil in quadruplicate. Lanes 17-20: samples of CONTROL soil in quadruplicate. Lane +: pHHV216, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

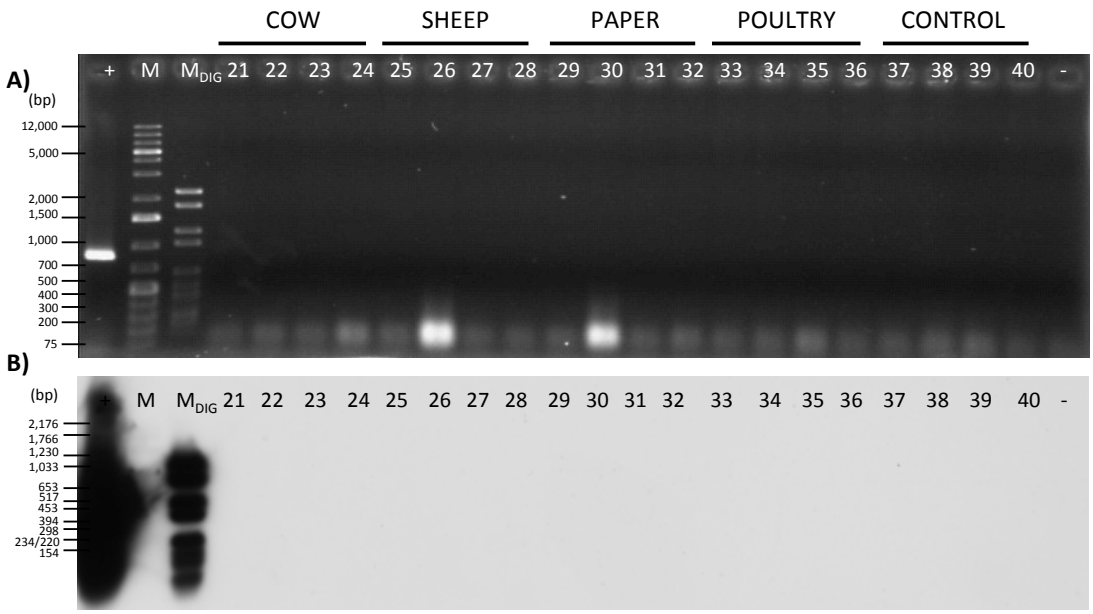


Figure 3.35. Detection of pV216 *rep* sequence in TC-DNA samples of the different soils in site 2. A) p*V216 rep* sequence (912 bp) was amplified by PCR and separated by agarose gel (1%, w/v) electrophoresis. TC-DNA from the different soils in site 2 was used as template for the PCR reactions. **B)** Amplicons of pV216 *rep* sequence were hybridized with DIG-labelled probe. Lane M: DNA molecular weight marker 1 Kb Plus DNA Ladder (Thermo Scientific). Lane M_{DIG}: DNA molecular weight marker VI, DIG-labeled (Roche). Lanes 21-24: samples of COW treated in quadruplicate. Lanes 25-28: samples of SHEEP treated soil in quadruplicate. Lanes 29-32: samples of PAPER treated soil in quadruplicate. Lanes 33-36: samples of POULTRY treated soil in quadruplicate. Lanes 37-40: samples of CONTROL soil in quadruplicate. Lane +: pHHV216, positive control. Lane -: negative control with ultrapure distilled water instead of DNA.

Results summarized in Table 3.36 show that characteristic sequences for IncP-1 and IncQ incompatibility groups were detected in both sites 1 and 2. When comparing the relative abundance of IncP-1 like sequences in both sites, IncP-1 like plasmid sequences were more present in site 1 than site 2. In contrast, in site 2 IncQ group-like sequences were detected more abundantly than in site 1. On the other hand, plasmids belonging to the incompatibility group IncN were not detected in any of the two sites. Finally, pHHV216-like plasmids, characterized by a low GC content (Heuer *et al.*, 2009), were present in site 1 but not in site 2.

Table 3.36. Detection of *intI1, qacEΔ1,* IncP-1 (all subgroups) *trfA,* IncP-1α *trfA,* IncP-1β *trfA,* IncQ *oriV,* IncN *rep,* pV216 *rep* sequences by PCR and Southern Blot assays. Each + represents a positive result for one of the four replicates of each of the soils studied.

	Site 1					Site 2				
Gene/Locus	**COW**	**SHEEP**	**PAPER**	**POULTRY**	**CONTROL**	**COW**	**SHEEP**	**PAPER**	**POULTRY**	**CONTROL**
intI1	++++	++	++++	+	+++	+		++	++	+
qacEΔ1	++++	+++	++++	++	++++	+++		+++	+	+
IncP-1 (all subgroups) *trfA*	+			+	++					
IncP-1α *trfA*			+							
IncP-1β *trfA*	+		++	+	+++		+			
IncQ *oriV*	+		+		+	++			+	+
IncN *rep*										
pV216 *rep*	+	+		+						

3.4. Discussion

When mining activity ceases, mines are often abandoned, leaving behind highly degraded areas characterized by the presence of elevated levels of environmental pollution. Among mine wastes, tailings are considered a long-term sink for heavy metals as they usually contain high concentrations of different toxic metals including Zn, Cu, Ni, Pb, Cr and Cd. Mine tailings are also highly susceptible to wind dispersal and water erosion (Wong *et al*., 1998) which makes them a dangerous source of heavy metal pollution for the surrounding areas.

In the area of study, Lanestosa's (Biscay, northern Spain) abandoned mine tailings, Zn and Pb showed the highest values of pseudo-total concentration in soil (Zn: 15,529 ± 179.3 mg kg^{-1} and 61,007 ± 352.2 mg kg^{-1}; Pb: 16,285 ± 188.0 mg kg^{-1} and 28,587 ± 330.1 mg kg^{-1} for site 1 and site 2, respectively). These pseudo-total concentrations are comparable with those reported previously for the same area (Galende *et al*., 2014b). And most important, they clearly exceed the Critical Reference Values (VIE-B values) for the protection of ecosystems in the Basque Country (120 mg kg^{-1} and 106 mg kg^{-1} for Pb and Zn, respectively) (IHOBE, 1998; BOPV, 2005). As a consequence, one can expect pollution-induced adverse environmental implications in these soils, including toxicity for the soil biota and the appearance of risk

factors, such as heavy metal resistance among the biological communities present in this mining site.

The soil physicochemical properties in both sites revealed a mine soil with a slightly acidic pH, a relatively high OM content, while a moderately low N concentration. The combination of these soil characteristics and the high pseudo-total concentrations of heavy metals explain the low vegetation cover and limited plant growth in many zones of the mine tailings. The vegetation naturally growing in our experimental area is dominated by *F. rubra*, previously identified as a metal excluder in this mining area (Epelde *et al*., 2010; Becerra-Castro *et al*., 2012; Galende *et al*., 2014a). These harsh conditions were especially remarkable in site 2 where the heavy metal contamination was considerably higher and the OM level lower, compared to site 1. The lack of vegetation, together with a concomitant low value of soil OM, usually results in an adverse effect on soil microbial biomass and activity.

To mitigate the potential negative effects of heavy metal contamination on soil functioning, an aided phytostabilization assay was started in 2010 (Galende *et al*., 2014a). The main goal of any aided phytostabilization procedure is to decrease the bioavailable fraction of heavy metals in soil. Besides, the incorporation of nutrients from the amendments helps overcome the frequently low nutrient content of mine soils and, hence, promote plant growth and restore soil quality in the mining area. Here, organic amendments were applied *in situ* to decrease the heavy metal bioavailability and, finally, add essential nutrients and OM to the soil, with concomitant positive effects for plant growth, soil microbial communities and soil physicochemical properties. The beneficial effect of the aided phytostabilization with different organic amendments on the heavy metal bioavailability was estimated through the determination of $CaCl_2$-extractable heavy metal fractions in soil (Houba *et al*., 2000). However, the 'bioavailable fraction' is context-specific and quantitatively elusive (Meyer, 2002); besides its chemical quantification through $CaCl_2$-extractability is just an estimation. In accordance with the pseudo-total concentration values mentioned above for both sites, a higher bioavailable fraction of Cd, Pb and Zn was observed in site 2 in comparison to site 1.

Along the same lines, Cd showed the lowest values of pseudo-total and $CaCl_2$-extractable concentration among the three heavy metals tested. On the other hand, Zn showed the highest values of $CaCl_2$-extractable concentration in both sites. However, in site 1 the pseudo-total concentration of Pb was higher than that of Zn. This suggests that the bioavailable fraction of Pb is much smaller than that of Zn.

Regarding the long-term effects of the application of the different organic amendments studied here, not all soils treated with amendments showed lower values of $CaCl_2$-extractable heavy metal concentrations, compared to the CONTROL soil. This finding contrast with what was previously observed by a short-term evaluation carried out by Galende *et al*. (2014a). COW and SHEEP treatments hardly modified Pb and Zn $CaCl_2$-extractable concentrations in soil whereas PAPER treatment efficiently reduced the $CaCl_2$-extractable fraction of Cd in both sites, which is one of the most toxic trace elements to living organisms (Dong *et al*., 2007). Additionally, PAPER treatment successfully reduced the values of Zn bioavailability, an essential element in low concentrations but highly toxic in excess. PAPER treated soils displayed the lowest $CaCl_2$-extractable concentration of Pb, a non-bioessential, persistent and hazardous heavy metal pollutant; nonetheless, this reduction was not statistically significant. These results contrast with the short-term (2 months) assessment obtained for the same study by Galende *et al*. (2014a), where COW was the most consistent treatment in terms of heavy metal (Pb and Zn) bioavailability reduction. However, after 6 months of amendment, while the reduction in heavy metal bioavailability caused by COW was approximately still the same, heavy metal bioavailability reduction caused by PAPER treatment, especially for Zn, was significantly higher and even surpass the values COW reduction in site 2. Although, at short-term, COW seemed to be the most beneficial treatment for reduction of heavy metal bioavailability, these initial effects were lost over time.

According to Adriano *et al*. (2004), organic residues can immobilize metals through adsorption, complexation, and redox reactions. Nevertheless, metal retention by OM has been reported to be case-specific (Kumpiene *et al*., 2008), as OM can mobilize or retain metals depending on soil pH, its degree of humification (the higher the degree of humification, the higher the metal retention), clay content (through the formation of clay-metal-OM complexes), and so on. In this study, the highest amount of oxidable OM added to the soil during amendment application corresponded to PAPER treatment (757 g m^2), a lower amount to COW (647 g m^2) and SHEEP (650 g m^2) treatments, and the lowest amount to POULTRY treatment (197 g m^2) (Galende *et al*., 2014a). Therefore, the high concentration of oxidable OM in the PAPER treatment could explain, at least partly, the observed long-term reduction of heavy metal $CaCl_2$-extractability.

Microorganisms are major determinants of the physical, chemical and biological characteristics of soil, and in general of the sustainability of the soil ecosystem. Given the crucial importance of maintaining soil functions and ecosystem services, there has been

considerable effort invested in understanding the response of soil ecosystems to disturbances including environmental pollution (Kuan *et al*., 2006; Griffiths *et al*., 2008; Gregory *et al*., 2009; Stres *et al*., 2010; Fernández-Calviño *et al*., 2011). Understanding the ecology of soil microorganisms (e.g. the forces shaping soil microbial communities, their response to environmental change and their physicochemical and biological interactions) is an extremely difficult task owing to their high degree of biological diversity and the inherent spatial heterogeneity present at scales of less than 1 mm in soils. Soil microbial biomass, composition and activity are directly influenced by many factors such as soil type, nutrient content and OM concentration, pH, moisture, and so on, as well as by plant growth, composition and physiological status (Grayston *et al*., 1998). High levels of heavy metals can adversely influence the number, diversity and activity of soil microorganisms, thus inhibiting OM decomposition and nitrogen mineralization. Numerous studies have shown a reduction in the density, metabolic activity and diversity of soil microbial communities after exposure to trace elements (Giller *et al*., 1998; Kozdrój and van Elsas, 2000; Lorenz *et al*., 2006). Toxic concentrations of heavy metals typically induce a shift in species composition and the selection of metal-tolerant microorganisms. Additionally, some works have shown that soil bacterial communities from abandoned mines tend to first stabilize and then increase its diversity (Guo *et al*., 2009; Bouskill *et al*., 2010; Reis *et al*., 2013). On the other hand, the nature and degree of the impact of heavy metals on soil enzymes is strongly dependent on soil type, pH, OM level, and other soil properties (Tate, 2002). The inhibition of enzyme activities in heavy metal contaminated soils frequently reflects the heavy metal bioavailability, as the mechanisms that protect soil enzymes are likely to be the same mechanisms limiting heavy metal uptake by plants and soil microorganisms (Speir and Ross, 2002).

In general terms, in our study sites, heavy metal contamination showed to be the most decisive factor in terms of microbial biomass, composition and activity. Since pseudo-total concentrations of Cd, Pb and Zn in soil were strongly correlated, it was challenging to disentangle and then rank the influence of individual heavy metals on soil microbial communities. In accordance to these observations, all biological indicators related to the C, N and S cycle studied here (e.g. enzyme activities involved in these cycles), as well as the more representative indicators of overall microbial activity such as FDA (catalytic activity) and dehydrogenase activity (respiratory activity) in soil, indicated a deterioration of the soil microbial communities in site 2.

Readily degradable organic substrates such as oxidable OM (the most accessible fraction of OM for soil microorganisms) can rapidly stimulate microbial growth and activity in soil (Ohm *et al*., 2011). Several studies have reported an increase in microbial activity in heavy metal contaminated soils as a result of the addition of organic amendments (Sastre *et al*., 1996; Moreno *et al*., 2002; Tejada *et al*., 2008). Pertaining to the effect of treatments on soil microbial properties, PAPER treatment in site 1 had a more pronounced positive impact on most parameters used here to estimate soil microbial activity. This observed recovery was a result of the reduction in heavy metal (mainly, Cd and Zn) bioavailability and, at least partly, to the highest input of easily biodegradable OM resulting from the application of the organic amendment.

As seen before, COW treatment was the less efficient in terms of heavy metal bioavailability reduction and presented a moderate input of easily biodegradable OM. Some amendments may immobilize essential nutrients, which together with the presence of toxic heavy metals, results in a restriction of overall soil microbial activity (Vangronsveld *et al*., 2009). In particular, referring to the C cycle, SOC values were significantly reduced by COW treatment in site 1. However, although lower values of β-glucosidase enzyme activity were indeed found in COW and SHEEP treated soils, this difference was not statistically significant for the COW treated soil. Nevertheless, in site 2, no amendment effect on the β-glucosidase enzyme activity was observed.

Pertaining to the N cycle, in site 1, COW and SHEEP treatments caused a significant reduction of urease enzyme activity, in comparison to the CONTROL soil. Therefore, as both COW and SHEEP treated soils showed low values of enzyme activities related to the C and N cycles, we speculate that bacterial activity in these soils decreased. Again, in site 2, values of urease enzyme activity and PMN were similar in all soils independently of the amendment type. Both C-cycling and N-cycling appear to be less resistant to high levels of heavy metal contamination. Also, this supports the idea that amendment application had a beneficial effect in moderately heavy metal contaminated soil but was not able to induce a long-term recovery of microbial activity in strongly heavy metal contaminated soil. In fact, most of the biological indicators studied here did not show any significant change among treated soils in site 2 where the level of heavy metal contamination was higher.

Regarding changes in overall enzyme activity over time, short-term OEA values were not clearly affected by any of the treatments (Galende *et al*., 2014a). Besides, amendment application had no significant effect on dehydrogenase enzyme activity and FDA activity in any

of the two sites. Therefore, in terms of long-term effect, SHEEP and COW treatments seem less suitable for the improvement of soil quality, as reflected by the values of the soil microbial parameters determined here. Our results highlight the importance of long-term monitoring on soil quality recovery.

Penicillin resilience was studied as an indicator of ecosystem stability, or the system's ability to maintain its structure and pattern of behaviour in the presence of a disturbance. Nowadays, there is evidence that biodiversity increases the stability of ecosystem processes through time (Tilman *et al*., 2006; Jiang and Pu, 2009; Griffin *et al*., 2009; Campbell *et al*., 2011; Loreau and de Mazancourt, 2013). However, the underlying mechanisms (e.g. asynchrony of species' intrinsic responses to environmental fluctuations, differences in the speed at which species respond to perturbations, reduction in the strength of competition) are still poorly understood (Loreau and de Mazancourt, 2013). In any case, stability is an emergent property of an ecosystem, coming from the interactions between the constituent species, instead of a property of the species themselves.

In the present study, the values obtained for untreated soils (CONTROL) suggest a stronger resilience of the soil bacterial community towards a penicillin (a broad-spectrum antimicrobial agent) perturbation when no amendment was applied. This could indicate that changes in the indigenous soil bacterial communities caused by the bacteria initially present in the amendments or other characteristics of the amendments themselves might disrupt soil stability. It seems that the application of COW, SHEEP or PAPER increased the degree of disturbance and reduced the resilience of soil bacterial communities.

Overall, resilience responses were faster and recovery values were higher in site 1 than in site 2. Once again, there is a clear correlation with the level of heavy metal contamination. More precisely, the lowest resilience values were observed for COW treated soils. Although differences in resilience might indeed reflect differences in microbial community structure (diversity and composition), they are also directly influenced by the soil´s physicochemical properties; in this respect, SOC concentration and clay content have been reported to correlate with penicillin resilience (Butler *et al*., 2011).

Microbial biomass carbon can be used as an early indicator of changes in total soil organic C (Powlson *et al*., 1987). Unlike total organic C, microbial biomass responds quickly to environmental changes (Hoyle *et al*., 2006). Concerning the effect of heavy metal contamination on microbial biomass, higher values of microbial biomass were observed in site

1 than in site 2, as expected from the higher heavy metal concentrations found in site 2. Particularly in site 1, COW and SHEEP treated soils showed a significantly lower microbial biomass. These results are in accordance with those obtained for the C cycle, where COW and SHEEP treated soils showed lower values of β-glucosidase enzyme activity and SOC concentration, respectively. By contrast, when 16S rRNA gene copy numbers were studied to estimate total bacterial biomass, this trend was not observed. In fact, while values of microbial biomass were reduced in COW treated soils, 16S rRNA gene copy numbers increased. This is probably due to the fact that unlike microbial biomass, 16S rRNA gene copy number is an estimation of only bacterial biomass; this discrepancy might also be due, at least partly, to methodological differences between both methods.

The heavy metal bioavailable ($CaCl_2$-extractable) concentration in both sites had the strongest influence on microbial community composition, as abovementioned for microbial activity. In accordance to this observation, previous studies, using DGGE and similar molecular fingerprinting techniques, have observed heavy metal-induced shifts in soil bacterial community profiles as well as a decrease in bacterial diversity in heavy metal-contaminated soils, congruent with the observation that many organisms can no longer thrive or compete due to heavy metal toxicity (Sandaa *et al*., 1999; Gremion *et al*., 2003; Bamborough and Cummings, 2009; Singh *et al*., 2014). In our DGGE data, soil bacterial communities from site 1 and site 2 formed separated clusters with only a 69.5% similarity, hence indicating once again the spatial heterogeneity of the mine tailings and the different level of heavy metal contamination in site 1 *versus* site 2. The application of amendments had a strong influence on the composition of dominant bacteria, as demonstrated by DGGE band patterns from soil samples. When comparing CONTROL soil (no amendment) with treated soils within the same site, the highest impact on the dominant bacterial species in soil was found for the PAPER treatment, with a difference of 74.3 and 54.9% for site 1 and site 2, respectively. On the contrary, the highest similarity between treated soils and CONTROL soil was found for COW treatment with a difference of only 6.5 and 7.1% for site 1 and site 2, respectively. So, according to our DGGE results, PAPER caused a strong long-term impact on the dominant bacterial species in both sites, whereas COW treated soils remained very similar to CONTROL soils in this respect.

Technological advances are enabling the characterization of environmental microbial communities at increasing depth. High-throughput sequencing analysis provides a more

complete look into soil microbial communities, compared to DGGE where only dominant species are observed.

Regarding our Illumina sequencing data, once again, an interrelationship between level of heavy metal contamination and microbial community structure was observed. Although several taxa were only encountered in site 1, most taxa in site 1 (less contaminated) were also present in site 2 (more contaminated). Long-term heavy metal contamination allows local adaptation and an increased diversity of endemic species. On the other hand, different mechanisms shaping the communities at higher contamination levels can occur, such as strain-specific traits, immigration and horizontal transfer of resistance genes. In relation to prokaryotes, there was a similar distribution of abundant groups in both sites. However, regarding fungi, there was a higher number of abundant groups in site 2 than in site 1.

In this manner, *Sphingobacteriales* were more abundant in the less contaminated site 1. *Sphingobacteriales* are Gram-negative bacteria normally associated to plants and root exudates that are found in a wide array of habitats and are known for their ability to utilize unusual compounds, including herbicides and antimicrobial compounds (Kämpfer, 2010). Following the same trend, *Gemmatimonadetes BD2-11 terrestrial group* showed a higher abundance in site 1 than site 2. The *Gemmatimonadetes* phylum is commonly found in soils and especially in arid soils (DeBruyn *et al*., 2011). Reis *et al.* (2013) studied sediments affected by heavy metals from mining areas in Brazil and found that the phylum *Gemmatimonadetes* was present in these contaminated environments. It is therefore possible that these bacteria were widely present in our mine soil and their abundance decreased slightly due to the prevalence of other bacteria more resistant to the high level of toxic heavy metals found in site 2.

Actinobacteria phylum was similarly distributed in both sites. Nevertheless, several taxa within this group followed opposite trends in relation to heavy metal contamination (e.g. *Thermoleophilia group AKIW543, Actinobacteria group PeM15* and *Rubrobacterales*). Other groups were encountered abundantly in the less contaminated site 1 (e.g. *Streptosporangiales, Solirubrobacterales* and *Micromonosporales*). *Actinobacteria* are known for their ability to maintain a high metabolic activity in heavy metal contaminated soils (Gremion *et al*., 2003; Bamborough and Cummings, 2009).

Terrabacteria possess important adaptations such as resistance to environmental hazards (desiccation, ultraviolet radiation, and high salinity) and oxygenic photosynthesis.

Some taxa belonging to this phylum increased notably at higher heavy metal concentrations (site 2) such as *Chloroflexi;* while other taxa were more abundant in the less contaminated site 1 (e.g. *Cyanobacteria group ML635J-21* and *SHA-109*). Taxa belonging to the *Proteobacteria* phylum were similarly found in both sites; however, secondary effects from decreased plant cover can probably explain the lower abundance of many microbial groups in the more contaminated site 2. An example is *Rhizobiales*, whose members are typically nitrogen-fixers living in the roots of legumes, and *Rhodospirillales*.

Acidobacteria are known to be adapted to an oligotrophic lifestyle. In the present study, the relative abundance of *Acidobacteria* was significantly higher in the more contaminated site 2, showing opposite trends in relation to contamination level (e.g. *Acidobacteria Group 11, 13, 15* and *18*). Nevertheless, other *Acidobacteria* groups were found in site 1 where there was a lower concentration of heavy metals (e.g. Group 4). Due to their high abundance and ubiquitous distribution in soils, *Acidobacteria* might play an important role in terrestrial ecosystems and consist of many diverse members with highly divergent lifestyles (Rappé and Giovannoni, 2003). Due to a lack of cultured representatives, there is still a limited knowledge about the differences between *Acidobacteria* groups (Epelde *et al*., 2015). In combination with the analysis of three genomes of acidobacterial isolates, Ward *et al*. (2009) suggested that *Acidobacteria* might significantly contribute to the terrestrial carbon cycle.

In the less contaminated site 1, SHEEP treated soil had lower values of 16S and 18S RR, H', J' diversity indices. However, in the more contaminated site 2, SHEEP treated soil presented higher 18S RR, H' and J' values instead. Based on the ANOSIM and NMSD combined results, PAPER treatment in site 1 showed the largest difference in soil composition for both prokaryotes and eukaryotes. This correlates with our DGGE results, based only on the dominant bacterial species where PAPER treated soils were the most different ones in comparison to CONTROL soils.

Based on taxonomic composition, only *Erysipelotrichales* and *Triplonchida* were more abundant in COW and POULTRY treated soils, respectively, than in SHEEP or PAPER treated soils and CONTROL soils regardless of the contamination level. Any other significant effect was observed in either the less or more contaminated site but not in both. In site 1, *Chlamydiales* were more abundant in COW treated soil. While *Chlamydiales* species are best known as human and animal pathogens, the recently described *Parachlamydia* species were also found to infect free-living amoebae (Corsaro and Greub, 2006). Free-living amoebae, which are

important members of soil and water ecosystems, are increasingly recognized as vectors for human pathogens.

Chloroflexi subdivision 10 group S085 was more abundant in the PAPER treated soil in site 1, while in site 2 it was more abundant in the POULTRY treated soil along with *Chloroflexi subdivision Gitt-GS-136*. Many species of *Chloroflexi* are slow growing, versatile and facultatively aerobic photoheterotrophs (Davies *et al*., 2011). They have been found in soil (Costello and Schmidt, 2006) and activated sludge (Björnsson *et al*., 2002; Kragelund *et al*., 2007) and have previously been reported as especially resistant to heavy metals (Rastogi *et al*., 2011).

Glomerales were more commonly present in the less contaminated site 1 rather than in site 2 and more specifically in the PAPER treated soil, which efficiently reduced heavy metal bioavailability. Arbuscular mycorrhizal fungi such as *Glomerales* usually colonize roots of plants (Colpaert, 1998). Extraradical hyphae of mycorrhizal fungi can enhance the metal tolerance of host plants by sequestering of toxic metals in soil (Leyval *et al*., 1997; Colpaert, 1998). Similarly, the highest abundance of *Rhizobiales* was found in the PAPER treated soil in site 1 as well as of *Pleosporomycetidae.*

Phycisphaerae and *SAR324* were more abundant in the POULTRY treated soil in site 2 whereas *Chlorobiales* were more abundant in COW treated soil in site 1. *Chlorobiales* have previously been observed in heavy metal contaminated mine tailings, specifically in an uranium mining waste pile (Selenska-Pobell, 2002) and in subsurface sediments from uranium contaminated sites in Tennessee (Akob *et al*., 2007; Barns *et al*., 2007) and Colorado (Barns *et al*., 2007). *Rhodospirillales*, an order of Proteobacteria, were less abundant in site 2 in all treated soils (but not significantly for SHEEP). Their decreased abundance in the more contaminated site 2 may be also explained by the relative lack of plant cover. Very likely the added nutrients present in the amendments favoured taxa with a more copiotrophic lifestyle.

On the other hand, the SQI can be used for a diagnosis of overall soil functionality. Nevertheless, using an index such as the SQI inevitably results in "information compression" and can lead to an oversimplification of available information. In consequence, as it has been done in this study, it is desirable to assess soil quality at both levels: the indicator (microbial parameter) level and the index level, since they provide complementary information. Here, from the following 13 soil microbial parameters, a SQI value for each soil was calculated: (1) β-glucosidase activity; (2) urease activity; (3) acid phosphatase activity; (4) dehydrogenase

activity; (5) FDA; (6) arylsulphatase activity; (7) potentially mineralizable nitrogen (PMN); (8) microbial biomass; (9) total bacteria from 16S rRNA; (10) RR-16S rRNA; (11) H'-16S rRNA; (12) RR-18S rRNA and (13) H'-18S rRNA.

According to the SQI values, in site 1, COW and SHEEP treatments significantly reduced soil quality. In turn, PAPER treatment significantly increased some parameters related to the N cycle (e.g. NMP and urease) as well as 16S and 18S diversity (RR and H'). However, the SQI did not reflect this beneficial effect caused by the PAPER treatment.

Contrarely, in site 2, SHEEP significantly improved soil quality, as reflected by the SQI value. In fact, in the AMOEBA plot corresponding to site 2, most of the parameters for SHEEP treated soil increased, in contrast to what was observed for the same treatment in the less contaminated site 1. However, when analysing the different microbial parameters separately, almost none of them displayed a statistically significant increase in comparison to the control soil, except for 16S rRNA gene copy numbers and 18S RR (neither confirmed by ANOSIM). This highlights the need of analysing the different parameters separately and in combination.

On the other hand, HGT in combination with selective pressure (e.g. antibiotic or heavy metals) promotes the widespread dissemination of antibiotic resistance genes not only in clinical microbial communities but also in nonclinical environments (Witte, 1998; Rhodes *et al*., 2000; Schmidt *et al*., 2001; Tennstedt *et al*., 2003). The PCR-Southern Hybridization detection system proved to be a rapid and specific approach in our study for the detection of plasmid specific sequences in soil. Although several biological replicates were indeed extracted from the same soil, we found important differences pertaining to the presence or absence of plasmid specific sequences among the replicates, most likely as a consequence of the high level of heterogeneity in our study area. As mentioned before, this is a typical characteristic of many mine tailings (García-Sánchez *et al*., 1999; Epelde *et al*., 2010; Barrutia *et al*., 2011; Becerra-Castro *et al*., 2012). As a consequence, we assumed that plasmid specific sequences were detected in a soil even though they might not be present in all or most replicates. This observation, along with the impossibility of quantifying these sequences by qPCR (they were below detection limit), was a clear indication that these genes although they were present in these soils, were not abundant or not homogeneously spread.

Regarding MGEs, the integrase gene of class 1 integrons (*intl1* gene) was found in all soils except for SHEEP treated soil in site 2. Same results were obtained for the QAC resistance gene, *qacEΔ1*, which provides resistance to QAC disinfectants (quaternary ammonium

compounds) and has been normally found associated to class 1 integrons (Jaglic and Červinková, 2012). These results are in accordance with the findings of previous studies where *intI1* genes were reported to be common in environmental samples (Gillings *et al*., 2008b). The application of organic amendments to the soil did not lead to a noticeable increase of *intI1* gene, however, Jechalke *et al*. (2014) reported a significantly higher abundance of *intI1* genes in soils with a short-term history of manure treatments compared to CONTROL soils. These integrons are typically integrated within plasmids flanked by insertion sequences, which facilitates their transfer and dissemination among bacterial communities. Class 1 integrons are able to incorporate gene cassettes and then express them (Heuer and Smalla, 2012). Moreover, these gene cassettes often contain antibiotic resistance genes contributing to the spread of antibiotic resistances among bacteria. Our results showed the presence of bacterial populations within both sites carrying *intI1* genes, which suggest a strong potential of the bacterial communities in our mine to acquire and exchange antibiotic resistance genes via gene cassettes.

Class 1 integrons have been previously described as integrated sequences in BHR plasmids belonging to the incompatibility group IncP-1 (Jechalke *et al*., 2014). HGT together with selective pressure foster the dissemination of BHR plasmids, thus facilitating the transfer of antibiotic resistance genes among genera, phyla and domains (Brown, 2003; Sørensen *et al*., 2005). IncP-1 *trfA* genes were detected in extracted community DNA from site 1 in accordance to the abundance of class 1 integrons. The detection of IncP *trfA* sequences by PCR amplification and hybridization in total DNA isolated from bacteria of contaminated environments has been previously described (Götz *et al*., 1996; Gstalder *et al*., 2003). Especially, IncP-1 plasmids are widely distributed in Gram-negative bacteria (e.g. in *E. coli, Pseudomonas spp., Klebsiella aerogenes* and *Sphingomonas*) (Thomas, 2000; Harada *et al*., 2006). These observations suggest a role for class 1 integrons and IncP-1 plasmids in the adaptation of soil microbial communities to heavy metals (De Lipthay *et al*., 2008).

On the contrary, IncP-1 plasmids were almost absent in site 2. As seen before, site 1 was characterized by a higher microbial activity, biomass and diversity that seem to promote also HGT and, hence, a higher abundance of IncP-1 genes, when compared to the more contaminated site 2. Surprisingly, IncP-1 *trfA* in site 2 was only found in the extracted community DNA from SHEEP treated soil where neither *intI1* nor QAC genes were detected. This could be explained by the above-mentioned spatial heterogeneity of the study area.

Additionally to the IncP-1 group of plasmids, most plasmids frequently detected in environments belong to IncN, IncQ and IncW incompability groups. Plasmids belonging to the IncQ family were also present in both sites; however, in contrast to IncP-1 plasmids, IncQ plasmids were observed almost at the same frequency in both sites. This supports the hypothesis that the IncQ plasmid group may play an important role in adaptation to high levels of heavy metal contamination as previously reported by Heuer *et al*. (2002), which suggested that IncQ plasmids are enriched in Cu-treated rhizosphere. In contrast to IncP-1 *trfA* and IncQ *oriV*, IncN *rep* could not be detected in the extracted community DNA from any of the two sites.

While IncQ type plasmids are usually mobilizable, IncP-1 associated plasmids are often conjugative, therefore able to self-transfer among bacterial communities (Smalla *et al*., 2006). Finally, pHHV216-like plasmids, characterized by a low GC content, were present in site 1 but not in site 2. Again, this could be a result of the lower heavy metal level in site 1, which results in a richer microbial community in terms of activity, biomass and diversity. Low GC-type plasmids are thought to play an important role in conferring sulfadiazine resistance in manure treated soils (Heuer *et al*., 2009; Jechalke *et al*., 2013; Kopmann *et al*., 2013).

The presence of conjugative and/or mobilizable plasmids in our study sites indicates that soil bacteria in the mine have gene-mobilizing capacity with implications for potential dissemination of antibiotic and heavy metal resistance genes from the native bacterial soil populations to pathogens, with the risk of being incorporated by humans via the food chain.

Overall, our results provide insight into the structure and function of soil microbial communities subjected to long-term heavy metal contamination.

Table A3.1. Overview of RNA-derived sequencing libraries, datasets and diversity statistics. RR: rarefied richness; H': Shannon diversity; J´: Pielou evenness.

Site	Soil	Reads 16S	OTUs 16S	RR 16S	H' 16S	J'16S	Reads 18S	OTUs 18S	RR 18S	H' 18S	J' 18S
1	COW	90,645	4,596	4,217	6.78	0.804	122,606	2,389	2,293	4.82	0.620
	COW	129,922	5,272	4,370	6.82	0.796	144,473	2,586	2,363	4.85	0.617
	COW	68,406	4,228	4,228	6.79	0.813	126,137	2,422	2,297	4.78	0.614
	COW	117,018	5,002	4,271	6.81	0.800	124,025	2,512	2,393	5.01	0.640
	SHEEP	129,518	4,314	3,533	6.12	0.731	116,107	2,315	2,246	4.83	0.623
	SHEEP	82,295	3,476	3,279	6.29	0.771	133,357	2,251	2,103	4.64	0.601
	SHEEP	119,144	4,322	3,651	6.35	0.759	121,869	2,334	2,234	4.74	0.612
	SHEEP	110,076	4,315	3,723	6.44	0.770	134,561	2,295	2,132	4.36	0.564
	PAPER	119,538	5,128	4,378	6.92	0.810	130,435	2,577	2,433	5.02	0.639
	PAPER	108,541	4,819	4,234	6.79	0.801	110,086	2,528	2,492	4.79	0.611
	PAPER	100,238	4,788	4,276	6.79	0.802	116,531	2,331	2,259	4.72	0.609
	PAPER	124,228	4,796	4,060	6.78	0.800	143,042	2,485	2,288	4.61	0.589
	POULTRY	106,166	4,925	4,311	6.70	0.788	173,344	2,564	2,251	4.95	0.630
	POULTRY	84,151	4,541	4,273	6.79	0.806	133,132	2,526	2,383	5.10	0.651
	POULTRY	80,769	4,563	4,341	6.74	0.799	138,102	2,681	2,485	4.91	0.622
	POULTRY	102,077	5,003	4,449	6.83	0.802	111,527	2,530	2,486	4.84	0.618
	CONTROL	96,413	4,944	4,454	6.83	0.803	111,270	2,502	2,459	4.77	0.610
	CONTROL	103,653	5,055	4,468	6.87	0.806	120,616	2,602	2,503	5.15	0.654
	CONTROL	112,082	4,589	3,945	6.55	0.777	163,960	2,704	2,400	4.78	0.605
	CONTROL	86,815	4,283	3,988	6.65	0.795	112,911	2,491	2,441	5.10	0.653
2	COW	114,044	3,431	2,901	5.91	0.726	155,809	1,783	1,578	3.66	0.489
	COW	107,779	3,704	3,180	5.98	0.728	117,217	1,593	1,539	3.78	0.513
	COW	109,957	3,747	3,223	6.10	0.742	145,908	1,615	1,471	3.42	0.463
	COW	95,102	3,457	3,104	6.08	0.746	186,976	1,687	1,422	3.77	0.508
	SHEEP	120,480	4,498	3,778	6.36	0.756	163,844	2,061	1,809	4.14	0.542
	SHEEP	105,801	3,988	3,494	6.31	0.760	129,076	2,052	1,926	4.20	0.551
	SHEEP	92,290	4,145	3,774	6.55	0.787	104,656	2,037	2,037	4.48	0.589
	SHEEP	132,022	4,521	3,715	6.57	0.780	128,426	2,145	2,022	4.33	0.564
	PAPER	131,875	4,448	3,679	6.41	0.764	145,372	1,836	1,652	4.03	0.536
	PAPER	111,324	4,003	3,416	6.20	0.748	191,112	2,135	1,790	4.17	0.544
	PAPER	123,099	4,386	3,687	6.48	0.772	171,298	1,976	1,722	3.73	0.492
	PAPER	116,255	3,708	3,120	6.02	0.733	181,214	1,793	1,519	3.86	0.515
	POULTRY	128,390	4,066	3,357	6.23	0.749	100	40	NaN	NaN	NaN
	POULTRY	123,313	4,133	3,525	6.60	0.793	110,772	1,719	1,691	4.22	0.566
	POULTRY	112,401	4,117	3,555	6.35	0.763	147,996	1,232	1,091	2.02	0.283
	POULTRY	102,211	4,306	3,815	6.53	0.781	138,995	1,739	1,598	3.86	0.517
	CONTROL	104,433	4,131	3,625	6.33	0.761	157,052	1,678	1,503	3.99	0.537
	CONTROL	110,050	3,664	3,166	6.08	0.741	153,126	1,759	1,579	4.06	0.543
	CONTROL	81,003	3,553	3,355	6.13	0.750	162,757	1,740	1,545	3.89	0.522
	CONTROL	120,472	3,889	3,262	6.18	0.747	191,675	1,950	1,640	3.92	0.518
TOTAL		3,431,723	10,120				4,408,999	5,798			

Chapter 4.
Horizontal transfer of antibiotic resistance plasmids

Chapter 4. Horizontal transfer of antibiotic resistance plasmids

4.1. Introduction

4.1.1. Mechanisms of action of antibiotics and antibiotic resistance

Since the first use of antibiotics to the present day, a great number of antibiotics have appeared on the market with more or less effectiveness. These antibiotics can be grouped based on the site of action (Table 4.1). Most antibiotics target cell structures (cell wall and membranes), DNA replication and transcription machinery, protein synthesis machinery or metabolic pathways.

Table 4.1. Target sites of antibiotics (Neu 1992)**.**

Site of action	**Antibiotic**
Cytoplasmic membrane structure	Polymyxin
Cell wall synthesis	Vancomycin β-Lactams Cephalosporin Carbapenemases
DNA replication and transcription machinery	Nalidixic acid Norfloxacin Rifampicin
Protein synthesis	Mupirocin Erythromycin Chloramphenicol Clindamycin Tetracycline Gentamicin Kanamycin Streptomycin
Folic acid metabolism	Trimethoprim Sulphonamides

Further characterization is based on whether antibiotics kill the bacteria (bactericidal activity) or stop bacteria from multiplying (bacteriostatic activity). Those that target the

bacterial structure or interfere with essential bacterial enzyme activities are considered bactericides. On the contrary, those that interfere with protein synthesis activities are usually bacteriostatic.

There are common defence mechanisms shared by Gram-positive and Gram-negative bacteria such as resistance mutations, disabling the antibiotic-binding site, inactivation of the antibiotic by covalent modification, activation of multidrug resistance efflux pumps or shut down protein channels through which antibiotics could otherwise enter the cell (Taubes, 2008) (Figure 1.3).

Misuse and overuse of antibiotics plays a critical role in development of resistance in the environment. Hence, a great number of resistance genes against different antibiotics have been detected in recent studies related to antibiotic resistance in the environment (Table 4.2).

Table 4.2. Most representative antibiotics, some of their corresponding antibiotic resistance genes and the environment where they were detected.

Antibiotic	**Antibiotic resistance genes**	**Environmental detection**	**References**
β-lactams	*ampC, bla$_{CTX-M}$, bla$_{TEM}$, bla$_{SHV-5}$, mecA*	Wastewater-irrigated fields, fertilized soils and municipal wastewater	Jechalke *et al*., 2015 Volkmann *et al*., 2004 Malik *et al*., 2008 Knapp *et al*., 2010
Chloramphenicol	*catI, catIII*	Surface seawater	Dang *et al*., 2008
Erythromycin	*ermB, ermC, ermE, ermF, ermV, ermX*	Fertilized soils, forest soil, compost, and agricultural soil	Knapp *et al*., 2010 Popowska *et al*., 2011
Fluoroquinolones	*qnrA, qnrB, qnrS*	Wastewater-irrigated fields	Dalkmann *et al*., 2012
Gentamicin	aac(6')-II/Ib, aph(2')-I	Sewage, faeces and coastal water polluted with wastewater	Heuer *et al*., 2002
Streptomycin	*strA, strB*	Forest soil, compost and agricultural soil	Popowska *et al*., 2011
Sulphonamides	*sul1, sul2, sul3*	Manured soils	Heuer and Smalla, 2007
Tetracycline	*tetM, tetO, tetQ, tetW*	Wastewater-irrigated fields, fertilized soils	Jechalke *et al*., 2015 Knapp *et al*., 2010
Vancomycin	*vanA, vanB*	Municipal wastewater	Volkmann *et al*., 2004

4.1.2. Heavy metal toxicity

Since early history, microorganisms have not only coexisted but also coevolved with heavy metals (Bruins *et al.*, 2000). However, increasing quantities of heavy metals are introduced into the environment through anthropogenic activities and they represent a long-term environmental hazard since they are not biodegradable or cannot be destroyed (Liao *et al.*, 2008). Heavy metals are a major cause of ecosystem dynamics disturbance. Most of these are toxic in high concentration but are essential for living organisms in lower concentrations such as Co, Cu, Ni and Zn. In these cases, they play important roles as co-factors for metal-dependent proteins and enzymes (active redox heavy metals) (Doelman *et al.*, 1994). However, other heavy metals such as Cd, Cr, Pb, Hg and Ag have not any acknowledged benefits for bacteria and are toxic even in low concentrations (non active redox heavy metals) (Nies, 2004).

Metal toxicity can occur by i) displacement of essential metals from their native bindings sites and ii) ligand interactions causing damage of cell membrane, alterations of enzymatic activity and DNA structure and disruption of cellular functions (Bruins *et al.*, 2000). Nevertheless, bacteria have developed a variety of metal resistance mechanisms that include extra and intracellular sequestrations of heavy metals in complexes, exclusion by permeability barriers, enzymatic detoxification and reduction in sensitivity of cellular targets and efflux pumps (Nies, 2003) (Figure 4.1). Metal resistance genes can be found on the chromosome, on transposons, and MGEs.

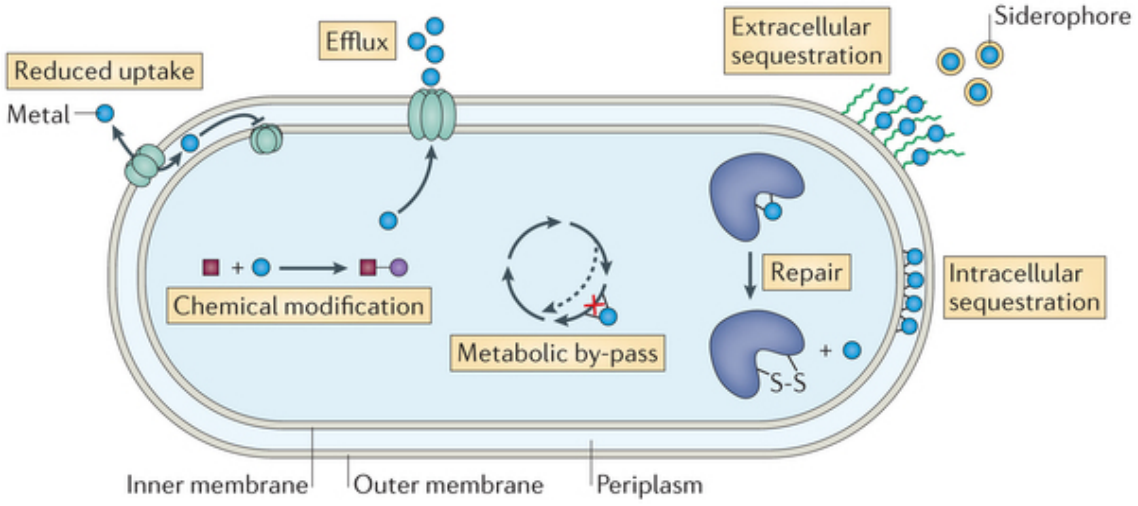


Figure 4.1. Mechanisms of bacterial resistance against heavy metals. The different mechanisms of action by which bacteria can fight against heavy metals are: reduced uptake and efflux to regulate the activity of heavy metal introduction into the cell by membrane transporters, extracellular sequestration in response to metal exposure or intracellular sequestration in the cytoplasm or periplasmic space,

repair of cellular molecules with redox-sensitive functional groups, metabolic by-pass avoiding metal disrupted enzymes and chemical modification altering the chemical reactivity of the metal (Lemire *et al*., 2013).

Metals like Cd, Cr, Cu, Fe, Hg, Ni and Ag possess the ability to produce reactive oxidative species (ROS). Increasing levels of ROS can exceed cell intrinsic antioxidant defence capacity, and result in a condition known as oxidative stress. Cells under oxidative stress display various dysfunctions due to lesions caused by these highly reactive oxygen-species to lipids, proteins, RNA and DNA. All these modifications are deleterious to the cell, since they lead to a loss of function of membranes and proteins, and block DNA replication or cause mutations. Consequently, it is suggested that metal-induced oxidative stress in cells can be partially responsible for the toxic effects of heavy metals.

4.1.3. Co-selection and cross-selection

As it was explained before, long-term exposure to antibiotics or heavy metals in soils at sub-inhibitory levels may favour HGT of resistance genes (Szczepanowski *et al*., 2008). The utilization of heavy metals for diverse activities may have selected genes encoding both heavy metal and antibiotic resistance (Baker-Austin, 2006) even before the antibiotic era. Co-selection of antibiotics and heavy metal resistance can be divided into two different types: co-resistance, different resistance determinants are harboured on the same genetic element, and cross-resistance, the same genetic determinant is responsible for resistance to antibiotics and heavy metals.

Co-resistance studies on mercury demonstrated that when transferring antibiotic resistance by conjugation between *Enterobacteriaceae* and recipient bacteria, mercury resistance was co-transferred within the same plasmid (Summers *et al*., 1993). Additionally, plasmids from bacteria in contaminated sites such as sewage treatment plants were shown to harbour genes that encode resistance to mercury (Szczepanowski *et al*., 2005), along with multiple antibiotic resistance genes. Furthermore, genome sequencing of a pathogenic *Salmonella enterica* strain revealed the presence of a conjugative plasmid that confers resistance to trimethoprim, sulphonamide, chloramphenicol, ampicillin and streptomycin which in addition containsed a mercury-resistance operon (Parkhill *et al*., 2001).

Conversely, cross-resistance can occur when antibiotic and heavy metals attack the same target within the cell, initiate a common pathway to cell death or share a common route

of access to their respective targets. In all these situations, and as a consequence of the development of resistance to one antibacterial agent (either antibiotic or metal), resistance to both agents is shown (Chapman, 2003). Cross-resistance mechanisms include reduction of membrane permeability or sequestration of heavy metals and antibiotics, drug and metal inactivation and/or modification, linked efflux of the metal and antibiotic and alteration of a cellular component to lower its sensitivity to the toxic metal and antibiotic.

A good example of cross-resistance is the MDR pump in *Listeria monocytogenes* that can export metals in addition to antibiotics (Mata *et al*., 2000). Mutants of the resistant strain without a functional efflux pump system were significantly more sensitive to a range of antibiotics and metals, including β-lactams, kanamycin, erythromycin, Cd and Zn (Hayashi *et al*., 2000).

Along the same lines, isolates exposed to Zn were also found to be resistant to other heavy metals (Cd and Co) and the carbapenem-class antibiotic imipenem (a drug of last resort). Analysis of the mechanisms that could underlie cross-resistance to both Zn and imipenem revealed a co-regulation system, thus, exposure to one toxicant (Zn or imipenem) led to both expression of metal efflux and imipenem resistance (Perron *et al*., 2004).

4.1.4. Cell fitness

Plasmids can provide their host with a large array of beneficial competitive phenotypes such as antibiotic or heavy metal resistance, the ability to grow quicker or even the ability to use a wider range of compounds as energy source (Top *et al.,* 1998; Riley and Wertz, 2002). Nevertheless, the acquisition of a MGE is commonly associated with a fitness or metabolic cost as a consequence of the replication, transcription and translation of the new genes (Bouma and Lenski, 1998; Martinez *et al*., 2009). These fitness costs will be therefore reflected in terms of reduced growth rate (lengthening of lag phase, lowering of maximum growth rates or cell densities) and/or competitive ability (due to the slower growth rate, absorption of carbon substrates is reduced) (Andersson, 2006; Andersson and Hughes, 2010).

Plasmids function as autonomous replicators whose intrinsic interests do not necessarily concur with those of their new host. Fitness cost due to the acquisition of resistance can be highly variable. In some systems, this can result in plasmids harbouring hosts being fitter than their plasmid-free ancestors (Dionisio *et al*., 2005; Starikova *et al*., 2013). Previous studies reported that, in general, the loss or partly deletion of any of the plasmids

resulted in changes in the metabolic capabilities (Stasiak *et al*., 2014). However, other studies reported cost of above 50% (Norström *et al*., 2007), while other studies have found little cost (Castaneda-Garcia *et al*., 2009).

In the context of antibiotic resistance, if the presence of an antibiotic determinant produces a high fitness cost, the resistance could be expected to disappear in the absence of antibiotic selective pressure (Morosini *et al*., 2000). This is due to the assumption that, in the absence of selective pressure from antibiotics, less-fit resistant bacteria would be outcompeted by their more-fit but susceptible counterparts in the population. However, recent studies pointed out that bacteria with acquired resistance kept the mechanisms of resistance and alleviated the fitness costs of such traits through compensatory evolution, instead of reverting to susceptibility (San Millan *et al*., 2014). Even if antibiotic pressure is occasional, compensatory mutations may evolve to reduce plasmid costs. This adaptation is due to mutations on the host's chromosome or on the plasmid itself (Bouma and Lenski, 1988; Dahlberg and Chao, 2003; Dionisio *et al*., 2005) and perhaps it explains why resistance plasmids are found in bacteria even when unexposed to antibiotic pressure (Hughes and Data, 1983; Svara and Rankin, 2011). However, bacteria can as well rapidly adapt to plasmids carrying resistance genes by eliminating the cost of plasmid carriage and integrating the antibiotic determinants in the chromosome. This host-plasmid coevolution could also explain why the cost of the same plasmid is so variable between different bacterial hosts.

There are several factors that can contribute to the cost of plasmid-mediated resistance: i) the degree of multidrug resistance or mechanism of action of resistance, which could be crucial as some mechanisms require more energy and gene/proteins involved than others; ii) plasmid size: small plasmids (less than 10 kb) may only carry a single resistance determinant and little else besides the genes involved in plasmid replication but large plasmids (more than 100 kb) can carry more than 10 resistance determinants as well as a wide variety of genes involved in other traits; iii) phenotypic changes due to chromosomal disruption by horizontally acquired regions: if horizontally transferred genes are incorporated into the chromosome, they must disrupt existing genomic sequences, and such changes could be deleterious; iv) energetic costs due to consumption of molecular building blocks or energy sources, as physiological processes require input of molecules such as nucleotides or ATP, and acquired regions can lower available amounts within the cell; v) sequestration of critical cellular processes, as fitness can be lowered if critical physiological processes require access to

molecular machinery (i.e. ribosomes), but transferred regions actively occupy this machinery (Baltrus, 2013; Vogwill and MacLean, 2015).

On the other hand, these newly acquired genes find themselves in a different metabolic context; therefore, their expression is highly associated to the machinery of the host. On some occasions, determinants may encounter a metabolic substrate/protein partner in the new host, so that specific changes in the host's metabolism become possible. Contrarily, sometimes the protein partners required for their activity or their original biochemical substrate or regulation are missing. When this occurs, bacteria would suffer the metabolic cost of replicating these genes or having misfolded proteins without obtaining any beneficial trait.

What has been demonstrated is that usually fitness cost increases with increasing plasmid resistance range and not necessarily with plasmid size. However, the number of total genes carried by a plasmid is directly correlated with its size. Therefore, it can be concluded that resistance genes are more costly than the majority of plasmid-encoded traits. This could be due to resistance genes being relatively recent additions due to the increased pressure caused by the use of antibiotics in the recent century (Hughes and Datta, 1983; Vogwill and MacLean, 2015).

The cost of resistance is known to play a key role in the evolutionary dynamics of antibiotic resistance because it generates selection against resistance (Austin *et al*., 1997; zur Wiesch *et al*., 2011). An increased understanding of how MGEs affect their bacterial hosts is fundamental to understand the different biological factors controlling the emergence, spread and persistence of antibiotic resistance determinants. Previous studies showed that phenotypic, physiological and biochemical characteristics, such as size (Matz and Kjelleberg, 2005), growth rate (Mächler and Altermatt, 2012) and resource utilization capability (Dawson *et al*., 2012), are important for colonization of new habitats. Of course, all these characteristics and bacterial invasiveness are often ecosystem dependent (Kolar and Lodge, 2001).

Plasmids are maintained in bacterial populations instead of always being integrated in the chromosome simply because they are ideal tools to create standing genetic variation within populations. This is by far more efficient for adaptation to environment challenges than genetic innovation by mutations (Hermisson and Pennings, 2005). Thus, the primary benefit of bacterial populations from maintaining transferable plasmids might be that they gain robustness in the uncertainty of irregular environments.

4.2. Materials and methods

4.2.1. Detachment of soil bacteria

Bacteria from the soil were detached by mixing 10 mg of soil and 1.5 ml of 7.5 mM sodium pyrophosphate and Tween 80 (0.5%, w/v) buffer. Consequently, the mix was shaken for 45 min at room temperature with the Intelli-Mixer RM-2M (ELMI, Riga, Latvia) at 900 rpm (Böckelmann *et al*., 2003). Then, it was allowed to settle for 5 min and the supernatant with the detached bacteria served as donor cell mixture for the following antibiotic sensitivity test and the exogenous plasmid isolation.

4.2.2. Antibiotic sensitivity test

To capture plasmids from soil bacteria two potential recipient strains were tested for resistance against potential selective antibiotics: *E. coli* 1030 (RIF^R) and *E. faecalis* OG1RF (RIF^R, Fusidic $Acid^R$). Along with these potential recipient strains, bacteria detached from soil were also tested for resistance against the following antibiotics (Table 4.3): ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin and tetracycline, in order to define the antibiotics that can be used as selection markers in the exogenous plasmid isolation experiments. These antibiotics were selected due to their relevance in promoting antibiotic resistant bacteria in soil environments (Götz *et al.*, 1997; Heuer *et al.*, 2002; Malik *et al.*, 2008; Bihn *et al.*, 2009; Allen *et al.*, 2009; Popowska *et al.*, 2011; You *et al.*, 2012 and Li *et al.*, 2013).

Table 4.3. Antibiotics used in the resistance tests of potential donors (bacteria detached from soil) and recipients [*E. coli* 1030 (RIF^R) and *E. faecalis* OG1RF (RIF^R, Fusidic $Acid^R$)].

Antibiotic	Abbreviation	Concentration (μg ml^{-1})	Supplier
Ampicillin	AMP	25	Sigma Aldrich, St. Louis, USA
Chloramphenicol	CHL	10	
Erythromycin	ERY	45	
Gentamicin	GEN	25	
Kanamycin	KAN	30	Gibco Life Technologies, Hercules, USA
Streptomycin	STR	10	Sigma Aldrich, St. Louis, USA
Tetracycline	TET	10	
Rifampicin	RIF	100	

First, to test the antibiotic resistance of the bacteria detached from soil 100 μl of the supernatant, obtained as described in section 4.2.1, were spread onto TSA (Tryptic Soy Agar) (Sigma Aldrich, St. Louis, USA) plates supplemented with one of the antibiotics at the concentration listed in Table 4.3. In addition, the agar plates were amended with 300 μg ml^{-1} of cycloheximide (Sigma Aldrich, St. Louis, USA) to reduce growth of co-detached fungi from soil. Furthermore, the detached bacteria were spread onto TSA plates supplemented with rifampicin to check their sensitivity to the antibiotic used for selection of the recipients. Plates were incubated overnight at 28 °C. All these tests were done in triplicate for each antibiotic and sample.

Second, to test the antibiotic resistance of the recipient strains, TSB (Tryptic Soy Broth) (Sigma Aldrich, St. Louis, USA) medium supplemented with rifampicin (100 μg ml^{-1}) was inoculated with the *E. coli* 1030 and *E. faecalis* OG1RF recipients and incubated overnight at 37 °C. Then, 100 μl of the overnight culture was spread on TSA plates supplemented with rifampicin (100 μg ml^{-1}) plus one of the antibiotics at the concentration listed in Table 4.3. All these tests were done in triplicate for each antibiotic and sample.

4.2.3. Exogenous plasmid isolation using *E. coli* 1030 as recipient

This technique was used to capture conjugative and/or mobilizable plasmids from bacterial communities present in the different soils. For each soil, 3 replicates were performed.

As described in Figure 4.2, recipient cells (*E. coli* 1030, RIF^R) were grown overnight at 37 °C on TSA plates supplemented with rifampicin (100 μg ml^{-1}). Next, a single recipient colony was picked from the plate with a sterile toothpick and introduced into 2 ml TSB medium supplemented with rifampicin (100 μg ml^{-1}). Again, the culture was allowed to grow overnight at 37 °C under constant shaking. 1 ml of the overnight recipient culture was centrifuged at 4,850 × *g* (13,000 rpm) at room temperature. Alongside, 1 ml of donor cells (detached soil-bacteria obtained as described in section 4.2.1) from each soil was centrifuged in the same manner. Both recipient and donor supernatant were discarded. Pellets were washed twice in 1 ml of sterile 1/10 TSB solution to eliminate remaining antibiotics from the recipient samples and detachment buffer from the donor samples. Pellets were resuspended in 1 ml of sterile 1/10 TSB solution.

Next, 500 μl of the recipient sample and 500 μl of the donor sample were mixed. Both, sample and control mixtures were centrifuged at 4,850 × *g* (13,000 rpm) at room temperature, supernatants were carefully removed. The pellet was resuspended in 50 μl of 1/10 TSB solution and applied to a nitrocellulose filter (0.22 μm pore size) (Millipore, Billerica, USA). The filters were incubated overnight at 28 °C on TSA plates supplemented with 300 μg ml^{-1} of cycloheximide. Additionally, control experiments were performed by incubating separately the donor fraction from the soil samples and the recipient strain.

After overnight incubation, the cell lawn was resuspended in 1 ml of sterile NaCl solution (0.85%, w/v) and stirred at 450 rpm for 20 min to release the bacteria from the filter. Serial 10-fold dilutions of detached bacteria were spread on TSA plates supplemented with cycloheximide (300 μg ml^{-1}) and rifampicin (100 μg ml^{-1}) and one selective antibiotic. After 48 h of incubation at 28 °C single colonies were picked for further characterization.

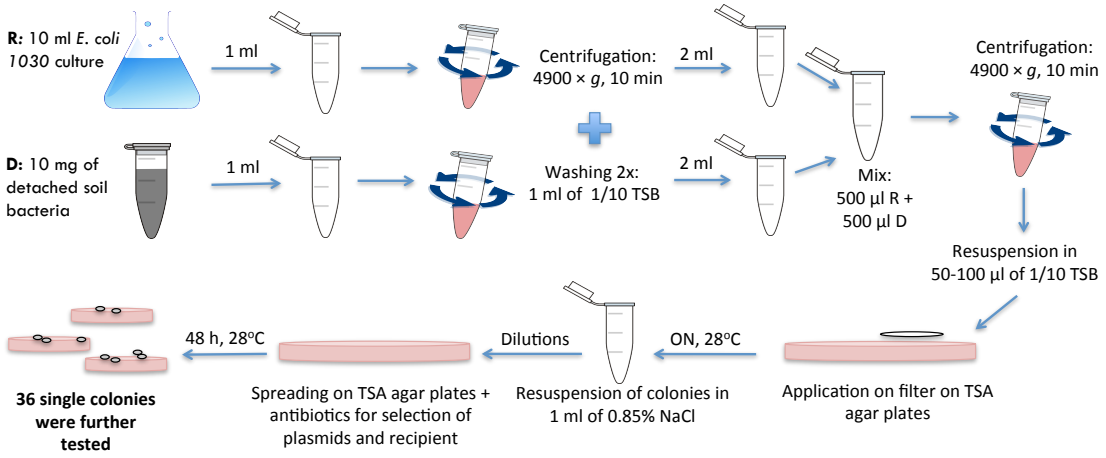


Figure 4.2. Exogenous plasmid isolation step-by-step procedure for detached bacteria using *E. coli* 1030 as recipient.

4.2.4. Exogenous plasmid isolation using *E. faecalis* OG1RF as recipient

The exogenous plasmid isolation using *E. faecalis* OG1RF as recipient was performed following the above-described protocol for the exogenous plasmid isolation using *E. coli* 1030 as recipient.

4.2.5. Growth conditions

Transconjugants cells were incubated overnight at 37 °C on Lysogeny Broth agar (LB) (Sigma Aldrich, St. Louis, USA) amended with rifampicin (100 μg ml^{-1}) plus the corresponding antibiotic previously used for their selection (Table 4.4). Recipient cells (*E. coli* 1030 and *E. faecalis* OG1RF) were incubated only with rifampicin (100 μg ml^{-1}).

Table 4.4. Antibiotics used to select for recipient and transconjugants.

Strains	**Selective antibiotics**
E. coli 1030 (recipient)	Rifampicin
1PAPER-AMP	Rifampicin and ampicillin
1COW-CHL	Rifampicin and chloramphenicol
1SHEEP-CHL	
1PAPER-CHL	
2COW-CHL	
2SHEEP-CHL	
2PAPER-CHL	
2CONTROL-CHL	
1SHEEP-ERY	Rifampicin and erythromycin
1PAPER-ERY	
1CONTROL-ERY	
2COW-ERY	
2SHEEP-ERY	
2PAPER-ERY	
2POULTRY-ERY	
1PAPER-GEN	Rifampicin and gentamicin
1CONTROL-GEN	
2COW-GEN	
2CONTROL-GEN	
1SHEEP-KAN	Rifampicin and kanamycin
1PAPER-KAN	
1POULTRY-KAN	
1CONTROL-KAN	
2COW-KAN	
2SHEEP-KAN	
2PAPER-KAN	
2POULTRY-KAN	
2CONTROL-KAN	
1COW-STR	Rifampicin and streptomycin
1SHEEP-STR	
1PAPER-STR	
1CONTROL-STR	
2COW-STR	
2POULTRY-STR	

4.2.6. DNA extraction from transconjugant cells

Cell lysis was carried out with the Genomic DNA Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Fresh transconjugants cells were picked from overnight-incubated colonies on LB agar amended with rifampicin (100 μg ml^{-1}) plus the corresponding antibiotic previously used for their selection (Table 4.4). Cells were resuspended in 1 ml of NaCl (0.85%, w/v) buffer and harvested by centrifugation at 13,000 × *g* for 2 min. The pellet was finally frozen at -20 °C.

Thawed cells were mixed with 200 µl of the pre-mixed lysis buffer (186.6 µl sterile pre-lysis buffer [50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), Tween 20 (0.5%, w/v) and Triton X-100 (0.5%, w/v), 4 µl lysozyme solution (QIAGEN), 9 µl protease solution (QIAGEN), 0.4 µl RNase A ready-to-use solution (QIAGEN)] and then vortexed vigorously before incubating for 30 min at 37 °C. After the incubation, 70 µl of sterile 3 M guanidine hydrochloride and Tween 20 (20%, w/v) were added and the mix was vortexed vigorously. Then, it was incubated for 30 min at 50 °C. Genomic DNA preparation of the transconjugants and the recipient strain was done using the Fermentas DNA Extraction Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions.

4.2.7. BOX – PCR

BOX-PCR assays were used to assess the presence in the transconjugants of naturally occurring, multi-copied, conserved and repetitive DNA sequences present in the genome of the original recipient *E. coli* 1030 strain. The BOX repeat consists of three discriminate regions: boxA, boxB, and boxC, which are 59, 45, and 50 base pairs in length, respectively. Various different combinations of these three elements are found to be present in different BOX loci and limited sequence heterogeneity is encountered among different elements from the same strain (Van Belkum *et al.*, 2001). Therefore, if the BOX fingerprinting profile was the same in the transconjugants as in the original *E. coli* 1030 recipient strain, they were considered as true transconjugants.

The oligonucleotide used to detect these highly conserved sequences was the BOX_A1R primer, which has the following sequence: 5'CTACGGCAAGGCGACGCTGACG3' (Martin *et al.*, 1992). Concentrations of reaction components and the thermal program for the PCR assay can be found in Table 4.5 and Table 4.6, respectively.

DNA amplifications were carried out in a CFX96 Real-Time System (BioRad Life Technologies, Hercules, USA). As positive control the *E. coli* 1030 recipient strain was used because, as it was mentioned before, the purpose of this was to compare the genetic profiles of the transconjugants with the one from the original recipient. As negative control, a PCR reaction mixture with ultrapure distilled water instead of template DNA was used.

Table 4.5. Reagents and respective concentrations for the amplification of the BOX repeat fragment in *E. coli* (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
13.25	Aqua ultrapure	
2.5	EP-Taq Buffer* (10×)	1×
2.5	2 mM dNTPs	0.2 mM
3.75	25 mM $MgCl_2$	3.75 mM
1.25	DMSO	5%
0.5	BOX primer	0.2 µM
0.25	EP-Taq polymerase* (5 U μl^{-1})	1.25 U/sample
1 µl	DNA template	Approx. 20 ng

*EP-Taq Buffer and Polymerase (JKI, Quedlinburg) (Dealtry *et al*., 2014).

Table 4.6. PCR thermal program for the molecular typing of *E. coli* 1030.

Cycles	Temperature (°C)	Time (s)	Step
1	94	420	Initial denaturation
30	94	60	Denaturation
	53	60	Annealing
	65	480	Elongation
1	65	960	Final elongation

PCR products were visualized by TBE agarose gel (1.5%, w/v) electrophoresis. 10 µl of PCR products were mixed with 2 µl of 6× loading dye prior to loading in the gel. As marker 5 µl of 1 Kb Plus DNA ladder (Thermo Scientific, Waltham, USA) was used. The gel was run at 50 V for 4-5 h. Once the electrophoresis was completed, the gels were stained with ethidium bromide solution for 30 min under continuous shaking and finally visualized under UV light.

4.2.8. Determination of minimum inhibitory concentrations (MIC) of antibiotics for the transconjugants

The minimum inhibitory concentration (MIC) of the following antibiotics was determined by the plate dilution method for each transconjugant following the guidelines by the National Committee for Clinical Laboratory Standards (NCCLS, 2004). The lowest concentration of the antibiotic that inhibited the growth of the bacteria was considered as MIC of the antibiotic for the transconjugant tested. The antibiotics selected were: ampicillin, chloramphenicol, erythromycin, gentamicin, imipenem, kanamycin, streptomycin, sulfadiazine, tetracycline and vancomycin.

Each antibiotic was prepared in a 5,120 μg ml^{-1} stock solution and filter-sterilized prior to the addition onto Mueller-Hinton agar plates (Sigma Aldrich, St. Louis, USA). These plates were prepared in varying antibiotic concentrations ranging from a maximum of 1,024 μg ml^{-1} to a minimum of 0.0625 μg ml^{-1} per plate. Mueller-Hinton agar is a non-selective microbiological growth medium, which presents properties that make it suitable for antibiotic testing. This agar contains starch, which is known to absorb toxins released from bacteria, so that they cannot interfere with the antibiotics. Additionally, it is a loose agar that allows for better diffusion of the antibiotics in order to lead to a truer zone of inhibition. *E. coli* ATCC 25299 strain was used as an internal reference to confirm the real concentration of the antibiotics on the plates (NCCLS, 2004).

Transconjugants and the *E. coli* 1030 recipient strain were pre-cultured in LB agar medium supplemented with the corresponding concentration of antibiotics for strain and MGEs selection, as listed in Table 4.4. The plates were incubated overnight at 37 °C to obtain single colonies. On the next day, single colonies were picked from the plates and introduced into 5 ml LB medium supplemented with the same concentration of antibiotics. Cells were incubated overnight at 37 °C under continuous shaking.

Next day, 50, 100 or 200 μl of the overnight cultures (dependent on the strain growth) were inoculated into 5 ml of fresh LB medium supplemented with the same concentration of antibiotics. They were incubated at 37 °C under continuous shaking until they reached an OD_{600} of 0.08 – 0.1. At this point, a dilution of 1/10 was prepared with fresh LB medium (450 μl LB and 50 μl of cell culture) and 1 μl of each sample was spread onto Mueller-Hinton agar plates supplemented with the different concentrations of the following antibiotics: ampicillin, chloramphenicol, erythromycin, gentamicin, imipenem, kanamycin, streptomycin, sulfadiazine,

tetracycline and vancomycin. By doing this, all cultures started with the same initial concentration of cells of 1×10^7 CFU ml^{-1}. Finally, these plates were grown overnight at 37 °C.

4.2.9. Modified Hodge Test

Carbapenemase production can be detected by the modified Hodge test (MHT), which allows growth of an indicator carbapenem susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk when the tested sample produces carbapenemases. Transconjugant 1PAPER-STR was tested for carbapenemase production.

First, an *E. coli* ATCC 25922 overnight culture equivalent to a 0.5 McFarland standard (Sigma Aldrich, St. Louis, USA) was prepared. Then, the culture was diluted 1:10 and it was applied as a lawn onto a Mueller-Hinton agar plate. After 5 minutes, a 10 µg meropenem susceptibility disk was then placed on the centre of the plate. Finally, in a straight line, fresh colonies from transconjugant 1PAPER-STR were streaked from the edge of the disk to the edge of the plate. Incubation was performed at 37 °C for 24 h and after this, the growth of the indicator strain (*E. coli* ATCC 25922) on the surrounding of the transconjugant 1PAPER-STR was studied. As positive control, a carbapenemase producer *Acinetobacter baumannii* strain was used and, as negative control, a carbapenem susceptible *E. coli* was employed. On the positive control the formation of a characteristic cloverleaf like indentation in the meropenem disc inhibition zone was expected due to an enhanced growth of the indicator strain (*E. coli* ATCC 25922). On the negative control instead, no growth of the indicator strain was predicted within the meropenem disc inhibition zone (Figure 4.3).

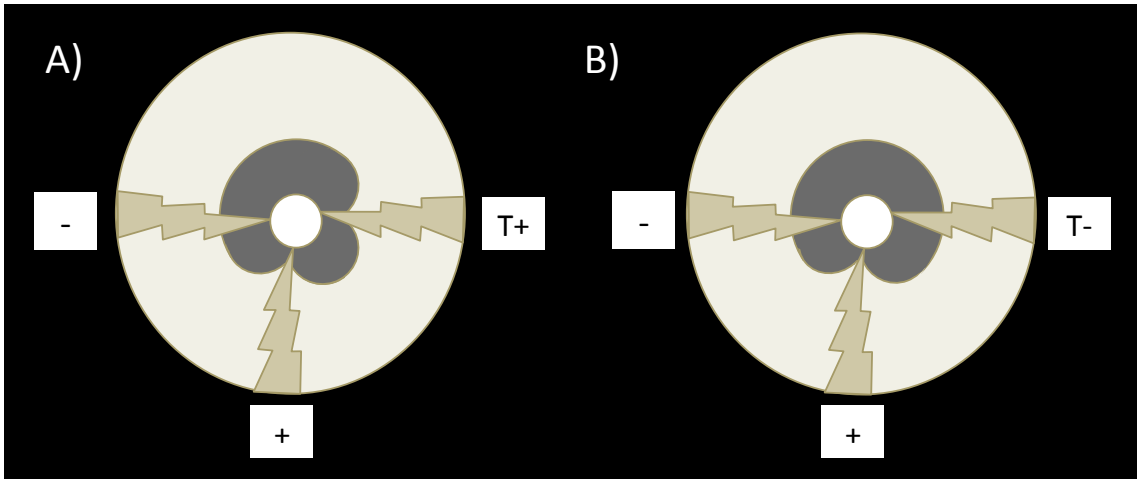


Figure 4.3. The MHT performed on a Mueller-Hinton agar plate. A) MHT positive result B) MHT negative result. The test relies on the formation of a characteristic cloverleaf like indentation in the zone of inhibition of a carbapenem susceptible strain (*E. coli* ATCC 25922) around a meropenem disc due to carbapenemase production. Negative control (-). Positive control (+). Carbapenemase-producing test strain (T+). No carbapenemase-producing test strain (T-).

4.2.10. Minimal inhibitory concentrations (MIC) of heavy metals

Minimal inhibitory concentration (MIC) of Cd, Cu, Zn and Pb was also tested. The MIC value was defined as the lowest concentration of heavy metal that inhibited cell growth as measured by absorbance at OD_{600}.

Single colonies from overnight cultures of *E. coli* 1030 recipient and transconjugant strains were added to 5 ml LB medium supplemented with the corresponding antibiotics for selection (Table 4.4). After overnight incubation at 37 °C, 50, 100 or 200 µl of the overnight cultures (based on strain growth) were inoculated into 5 ml of fresh LB medium supplemented with the same concentration of antibiotics. Cells were incubated at 37 °C until they reached an OD_{600} of 0.08 – 0.1. At this point, 10 µl of each culture were inoculated into 150 µl of LB medium containing increasing concentrations of Cd, Cu, Zn or Pb ($CdCl_2$, $CuCl_2$, $ZnCl_2$ or $Pb(NO_3)_2$ on a 96-well plate. Plates were incubated overnight at 37 °C under continuous shaking. Next day, cell growth was visualized with a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, USA). At least 3 replicates per sample and for each metal were performed.

As an exception, due to the high absorbance of $Pb(NO_3)_2$ at 600 nm, a 1:4 dilution with LB medium was carried out prior to cell growth determination at 600 nm for cultures with

$Pb(NO_3)_{2,}$. As shown in Figure 4.4, when diluted to a 1:4 ratio, the *E. coli* 1030 cells absorbance exhibited a higher absorbance than $Pb(NO_3)_2$.

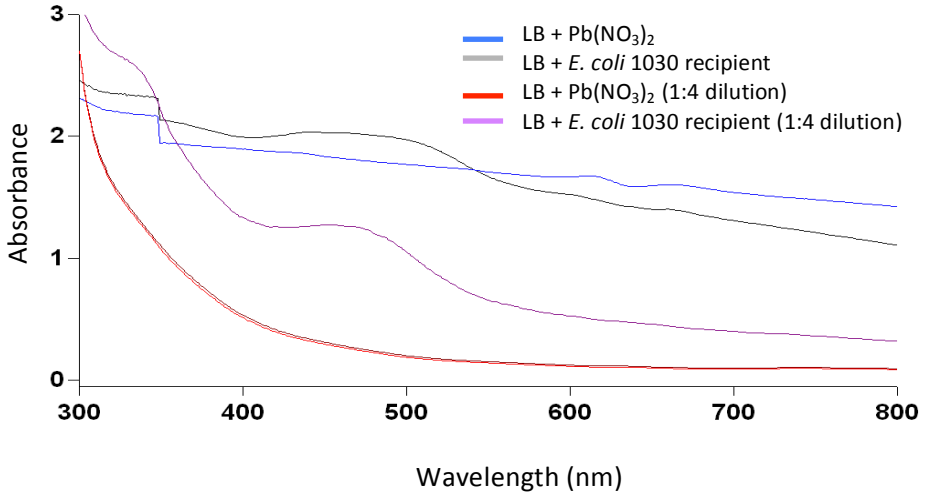


Figure 4.4. Spectra of $Pb(NO_3)_2$ and *E. coli* 1030 in LB. Both top spectrums, blue and grey, represent $Pb(NO_3)_2$ in LB and *E. coli* Nr 1030 (recipient) cells in LB with no dilution, respectively. Red and violet spectrums represent $Pb(NO_3)_2$ in LB and *E. coli* 1030 (recipient) cells in LB with a 1:4 dilution, respectively.

4.2.11. Effect of Cd and Cu on the expression of genes related to oxidative stress in *E. coli* transconjugants

This assay is based on the relative quantification by quantitative reverse transcription PCR (RT-qPCR) of the differential oxidative stress related gene expression in transconjugants and in the original *E. coli* 1030 recipient strain when exposed to Cd (non-active redox heavy metal) and Cu (active redox heavy metal). Specifically, an alkyl hydroperoxide reductase (ahpF), a catalase (katG) and a superoxide dismutase (sodA) were studied in this assay. The 3-phenyl propionic transporter (*hcaT*) reference gene was used for expression control. In this manner, the level of expression of the target genes was normalized relative to the expression obtained for the reference gene.

First, a fresh colony of each of the transconjugants and the *E. coli* 1030 recipient strain were picked to inoculate 10 ml of LB medium supplemented with the corresponding antibiotics for their selection listed in Table 4.4. The cultures were grown overnight at 37 °C under continuous shaking. From this point onwards the assay for each transconjugant and the recipient was done in triplicate.

Next day, transconjugants and the recipient strain were allowed to grow on fresh LB medium supplemented with the same antibiotic pressure until they reached an OD_{600} of approximately 0.5. At this point, 50 ml of the samples were mixed with 500 µl of 20 mM $CdCl_2$ or $CuCl_2$. The reaction mix was incubated for 15 min at 37 °C under constant shaking. After the Cd exposure, 20 ml of the samples were mixed with 2 ml of the cold expression stop buffer (95% ethanol: 5% phenol). Finally, cells were harvested after centrifugation at 9,000 rpm at 4 °C for 10 min. In analogy, a negative control for each of the transconjugants and the recipient strain was prepared with no exposure to 0.2 mM Cd or Cu.

In vivo stabilization of total RNA

Immediately after cell harvest, samples were treated with RNAProtect Bacteria Mini kit (Qiagen, Hilden, Germany) for *in vivo* stabilization of total RNA in bacteria to ensure a more accurate analysis of gene expression. RNA stabilization was performed according to manufacturer's instructions for enzymatic lysis, proteinase K digestion of bacteria and subsequent purification of total RNA from bacterial lysate.

RNA extraction

First, samples were mixed in a 1:1 ratio with 1:1 phenol/chloroform solution. Then, the mix was centrifuged at 12,000 rpm at 4 °C for 2 min. The formation of two layers was observed after centrifugation. In order to extract only the RNA, the aqueous layer was transferred to a clean tube, avoiding the interface. A 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol were added to the samples. Then, they were incubated at -20°C for 1.5 h. After the incubation, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was decanted and two washes were performed with 70% ethanol. The final pellet was resuspended in 50 µl of ultrapure distilled water.

DNAse I treatment

DNAse I treatment was carried out with the DNase I (RNase-free) (Life Technologies, Hercules, USA) from bovine pancreas according to the manufacturer's instructions.

cDNA reverse transcription

The cDNA reverse transcription was carried out with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA) according to the manufacturer's instructions.

Oxidative stress related gene expression in transconjugants by RT-qPCR assays

To study the gene expression of *ahpF, katG* and *sodA* genes, RT-qPCR assays were performed in a CFX96 Real-Time System with the 7500 Software V2.0.6 (BioRad Life Technologies, Hercules, USA). The primers are listed in Table 4.7 (Gómez-Sagasti *et al*., 2015). Concentrations of reaction components and the thermal program for the RT-qPCR assays can be found in Table 4.8 and Table 4.9, respectively. The Taq polymerase and buffer used were the SYBR Premix ExTaq (Takara, Japan), which also comes with ROX Reference Dye designed to normalize the fluorescent reporter signal. As a reference gene to control gene expression, *hcaT* gene was used. Bovine Serum Albumin (BSA) (Roche, Mannheim, Germany) was used to increase the efficiency of the reaction. The study of the gene expression results obtained by RT-qPCR was performed with the GenEx qPCR v.5.4.3 (MultiD Analyses AB, Göteborg, Sweden) software.

Table 4.7. Primers used for the quantification of highly conserved *ahpF, katG* and *sodA* sequences by RT-qPCR. *hcaT* gene was used as a reference gene for expression control.

Gene	Primer/Probe*	Primer Sequence (5'-3')	Product Size (bp)	Reference
hcaT	*hcaT - F*	CTGGCCTGCGTTTGTTTATT	281	Gómez-Sagasti *et al*., 2015
	hcaT - R	CAATGCAGAATTTGCACCAC		
ahpF	*ahpF - F*	CACGTAAAGGCATCCGTACC	263	
	ahpF - R	GATAATGCTGCGTGCTTTCA		
katG	*katG - F*	AAAACGGCGTCTTCACTGAC	289	
	katG - R	AAACGGTCGAGGTTCATCAC		
sodA	*sodA - F*	GCCTGTTCTGGAAAGGTCTG	232	
	sodA - R	GATGCGCCAGAAATAGCTTC		

*F: forward primer, R: reverse primer.

Table 4.8. Reagents and respective concentrations for the amplification of highly conserved *ahpF, katG* and *sodA* sequences (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
5.25	Aqua ultrapure	
12.5	SYBR Premix ExTaq (2×)	1×
1.25	BSA (2 mg ml^{-1})	0.1 mg ml^{-1}
2.5	Forward primer (10 µM)	1 µM
2.5	Reverse primer (10 µM)	1 µM
0.5	ROX (50×)	1×
0.5	DNA template	Approx. 20 ng

Table 4.9. RT-qPCR thermal program for the amplification of highly conserved *ahpF, katG* and *sodA* sequences.

Cycles	Temperature (°C)	Time (s)	Step
1	95	600	Initial denaturation
40	95	15	Denaturation
	60	60	Annealing
1	95	15	Melting curve
	60	60	
	95	30	
	60	15	

4.2.12. Cross-resistance against Cu and imipenem

As observed in Caille *et al*., 2007, Cu treatment in *Pseudomonas aeruginosa* could induce resistance not only to this metal, but also result in resistance to imipenem, a carbapenem antibiotic. CopR appears to be a key regulator involved in resistance against Cu in *P. aeruginosa.* OprD is a protein whose primary role is the passive uptake of basic amino acids, but it forms pores that are also permeable to imipenem (Ochs *et al*., 1999). Under Cu exposure, *copR* gene expression was found strongly enhanced while, at the same time, CopR negatively regulated the *oprD* system. Therefore the uptake of imipenem was also inhibited. This co-regulation was responsible for the observed cross-resistance against Cu and imipenem.

RT-qPCR analysis

As explained before in section 4.2.11, *E. coli* 1030 recipient and transconjugant 1PAPER-STR cells were first growth until they reached and OD_{600} of 0.5. Then, cells were exposed to 0, 1 and 5 mM of $CuCl_2$ by mixing 50 ml of the samples with 500 μl of 100 mM and 500 mM $CuCl_2$, respectively. The reaction mix was incubated for 15 min at 37 °C under constant shaking.

After Cu exposure, 20 ml of the incubated samples were mixed with 2 ml of the pre-refrigerated stop expression buffer (95% ethanol: 5% phenol). Finally, cells were harvested after centrifugation at 9,000 rpm at 4 °C for 10 min. Subsequent RNA extraction, DNAse I treatment and cDNA reverse transcription were carried out as described in section 4.2.11.

RT-qPCR of *copR*, *copS* and *oprD* were performed in a CFX96 Real-Time System with the 7500 Software V2.0.6 (BioRad Life Technologies, Hercules, USA). The primers are listed in Table 4.10 (Caille *et al*., 2007). Concentrations of reaction components and the thermal program for the RT-qPCR assays can be found in Table 4.11 and Table 4.12, respectively. The Taq polymerase and buffer used were the SYBR Premix ExTaq (Takara, Japan), which also comes with ROX Reference Dye designed to normalize the fluorescent reporter signal. As a reference gene to control gene expression, *hcaT* gene was used. BSA (Roche, Mannheim, Germany) was used to increase the efficiency of the reaction.

Table 4.10. Primers used for the quantification of the *copR*, *copS* and *oprD* sequences by RT-qPCR. *hcaT* gene was used as a reference gene for expression control.

Gene	Primer/Probe*	Primer Sequence (5'-3')	Product Size (bp)	Reference
hcaT	hcaT - F	CTGGCCTGCGTTTGTTTATT	281	Caille *et al*., 2007
	hcaT - R	CAATGCAGAATTTGCACCAC		
copR	*copR* - F	ACCTCGAACTCGATTTGCTG	166	
	copR - R	TCGCTGTCGAAGTTCATGTC		
copS	*copS* - F	GTTCGACCGGTTCTATCGTG	160	
	copS - R	AGCGAAATCGATGACGAAAC		
oprD	*oprD* - F	ATCTACCGCACAAACGATGAAGG	156	
	oprD - R	GCCGAAGCCGATATAATCAAACG		

*F: forward primer, R: reverse primer.

Table 4.11. Reagents and respective concentrations for the amplification of highly conserved *copR*, *copS* and *oprD* sequences (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
5.25	Aqua ultrapure	
12.5	SYBR Premix ExTaq (2×)	1×
1.25	BSA (2 mg ml^{-1})	0.1 mg ml^{-1}
2.5	Forward primer (10 µM)	1 µM
2.5	Reverse primer (10 µM)	1 µM
0.5	ROX (50×)	1×
0.5	DNA template	Approx. 20 ng

Table 4.12. RT-qPCR program for the amplification of *copR*, *copS* and *oprD* sequences.

Cycles	Temperature (°C)	Time (s)	Step
1	95	600	Initial denaturation
40	95	15	Denaturation
	60	60	Annealing
1	95	15	Melting curve
	60	60	
	95	30	
	60	15	

MIC analysis

MIC test of imipenem was also performed under Cu treatment. The MIC value was defined by the plate dilution method for the transconjugant 1PAPER-STR following the guidelines by the NCCLS (2004). The lowest concentration of the antibiotic that inhibited the growth of bacteria was considered as MIC.

Mueller-Hinton agar plates (Sigma Aldrich, St. Louis, USA) were prepared in different antibiotic concentrations ranging from a maximum of 64 µg ml^{-1} to a minimum of 0.0625 µg ml^{-1} of imipenem per plate with the addition of 1 mM of $CuCl_2$. As a control, Mueller-Hinton agar plates without Cu treatment were also prepared.

Transconjugant 1PAPER-STR and *E. coli* 1030 recipient cells were pre-cultured in 5 ml LB agar medium supplemented with the corresponding concentration of antibiotics for strain and MGEs selection listed in Table 4.4 plus 1 mM of $CuCl_2$. After an overnight incubation at 37 °C, 200 μl of the overnight cultures were inoculated into 5 ml of fresh LB medium supplemented with the same concentration of antibiotics and $CuCl_2$. As a control, cultures without the addition of 1 mM of $CuCl_2$ were also prepared.

Fresh cultures were incubated at 37 °C under continuous shaking until they reached an OD_{600} of 0.08 – 0.1. At this point, a dilution of 1/10 was prepared with fresh LB medium (450 μl LB and 50 μl of cell culture) and 1 μl of each sample was spread onto Mueller-Hinton agar plates supplemented with the different concentrations of imipenem. By doing this, all cultures started with the same initial concentration of cells of 1×10^7 CFU ml^{-1}. Finally, plates were grown overnight at 37 °C.

4.2.13. Transconjugants growth pattern

To standardize the growth curves with the same initial concentration of cells, all cultures were adjusted to an initial OD_{600} of 0.10. In this manner, the required volume of overnight culture was diluted into 25 ml of fresh LB medium supplemented with rifampicin (100 μg ml^{-1}). This OD_{600} value corresponded to time 0 of the growth curve. Next, cells were incubated at 37 °C and OD_{600} values were measured every hour and for at least the first 8 h of growth. Then, a final measure was taken after 24 h of incubation.

OD_{600} measurements were done using LB medium supplemented with rifampicin (100 μg ml^{-1}) as blank. For a more reliable measurement, when the OD_{600} values of the cultures exceeded a value of 1, samples were diluted with LB plus rifampicin (100 μg ml^{-1}). Cell concentrations were calculated from the OD_{600} values using the following relation: OD_{600}=1 represents 8×10^8 cells ml^{-1} (Myers *et al*., 2013). Finally, the maximum growth rate (MGR) was estimated from the slope of the inflection point of each growth curve (Perni *et al*., 2005).

4.2.14. Phenotype fingerprint: Gen III MicroPlate™

The GEN III MicroPlate™ (Biolog, Hayward, USA) is a system used to identify a broad range of Gram-negative and Gram-positive bacteria. It studies the phenotype fingerprint of a

bacteria in a standardized micromethod using 94 biochemical tests to profile 71 carbon source utilization assays (Figure 4.5, columns 1-9) and 23 chemical sensitivity assays (Figure 4.5, columns 10-12); such as antibiotic resistance, salinity and pH tolerance assays. All nutrients and biochemical needed to incubate the bacteria came already prefilled and dried into the 96 wells of the GEN III MicroPlate™. The GEN III MicroPlate™ was used to compare the transconjugants and the *E. coli* 1030 recipient at phenotypic level.

GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 4.5. Layout of the MicroPlate assays. Columns 1-9: 71 carbon source utilization assays. Columns 10-12 (grey): 23 chemical sensitivity assays.

Fresh colonies from transconjugants and *E. coli* 1030 recipient strain were suspended in a special "gelling" inoculating fluid following the manufacture's instructions (protocol A) for the GEN III MicroPlate™ (Biolog, Hayward, USA). Then, 100 µl of the cell suspension was inoculated per well into the GEN III MicroPlate™. A single MicroPlate™ was performed for each transconjugant and the *E. coli* 1030 recipient strain. MicroPlates™ were incubated at 33 °C for at least 20 h.

Tetrazolium redox dye was the system employed to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals. All the wells started out colourless when inoculated. During incubation there was an increase in respiration only in the wells where cells were able to use the specific carbon source present in that well or where cells were able to grow despite the presence of antibiotics, acid pH or salinity. This increased

respiration causes reduction of the tetrazolium redox dye, forming a purple colour. Negative wells where cells were not able to use the specific carbon source present in that well or where they were not able to grow in the presence of antibiotics, acid pH or salinity, remained colourless. In the MicroPlate™ there are also a negative control well (A-1) with no carbon source and a positive control well (A-10) used as a reference for the chemical sensitivity assays in columns 10-12 (Figure 4.5).

4.2.15. Ratio RNA: DNA

The RNA: DNA ratio is a physiological index of activity that gives a measure of the synthetic capacity of the bacterial cell and usually correlates with growth and nutritional status. The relative abundance of RNA compared with DNA in the cell has been used to indicate recent growth in a wide range of organisms (Kennell and Magasanik, 1962; Elser *et al*., 2006).

Following the same steps as for the determination of growth rate in section 4.2.12, fresh colonies added to a 25 ml of fresh LB medium supplemented with rifampicin (100 μg ml) were adjusted to an initial OD_{600} nm of 0.10. Cells were incubated at 37 °C until they reached an OD_{600} of 2.0. Then, samples were divided in two parts for RNA and DNA extraction**.**

DNA extraction was performed with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and RNA extraction was carried out as described in section 4.2.11.

4.2.16. Biofilm formation screening

Transconjugants and the *E. coli* 1030 recipient strain were tested for biofilm formation, by an *in vitro* quantitative adherence assay (Christensen *et al*., 1985) with some modifications. Following the same steps as for the determination of growth rate in section 4.2.12, overnight-grown cells were first adjusted to an initial OD_{600} of 0.10. Then, 10 μl of the cultures were inoculated into 200 μl of LB medium in 96-well flat-bottom polystyrene plates and grown without shaking at 37 °C for 24 h.

Planktonic bacteria were removed by washing 3 times per well with sterile distilled water. To stain biofilm-forming cells, 125 μl of crystal violet solution (0.1%, w/v) were added to

each well and incubated 10 min at room temperature. Subsequently, 3 washes with sterile distilled water were done. To solubilize the dye, 200 µl of glacial acetic acid solution (33%, v/v) were added to each stained well and incubated 10 min at room temperature.

As negative control LB medium was used. The optical density of biofilms was measured in a microplate reader at 570 nm in triplicate for each strain. The ability to form a biofilm was scored as follows: OD < 0.120, non-biofilm-forming; 0.120 < OD < 0.240, weak biofilm-forming; OD > 0.240, strong biofilm-forming strains (Christensen *et al*., 1985; Di Rosa *et al*., 2006).

4.2.17. Quantification of *gyrA* gene

Bacterial gyrase (*gyrA* gene) copy numbers were determined by qPCR for all transconjugants as well as for the recipient strain *E. coli* 1030. Quantification of *gyrA* gene was carried out under two different conditions: transconjugants exposed to 0.2 mM of Cd and control conditions (without Cd exposure) as described previously in section 4.2.11.

Quantification of *gyrA* was performed in a CFX96 Real-Time System with the 7500 Software V2.0.6 (BioRad Life Technologies, Hercules, USA). The primers are listed in Table 4.13 (Fajardo *et al*., 2012). Concentrations of reagents and the cycle program of the qPCR assay are listed in Table 4.14 and Table 4.15, respectively. Taq polymerase and buffer used were the SYBR Premix ExTaq (Takara, Japan), which also comes with ROX Reference Dye designed to normalize the fluorescent reporter signal. BSA was used to increase the efficiency of the reaction. To determine the copy number of the *gyrA* gene, the serially diluted gene from *Bacillus subtilis* served as template for the generation of standard curves.

Table 4.13. Primers used for determination of *gyrA* gene copy numbers.

Gene	Primer/Probe*	Primer Sequence (5'-3')**	Product Size (bp)	Reference
gyrA	gyrA - *F*	TTTTATATGCGATGAATGATTTAGG	291	Fajardo *et al*., 2012
	gyrA - *R*	GACATTCTTGCTTCTGTATAACGCAT		

*F: forward primer, R: reverse primer.

Table 4.14. Reagents and respective concentrations for the determination of *gyrA* gene copy numbers (25 µl PCR reaction each)**.**

Volume (µl)	Reagent	Final Concentration
5.25	Aqua ultrapure	
12.5	SYBR Premix ExTaq (2×)	1×
1.25	BSA (2 mg ml^{-1})	0.1 mg ml^{-1}
2.5	Forward primer (10 µM)	1 µM
2.5	Reverse primer (10 µM)	1 µM
0.5	ROX (50×)	1×
1	DNA template	

Table 4.15. qPCR program for the determination of *gyrA* gene copy number.

Cycles	Temperature (°C)	Time (s)	Step
1	95	600	Denaturation
40	95	15	Annealing
	55	60	Elongation
1	60-95	0.2 °C sec^{-1}	Denaturation curve

4.2.18. Exogenous plasmid isolation from transconjugants using *E. coli* UB 1637 as recipient strain.

Most likely transconjugants obtained by exogenous plasmid isolation from the bacterial community in soils (section 4.2.3) adquired conjugative and/or mobilizable plasmids that encoded the phenotypically shown antibiotic resistance. To test this, transconjugants 1PAPER-AMP, 1SHEEP-CHL, 1PAPER-CHL and 2CONTROL-CHL were used as donnors in new exogenous plasmid isolation experiments using a new *E. coli* recipient strain: *E. coli* UB 1637 (STR^R) that was resistant to streptomycin.

Conjugation assays were performed as described in section 4.2.3 with a few modifications. Recipient cells (*E. coli* UB 1637, STR^R) were grown overnight at 37 °C on LB plates supplemented with streptomycin (60 µg ml^{-1}). Simultaneously, donor cells (transconjugants 1PAPER-AMP, 1SHEEP-CHL, 1PAPER-CHL and 2CONTROL-CHL) were grown

overnight at 37 °C on LB plates supplemented with the antibiotics that were used for their selection (Table 4.4). Next, single colonies from either recipient or donor cells were innoculated into 2 ml LB medium supplemented with the corresponding antibiotic and incubated at 37 °C for 24h.

4.2.19. Statistical analysis

ANOVA was applied to the following tests: MIC of heavy metals, effect of Cd and Cu on the expression of genes related to oxidative stress in *E. coli* transconjugants, growth pattern of transconjugants, RNA: DNA ratio, biofilm formation and *gyrA* gene copy number quantification. Where more than two groups were compared, post-hoc pairwise comparisons were performed with Tukey-Kramer adjustment ($p < 0.05$) to refine *p*-values. All statistical analyses were carried out with the Statview software (SAS Institute Inc. Software Informer).

4.3. Results

4.3.1. Exogenous plasmid isolation from soils

Antibiotic sensitivity test

To design the conjugation experiments for the exogenous plasmid isolation an antibiotic sensitivity test in the soil community was performed. It was observed that soil bacteria from the different soils studied were sensitive to rifampicin (100 μg ml^{-1}) and they were resistant to ampicillin (25 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), erythromycin (45 μg ml^{-1}), gentamicin (25 μg ml^{-1}), kanamycin (30 μg ml^{-1}), streptomycin (10 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). The Gram-negative strain *E. coli* 1030 (RIF^R) was selected as recipient as it proved to be sensitive to the abovementioned antibiotics at the indicated concentrations. As Gram-positive recipient, *E. faecalis* OG1RF (RIF^R, fusidic $acid^R$) strain was used. Since this strain was resistant to kanamycin (30 μg ml^{-1}), streptomycin (10 μg ml^{-1}) and gentamicin (25 μg ml^{-1}), only ampicillin (25 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}) and erythromycin (45 μg ml^{-1}) were used

as selection markers in the exogenous plasmid isolation assays when *E. faecalis* OG1RF was applied as recipient.

Exogenous plasmid isolation using E. coli 1030 as recipient

E. coli 1030 transconjugants were obtained from every soil under the selection of at least three different antibiotics, except for the POULTRY treated soil in site 1 that rendered only one transconjugant under kanamycin selection (Table 4.16). Conversely, the PAPER treated soil in site 1 showed the broadest variety of transconjugants as colonies were obtained for six of the seven antibiotics used for selection: ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin and streptomycin. However, no transconjugant was obtained under tetracycline selection from any of the soils studied.

Altogether in this study, a total of 36 transconjugants were obtained from the different soils and under the selection of six different antibiotics. From this step on, a code consisting of numbers and letters will be used to denominate each transconjugant. The first number refers to the site (site 1 or site 2) and following is stated the treatment that received the soil from where the transconjugants was obtained (COW: Cow manure; SHEEP: Sheep manure; PAPER: Paper mill sludge and poultry manure (2:1, v/v); POULTRY: Poultry manure and CONTROL: No amendment). The last letters refer to the antibiotic used to select for transconjugants: ampicillin (AMP), chloramphenicol (CHL), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN) and streptomycin (STR).

Table 4.16. Resistance of *E. coli* 1030 transconjugants obtained by exogenous plasmid isolation. The following concentrations (μg ml^{-1}) of antibiotics were used: rifampicin (RIF 100); ampicillin (AMP 25); kanamycin (KAN 30); tetracycline (TET 10); chloramphenicol (CHL 10); erythromycin (ERY 45); gentamicin (GEN 25) and streptomycin (STR 10).

		Antibiotics used for selection						
Site	**Soil**	**AMP + RIF**	**CHL + RIF**	**ERY + RIF**	**GEN + RIF**	**KAN + RIF**	**STR + RIF**	**TET + RIF**
1	COW	-	**+**	-	**+**	-	**+**	-
	SHEEP	-	**+**	**+**	**+**	**+**	**+**	-
	PAPER	**+**	**+**	**+**	**+**	**+**	**+**	-
	POULTRY	-	-	-	-	**+**	-	-
	CONTROL	-	-	**+**	**+**	**+**	**+**	-
2	COW	-	**+**	**+**	**+**	**+**	**+**	-
	SHEEP	-	**+**	**+**	-	**+**	-	-
	PAPER	-	**+**	**+**	-	**+**	-	-
	POULTRY	-	-	**+**	-	**+**	**+**	-
	CONTROL	-	**+**	-	**+**	**+**	-	-

+: transconjugants were obtained; -: no transconjugants were obtained.

At this point it must be indicated that transconjugants 1COW-GEN and 1SHEEP-GEN did not grow after restreaking on LB medium supplemented with rifampicin (100 μg ml^{-1}) and gentamicin (10 μg ml^{-1}), so they were not taken into consideration for further studies.

Exogenous plasmid isolation using E. faecalis OG1RF as recipient

When *E. faecalis OG1RF* was used as recipient no transconjugants were obtained from any of the soils studied and for any of the antibiotics used for selection.

4.3.2. *E. coli*-specific genomic sequences are present in most of the transconjugants

The band pattern of each transconjugant was compared to the original *E. coli* 1030 recipient strain (Figure 4.6).

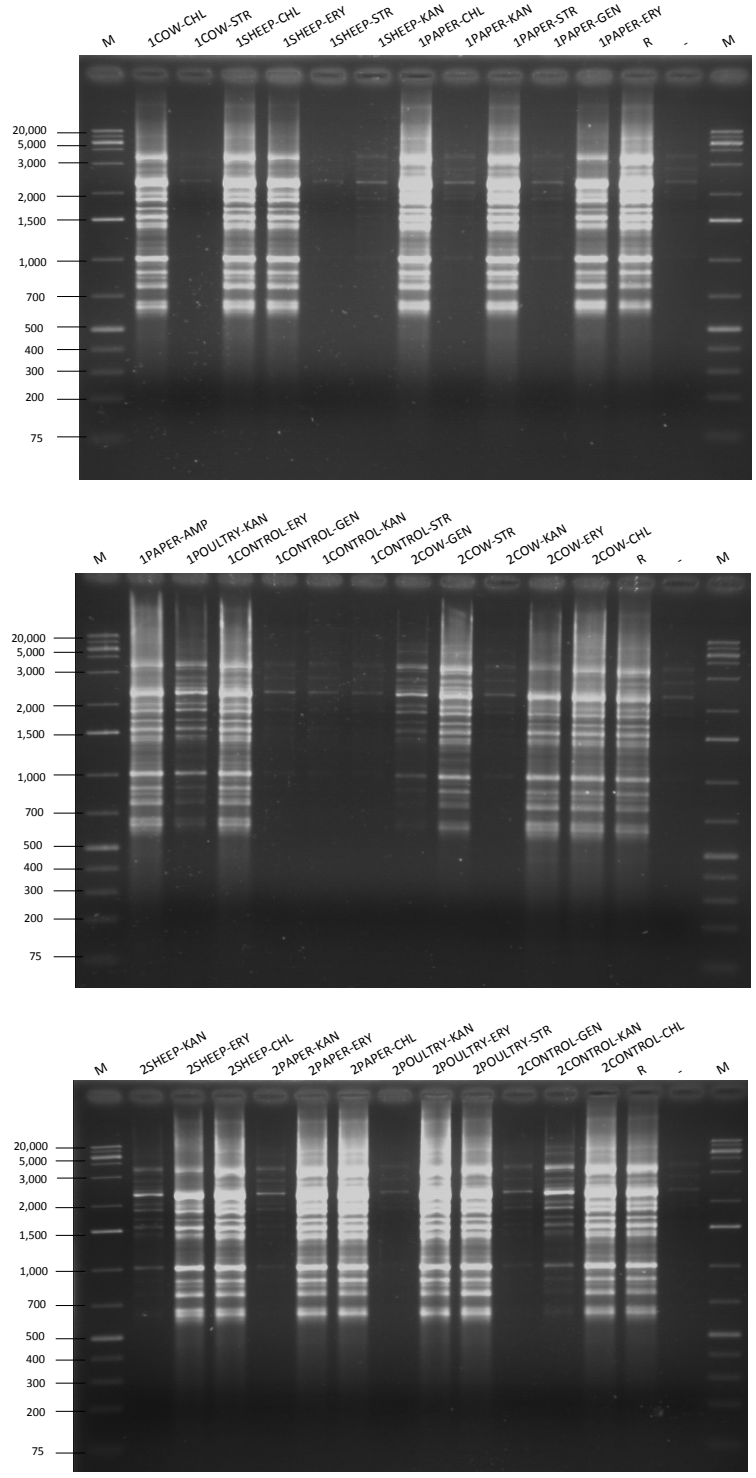


Figure 4.6. Band patterns of the transconjugants analysed by BOX-PCR in comparison with *E. coli* 1030 recipient strain. Positive control (R): *E. coli* 1030; negative control (-): no DNA; M: 1 kb Plus DNA Ladder DNA molecular weight marker (Thermo Scientific).

It was observed that transconjugants 1COW-STR, 1SHEEP-STR, 1SHEEP-KAN, 1PAPER-KAN, 1PAPER-GEN, 1POULTRY-KAN, 1CONTROL-GEN, 1CONTROL-KAN, 1CONTROL-STR, 2COW-GEN, 2COW-KAN, 2SHEEP-KAN, 2PAPER-KAN, 2POULTRY-KAN, 2CONTROL-GEN and 2CONTROL-KAN did not show the same profile as the recipient strain. Thus, they were not considered as true transconjugants and not taken into consideration for further studies.

4.3.3. Detection of multiple antibiotic resistant transconjugants

Determination of minimum inhibitory concentrations (MIC) of antibiotics for the transconjugants

The MIC of ampicillin, chloramphenicol, erythromycin, gentamicin, imipenem, kanamycin, streptomycin, sulfadiazine and tetracycline were determined by the plate dilution method for each transconjugant and the recipient strain (section 4.2.8). As shown in Table 4.17, all transconjugants presented a new resistance phenotype to at least one additional antibiotic in comparison with the recipient strain.

In terms of antibiotic resistance, transconjugants 1COW-CHL, 1SHEEP-CHL, 2COW-CHL, 2SHEEP-CHL, 2PAPER-CHL and 2CONTROL-CHL shared the same resistance phenotype. They showed a two-fold higher resistance to ampicillin, gentamicin and streptomycin, a four-fold higher resistance to erythromycin, kanamycin and tetracycline and an eight-fold higher resistance to chloramphenicol. In addition to all these resistances, the transconjugant 1PAPER-CHL also had a 2-fold higher resistance to sulfadiazine, thus being the transconjugant that presented adquired resistance against the highest number of antibiotics tested in this study.

The transconjugant 1PAPER-STR presented a two-fold higher resistance to sulfadiazine, an eight-fold resistance to gentamicin and streptomycin, a 32-fold higher resistance to kanamycin, and a four-fold higher resistance to imipenem. This is the only transconjugant that acquired a resistance to imipenem. The transconjugant 2POULTRY-STR exhibited a 16-fold higher resistance to kanamycin and a 512- fold higher resistance to streptomycin. This is the transconjugant that presented the highest increase in resistance against any of the antibiotics tested in this study.

Transconjugants 1SHEEP-ERY, 1PAPER-ERY, 1CONTROL-ERY, 2COW-ERY, 2SHEEP-ERY and 2PAPER-ERY presented a four-fold higher resistance to erythromycin. In addition to this

resistance, the 1PAPER-ERY and 2COW-ERY transconjugants showed a two-fold higher resistance to chloramphenicol while transconjugant 1SHEEP-ERY had an additional four-fold resistance to kanamycin. The transconjugant 2PAPER-ERY showed also an additional two-fold higher resistance to chloramphenicol and gentamicin. The transconjugant 1CONTROL-ERY presented a two-fold higher resistance to chloramphenicol, a four-fold higher resistance to streptomycin, an eight-fold higher resistance to gentamicin and a 16-fold higher resistance to kanamycin.

In contrast, transconjugant 2POULTRY-ERY had acquired a higher resistance to erythromycin (16-fold more) but did not show any additional resistance to any other antibiotic. This was also observed for the transconjugant 1PAPER-AMP, which only exhibited an eight-fold higher resistance to ampicillin. Additionally, none of the transconjugants showed an increased or acquired resistance to vancomycin.

Table 4.17. Minimum inhibitory concentrations (MIC) of selected antibiotics for the transconjugants and the *E. coli* 1030 recipient strain. The concentrations listed are given in μg ml^{-1}. All the transconjugants with antibiotic resistance levels above those of the recipient strain are shown in bold.

Antibiotic/ Strain	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Imipenem	Kanamycin	Streptomycin	Sulfadiazine	Tetracycline	Vancomycin
E. coli 1030 (R)	4-8	2-4	32-64	0.125-0.25	0.25-0.5	0.125-0.25	1-2	4-8	1-2	64-128
1PAPER-AMP	**32-64**$^{++}$	2-4	32-64	0.125-0.25	0.25-0.5	0.125-0.25	1-2	4-8	1-2	32-64
1COW-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.25-0.5	**0.5-1**$^{++}$	**2-4**$^{+}$	2-4	**4-8**$^{++}$	64-128
1SHEEP-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.125-0.25	**0.5-1**$^{++}$	**2-4**$^{+}$	1-2	**4-8**$^{++}$	32-64
1PAPER-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.125-0.25	**0.5-1**$^{++}$	**2-4**$^{+}$	**8-16**$^{+}$	**4-8**$^{++}$	16-32
2COW-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.125-0.25	**0.5-1**$^{++}$	**2-4**$^{+}$	2-4	**4-8**$^{++}$	64-128
2SHEEP-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.125-0.25	**0.5-1**$^{++}$	**2-4**$^{+}$	2-4	**4-8**$^{++}$	32-64
2PAPER-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.125-0.25	**0.5-1**$^{++}$	**2-4**$^{+}$	1-2	**4-8**$^{++}$	64-128
2CONTROL-CHL	**8-16**$^{+}$	**16-32**$^{+++}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.062-0.125	**0.5-1**$^{++}$	**2-4**$^{+}$	1-2	**4-8**$^{++}$	64-128
1SHEEP-ERY	4-8	**4-8**$^{+}$	**128-256**$^{++}$	0.125-0.25	0.25-0.5	**0.5-1**$^{++}$	1-2	4-8	1-2	32-64
1PAPER-ERY	4-8	**4-8**$^{+}$	**128-256**$^{++}$	0.125-0.25	0.25-0.5	0.25-0.5	1-2	2-4	1-2	32-64
1CONTROL-ERY	4-8	**4-8**$^{+}$	**128-256**$^{++}$	**1-2**$^{+++}$	0.125-0.25	**2-4**$^{++++}$	**4-8**$^{++}$	2-4	0.5-1	32-64
2COW-ERY	2-4	**4-8**$^{+}$	**128-256**$^{++}$	0.125-0.25	0.25-0.5	0.25-0.5	1-2	2-4	1-2	16-32
2SHEEP-ERY	2-4	2-4	**128-256**$^{++}$	0.125-0.25	0.25-0.5	0.25-0.5	1-2	4-8	1-2	16-32
2PAPER-ERY	4-8	**4-8**$^{+}$	**128-256**$^{++}$	**0.25-0.5**$^{+}$	0.25-0.5	**0.5-1**$^{++}$	0.5-1	1-2	1-2	16-32
2POULTRY-ERY	2-4	1-2	**512-1024**$^{++++}$	0.0625-0.125	0.125-0.25	0.25-0.5-	0.5-1	1-2	0.5-1	16-32
1PAPER-STR	4-8	1-2	32-64	**1-2**$^{+++}$	**1-2**$^{++}$	**4-8**$^{+++++}$	**8-16**$^{+++}$	**8-16**$^{+}$	1-2	16-32
2POULTRY-STR	4-8	1-2	32-64	**0.5-1**$^{++}$	0.25-0.5	**2-4**$^{++++}$	**>1024**$^{+++++++++}$	4-8	0.5-1	16-32

+, ++, +++, ++++, +++++, +++++++++: MIC-transconjugant 2-, 4-, 8-, 16-, 32- and 512-fold higher than MIC-recipient, respectively.

Additionally to this, the study of resistance against aztreonam, fusidic acid, lincomycin, minocycline, nalidix acid, rifampicin, troleandomycin and vancomycin was done, as part of phenotype fingerprint test with the GEN III MicroPlate™.

All transconjugants as well as the *E. coli* 1030 recipient strain showed resistance to six of the eight antibiotics tested: aztreonam, fusidic acid, lincomycin, rifampicin, troleandomycin and vancomycin. Some of the transconjugants showed also an increased resistance to either minocycline or nalidix acid or both of them. Transconjugant 2CONTROL-CHL was the only one that showed a higher resistance to minocycline. On the contrary, transconjugants 2COW-ERY and 2PAPER-ERY were more resistant to nalidix acid. Finally, transconjugants 1COW-CHL, 1SHEEP-CHL, 2COW-CHL, 2SHEEP-CHL, 2PAPER-CHL, 1SHEEP-ERY and 1PAPER-ERY exhibited a higher resistance to both of them while transconjugants 1PAPER-AMP, 1PAPER-CHL, 1CONTROL-ERY, 2SHEEP-ERY, 2POULTRY-ERY, 1PAPER-STR and 2POULTRY-STR showed no additional resistance to minocycline or nalidix acid.

Taking all this into account transconjugants were classified as resistant when they showed a MIC value higher than the recipient for at least one of the antibiotics tested. In Figure 4.7 the number of transconjugants resistant to each antibiotic is shown. More than half of the transconjugants were resistant to nalidix acid, streptomycin, gentamicin, chloramphenicol, kanamycin and erythromycin with 9, 10, 11, 12, 12 and 14 transconjugants, respectively. On the contrary, few transconjugants showed acquired resistance to sulfadiazine, imipenem and vancomycin (2, 1 and 0, respectively).

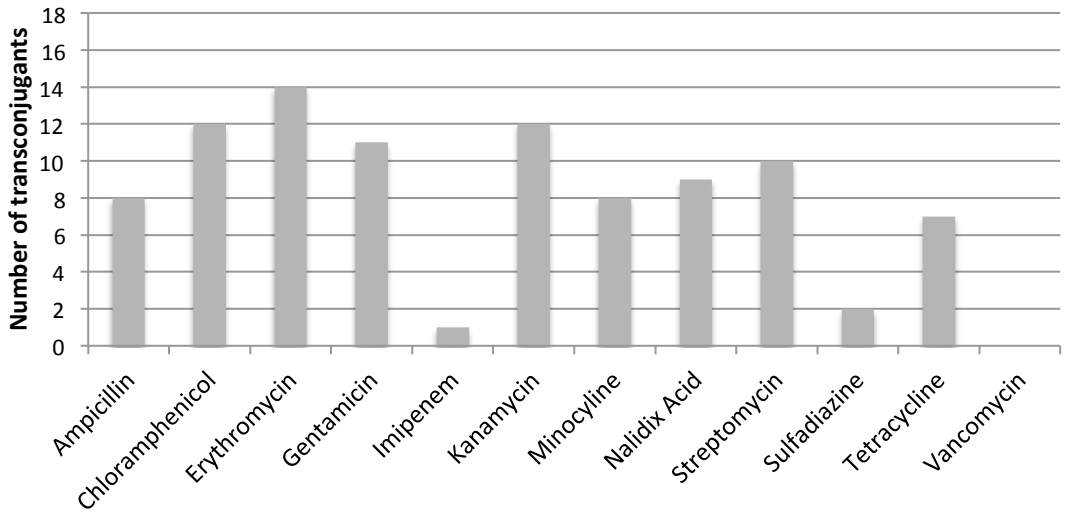


Figure 4.7. Number of transconjugants that presented acquired resistance to the selected antibiotics. All transconjugants with MIC values above those of the recipient strain were considered resistant.

Modified Hodge Test

Transconjugant 1PAPER-STR was tested for carbapenemase production by the modified Hodge test (MHT) to see if the resistance encountered for imipenem was due to a carbapenemase production mechanism. After 24 h of incubation at 37 °C, the indicator strain (*E. coli* ATCC 25922) showed no growth within the meropenem disc inhibition zone on the surroundings of the transconjugant 1PAPER-STR, as shown in Figure 4.8. This result correlated with the one observed for the negative control. The positive control (*Acinetobacter baumannii*) showed a small clover leaf-like indentation of *E. coli* ATCC 25922 growing within the meropenem disc inhibition zone instead. This implies that transconjugant 1PAPER-STR does not produce carbapenemases as a resistance mechanism for imipenem.

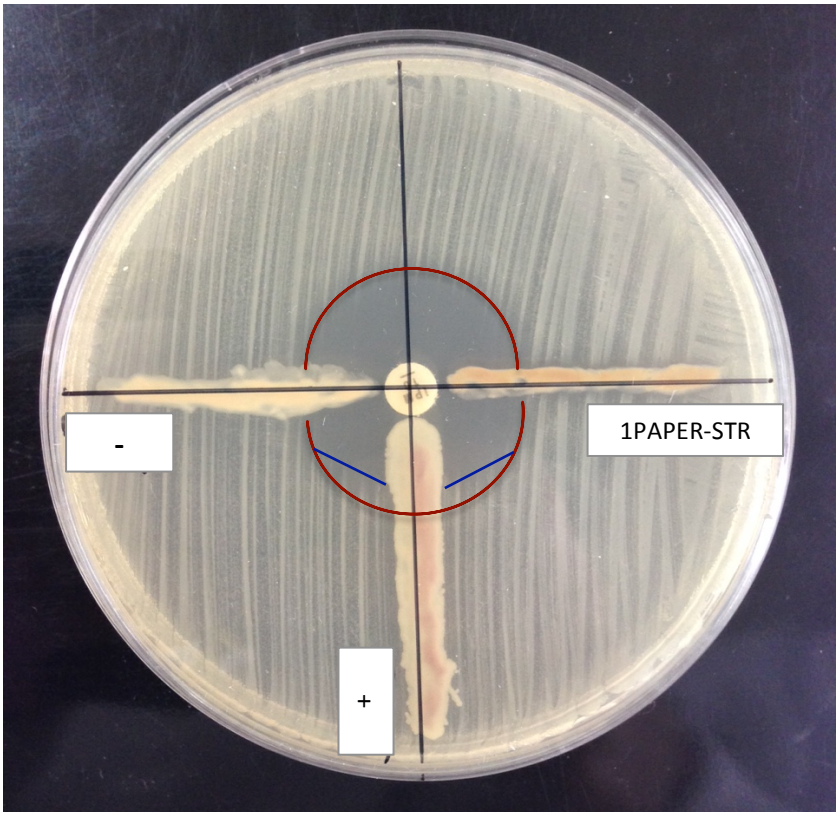


Figure 4.8. The MHT performed for detection of carbapenemase production. The plate was incubated during 24 h at 37 °C. The meropenem disc inhibition zone is marked in red while the cloverleaf like indentation due to the enhanced growth of the indicator strain (*E. coli* ATCC 25922) is marked in blue. Negative control (-): susceptible strain. Positive control (+): *Acinetobacter baumannii*.

4.3.4. Detection of heavy metal resistance and tolerance in transconjugants

Heavy metal minimal inhibitory concentration

Transconjugants along with the recipient strain showed a MIC of 1-2, 2.5-5, 1-2.5 and 5-7.5 mM for Cd, Cu, Zn and Pb, respectively. Nevertheless, significantly lower tolerances to heavy metals ($p < 0.05$) were represented as * in Table A4.1-4.4 in Appendix, in comparison to the recipient strain. At concentrations below the MIC, 41%, 47%, 88% and 59% of the 17 transconjugants were less tolerant to Cd, Cu, Zn and Pb, respectively, when compared to the recipent. Transconjugants 1PAPER-AMP and 2POULTRY-STR were the only two transconjugants that did not decrease their tolerance against any of the four heavy metals tested at any concentration.

On the contrary, transconjugant 1PAPER-STR presented a higher tolerance to Cu at 5 mM (MIC). It was the only one that exhibited a higher tolerance to at least one of the four heavy metals tested than the *E. coli* 1030 recipient strain. Statistically significant higher tolerance ($p < 0.05$) in comparison to the recipient strain were represented as ** in Table A4.1-4.4 in the Appendix.

4.3.5. Effect of Cd and Cu on the expression of genes related to oxidative stress in *E. coli* transconjugants.

Toxic metals (such as Cd) or toxic concentrations of redox-active metals (such as Cu) can produce ROS that harm the cell. *AhpF* and *katG* genes encoding alkyl hydroperoxide reductase and hydroperoxidase I, respectively, are related to oxidative stress: peroxide metabolism and protection. AhpF and katG are found frequently overexpressed as part of a transcriptional strategy against the deleterious effects of H_2O_2. Similarly, *sodA* gene encoding superoxide dismutase is involved in the protection of the cell against the superoxide free radicals.

According to the RT-qPCR analysis, the *ahpF*, *katG* and *sodA* genes were differentially expressed among the different transconjugants ($p < 0.05$) (Figure 4.9-4.11). In general, the expression of the *ahpF* gene showed the highest fold-change value under both Cd and Cu treatment while *katG* showed the lowest fold-change value and *sodA* exhibited a moderate expression.

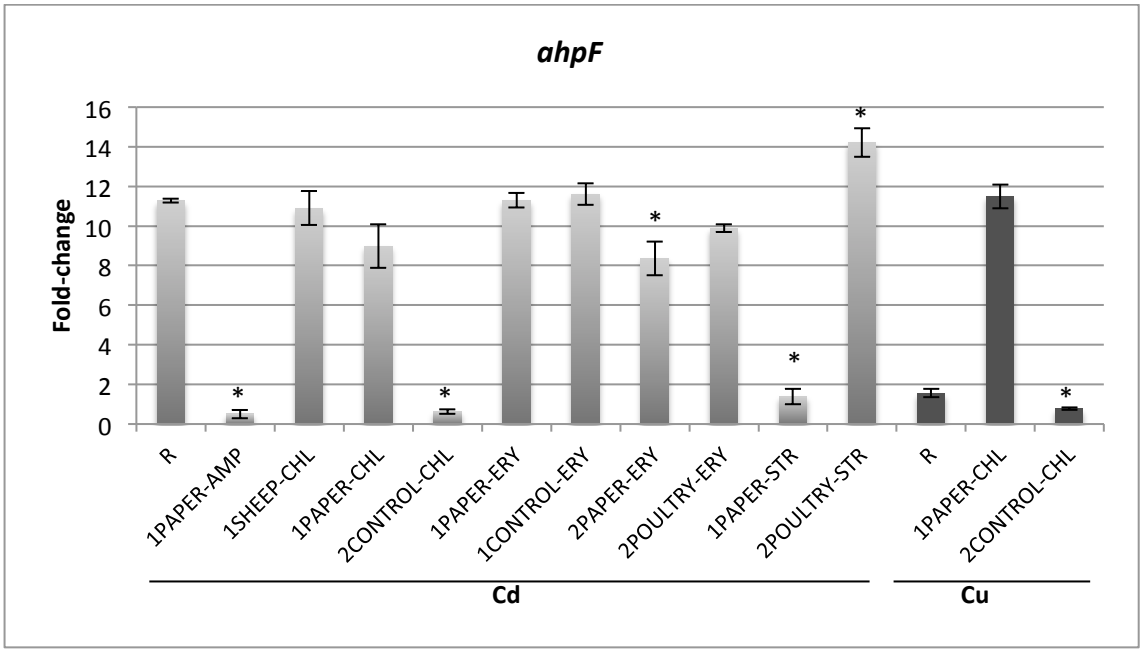


Figure 4.9. Fold-change value of the *ahpF* gene expression for the different transconjugants and the *E. coli* 1030 recipient strain. Results are the mean ± SEM of at least three independent biological experiments. Cd and Cu indicate a cadmium or copper exposure. R: *E. coli* 1030 recipient strain. *Asterisks indicate significance ($p \leq 0.05$).

There was clearly a group of transconjugants formed by 1PAPER-AMP, 2CONTROL-CHL and 1PAPER-STR that seemed to underexpress the *ahpF* gene under Cd exposure. A similar result was obtained for the transconjugant 2CONTROL-CHL under Cu exposure. Less obvious but still statistically significant ($p \leq 0.05$) was the lower expression of the *ahpF* gene by the transconjugant 2PAPER-ERY under Cd exposure compared to the *E. coli* 1030 recipient strain (R). On the contrary, transconjugants 2POULTRY- STR and 1PAPER-CHL overexpressed the *ahpF* gene when exposed to Cd or Cu, respectively.

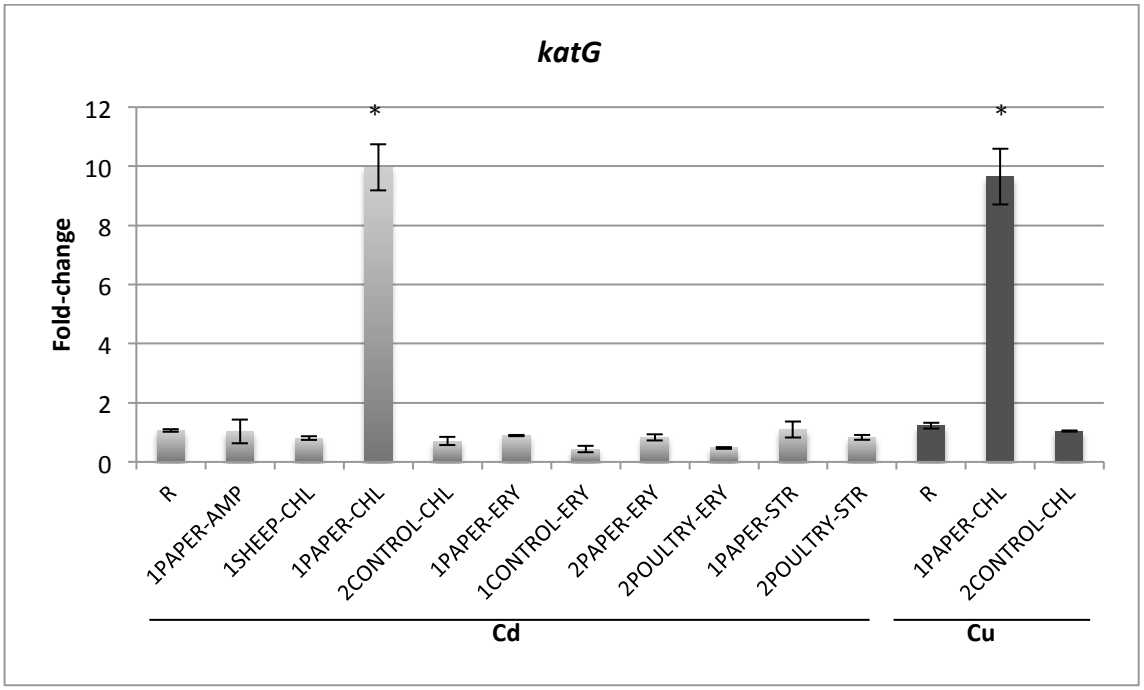


Figure 4.10. Fold-change value of the *katG* gene expression for the different transconjugants and the *E. coli* 1030 recipient strain. Results are the mean ± SEM of at least three independent biological experiments. Cd and Cu indicate a cadmium or copper exposure. R: *E. coli* 1030 recipient strain. *Asterisks indicate significance ($p \leq 0.05$).

Transconjugant 1PAPER-CHL was the only transconjugant that showed a higher expression of the *katG* gene under both Cd and Cu exposure.

There were two transconjugants that displayed an overexpression of the *sodA* gene. Transconjugant 2POULTRY-ERY had the highest expression when exposed to Cd while transconjugant 1PAPER-CHL presented a significantly higher expression under Cu exposure but not under Cd exposure. Once again, as seen for the *ahpF* gene, the group of transconjugants formed by 1PAPER-AMP, 2CONTROL-CHL (Cd or Cu) and 1PAPER-STR underexpressed the *sodA* gene in comparison to the expression of the *E. coli* 1030 recipient strain.

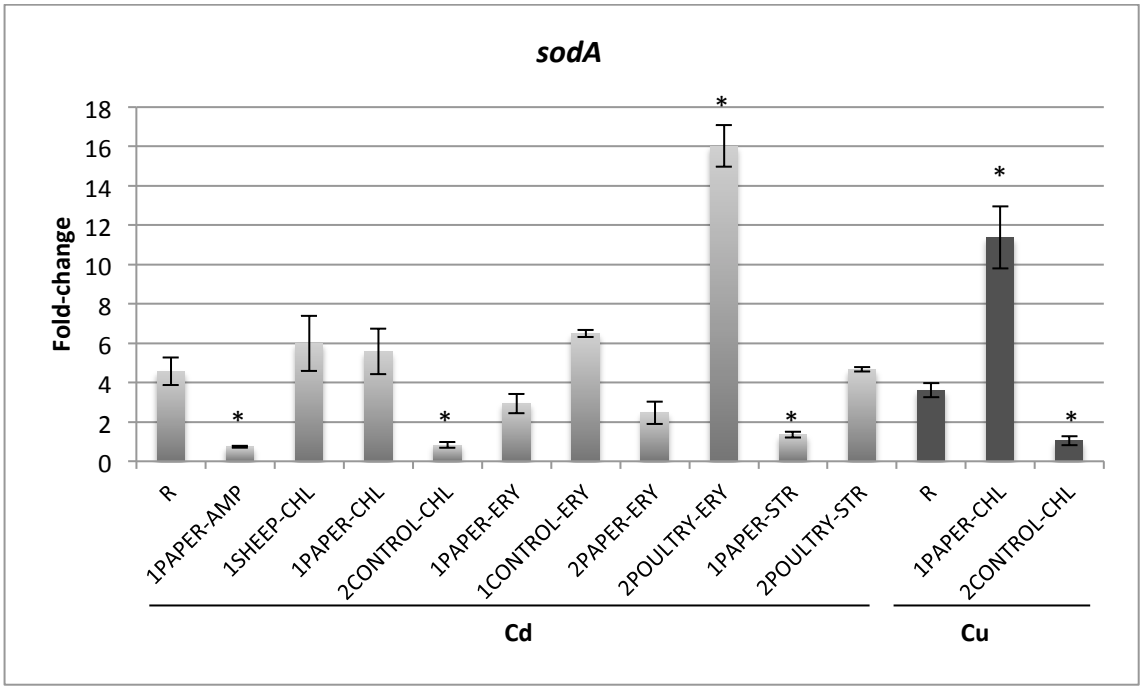


Figure 4.11. Fold-change value of the *sodA* gene expression for the different transconjugants and the *E. coli* 1030 recipient strain. Results are the mean ± SEM of at least three independent biological experiments. Cd and Cu indicate a cadmium or copper exposure. R: *E. coli* 1030 recipient strain. *Asterisks indicate significance ($p \leq 0.05$).

4.3.6. Cu does not induce resistance to the carbapenem antibiotic imipenem

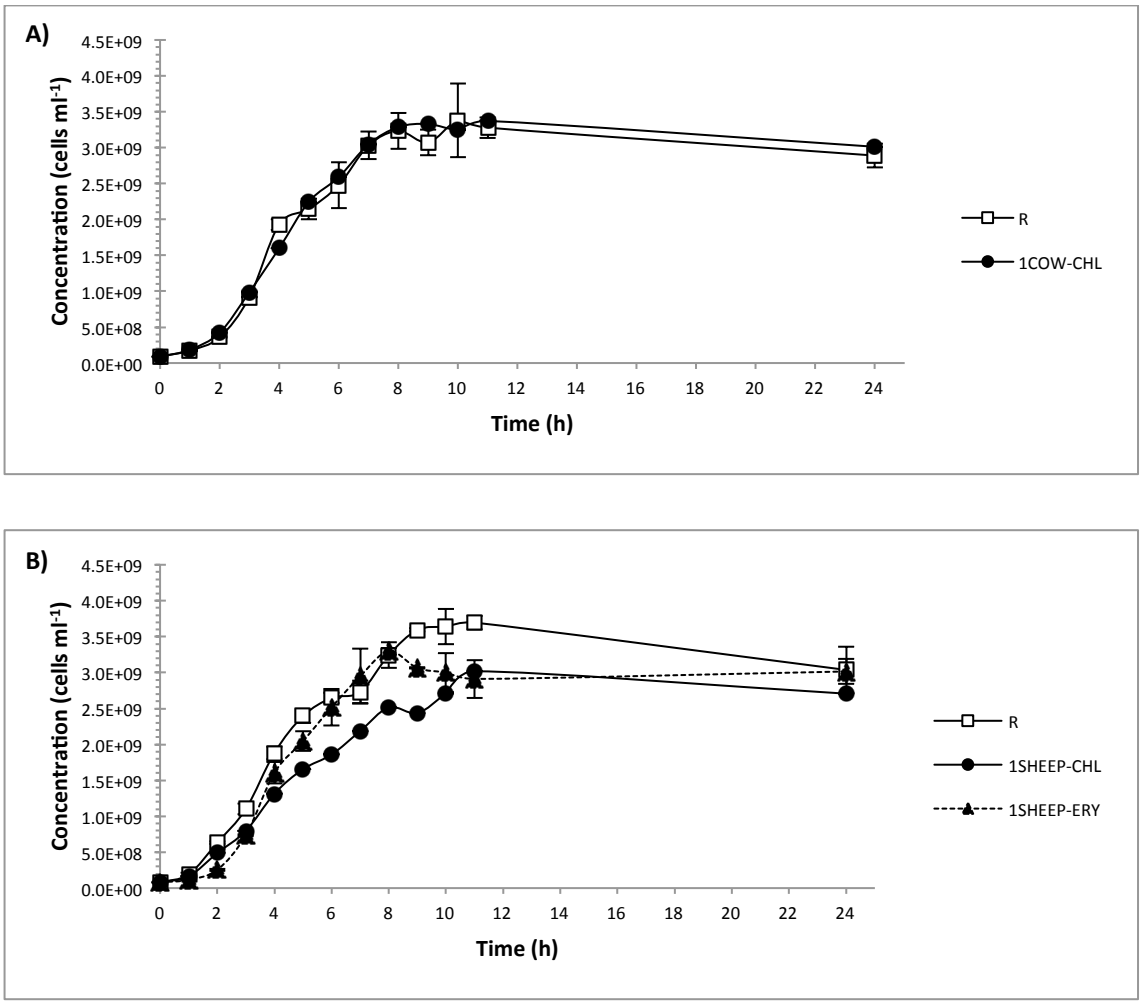
Quantitative reverse transcription-PCR (qRT-PCR) revealed that after Cu treatment (0, 1 and 5 mM of $CuCl_2$) neither the recipient strain nor the 1PAPER-STR transconjugant showed expression of the *copR*/*copS* or *oprD* system.

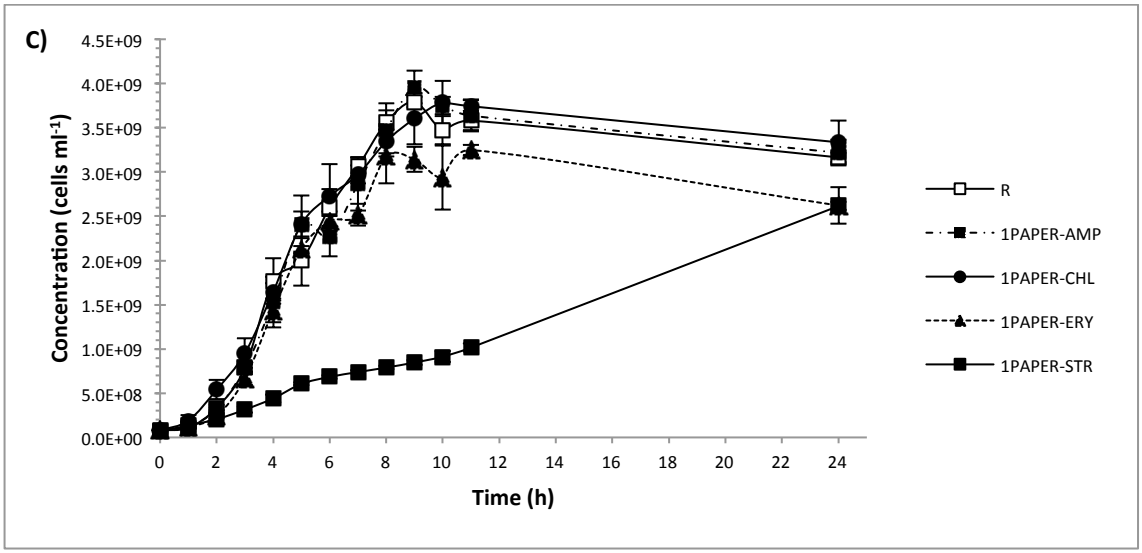
Similarly, the MIC of imipenem was studied under 1 mM Cu exposure by the plate dilution method in Mueller-Hinton agar plates at 37 °C. Once again, imipenem resistance was not enhanced for the transconjugant 1PAPER-STR under Cu treatment.

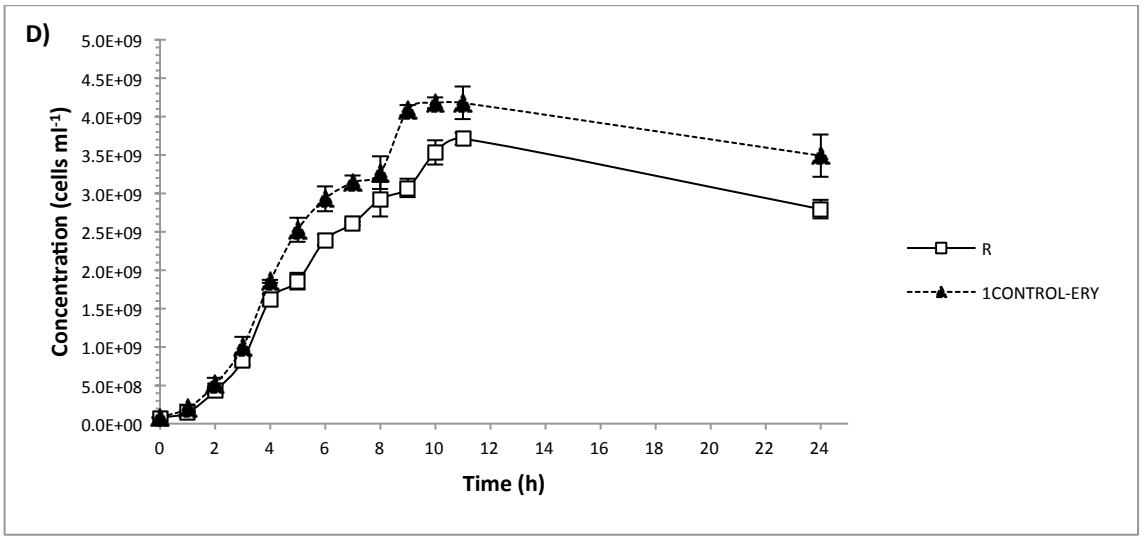
4.3.7. Fitness affected by plasmid uptake in many transconjugants

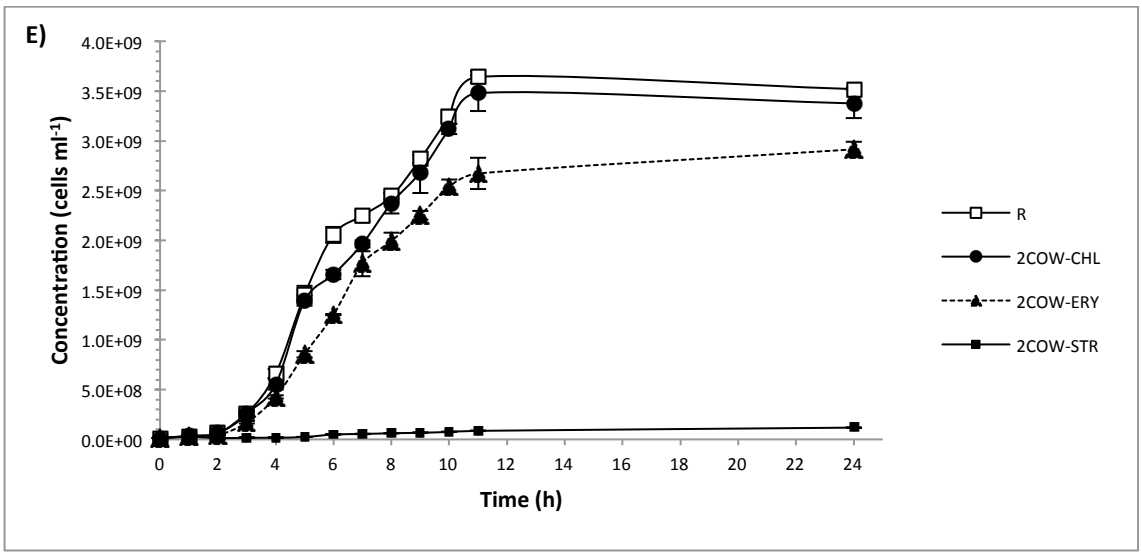
Growth pattern of transconjugants

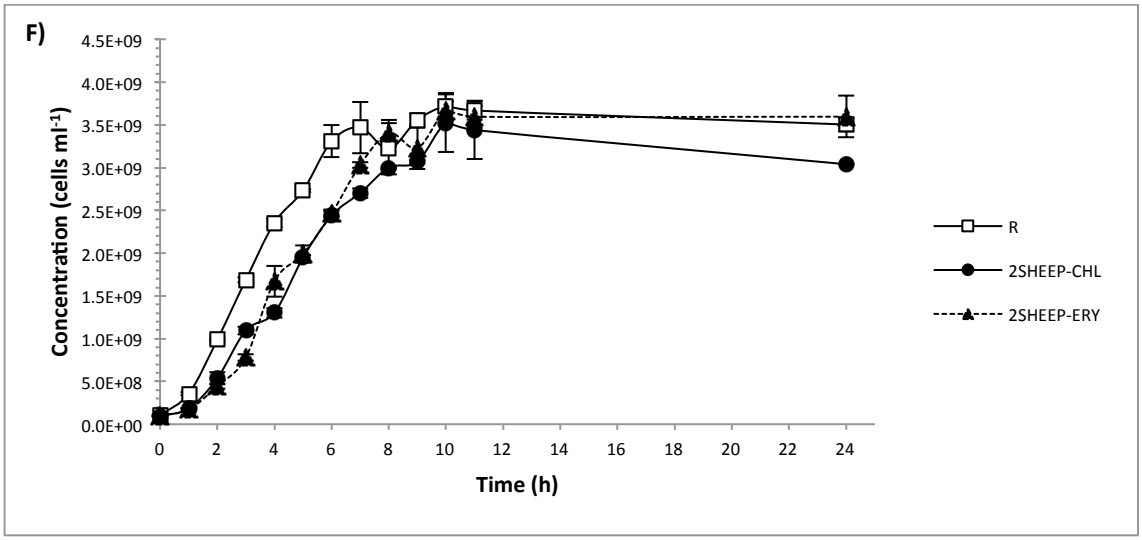
The adquisition of MGEs normally entails a fitnest cost. To study this effect, differences in the growth pattern of the transconjugants were analyzed by comparing their growth curves to the growth curve of the *E. coli* 1030 recipient strain (Figure 4.12).

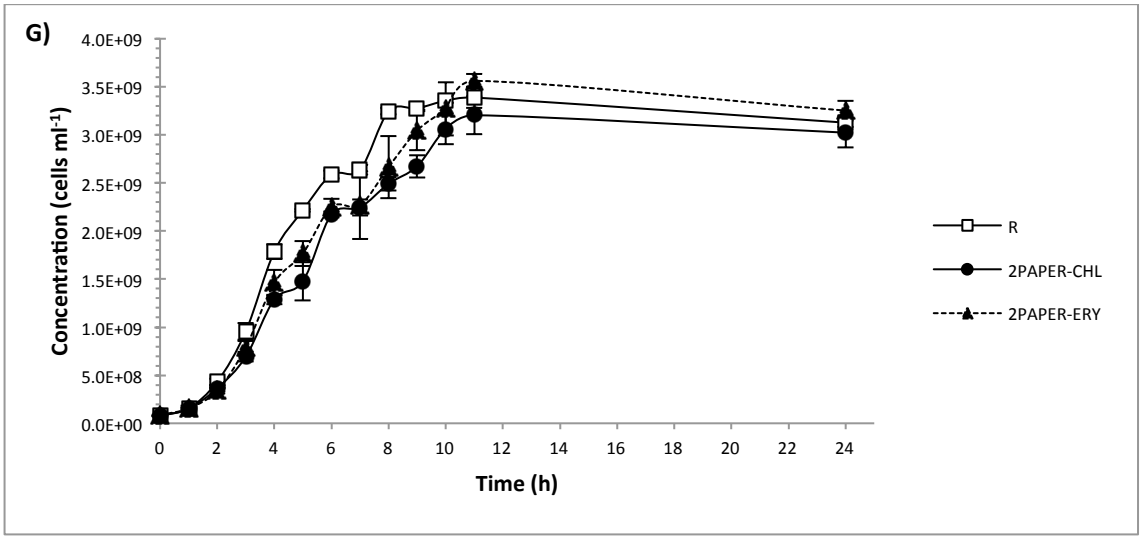


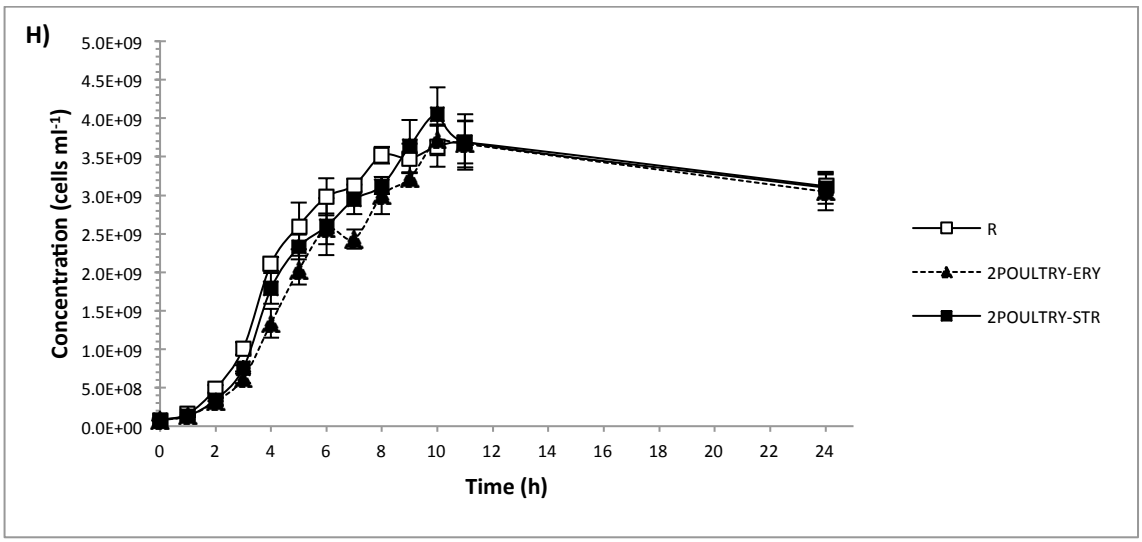

C)
Concentration (cells ml-1)
4.5E+09
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
R
1PAPER-AMP
1PAPER-CHL
1PAPER-ERY
1PAPER-STR


D)
Concentration (cells ml-1)
5.0E+09
4.5E+09
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
R
1CONTROL-ERY


E)
Concentration (cells ml-1)
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
R
2COW-CHL
2COW-ERY
2COW-STR


F)
Concentration (cells ml-1)
4.5E+09
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
R
2SHEEP-CHL
2SHEEP-ERY


G)
Concentration (cells ml-1)
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
R
2PAPER-CHL
2PAPER-ERY


H)
Concentration (cells ml-1)
5.0E+09
4.5E+09
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
R
2POULTRY-ERY
2POULTRY-STR

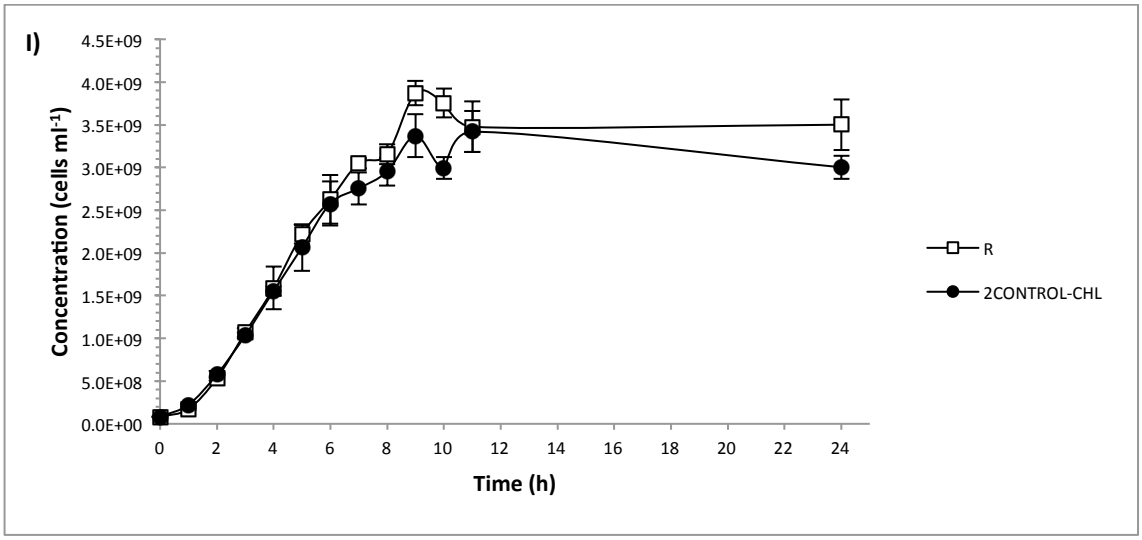


Figure 4.12. Growth curves of transconjugants from (A) COW treated soil in site 1, (B) SHEEP treated soil in site 1, (C) PAPER treated soil in site 1, (D) CONTROL soils in site 1, (E) COW treated soil in site 2, (F) SHEEP treated soil in site 2, (G) PAPER treated soil in site 2, (H) POULTRY treated soil in site 2 and (I) CONTROL soil in site 2. Each diagram shows the growth curves of the transconjugants obtained from each soil along with the growth curve of the *E. coli* 1030 recipient strain (R).

Transconjugants obtained by exogenous plasmid isolation were divided into three groups depending on whether they showed a statistically significant ($p < 0.05$) higher, similar or lower MGR in comparison to the *E. coli* 1030 recipient strain (Table 4.18). Transconjugants isolated using chloramphenicol and erythromycin as selective marker presented a wide spectrum of MGR, reaching all of them the stationary phase after 24 h. Transconjugants 2COW-CHL and 1SHEEP-ERY showed a higher MGR, transconjugants 1COW-CHL, 1PAPER-CHL, 2SHEEP-CHL, 2CONTROL-CHL, 1CONTROL-ERY, 2SHEEP-ERY and 2POULTRY-ERY showed a similar MGR and transconjugants 1SHEEP-CHL, 2PAPER-CHL, 1PAPER-ERY, 2COW-ERY and 2PAPER-ERY showed a lower MGR. Ampicillin isolated transconjugant 1PAPER- AMP exhibited a higher MGR in comparison to the *E. coli* 1030 recipient strain.

On the contrary, transconjugants obtained using streptomycin, kanamycin or gentamicin as selective antibiotic showed statistically significant slower growth rates, except for the transconjugant 2POULTRY-STR (Table 4.18), which presented a similar MGR in comparison to the recipient. In particular, the transconjugant 2COW-STR stayed in lag phase after 24 h and transconjugant 1PAPER-STR stayed in log phase after 24 h. In fact, none of these transconjugants reached the stationary stage after 24 h of incubation. Due to its slow growth transconjugant 2COW-STR was not selected for further studies.

Table 4.18. Summary table of the maximum growth rates (MGR) of the transconjugants in comparison to the *E. coli* 1030 recipient strain. Transconjugants were classified based on whether they presented a higher, similar or lower MGR in comparison to the MGR of the *E. coli* 1030 recipient strain. Transconjugants with a lower MGR were as well separated into three groups depending on whether after 24 h they reached the stationary phase, stayed on the log phase or did not pass from the lag phase.

	Growth Pattern				
Transconjugants	**Higher MGR**	**Similar MGR**	**Lower MGR**		
			Stationary phase	**Log phase**	**Lag phase**
1PAPER-AMP	x				
1COW-CHL		x			
1SHEEP-CHL			x		
1PAPER-CHL		x			
2COW-CHL	x				
2SHEEP-CHL		x			
2PAPER-CHL			x		
2CONTROL-CHL		x			
1SHEEP-ERY	x				
1PAPER-ERY			x		
1CONTROL-ERY		x			
2COW-ERY			x		
2SHEEP-ERY		x			
2PAPER-ERY			x		
2POULTRY-ERY		x			
1PAPER- STR				x	
2COW-STR					x
2POULTRY-STR		x			

Phenotype fingerprint: Gen III MicroPlate™

The original purpose of the GEN III MicroPlate™ assay is identify a broad range of Gram-negative and Gram-positive bacteria by analyzing the metabolic profile of a broad range of biochemicals. In this particular study, we used this technique to analyze the phenotypic fingerprints of the transconjugants in comparison to the *E. coli* 1030 recipient strain. In this regard, all of the transconjugants showed a different carbon substrate utilization pattern in comparison to the original recipient strain.

The first criteria used to establish positive and negative results for using the carbon substrate were as follows: a transconjugant was considered positive when the color intensity of the transconjugant was ≥ 50% of the intensity of the negative control; a transconjugant was considered negative when the color intensity of the transconjugant tested was below 50% of that of the negative control. Almost all transconjugants increased their use of at least one carbon substrate, except for 1PAPER-CHL, 2COW-CHL and 1SHEEP-ERY, in comparison to the original pattern of carbon substrate of the recipient strain. However, the majority of the observed trait changes in the transconjugants involved the loss or decreased use of some carbon substrates that the original recipient strain could use.

The transconjugant that showed the biggest phenotypic change concerning its carbon substrate utilization pattern was the transconjugant 1PAPER-STR with the loss or decreased use of 15 carbon substrates that the original recipient strain could use and only the acquisition of the capacity to use one additional carbon substrate. On the contrary, transconjugants 1PAPER-AMP, 2COW-CHL, 1SHEEP-ERY, 2PAPER-ERY and 2POULTRY-STR were the most similar ones to the recipient strain. In particular, transconjugant 1PAPER-AMP showed the highest number of carbon substrates metabolized without any loss of the characteristic traits of the original recipient strain.

Based on these trait changes, a similarity index (Jaccard) was obtained for each of the transconjugants (Table 4.20). The index range goes from 1 (most similar) to 0 (least similar). On one side, the transconjugants most similar to the *E. coli* 1030 recipient strain were 1PAPER-AMP, 2POULTRY-STR and 2PAPER-ERY with a Jaccard value of 0.92. On the other side, transconjugants 1PAPER-STR, 2PAPER-CHL and 1PAPER-CHL showed the most different pattern of carbon substrate metabolism, whith Jaccard values of 0.67, 0.71 and 0.74, respectively.

Table 4.19. Carbon substrate utilization patterns of transconjugants compared to the *E. coli* 1030 recipient strain. The first lane indicates the number of either acquired or lost traits of each transconjugant in comparison to the carbon substrate utilization pattern of the *E. coli* 1030 strain. The second lane shows how many of those traits changes in the use of carbon substrates are due to an acquisition of new traits. . The third lane indicates how many of those traits changes in the use of carbon substrates are due to a lost of traits. The following lanes indicate, in detail, the use of different carbon sources for each of the transconjugants. Taking into account the carbon substrate utilization patterns of the transconjugants, the most similar transconjugants are marked in red while the least similar transconjugants are shown in pink, as compared to the recipient.

	1PAPER-AMP	1COW-CHL	1SHEEP-CHL	1PAPER-CHL	2COW-CHL	2SHEEP-CHL	2PAPER-CHL	2CONTROL-CHL	1SHEEP-ERY	1PAPER-ERY	1CONTROL-ERY	2COW-ERY	2SHEEP-ERY	2PAPER-ERY	2POULTRY-ERY	1PAPER-STR	2POULTRY-STR
Number of acquired/lost traits	3	6	4	11	3	10	14	10	3	7	10	7	5	3	6	16	3
Number of acquired traits	3	2	2	0	0	1	2	2	0	2	2	1	2	2	4	1	2
Number of lost traits	0	4	2	11	3	9	12	8	3	5	8	6	3	1	2	15	1
Acquired traits																	
L-Alanine	+		+			+					+		+		+		+
α-Hydroybutyric Acid	+		+														
α- D-Glucose		+					+	+						+	+		+
N- Acetyl-Glucosamine		+					+	+							+		
Glucuronamide										+							

Inosine																				
D-Lactic Acid Methylester																				+
Propionic Acid	+																			
L-aspartic Acid																			+	
Lost traits																		+		
Dextrin		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Maltose			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Trehalose			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
α- D-Lactose			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Melibiose																				-
N-Acetyl-Neuraminic Acid					-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannose						-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fructose							-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose																				-
L-Rhamnose																				-

D-Sorbitol		-	-	-	-						-	-
D-Mannitol					-	-	-	-	-	-		
Glycyl-L-Proline		-									-	
L-Serine		-									-	
Mucic Acid		-									-	
Methyl Pyruvate		-									-	-
L-Lactic Acid Methyl Ester											-	-
α – Keto-Glutaric Acid	-										-	-
D-Malic Acid											-	-
L-Malic Acid		-									-	-
Bromo-Succinic Acid	-	-									-	-
Acetic Acid	-	-									-	-

Table 4.20. Similarity based on the carbon substrate utilization pattern. The similarity between the transconjugants and the *E. coli* 1030 (recipient) strain is represented in the table with the Jaccard index (from 1 to 0). The transconjugants were sorted from the highest Jaccard index (highest similarity) to the lowest. The total number of carbon substrates used (out of the 71 carbon sources available) is as well listed on the table.

Strain	Jaccard index	Number of carbon substrates used
E. coli 1030 (R)	-	32
1PAPER-AMP	0.92	35
2POULTRY-STR	0.92	33
2PAPER-ERY	0.92	33
2COW-CHL	0.91	29
1SHEEP-ERY	0.91	29
1SHEEP-CHL	0.89	32
2SHEEP-ERY	0.87	31
2POULTRY-ERY	0.86	34
1COW-CHL	0.85	30
1PAPER-ERY	0.83	29
2COW-ERY	0.83	27
2CONTROL-CHL	0.77	26
1CONTROL-ERY	0.77	26
2SHEEP-CHL	0.77	24
1PAPER-CHL	0.74	21
2PAPER-CHL	0.71	22
1PAPER-STR	0.67	18

The second criteria used to establish positive and negative results for using the carbon substrate were as follows: a transconjugant was considered positive when the color intensity of the transconjugant was ≥ 75% of the intensity of the negative control; a transconjugant was considered negative when the color intensity of the transconjugant was below 25% of that of the negative control. In this case, only the transconjugant 1PAPER-STR showed a strong reduced use of L-Fucose (6-deoxy-L-galactose) as carbon source in comparison to the *E. coli* 1030 recipient strain. Once again, the transconjugants 1PAPER-STR seemed to have the biggest phenotypic change among all transconjugants.

As part of the GEN III MicroPlate^{TM} test, tolerance assays to pH and sodium chloride were also performed. In this regard, pH 5 and 6 were the pH values included in the GEN III MicroPlate^{TM} test. Positive results were considered when the tolerance was above the 50%

and negative results when the tolerance was below the 50% in comparison to the positive control included in the GEN III MicroPlate™. It was observed that neither the recipient nor the transconjugants tolerated pH 5. In contrast, the original recipient strain tolerated pH 6 (Table 4.21) as well as all transconjugants except for transconjugant 1PAPER-ERY.

Similarly, the recipient strain and all the transconjugants tolerated 1% (w/v) sodium chloride (Table 4.21). However, transconjugants 1COW-CHL, 1PAPER-ERY, 2COW-ERY, 2CONTROL-CHL, 1CONTROL-ERY, 1PAPER-CHL and 2PAPER-CHL lose their tolerance to 4% (w/v) NaCl. In contrast, transconjugant 2SHEEP-ERY gained tolerance to 8% (w/v) NaCl.

Table 4.21. pH and sodium chloride (NaCl) tolerance. For all the transconjugants, tolerance to pH 6 was studied as well as tolerance to 1%, 4% and 8% NaCl. Positive results are based on a tolerance of above the 50%.

Strain	pH tolerance	NaCl tolerance		
	pH 6	1%	4%	8%
E. coli 1030 (R)	+	+	+	
1PAPER-AMP	+	+	+	
1COW-CHL	+	+		
1SHEEP-CHL	+	+	+	
1PAPER-CHL	+	+		
2COW-CHL	+	+	+	
2SHEEP-CHL	+	+	+	
2PAPER-CHL	+	+		
2CONTROL-CHL	+	+		
1SHEEP-ERY	+	+	+	
1PAPER-ERY		+		
1CONTROL-ERY	+	+		
2COW-ERY	+	+		
2SHEEP-ERY	+	+	+	+
2PAPER-ERY	+	+	+	
2POULTRY-ERY	+	+	+	
1PAPER-STR	+	+	+	
2POULTRY-STR	+	+	+	

Ratio RNA: DNA

RNA: DNA ratio was used to test the growth and nutritional state of the transconjugants. As seen in Figure 4.13, all transconjugants exhibited a higher RNA: DNA ratio when compared to the original *E. coli* 1030 recipient strain. Transconjugants 1PAPER-AMP and 1SHEEP-CHL presented the lowest increase of the RNA: DNA ratio. In fact, they were very similar to the recipient strain. Transconjugants 1PAPER-ERY, 1CONTROL-ERY and 1PAPER-STR showed the largest increase of the ratio, indicating a higher metabolic activity. However, these differences were not statistically significant when compared to the result obtained for the recipient strain.

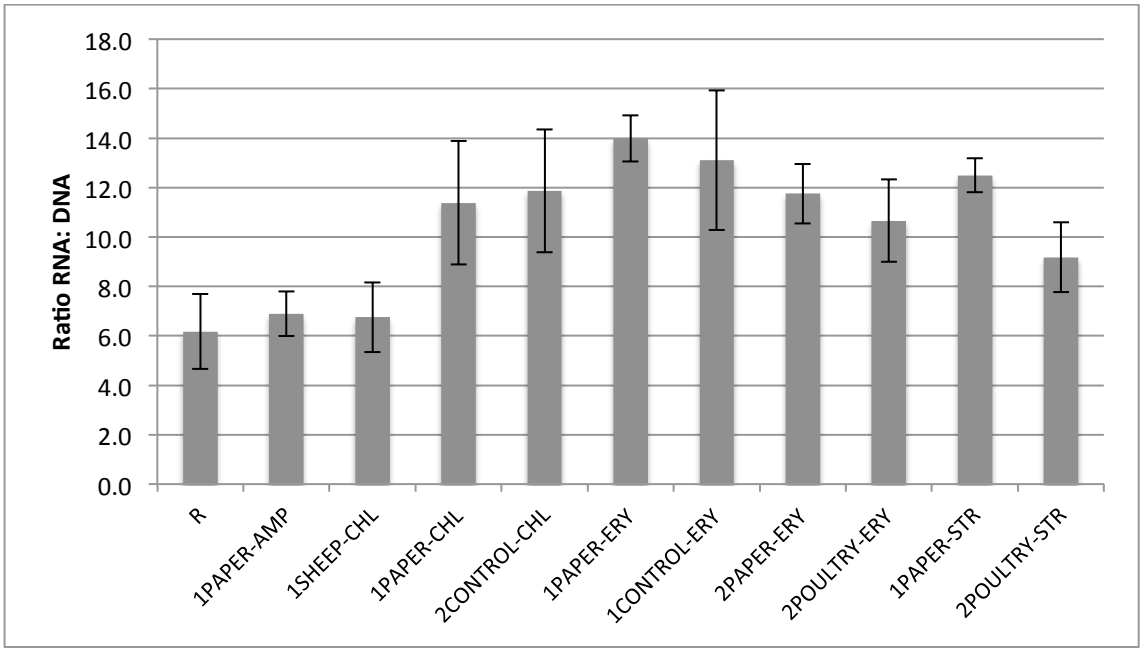


Figure 4.13. RNA: DNA ratio of transconjugants. Results are the mean ± SEM of three independent biological experiments. R: *E. coli* 1030 recipient strain.

Biofilm-forming capacity

An *in vitro* assay based on the staining of biofilm-forming bacteria with crystal violet after 24 h of incubation was used to evaluate the biofilm forming capacity of the transconjugants. As shown in Figure 4.14, transconjugants had different capacities to form biofilms *in vitro.* According to these results, transconjugants 1PAPER-AMP (0.151 ± 0.023), 1PAPER-ERY (0.138 ± 0.013), 1CONTROL-ERY (0.177 ± 0.032) and 2PAPER-ERY (0.163 ± 0.024) were classified as weak biofilm-forming ($0.120 < OD_{570} < 0.240$), whereas the remaining

transconjugants and the recipient strain *E. coli* 1030 were classified as non-biofilm-forming (OD_{570} < 0.120).

There were clearly two statistically (p < 0.05) different groups according to their biofilm formation capacity. The first group was composed by transconjugants with a higher biofilm formation capacity; transconjugants 1PAPER-AMP, 1CONTROL-ERY and 2PAPER-ERY. The second group was represented by transconjugants with a low biofilm formation capacity; transconjugants 1SHEEP-CHL, 2CONTROL-CHL and 1PAPER-STR.

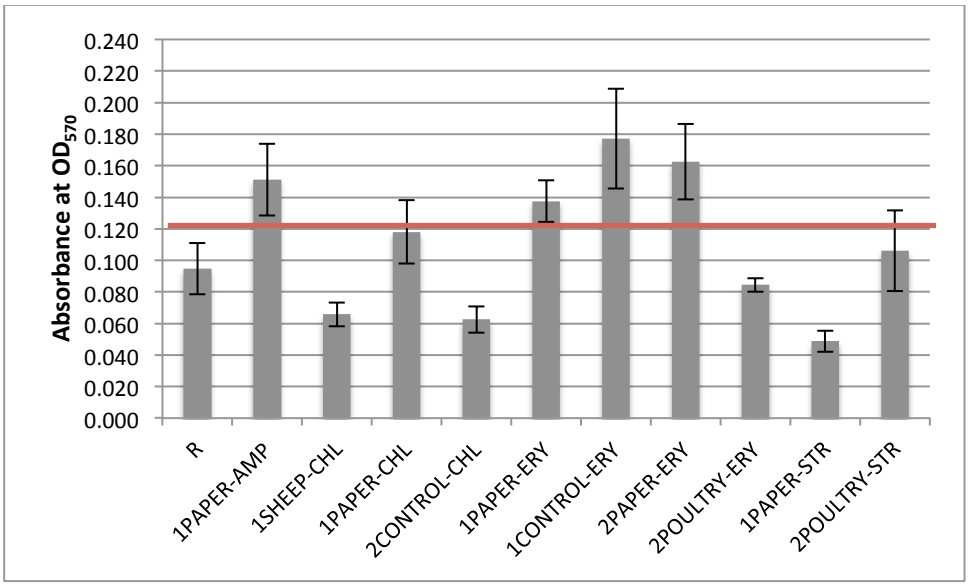


Figure 4.14. Biofilm formation capacity of transconjugants tested in 96-well flat-bottom polystyrene plates. The OD_{570} was measured to quantify the biofilm formation. The red line represents OD_{570} of 0.120 to discriminate between non-biofilm-forming (OD_{570} < 0.120) and weak biofilm-forming transconjugants (0.120 < OD_{570} < 0.240). Results are the mean ± SEM of at least 4 independent biological experiments performed in triplicate. R: *E. coli* 1030 recipient strain.

DNA replication capacity

The quantification of the copy number of the *gyrA* gene in the transconjugants can be approximated to total DNA replication capacity and it can be considered an indicator of the biological activity under stress conditions.

As seen in Figure 4.15, the *E. coli 1030* recipient strain (R) showed a statistically significant increase of the DNA replication activity (p < 0.05) when exposed to 0.2 mM Cd and compared to control conditions. The same situation was observed for transconjugants 1SHEEP-

CHL, 2POULTRY-STR, 1CONTROL-ERY, 2POULTRY-ERY and 1PAPER-STR, although the last one was not statistically significant. Contrarely, transconjugants 1PAPER-AMP, 1PAPER-CHL, 2CONTROL-CHL and 2PAPER-ERY decreased the *gyrA* gene copy number when exposed to Cd. Nevertheless, these reductions were not considered statistically significant ($p > 0.05$), except for 1PAPER-AMP.

When comparing the DNA replication activity among the different transconjugants, transconjugant 1PAPER-CHL had the highest activity, being the only transconjugant that was significantly different to the rest of transconjugants and the recipient strain *E. coli 1030* (R). However, after Cd exposure, transconjugant 2POULTRY-ERY presented then the highest number of *gyrA* gene copies and was significantly different to the rest of transconjugants and the recipient strain. None of the other transconjugants exhibited any significant difference with the recipient strain *E. coli 1030* (R).

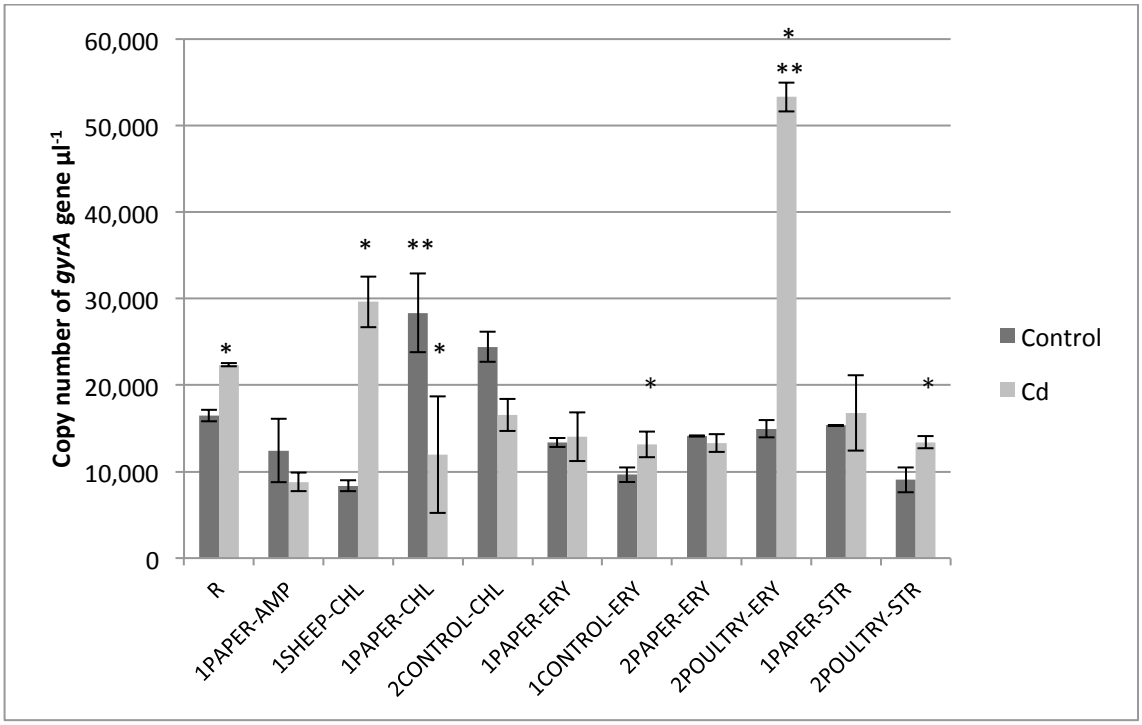


Figure 4.15. Copy number of *gyrA* gene (µl-^{1}) of each transconjugants. Results are the mean ± SEM of three independent biological experiments performed under standard cultivation characteristics (Control) and under exposure to 0.2 mM Cd as stress factor. R: *E. coli* 1030 recipient strain. *Asterisks indicate significance ($p \leq 0.05$) in comparison to control conditions. **Asterisks indicate significance ($p \leq 0.05$) in comparison to *E. coli* 1030 recipient strain.

4.3.8. Conjugative and/or mobilizable plasmids in antibiotic resistant transconjugants

Transconjugants obtained by exogenous plasmid isolation from the bacterial community in soil (section 4.3.1) adquired conjugative and/or mobilizable plasmids that encode the antibiotic resistance genes. To test the capability of transfer of the conjugative and/or mobilizable plasmids in the transconjugants 1PAPER-AMP, 1SHEEP-CHL, 1PAPER-CHL and 2CONTROL-CHL, exogenous plasmid isolation assays were performed using *E. coli* UB 1637 as recipient.

Out of the four transconjugants tested, 1PAPER-AMP, 1SHEEP-CHL, 1PAPER-CHL and 2CONTROL-CHL, only transconjugant 1PAPER-CHL showed the capacity to transfer conjugative and/or mobilizable plasmids into the *E. coli* UB 1637 recipient cells.

4.4. Discussion

Large amounts of manure have been applied to soils worldwide as fertilizer. Manure is often contaminated with veterinary antibiotics, which enter the soil together with antibiotic resistant bacteria. In our study soils, apart from the application of organic amendments as part of an aided phytostabilization assay, a perpetual heavy metal contamination was present. Conjugative transfer of plasmids is the most effective mechanism of HGT by which bacteria can share advantageous genetic information (e.g. antibiotic or heavy metal resistance genes) to thrive in adverse conditions. However, little information is available regarding how bacterial communities in soil are affected by HGT.

Exogenous plasmid isolation assays allow the transfer of conjugative and/or mobilizable plasmids from soil bacteria to a well-known recipient strain that can easily be grown under laboratory conditions. This technique relies upon the selection of an appropriate strain to act as a recipient to capture MGEs (Smalla *et al.*, 2000). Also, it requires the expression of selection markers (such as antibiotic resistance) from the donor in the selected recipient strain (Heuer *et al.*, 2008; Jorquera *et al.*, 2010). Most importantly, this method allows the isolation of plasmids independently of the cultureability of the original host providing information on the occurrence of these traits on self-tramissible MGEs in the entire bacterial community.

A total of 17 conjugative plasmids were obtained from the mine tailings in Lanestosa when using *E. coli* 1030 as recipient and ampicillin (AMP), chloramphenicol (CHL), erythromycin (ERY), or streptomycin (STR) resistance as selection markers. As *E. coli* is not well adapted to thrive in soil habitats, it is likely that the plasmids were captured from other species (Pukall *et al*., 1996). Anjum *et al*. (2012) also successfully performed exogenous plasmid isolation assays in biparental mating with bacteria detached from soil as donor and a rifampicin resistant *E. coli* (HMS) strain as recipient. They obtained ampicillin, tetracycline, kanamycin and chloramphenicol resistant transconjugants. As seen before, IncP-1 plasmids were frequently detected in our study sites, especially in site 1, which was characterized by a higher microbial activity, quantity and diversity, as a result of lower heavy metal contamination in soil. As already mentioned, the self-transmissible IncP-1 plasmids are widely distributed in Gram-negative bacteria (Thomas, 2000; Harada *et al*., 2006), which suggest that IncP-1 plasmids may have been involved in the transfer among Gram-negative bacteria and the *E. coli* recipient strain.

Contrarily, when *E. faecalis* OG1RF served as recipient in the biparental matings, no transconjugant was obtained by using the same selective drugs/agents. This contrasting result may be due to the lack or low concentration of Gram-positive (*Enterococcus* and *Streptococcus*) bacteria closely related to *E. faecalis* to act as donors in our soils (Köhler, 2007). An important prerequisite for conjugative transfer is an intimate association between the cell surfaces of the interacting donor and recipient cells. *E. coli* 1030, a Gram-negative bacteria, presents a two-membrane configuration with a periplasmic space and a thin peptidoglycan layer between the two membranes whereas *E. faecalis* OG1RF, a Gram-positive bacteria, has a cytoplasmic membrane followed by a thick multi-layered peptidoglycan. *Bacillus* and *Clostridia* are Gram-positive bacteria typically present in soils (Janssen, 2006). Previously, it was shown that their relative abundance also increased after the amendment of soil with manure (Ding *et al*., 2014). However, *Bacillus* and *Clostridia* are not as closely related to *Enterococcus* as *Streptococcus*. This makes the chances of establishing a successful transfer channel with *E. faecalis* less likely, and in addition, the newly acquired gene has a better chance of being integrated into the recipient's regulatory and metabolic networks if it comes from bacteria with similar regulatory signals (Vogan and Higss, 2011). As a consequence, plasmid-transfer frequencies to *E. faecalis* were not high enough to be detected by the *in vitro* exogenous plasmid isolation technique.

There was a similar isolation of plasmids from both sites using *E. coli* 1030 as a recipient: 53% of the transconjugants were obtained from site 2, whereas the remaining 47% was isolated from site 1. Regarding the different amendment application, PAPER treated soils allowed the recovery of the largest fraction of conjugative and/or mobilizable plasmids (35%). These results are in accordance with those obtained for the soil quality of the different soils after the amendment treatment. A remarkably diverse bacterial community characterized the soils treated with PAPER showing also higher bacterial activity in comparison to the other soils. Therefore, this microbial-activity rich environment seems to promote HGT.

The lowest portion of transconjugants (12%) was obtained from POULTRY treated soils and CONTROL soils (12%). The remaining 42% of transconjugants was distributed equally among the other treated soils: COW (18%) and SHEEP (24%). The application of PAPER, SHEEP and COW amendments to the soil leaded to a noticeable increase of conjugative and/ or mobilizable plasmid transfer in comparison to CONTROL soils. Heuer and Smalla (2007) reported as well a significant increase of transfer frequencies in soils with a short-term history of manure treatments compared to CONTROL soils.

It has been suggested that application of manure contributes significantly to the occurrence of antibiotic resistance genes among bacterial populations in soil (Binh *et al*., 2008; Chee-Sanford *et al*., 2009; Heuer *et al*., 2011). Strong selective pressure due to anthropogenic pollution was shown to stimulate the presence of resistance determinants in MGEs, so that they can efficiently spread among bacterial populations (Nies, 2003; Silver and Phung, 2005). Nevertheless, in our study soils, the occurrence of antibiotic resistance genes in transconjugants obtained from the CONTROL soils indicates a natural population carrying antibiotic resistance genes on MGEs in these soils with no history of previous manure amendment. This supports the idea that *in situ* conditions under high heavy metal contamination are selective enough to drive the development of antibiotic resistance mechanisms as previously seen by Berg *et al*. (2005) and Stepanauskas *et al*. (2006). This resistance against antibiotics may be a consequence of a cross- or co-selection to heavy metal resistance (Summers *et al*., 1993; Mata *et al*., 2000; Baker-Austin, 2006).

Regarding the selection markers used in the exogenous plasmid isolation assay, 41% of the transconjugants were obtained under chloramphenicol selection, the same fraction as for erythromycin selection. Ampicillin selection only allowed isolation of 6% of the transconjugants while streptomycin selection resulted in 12% of the transconjugants. No transconjugant was isolated with tetracycline, gentamicin or kanamycin amendment.

Although plasmids were isolated by conjugation based on their ability to confer a specific resistance encoded on the transferred plasmid to the *E. coli* 1030 recipient strain, most of them harboured additional resistance to a diverse spectrum of antibiotics. Almost all transconjugants displayed a multi-resistance phenotype except transconjugants 1PAPER-AMP, 2SHEEP-ERY and 2POULTRY-ERY, which only showed resistance to ampicillin and erythromycin, respectively. Also, there was no antibiotic, to which all transconjugants were resistant.

Previous studies on agricultural soils irrigated with wastewater discarded from a pesticide industry in India also reported antibiotic resistant transconjugants to most of the antibiotics tested here: ampicillin, chloramphenicol, erythromycin, kanamycin, sulfadiazine, streptomycin and tetracycline among others (Anjum *et al*., 2012). Along the same lines, resistance genes for the respective antibiotics were often encountered in soil bacterial communities (Heuer and Smalla, 2007; Knapp *et al*., 2010; Popowska *et al*., 2011). Although no transconjugant was obtained under tetracycline or gentamicin selection, tetracycline and gentamicin resistance was indeed detected in the transconjugants' phenotype. Tetracycline and gentamicin are broad-spectrum antibiotics often used in animal husbandry and human therapy (Heuer *et al*., 2002; Popowska *et al.,* 2011). Due to their relative safety and low cost, these antibiotics have been used worldwide. In particular, tetracycline is considered the second antibiotic after penicillin in world consumption (Van Hoek *et al*., 2011).

Apart from these general features, all transconjugants isolated under chloramphenicol selection, showed the same pattern of resistance except 1PAPER-CHL and 2CONTROL-CHL. Transconjugants 1COW-CHL, 1SHEEP-CHL, 1PAPER-CHL, 2COW-CHL, 2SHEEP-CHL, 2PAPER-CHL and 2CONTROL-CHL presented a two-fold higher resistance to ampicillin, gentamicin and streptomycin, a four-fold higher resistance to erythromycin, kanamycin and tetracycline and an eight-fold higher resistance to chloramphenicol as compared to the recipient strain. Krauland et al. (2010) reported several gene cassettes conferring resistance to *Salmonella enterica* isolates to the majority of the antibiotics for which resistance was encountered in our transconjugants isolated by chloramphenicol selection: an aac(6')IIc/ereA2/IS1247/aac3/arr/ereA2 gene cassette array which confers resistance to rifampicin, nalidixic acid, gentamicin, aztreonam, and trimethoprim-sulfamethoxazole as well as ampicillin, ampicillin/sulbactam, tetracycline, chloramphenicol, and streptomycin; and the qacH/dfrA32/ereA1/aadA2/cmlA/aadA1 gene cassette array conferring resistance to erythromycin, streptomycin, chloramphenicol, and trimethoprim/sulfamethoxazole. These isolates were obtained from a global collection comprising >1,900 isolates of non-typhoid *S.*

enterica collected by laboratories from 13 countries and 6 continents. Krauland *et al*. (2010) also found *S. enterica* isolates with the *arr2/cmlA5/blaOXA10/aadA1* gene cassette array exhibiting resistance to rifampicin, chloramphenicol, ampicillin, and streptomycin, which were additionally resistant to tetracycline, ceftazidime, gentamicin, aztreonam, and trimethoprim-sulfamethoxazol.

Tetracycline and ampicillin resistance were only detected in transconjugants originally isolated under chloramphenicol selection. This could indicate that tetracycline, ampicillin and chloramphenicol resistance in transconjugants 1COW-CHL, 1SHEEP-CHL, 1PAPER-CHL, 2COW-CHL, 2SHEEP-CHL, 2PAPER-CHL and 2CONTROL-CHL could be encoded together in the same gene cassette or on the same plasmid (Krauland *et al*., 2010). Moreover, transconjugant 1PAPER-CHL demonstrated additionally a two-fold higher resistance to sulfadiazine, and transconjugant 2CONTROL-CHL exhibited a higher resistance to minocycline. This suggests that almost all transconjugants isolated under chloramphenicol selection, 1COW-CHL, 1SHEEP-CHL, 2COW-CHL, 2SHEEP-CHL and 2PAPER-CHL (except 1PAPER-CHL and 2CONTROL-CHL) could harbor a similar gene content. In contrast, transconjugants isolated under erythromycin selection did not share a similar pattern of antibiotic resistance, which could indicate the presence of different plasmids. The same scenario/picture was observed for the streptomycin-isolated transconjugants. Different levels of antibiotic resistance also could suggest different mechanism of resistance and therefore different antibiotic resistance genes. The most remarkable case was the observed for the streptomycin-isolated transconjugants where transconjugants 1PAPER-STR and 2POULTRY-STR presented an eight-fold increase and a 512-fold higher resistance to streptomycin, respectively.

Erythromycin, gentamicin, kanamycin and streptomycin resistance were observed independently of the amendment treatment. Resistance genes/phenotype against erythromycin (Knapp *et al*., 2010; Popowska *et al*., 2011; Anjum *et al*., 2011), gentamicin (Heuer *et al*., 2002; Heuer and Smalla, 2007), kanamycin (Ansari *et al*., 2008; Anjum *et al*., 2011) and streptomycin (Popowska *et al*., 2011; Anjum *et al*., 2012) have already been reported in environmental samples contaminated by anthropogenic activities. As it was demonstrated before, PAPER treated soils showed lower Zn and Cd bioavailability, a higher OM concentration and a higher microbial biodiversity than the other treated soils which seems to correlate with an increased diversity of transconjugants with different antibiotic resistance phenotypes.

In particular, sulfadiazine resistance was encountered only in site 1 where LowGC-type plasmids were found in this work. LowGC-type plasmids seem to play an important role in conferring sulfadiazine resistance in organic amended soils as observed by Heuer *et al*. (2009), Jechalke *et al*. (2013) and Kopmann *et al*. (2013). In these works, LowGC-type plasmids also conferred resistance to sulfadiazine in soils treated with organic amendments. In this regard, it would be interesting to extract plasmids from transconjugants isolated from site 1 to compare them with the plasmids found by Heuer *et al*. (2009), Jechalke *et al*. (2013) and Kopmann *et al*. (2013).

Our results demonstrated that conjugative and/or mobilizable plasmids with multiple antibiotic resistance genes are likely present in these populations and have the potential to persist and mobilize resistance genes among the community. It was shown that very low concentrations of antibiotics, after amendment, could select for antibiotic resistant bacteria (Gullberg *et al*., 2011). Nonetheless, we cannot exclude that some bacteria might be intrinsically resistant to these antibiotics (Quinn, 1998; Gómez and Neyfakh, 2006; Wright, 2007). To identify the genes responsible for the antibiotic resistance phenotype in our transconjugants, a PCR-Southern Hybridization detection system could be used. This technique is limited to the detection of already-known gene sequences. However, there is still a large proportion of antibiotic resistance genes in the environment that remain unknown.

The Lanestosa mine-tailings habitat, especially after PAPER treatment, might become a reservoir of antibiotic resistance genes. Run-off events due to heavy rain may allow further spread of bacteria carrying antibiotic resistance plasmids into the surroundings; including water sources (Rahube and Yost, 2012). Finally, these self-transmissible plasmids harbouring antibiotic resistance genes could be acquired by clinical pathogens (Forsberg *et al*., 2012; Yang *et al*., 2013). Therefore, environmental reservoirs of antibiotic resistance genes might become a major contamination risk for human health.

At the moment, a small number of antimicrobials including imipenem and vancomycin are still considered drugs of last resort for treating antibiotic resistant pathogens. Although vancomycin resistance has been previously recovered from environmental samples by conjugation using *Enterococcus* as recipient (Manson *et al*., 2003), when using *E. coli* as the recipient strain in our study, none of the transconjugants was resistant to vancomycin. Still, almost 30 years after its introduction, resistance to this glycopeptide antibiotic was rarely reported until the late 1980s when emergence of acquired resistance to this antibiotic was observed for the first time (Van Hoeck *et al*., 2011). Vancomycin resistant pathogens represent

now a severe problem in health care worldwide (Gold, 2001; Tarai *et al*., 2013; Schröder *et al*., 2015).

Interestingly, transconjugant 1PAPER-STR showed enhanced resistance to imipenem. Imipenem is a broad-spectrum carbapenem antibiotic that was designed semi synthetically in 1980 and it is still considered a drug of last resort due to the slow emergence of resistant strains since then. Imipenem resistance is still rare in nature but a few studies found imipenem resistant bacterial isolates in soil or water from different environmental sites (Girlich *et al*., 2010; Ash *et al*., 2002; Jones-Dias *et al*., 2016); including farm soil treated with manure (Rossolini *et al*., 2001). This suggests that there is a potentially large and divergent gene pool encoding imipenem resistance within natural environments, and that soils are important reservoirs of antibiotic resistance with clinical relevance. In like manner, Girlich *et al*. (2010) discovered a possible connection between an imipenem-resistant *Acinetobacter baumannii* in hospital settings and in the environment. When comparing the environmental and clinical isolates they found evidence for genetic exchange between them, indicating the possibility that carbapenemase resistance could have been transferred from the clinical settings to the environment.

To identify if imipenem resistance was due to carbapenemase production, the modified Hodge Test was performed for the transconjugant 1PAPER-STR. Carbapenemases represent the most versatile family of β-lactamases that hydrolyze not only carbapenems but other β-lactam antibiotics as well (Queenan and Bush, 2007). Furthermore, carbapenemases are found commonly encoded on MGEs (Datta and Wattal, 2010). The modified Hodge Test demonstrated that carbapenemase production is not the mechanism of imipenem resistance in transconjugant 1PAPER-STR.

Moreover, transconjugant 1PAPER-STR was the only one that showed increased resistance to at least one of the four heavy metals tested (Cu, 5 mM). Many plasmids conferring resistance to antibiotics have been frequently found in association with genes coding for resistance to heavy metal (Tennstedt *et al*., 2003; Stokes *et al*., 2006; Moura *et al*., 2007). Heavy metals, including As, Cu and Zn, have been detected before in pig manure slurry and agricultural soils and shown to co-select and enhance the spread of antibiotic resistance genes among soil microbial populations (Bolan *et al*., 2004; Marcato *et al*., 2009; Berg *et al*., 2010; Heuer *et al*., 2011). Previously, *P. aeruginosa* isolates exposed to Zn were found to be resistant to other heavy metals (Cu) as well and to the carbapenem-class antibiotic imipenem. *P. aeruginosa* is a Gram-negative bacteria that commonly thrives in environments polluted

with organic matter but it is also an opportunistic pathogen frequently encountered in hospitals, causing morbidity and mortality in immunocompromised patients (Rosenfeld *et al*., 2003). Analysis of the mechanisms that could underlie cross-resistance to both Zn and imipenem revealed a co-regulation system, thus, exposure to one toxicant (heavy metal or imipenem) leads to expression of both, metal efflux and imipenem resistance (Perron *et al*., 2004; Caille *et al*., 2007). The CopR/CopS and OprD system that promotes the cross-resistance of heavy metals and imipenem is involved in the down-regulation of primary metabolism and up-regulation of protective mechanisms to cope with the stress caused by antibiotics or toxic concentrations of heavy metals. Our findings suggest that this mechanism is not responsible for the resistance to imipenem and Cu in transconjugant 1PAPER-STR. Transcription of the *copR*/*copS* or *oprD* system was not observed and imipenem resistance of the transconjugant was not enhanced when exposed to different concentrations of Cu. These results suggest the existence of independent resistance mechanisms to imipenem and Cu on the transferred genes of transconjugant 1PAPER-STR. This might be due to co-selection of antibiotic and heavy metal resistance instead of cross-selection of mechanisms.

Regarding heavy metal resistance, the rest of the transconjugants did not show increased resistance to any of the heavy metals tested (Cd, Cu, Pb and Zn) which suggests that resistance to heavy metals may not be encoded in MGEs but in the chromosome of the original donor bacteria. Previous studies based on whole genome sequencing revealed that genetic determinants for heavy metal resistance are frequently encoded on the chromosome (Mergeay *et al*., 2003; Perron *et al*., 2004; Janssen *et al*., 2010). This was the case for *Cupriavidus metallidurans* CH34, which was isolated in the 1970s from a metallurgical site in Belgium with high levels of bioavailable heavy metals (Janssen *et al*., 2010). The fact that a gene is encoded on the chromosome is often a result of evolutionary adaptation and specialization of soil bacteria to a strong and constant selective pressure (Janssen *et al*., 2010). In our soils, the input of organic amendments was a recent and one-time event in contrast to the long-term and constant contamination by heavy metals due to previous mining activities in the area. These findings together could explain why while antibiotic resistance determinants were often encountered on plasmids, heavy metal resistance determinants were hardy ever detected on them.

Stress response under metal exposure was further studied by RT-qPCR as the relative expression of *ahpF*, *katG* and *sodA* genes, which have been previously reported to be involved in cellular defence against oxidative stress. Oxidative stress, referred to as the elevated

intracellular levels of ROS, affects the status of redox-active compounds such as enzymes, causes lipid peroxidaton and modulates gene expression (Foyer and Noctor 2009; Potters *et al*., 2010). In living cells, elimination of alkyl hydroperoxides is particularly important since they can initiate lipid peroxidation chain reaction and consequently propagate free radicals, leading to DNA and membrane damage (Ayala *et al*., 2014).

KatG (catalase) and Ahp (alkyl hydroperoxide reductase), among others (Rocha and Smith, 1999), are controlled by a single regulator, OxyR (Rocha and Smith, 1999), responsible for the synthesis of proteins against oxidative stress. Overexpression of *katG* and *ahpF* genes in *P. aeruginosa* and *Nitrosomonas europaea* was previously seen by Teitzel *et al*. (2006) and Park and Ely (2008) when exposed to 10 mM $CuSO_4$ and 200 μM $ZnSO_4$, respectively. It has been also reported (Seaver and Imlay, 2001; Zheng *et al*., 2001; Asad *et al*., 2004) that *katG* and a*hpF* genes are frequently overexpressed as part of a transcriptional strategy against the deleterious effects of H_2O_2. In this manner, *katG* and *ahpF* genes could be used as potential biomarkers for the effect of metal exposure on the early gene expression profile of *E. coli* cells (Wang *et al*., 2009; Liu *et al*., 2011).

The gene expression profiles obtained here suggest an up-regulation of the expression of *ahpF* in the transconjugant 2POULTRY-STR upon Cd exposure and of *katG* in the transconjugant 1PAPER-CHL upon Cd and Cu exposure. Thus, it could be assumed that both transconjugants have upgraded either their Ahp or KatG defence against oxidative stress under metal exposure. On the other hand, in transconjugants 1PAPER-AMP, 2CONTROL-CHL (Cd and Cu exposure), 1PAPER-STR and 2PAPER-ERY the *ahpF* gene was repressed while the expression level of the *katG* gene was similar to the *katG* gene expression in the *E. coli* recipient strain. However, we cannot exclude the possibility that they may increase the expression of other genes controlled by the same regulator as disruption of the *ahpF* normally causes compensatory up-regulation of other genes controlled by the same regulator in *E. coli* (Rosner and Storz, 1994).

Regarding *sodA* expression levels, again, transconjugants 1PAPER-AMP, 2CONTROL-CHL (Cd and Cu exposure) and 1PAPER-STR showed a lower expression of this gene compared to the *E. coli* recipient strain. In contrast, 2POULTRY-ERY and 1PAPER-CHL (only when previously exposed to Cu and not to Cd) showed a higher expression in comparison to the *E. coli* recipient strain.

The different *sodA* expression behaviour observed for transconjugant 1PAPER-CHL when exposed to Cu and Cd could be a result of the distinct redox character of these two heavy metals. Cu influences the redox homeostasis in the cell, due to the accumulation of ROS. Kimura and Nishioka (1997) found Cu to be a powerful inducer of *sodA* transcription in *E. coli*. In contrast, Cd itself is unable to generate free radicals directly, however, indirect generation of radicals by Cd has been reported (Galan *et al*., 2001). Nevertheless, Cd causes toxicity primarely by deactivating an essential DNA repair activity (McMurray and Tainer, 2003). Hu *et al*. (2005) carried out a study on the transcriptional response of *Caulobacter crescentus* cells exposed to 6 µM $CdSO_4$ for 30 min. They observed overexpression of *sodA*, together with induction of Cu-binding proteins. Moreover, while studying ROS behaviour under Cd and Cu stress, Geslin *et al*. (2001) suggested that bacterial superoxide dismutases (SODs) might also reduce the toxicity of heavy metals by a process of metal storage.

Study of the fitness of the different transconjugants, and the changes after the acquisition of new plasmids by HGT, could provide a comprehensive understanding of the impact of this mechanism on bacterial adaptation and diversification. HGT is one of the most important evolutionary forces for microbial populations. However, in order to maintain plasmids within a population, the acquired genes must provide enough benefits or poor fitness costs for the host to avoid their loss due to genetic drift or selection. Identifying and exploring costs is essential to explain how HGT affects evolutionary genetic diversity among populations.

The first indication that changes in the fitness of transconjugants have occurred is to observe changes in its maximum growth rate (MGR) in comparison to the recipient. Transfer of plasmids into new cells was reported to often alter growth properties by extending the lag phase, lowering maximum growth rates or cell densities (Diaz-Ricci and Hernandez, 2000; van Rensburg *et al*., 2012). The most remarkable decrease in growth rate was observed for the transconjugant 1PAPER-STR. This could suggest that the DNA content captured in this transconjugant is larger than the others. DNA content has a strong influence on the ecophysiological traits (adaptive traits to environemental changes) of bacteria, and as it was previously observed, it has a key role in setting the MGR of a strain (Wyckham and Lynn, 1990). Despite this assumption of the fitness cost for maintaining extra-DNA, previous experimental studies have demonstrated that this cost is not due to the increment in the DNA content itself or its replication. Instead, it is predominantly due to transcription and translation of the extra-DNA (Bragg *et al*., 2009; Shachrai *et al*., 2010).

Along the same lines, RNA: DNA ratio is another parameter that was successfully used to predict growth and nutritional state of a variety of organisms (Kennell and Magasanik, 1962; Bulow, 1970; Dortch *et al.*, 1983; Buckley, 1984; Clemmesen *et al*., 2003; Islam and Tanaka, 2005; Elser *et al*. 2007) as it provides an index of protein synthetis capacity during active growth and cell enlargement. The increment of the RNA: DNA ratio was observed for all transconjugants in different degrees and, as expected, the RNA: DNA ratio of transconjugant 1PAPER-STR was significantly up-regulated. This finding correlates with the idea that the fitness costs of the transconjugants are highly related to transcription and translation of the extra genes encoded in the collected MGEs.

Although evidence of beneficial fitness effects by HGT is overwhelming, it has been noticed that acquired regions often function inefficiently within new genomic backgrounds so that, despite great evolutionary benefits, they can be energetically or physiologically costly (Chou *et al*., 2011; Park and Zhang, 2012). Whereas gene transfer within a single species results in the propagation of specific traits, interspecific gene transfer may lead to entirely new genetic combinations. *E. coli* is not an indigenous bacteria of these soils so we can assume that the *in vitro* exogenous plasmid isolation was based on interspecific gene transfer events. This implies, that plasmids transferred into the *E. coli* recipient encountered a new genomic background as they came from different bacterial species from soil. Therefore, this transfer event has the potential to disrupt existing regulatory and physiological networks within the new host, costing sometimes more than the actual benefits (Baltrus, 2013).

Paired with this, transconjugant 1PAPER-STR seemed to have the most significantly changed physiological state as referring to the capacity of using carbon substrates which the original strain was able to grow on or not. HGT-dependent changes can affect cellular networks to shift a wide variety of pathways in global regulation of the host and cause phenotypic changes as collateral damage. The new plasmid could sequester carbon, nitrogen, phosphorous or even impede the cellular machinery from performing critical housekeeping processes (Shachrai *et al*., 2010), as well as consume molecular fuel (e.g. ATP) to carry out basic cellular processes. Because these plasmids encode genes controlling their own replication and transmission, they are also subject to natural selection, with fitness interests that need not necessarily be in line with those of their bacterial host. Therefore, there exist two options, conflict or reciprocal collaboration between bacterial host and plasmids (Harrison and Brockhurst, 2012).

On the other hand, transconjugant 1PAPER-AMP presented one of the highest MGR values along with one of the lowest increments in the RNA: DNA ratio. Also, the analysis of the capacity of using carbon substrates confirmed that this transconjugant has the less affected fitness or physiological state of all of them. However, the validity of Biolog assays as a proxy for strain improvement is still controversial (Leiby and Marx, 2014). Along with this, transconjugant 1PAPER-AMP was the only one that acquired just a single antibiotic resistance (ampicillin) by HGT. So, it seems to have acquired a plasmid, which does not require hardly any additional energy giving more benefits than costs to the original strain (Vedler *et al*., 2004; Stasiak *et al*., 2014; Hall *et al*., 2015).

Genes that encode functions, which enhance the fitness of the plasmid's host under a given selective pressure, are typically described as accessory genes (Smillie *et al*., 2010). Apart from the above-discussed antibiotic or metal resistance, another example of functions of accessory genes includes attachment to specific surfaces (Norman *et al*., 2009) by biofilm-associated factors. A growing number of studies indicate that HGT and biofilm community structure and functions are connected through numerous interactions, ranging from the genetic level to the community level (Madsen *et al*., 2012). Improved cell protection induced by biofilms has been previously shown against antimicrobial exposure (Høiby *et al*., 2010) and oxidative stress (Burmølle *et al*., 2006; Geier *et al*., 2008). Other studies have catalogued a library of phenotypic effects, including alterations in biofilm formation and thermal tolerance, as byproducts of HGT (Sato and Kuramitsu, 1998; Gaillard *et al*., 2008). The process of biofilm formation has an important role, as well, as a provider of a spatially structured community where plasmids can be shared through HGT (Jeffterson, 2004). HGT stimulation in biofilms will therefore ensure maintaining genetic heterogeneity.

Therefore, cooperative traits such as adhesive properties are more likely maintained because they provide conditions for their own persistence. Transconjugants 1PAPER-AMP, 1CONTROL-ERY and 2PAPER-ERY were more effective in terms of formation of biofilm structures *in vitro* after 24 h of incubation whereas transconjugants 1SHEEP-CHL, 2CONTROL-CHL and 1PAPER-STR were less competent in generating attached structures after plasmid acquisition. In this case, it can be speculated that transconjugants 1PAPER-AMP, 1CONTROL-ERY and 2PAPER-ERY may have accessory genes related to adhesion encoded in the acquired plasmids. Nevertheless, further research at molecular level would be needed to confirm this by PCR-Southern Hybridization detection.

Gyrases belong to a class of essential and highly conserved enzymes that are present in all living organisms. These enzymes relieve topological constraints caused by transcription and replication complexes that move along the DNA. The *gyrA* subunits form the catalytic core of the enzyme and ensure the DNA breaking and re-joining reactions. The measure of the *gyrA* activity can be approximated to total DNA replication capacity and can be considered a broad-spectrum indicator of biological activity (Couturier *et al*., 1998). In this study, the number of *gyrA* gene copies was measured under normal and under stress conditions (Cd exposure). Under normal conditions, transconjugant 1PAPER-CHL was characterized by a higher copy number of *gyrA* gene in comparison to the recipient strain. As it was mentioned above, the MGR did not show any significant increase for this transconjugant although the increment of RNA: DNA ratio was one of the highest. Together these results might indicate the capture of a large plasmid (with a low fitness cost) by the transconjugant 1PAPER-CHL as there is a notable increase of the DNA replication activity.

Furthermore, when transconjugants were grown with Cd, transconjugant 1PAPER-CHL along with transconjugants 1PAPER-AMP, 2CONTROL-CHL and 2PAPER-ERY showed a lower copy number of *gyrA* gene while 2POULTRY-ERY presented the highest DNA replication activity. These results seems to be strongly related with the ones observed for the expression of genes involved in cellular defence against oxidative stress when exposed to Cd. Transconjugants 1PAPER-AMP, 2CONTROL-CHL and 2PAPER-ERY (although not statistically significant) down-regulated the expression of *ahpF* and s*odA* gene. Therefore, when exposed to Cd the low copy number of *gyrA* gene is clearly a consequence of the weaker response of these transconjugants to Cd; either due to cell death or to the need of centralizing the energy on defence against the oxidative stress rather than in replication.

Secondary *in vitro* exogenous plasmid isolation was performed using transconjugants 1PAPER-AMP, 1SHEEP-CHL, 1PAPER-CHL and 1CONTROL-CHL as donors and *E. coli* UB 1637 as the new recipient. According to the results, transconjugant 1PAPER-CHL still harboured a conjugative plasmid, as it was able to transfer a plasmid to the new *E. coli* recipient strain. As it was seen before, transconjugant 1PAPER-CHL was characterized by a high replication activity, which seems to be a consequence of acquisition of a large MGE. Previous studies have repeatedly obtained broad-host-range IncP-1 conjugative plasmids from soil bacteria when performing the exogenous plasmid isolation technique (Top *et al*., 1994; Dröge *et al*., 2000; Smalla *et al*., 2000). Bearing in mind that IncP-1 specific genes were also detected in the PAPER

treated soil in site 1, it can be expected that transconjugant 1PAPER-CHL acquired an IncP-1 conjugative plasmid.

According to mobility, plasmids can be classified into three categories: conjugative, mobilizable and nonmobilizable (Smillie *et al*., 2010). Conjugative plasmids have a size, on average, of 100 kb; whereas mobilizable plasmids are, on average, 5 kb long. Therefore, it is likely that transconjugant 1PAPER-CHL harbours a large conjugative plasmid.

Contrarily, transconjugants 1PAPER-AMP, 1SHEEP-CHL and 1CONTROL-CHL were not able to perform horizontal transfer. There are several reasons that could explain this event. It may be due to the fact that i) the entire plasmid/s or some of its genes integrated into the chromosome, ii) the plasmid/s are mobilizable plasmids once transferred with the help of another conjugative plasmid or iii) the bacterial host reduced the conjugation rate. An *in vitro* triparental-mating assay with a helper strain harbouring a conjugative plasmid that can mobilize the non-self transmissible plasmid could demonstrate/show if these transconjugants harbour a mobilizable plasmid, once transferred with the help of a conjugative plasmid (Götz and Smalla, 1997). Furthermore, transconjugant 1PAPER-AMP was characterized by fast growth rate, resistance against only ampicillin and the lowest increase in RNA: DNA ratio. These findings support the assumption that transconjugant 1PAPER-AMP had either a well-adapted or integrated plasmid, or a small mobilizable plasmid.

Dahlberg and Chao (2003) observed that the bacterial host can entirely loose the ability to conjugate the acquired plasmid or reduce the conjugation rate, to cope with the fitness costs imposed by the transferability of the plasmid. Turner *et al*. (1998) found that plasmids that evolved lower conjugation rates imposed lower fitness costs; whereas those that had evolved increased conjugation rates imposed greater costs. Amelioration of the cost-of-carriage may also be achieved through the loss of the nonessential portion of the plasmid genome. When not under positive selection, accessory genes represent 'excess baggage' increasing the number of genes requiring transcription and translation by the host (Modi *et al*., 1991). In that instance, the loss of MGE structures may allow the retention of beneficial genes by integration into the bacterial genome (Modi *et al*., 1992; Bergstrom *et al*., 2000) and reduce the fitness costs.

In conclusion, transconjugants isolated by *in vitro* exogenous plasmid isolation in biparental matings indicate that genes for heavy metal/antibiotic resistance can be distributed by conjugative and/or mobilizable plasmids in the natural environment with a wide range of

fitness costs. Our observations contribute to the understanding of gene exchange between bacteria in dynamically developing ecosystems.

Table A4.1: **Average OD_{600} values and standard error of the mean (mean ± SEM) for transconjugants after 24 h of incubation with $CdCl_2$.** Different letters indicate statistically significant ($p < 0.05$) different groups among means.

$CdCl_2$ (mM)	R	1PAPER-AMP	1COW-CHL	1.SHEEP-CHL	1PAPER-CHL	2COW-CHL	2SHEEP-CHL	2PAPER-CHL	2CONTROL-CHL
0	0.771 ± 0.075^{a}	0.702 ± 0.060^{a}	0.465 ± 0.133^{a}	0.507 ± 0.125^{a}	0.470 ± 0.138^{a}	0.498 ± 0.118^{a}	0.497 ± 0.108^{a}	0.522 ± 0.117^{a}	0.552 ± 0.088^{a}
0.5	0.626 ± 0.025^{ab}	0.547 ± 0.030^{b}	0.402 ± 0.082^{a}	0.390 ± 0.062^{a}	$0.205 \pm 0.073^{ab*}$	0.439 ± 0.045^{a}	0.446 ± 0.064^{b}	0.472 ± 0.061^{ab}	$0.392 \pm 0.040^{b*}$
1	0.398 ± 0.031^{b}	0.376 ± 0.018^{c}	$0.285 \pm 0.016^{ab*}$	0.328 ± 0.007^{a}	$0.234 \pm 0.008^{ab*}$	$0.295 \pm 0.050^{a*}$	$0.266 \pm 0.004^{ab*}$	$0.244 \pm 0.014^{b*}$	$0.164 \pm 0.024^{c*}$
2	0.022 ± 0.005^{c}	0.024 ± 0.005^{d}	0.023 ± 0.006^{b}	0.026 ± 0.005^{b}	0.023 ± 0.005^{b}	0.024 ± 0.007^{b}	0.019 ± 0.005^{b}	0.021 ± 0.003^{c}	0.026 ± 0.005^{c}
3	0.024 ± 0.005^{c}	0.021 ± 0.004^{d}	0.022 ± 0.006^{b}	0.027 ± 0.008^{b}	0.025 ± 0.007^{b}	0.027 ± 0.006^{b}	0.024 ± 0.010^{b}	0.021 ± 0.005^{c}	0.020 ± 0.003^{c}
4	0.025 ± 0.006^{c}	0.019 ± 0.003^{d}	0.023 ± 0.008^{b}	0.052 ± 0.027^{b}	0.026 ± 0.007^{b}	0.036 ± 0.009^{b}	0.018 ± 0.009^{b}	0.012 ± 0.012^{c}	0.018 ± 0.002^{c}
5	0.022 ± 0.006^{c}	0.016 ± 0.003^{d}	0.031 ± 0.009^{b}	0.034 ± 0.005^{b}	0.031 ± 0.008^{b}	0.025 ± 0.004^{b}	0.020 ± 0.007^{b}	0.025 ± 0.003^{c}	0.012 ± 0.003^{c}
6	0.032 ± 0.008^{c}	0.019 ± 0.003^{d}	0.033 ± 0.002^{b}	0.024 ± 0.001^{b}	0.028 ± 0.004^{b}	0.023 ± 0.001^{b}	0.018 ± 0.003^{b}	$0.010 \pm 0.006^{c*}$	0.017 ± 0.002^{c}

$CdCl_2$ (mM)	R	1SHEEP-ERY	1PAPER-ERY	1CONTROL-ERY	2COW-ERY	2SHEEP-ERY	2PAPER-ERY	2.POULTRY-ERY	1PAPER-STR	2POULTRY-STR
0	0.771 ± 0.015^{a}	$0.537 \pm 0.017^{a*}$	$0.597 \pm 0.040^{a*}$	0.514 ± 0.035^{a}	$0.556 \pm 0.031^{a*}$	$0.746 \pm 0.030^{*}$	$0.557 \pm 0.011^{a*}$	$0.674 \pm 0.020^{a*}$	$0.504 \pm 0.038^{a*}$	0.819 ± 0.010^{a}
0.5	0.626 ± 0.022^{b}	$0.379 \pm 0.012^{b*}$	$0.375 \pm 0.026^{b*}$	0.315 ± 0.009^{b}	$0.373 \pm 0.011^{b*}$	$0.466 \pm 0.022^{b*}$	$0.389 \pm 0.011^{b*}$	$0.437 \pm 0.026^{b*}$	$0.343 \pm 0.019^{b*}$	0.572 ± 0.015^{b}
1	0.398 ± 0.057^{c}	0.213 ± 0.064^{c}	0.234 ± 0.078^{b}	0.019 ± 0.002^{c}	0.226 ± 0.047^{c}	0.207 ± 0.073^{c}	0.232 ± 0.042^{c}	0.063 ± 0.023^{c}	0.163 ± 0.041^{c}	0.282 ± 0.060^{c}
2	0.022 ± 0.004^{d}	0.025 ± 0.003^{d}	0.021 ± 0.002^{c}	$0.017 \pm 0.002^{c*}$	$0.018 \pm 0.002^{d*}$	$0.016 \pm 0.001^{d*}$	0.024 ± 0.005^{d}	0.021 ± 0.002^{c}	0.029 ± 0.006^{d}	0.025 ± 0.004^{d}
3	0.024 ± 0.008^{d}	0.032 ± 0.012^{d}	0.033 ± 0.015^{c}	0.025 ± 0.008^{c}	0.035 ± 0.017^{d}	0.024 ± 0.008^{d}	0.051 ± 0.032^{d}	0.033 ± 0.010^{c}	0.026 ± 0.003^{d}	0.022 ± 0.002^{d}
4	0.025 ± 0.003^{d}	0.016 ± 0.003^{d}	0.023 ± 0.007^{c}	0.035 ± 0.011^{c}	0.027 ± 0.006^{d}	0.019 ± 0.003^{d}	0.021 ± 0.005^{d}	0.023 ± 0.002^{c}	0.019 ± 0.002^{d}	0.016 ± 0.001^{d}
5	0.022 ± 0.004^{d}	0.027 ± 0.014^{d}	0.026 ± 0.013^{c}	0.042 ± 0.017^{c}	0.018 ± 0.005^{d}	0.018 ± 0.006^{d}	0.014 ± 0.004^{d}	0.021 ± 0.004^{c}	0.024 ± 0.007^{d}	0.015 ± 0.003^{d}
6	0.032 ± 0.008^{d}	0.026 ± 0.011^{d}	0.020 ± 0.012^{c}	0.026 ± 0.009^{c}	0.017 ± 0.011^{d}	0.015 ± 0.005^{d}	0.018 ± 0.007^{d}	0.023 ± 0.007^{c}	0.020 ± 0.006^{d}	0.015 ± 0.004^{d}

*Significantly less tolerance in comparison to the recipient strain (R).

** Significantly more tolerance in comparison to the recipient strain (R).

Table A4.2: **Average OD_{600} values and standard error of the mean (mean ± SEM) for transconjugants after 24 h of incubation with $CuCl_2$.** Different letters indicate statistically significant ($p < 0.05$) different groups among means.

$CuCl_2$ (mM)	R	1PAPER-AMP	1COW-CHL	1.SHEEP-CHL	1PAPER-CHL	2COW-CHL	2SHEEP-CHL	2PAPER-CHL	2CONTROL-CHL
0	0.875 ± 0.029^{a}	0.858 ± 0.014^{a}	0.681 ± 0.020^{a}	$0.662 \pm 0.027^{a*}$	0.667 ± 0.018^{a}	$0.720 \pm 0.010^{a*}$	0.711 ± 0.034^{a}	0.732 ± 0.025^{a}	$0.697 \pm 0.028^{a*}$
0.5	0.851 ± 0.043^{ab}	0.778 ± 0.024^{ab}	$0.651 \pm 0.028^{a*}$	0.606 ± 0.051^{a}	$0.618 \pm 0.020^{ab*}$	0.688 ± 0.036^{ab}	0.787 ± 0.025^{a}	0.721 ± 0.032^{a}	$0.658 \pm 0.032^{ab*}$
1	0.787 ± 0.046^{ab}	0.726 ± 0.027^{ab}	$0.612 \pm 0.027^{ab*}$	0.580 ± 0.049^{a}	$0.581 \pm 0.029^{b*}$	0.644 ± 0.033^{ab}	0.790 ± 0.023^{a}	0.675 ± 0.035^{a}	$0.626 \pm 0.037^{ab*}$
2.5	0.744 ± 0.053^{b}	0.670 ± 0.029^{b}	$0.526 \pm 0.029^{b*}$	0.540 ± 0.036^{a}	$0.433 \pm 0.028^{c*}$	0.605 ± 0.035^{b}	0.718 ± 0.040^{a}	$0.586 \pm 0.051^{a*}$	$0.542 \pm 0.045^{b*}$
5	0.147 ± 0.009^{c}	0.182 ± 0.076^{c}	0.054 ± 0.010^{c}	0.041 ± 0.007^{b}	0.045 ± 0.010^{d}	0.039 ± 0.008^{c}	0.049 ± 0.011^{b}	0.121 ± 0.052^{b}	0.162 ± 0.045^{c}
7.5	0.065 ± 0.010^{c}	0.040 ± 0.013^{c}	0.052 ± 0.009^{c}	0.068 ± 0.016^{b}	0.046 ± 0.012^{d}	0.062 ± 0.017^{c}	0.046 ± 0.011^{b}	0.114 ± 0.071^{b}	0.052 ± 0.014^{c}
10	0.059 ± 0.012^{c}	0.049 ± 0.012^{c}	0.059 ± 0.011^{c}	0.073 ± 0.018^{b}	0.047 ± 0.013^{d}	0.072 ± 0.018^{c}	0.057 ± 0.013^{b}	0.111 ± 0.068^{b}	0.051 ± 0.012^{c}
15	0.068 ± 0.011^{c}	0.052 ± 0.009^{c}	0.069 ± 0.010^{c}	0.081 ± 0.013^{b}	0.067 ± 0.010^{d}	0.085 ± 0.016^{c}	0.068 ± 0.011^{b}	0.070 ± 0.016^{b}	0.058 ± 0.010^{c}

$CuCl_2$ (mM)	R	1SHEEP-ERY	1PAPER-ERY	1CONTROL-ERY	2COW-ERY	2SHEEP-ERY	2PAPER-ERY	2.POULTRY-ERY	1PAPER-STR	2POULTRY-STR
0	0.854 ± 0.014^{a}	$0.584 \pm 0.013^{a*}$	$0.632 \pm 0.042^{a*}$	0.489 ± 0.014^{a}	0.567 ± 0.027^{a}	0.754 ± 0.021^{a}	0.564 ± 0.016^{a}	$0.697 \pm 0.019^{a*}$	$0.557 \pm 0.027^{a*}$	0.838 ± 0.017^{a}
0.5	0.789 ± 0.032^{ab}	$0.513 \pm 0.030^{ab*}$	0.598 ± 0.099^{a}	$0.496 \pm 0.015^{a*}$	$0.528 \pm 0.031^{a*}$	0.830 ± 0.025^{b}	$0.525 \pm 0.015^{a*}$	$0.656 \pm 0.012^{ab*}$	$0.469 \pm 0.038^{a*}$	0.736 ± 0.021^{b}
1	0.741 ± 0.035^{b}	0.488 ± 0.028^{b}	0.605 ± 0.104^{a}	$0.508 \pm 0.014^{a*}$	$0.510 \pm 0.028^{a*}$	0.828 ± 0.024^{b}	$0.534 \pm 0.014^{a*}$	0.645 ± 0.015^{ab}	$0.447 \pm 0.063^{a*}$	0.686 ± 0.020^{b}
2.5	0.677 ± 0.036^{b}	0.496 ± 0.019^{b}	0.610 ± 0.097^{a}	$0.508 \pm 0.017^{a*}$	0.522 ± 0.023^{a}	0.790 ± 0.015^{ab}	0.541 ± 0.012^{a}	0.618 ± 0.022^{b}	0.549 ± 0.132^{a}	0.647 ± 0.015^{b}
5	0.039 ± 0.003^{c}	0.048 ± 0.007^{c}	0.042 ± 0.009^{b}	0.042 ± 0.009^{b}	0.041 ± 0.007^{b}	0.041 ± 0.009^{c}	0.121 ± 0.052^{b}	0.058 ± 0.026^{c}	$0.068 \pm 0.005^{b**}$	0.086 ± 0.047^{c}
7.5	0.044 ± 0.005^{c}	0.065 ± 0.012^{c}	0.058 ± 0.014^{b}	0.040 ± 0.006^{b}	0.046 ± 0.011^{b}	0.030 ± 0.005^{c}	0.030 ± 0.007^{b}	0.018 ± 0.006^{c}	0.031 ± 0.009^{b}	0.028 ± 0.002^{c}
10	0.045 ± 0.004^{c}	0.058 ± 0.004^{c}	$0.047 \pm 0.009^{b*}$	0.037 ± 0.004^{b}	0.040 ± 0.005^{b}	0.032 ± 0.005^{c}	0.037 ± 0.007^{b}	0.028 ± 0.005^{c}	$0.028 \pm 0.003^{b*}$	0.029 ± 0.003^{c}
15	0.048 ± 0.004^{c}	$0.054 \pm 0.008^{c*}$	$0.059 \pm 0.007^{b*}$	0.047 ± 0.005^{b}	0.044 ± 0.004^{b}	0.042 ± 0.006^{c}	0.043 ± 0.004^{b}	0.029 ± 0.004^{c}	$0.040 \pm 0.005^{b*}$	0.044 ± 0.004^{c}

*Significantly less tolerance in comparison to the recipient strain (R).

** Significantly more tolerance in comparison to the recipient strain (R).

Table A4.3: **Average OD_{600} values and standard error of the mean (mean ± SEM) for transconjugants after 24 h of incubation with $ZnCl_2$.** Different letters indicate statistically significant ($p < 0.05$) different groups among means.

$ZnCl_2$ (mM)	R	1PAPER-AMP	1COW-CHL	1.SHEEP-CHL	1PAPER-CHL	2COW-CHL	2SHEEP-CHL	2PAPER-CHL	2CONTROL-CHL
0	0.817 ± 0.079^{a}	0.753 ± 0.088^{a}	0.650 ± 0.075^{a}	0.595 ± 0.094^{a}	0.445 ± 0.183^{a}	0.651 ± 0.097^{a}	0.608 ± 0.086^{a}	0.625 ± 0.089^{a}	0.569 ± 0.108^{a}
0.5	0.820 ± 0.017^{a}	0.797 ± 0.017^{a}	$0.624 \pm 0.020^{a*}$	$0.608 \pm 0.019^{a*}$	$0.586 \pm 0.012^{a*}$	$0.682 \pm 0.025^{a*}$	$0.607 \pm 0.032^{a*}$	$0.636 \pm 0.028^{a*}$	$0.572 \pm 0.025^{a*}$
1	0.723 ± 0.038^{a}	0.701 ± 0.045^{a}	0.494 ± 0.026^{a}	$0.526 \pm 0.034^{ab*}$	$0.478 \pm 0.022^{a*}$	$0.553 \pm 0.051^{a*}$	$0.525 \pm 0.045^{a*}$	$0.545 \pm 0.052^{a*}$	$0.501 \pm 0.043^{a*}$
2.5	0.32 ± 0.007^{b}	0.028 ± 0.006^{b}	0.039 ± 0.014^{b}	0.025 ± 0.004^{c}	0.022 ± 0.003^{b}	0.029 ± 0.011^{b}	0.024 ± 0.007^{b}	0.022 ± 0.006^{b}	0.029 ± 0.012^{b}
5	0.040 ± 0.006^{bc}	$0.020 \pm 0.003^{b*}$	0.031 ± 0.005^{b}	0.027 ± 0.003^{c}	0.040 ± 0.006^{b}	0.030 ± 0.007^{b}	0.025 ± 0.002^{b}	0.024 ± 0.003^{b}	0.027 ± 0.008^{b}
10	0.081 ± 0.018^{bc}	0.086 ± 0.042^{b}	0.052 ± 0.009^{b}	0.067 ± 0.013^{c}	0.105 ± 0.017^{b}	0.087 ± 0.014^{b}	0.186 ± 0.123^{ab}	0.159 ± 0.092^{b}	0.166 ± 0.110^{ab}
15	0.197 ± 0.064^{bc}	0.098 ± 0.024^{b}	0.148 ± 0.062^{bc}	0.267 ± 0.088^{bc}	0.140 ± 0.039^{ab}	0.131 ± 0.019^{b}	0.293 ± 0.166^{ab}	0.134 ± 0.024^{b}	0.335 ± 0.190^{ab}
25	0.235 ± 0.064^{c}	0.207 ± 0.105^{b}	0.279 ± 0.076^{c}	0.162 ± 0.104^{c}	0.058 ± 0.041^{b}	0.236 ± 0.118^{b}	0.338 ± 0.168^{ab}	0.202 ± 0.108^{b}	0.372 ± 0.140^{ab}

$ZnCl_2$ (mM)	R	1SHEEP-ERY	1PAPER-ERY	1CONTROL-ERY	2COW-ERY	2SHEEP-ERY	2PAPER-ERY	2.POULTRY-ERY	1PAPER-STR	2POULTRY-STR
0	0.890 ± 0.017^{a}	0.586 ± 0.008^{a}	0.621 ± 0.042^{a}	0.491 ± 0.019^{a}	0.555 ± 0.027^{ab}	0.744 ± 0.006^{a}	0.559 ± 0.009^{a}	0.689 ± 0.013^{a}	0.489 ± 0.031^{a}	0.855 ± 0.019^{a}
0.5	0.876 ± 0.012^{a}	$0.545 \pm 0.014^{a*}$	$0.621 \pm 0.059^{a*}$	$0.445 \pm 0.014^{ab*}$	$0.568 \pm 0.028^{a*}$	$0.693 \pm 0.022^{a*}$	$0.555 \pm 0.012^{a*}$	$0.656 \pm 0.009^{a*}$	$0.401 \pm 0.040^{a*}$	0.794 ± 0.014^{a}
1	0.694 ± 0.087^{a}	$0.384 \pm 0.021^{ab*}$	$0.428 \pm 0.049^{ab*}$	$0.336 \pm 0.045^{b*}$	$0.416 \pm 0.055^{b*}$	0.537 ± 0.104^{ab}	$0.375 \pm 0.048^{b*}$	$0.442 \pm 0.058^{b*}$	$0.350 \pm 0.051^{a*}$	0.560 ± 0.114^{b}
2.5	0.030 ± 0.004^{b}	0.024 ± 0.001^{c}	0.028 ± 0.003^{c}	0.027 ± 0.007^{c}	0.027 ± 0.005^{c}	0.026 ± 0.004^{c}	0.028 ± 0.006^{c}	0.034 ± 0.010^{c}	0.030 ± 0.004^{b}	0.021 ± 0.003^{c}
5	0.051 ± 0.013^{b}	$0.070 \pm 0.017^{bc*}$	$0.135 \pm 0.022^{cd*}$	0.067 ± 0.030^{c}	0.051 ± 0.015^{c}	0.071 ± 0.026^{c}	0.043 ± 0.015^{c}	0.029 ± 0.008^{c}	0.035 ± 0.008^{b}	0.019 ± 0.002^{c}
10	0.095 ± 0.027^{b}	0.290 ± 0.099^{ac}	0.177 ± 0.044^{cd}	0.094 ± 0.030^{c}	0.087 ± 0.028^{c}	0.138 ± 0.046^{c}	0.076 ± 0.022^{c}	0.046 ± 0.019^{c}	0.040 ± 0.007^{b}	0.040 ± 0.020^{c}
15	0.232 ± 0.095^{b}	0.390 ± 0.118^{ac}	0.279 ± 0.082^{bd}	0.115 ± 0.029^{c}	0.124 ± 0.029^{c}	0.299 ± 0.131^{bc}	0.091 ± 0.041^{c}	0.069 ± 0.047^{c}	0.061 ± 0.013^{b}	0.041 ± 0.007^{c}
25	0.089 ± 0.033^{b}	0.292 ± 0.122^{ac}	0.195 ± 0.022^{cd}	0.079 ± 0.035^{c}	0.160 ± 0.048^{c}	0.113 ± 0.038^{c}	0.075 ± 0.037^{c}	0.044 ± 0.032^{c}	0.134 ± 0.081^{b}	0.019 ± 0.030^{c}

*Significantly less tolerance in comparison to the recipient strain (R).

** Significantly more tolerance in comparison to the recipient strain (R)

Table A4.4: **Average OD_{600} values and standard error of the mean (mean ± SEM) for transconjugants after 24 h of incubation with $Pb(NO_3)_2$.** Different letters indicate statistically significant ($p < 0.05$) different groups among means.

$Pb(NO_3)_2$ (mM)	R	1PAPER-AMP	1COW-CHL	1.SHEEP-CHL	1PAPER-CHL	2COW-CHL	2SHEEP-CHL	2PAPER-CHL	2CONTROL-CHL
0	0.230 ± 0.018^{a}	0.205 ± 0.007^{a}	0.181 ± 0.007^{a}	0.134 ± 0.011^{a}	0.172 ± 0.008^{a}	0.172 ± 0.007^{a}	0.182 ± 0.014^{a}	0.164 ± 0.017^{a}	0.160 ± 0.008^{a}
0.25	0.221 ± 0.010^{a}	0.192 ± 0.008^{a}	0.161 ± 0.012^{a}	$0.122 \pm 0.003^{a*}$	$0.131 \pm 0.004^{ab*}$	0.143 ± 0.002^{a}	0.168 ± 0.009^{a}	$0.129 \pm 0.016^{ab*}$	0.142 ± 0.009^{a}
0.5	0.218 ± 0.012^{a}	0.180 ± 0.006^{a}	0.142 ± 0.007^{a}	$0.113 \pm 0.009^{a*}$	$0.131 \pm 0.008^{ab*}$	0.155 ± 0.010^{a}	0.160 ± 0.007^{a}	0.134 ± 0.015^{ab}	$0.125 \pm 0.010^{a*}$
1	0.209 ± 0.012^{a}	0.172 ± 0.009^{ab}	0.142 ± 0.007^{a}	$0.099 \pm 0.013^{ab*}$	$0.108 \pm 0.011^{b*}$	0.133 ± 0.005^{a}	0.147 ± 0.005^{ab}	$0.113 \pm 0.012^{abc*}$	$0.119 \pm 0.011^{a*}$
2.5	0.225 ± 0.014^{a}	0.192 ± 0.012^{a}	0.141 ± 0.030^{a}	$0.112 \pm 0.020^{a*}$	0.121 ± 0.019^{ab}	0.133 ± 0.019^{a}	0.163 ± 0.017^{a}	0.128 ± 0.019^{ab}	$0.128 \pm 0.022^{a*}$
5	0.222 ± 0.018^{a}	0.205 ± 0.011^{a}	0.160 ± 0.021^{a}	$0.139 \pm 0.013^{a*}$	0.153 ± 0.019^{ab}	0.156 ± 0.019^{a}	0.163 ± 0.011^{a}	0.149 ± 0.015^{ab}	$0.138 \pm 0.014^{a*}$
7.5	0.096 ± 0.024^{b}	0.122 ± 0.024^{bc}	0.040 ± 0.007^{b}	0.046 ± 0.015^{b}	0.032 ± 0.004^{c}	0.031 ± 0.009^{b}	0.072 ± 0.039^{bc}	0.065 ± 0.037^{bc}	0.079 ± 0.037^{ab}
10	0.075 ± 0.011^{b}	0.075 ± 0.014^{c}	0.040 ± 0.007^{b}	0.043 ± 0.010^{b}	0.042 ± 0.013^{c}	$0.025 \pm 0.008^{b*}$	$0.016 \pm 0.008^{c*}$	$0.030 \pm 0.006^{c*}$	$0.023 \pm 0.007^{b*}$

$Pb(NO_3)_2$ (mM)	R	1SHEEP-ERY	1PAPER-ERY	1CONTROL-ERY	2COW-ERY	2SHEEP-ERY	2PAPER-ERY	2.POULTRY-ERY	1PAPER-STR	2POULTRY-STR
0	0.251 ± 0.013^{a}	0.111 ± 0.006^{ab}	0.116 ± 0.012^{ab}	0.103 ± 0.010^{ab}	0.112 ± 0.009^{ab}	0.219 ± 0.006^{a}	$0.151 \pm 0.014^{a*}$	$0.156 \pm 0.004^{ab*}$	0.095 ± 0.011^{a}	0.197 ± 0.003^{a}
0.25	0.220 ± 0.010^{a}	$0.094 \pm 0.004^{ab*}$	$0.101 \pm 0.010^{ab*}$	$0.086 \pm 0.006^{ab*}$	$0.101 \pm 0.009^{ab*}$	0.199 ± 0.006^{ab}	$0.136 \pm 0.012^{a*}$	0.146 ± 0.005^{abv}	$0.080 \pm 0.010^{a*}$	0.176 ± 0.002^{a}
0.5	0.218 ± 0.012^{a}	$0.095 \pm 0.003^{ab*}$	$0.096 \pm 0.010^{ab*}$	$0.088 \pm 0.007^{ab*}$	$0.095 \pm 0.005^{a*}$	0.197 ± 0.006^{ab}	$0.129 \pm 0.010^{a*}$	$0.150 \pm 0.006^{ab*}$	$0.087 \pm 0.012^{a*}$	0.168 ± 0.003^{a}
1	0.219 ± 0.009^{a}	$0.100 \pm 0.006^{ab*}$	$0.098 \pm 0.004^{ab*}$	$0.085 \pm 0.007^{ab*}$	$0.095 \pm 0.003^{a*}$	$0.168 \pm 0.005^{bc*}$	$0.126 \pm 0.009^{a*}$	$0.158 \pm 0.007^{ab*}$	$0.102 \pm 0.013^{a*}$	0.173 ± 0.006^{a}
2.5	0.224 ± 0.013^{a}	$0.104 \pm 0.006^{ab*}$	$0.106 \pm 0.008^{ab*}$	$0.095 \pm 0.011^{ab*}$	$0.104 \pm 0.008^{ab*}$	$0.166 \pm 0.011^{bc*}$	$0.130 \pm 0.005^{a*}$	$0.164 \pm 0.008^{ab*}$	$0.106 \pm 0.014^{a*}$	0.174 ± 0.005^{a}
5	0.239 ± 0.017^{a}	$0.140 \pm 0.016^{b*}$	$0.137 \pm 0.014^{a*}$	$0.124 \pm 0.013^{a*}$	$0.156 \pm 0.019^{b*}$	0.197 ± 0.018^{ab}	$0.160 \pm 0.010^{a*}$	0.181 ± 0.010^{b}	$0.111 \pm 0.015^{a*}$	0.186 ± 0.023^{a}
7.5	0.115 ± 0.022^{b}	0.092 ± 0.023^{ab}	0.086 ± 0.019^{ab}	0.082 ± 0.022^{ab}	0.090 ± 0.021^{a}	0.132 ± 0.007^{c}	0.128 ± 0.008^{a}	0.140 ± 0.018^{b}	0.077 ± 0.020^{a}	0.080 ± 0.026^{b}
10	0.079 ± 0.016^{b}	0.053 ± 0.016^{c}	0.064 ± 0.025^{b}	0.056 ± 0.019^{b}	0.066 ± 0.019^{a}	0.067 ± 0.013^{d}	0.067 ± 0.010^{b}	0.113 ± 0.020^{a}	0.086 ± 0.037^{a}	0.041 ± 0.009^{b}

*Significantly less tolerance in comparison to the recipient strain (R).

** Significantly more tolerance in comparison to the recipient strain (R).

Chapter 5.
Conclusions

Chapter 5. Conclusions

Conclusion 1. PAPER was the most efficient treatment regarding the long-term reduction of heavy metal bioavailability (Cd, Pb and Zn) in soil as part of an aided phytostabilization field assay.

Conclusion 2. PAPER treatment displayed the most consistent recovery of soil quality especially in the less contaminated site. The microbial activity, biomass and diversity were clearly enhanced as a result of the reduction in heavy metal bioavailability and, at least partly, due to the highest input of easily biodegradable OM.

Conclusion 3. The detection of conjugative/mobilizable IncP and IncQ plasmids, class 1 integron specific sequence and QAC resistance gene in the heavy metal contaminated soils (treated or untreated with organic amendments) indicates a gene-mobilizing capacity of the bacterial community in these soils with implications for potential antibiotic resistance dissemination.

Conclusion 4. Out of these soils, 17 *E. coli* transconjugants were obtained by HGT under ampicillin, erythromycin, chloramphenicol or streptomycin selection. Most of the transconjugants displayed a multi-resistance phenotype against a broad spectrum of antibiotics. Additionally, transconjugant 1PAPER-STR showed an acquired resistance to Cu.

Conclusion 5. The acquisition of plasmids by HGT affected the fitness of the host to different extents depending, primarily, on the phenotypic changes and the interaction of the new acquired genes with the host genome. Transconjugant 1PAPER-STR seemed to have the worst physiological state as result of fitness costs of acquiring additional plasmid/s.

Chapter 6.
References

Chapter 6. References

Adamczyk M. and Jagura-Burdzy G. (2003) Spread and survival of promiscuous IncP-1 plasmids. *Acta Biochim Pol*; 50: 425-453.

Adriano D. C., Wenzel W. W., Vangronsveld J. and Bolan N. S. (2004) Role of assisted natural remediation in environmental cleanup. *Geoderma*; 122: 121-142.

Akob D. M., Mills H. J. and Kostka J. E. (2007) Metabolically active microbial communities in uranium-contaminated subsurface sediments. *FEMS Microbiol Ecol*; 59: 95-107.

Alkorta I., Becerril J. M. and Garbisu C. (2010) Phytostabilization of metal contaminated soils. *Rev Environ Health*; 25: 135-146.

Allen H. K., Moe L. A., Rodbumrer J., Gaarder A. and Handelsman J. (2009) Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. *ISME J*; 3: 243-251.

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

Allison K. R., Brynildsen M. P. and Collins J. J. (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature*; 473: 216-220.

Alonso A., Rojo F. and Martinez J. L. (1999) Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin. *Environ Microbiol*; 1: 421-430.

Alvarenga P., Gonçalves A. P., Fernandes R. M., de Varennes A., Vallini G., Duarte E. and Cunha-Queda A. C. (2008) Evaluation of composts and liming materials in the phytostabilization of a mine soil using perennial ryegrass. *Sci Total Environ*; 406: 43-56.

Alvarenga P., Gonçalves A. P., Fernandes R. M., de Varennes A., Vallini G., Duarte E., Cunha-Queda A. C. (2009) Organic residues as immobilizing agents in aided phytostabilization: (I) Effects on soil chemical characteristics. *Chemosphere*; 74: 1292-1300.

Aminov R. I. and Mackie R. I. (2007) Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol Lett*; 271: 147-161.

Aminov R. I. (2009) The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol;* 11: 2970-2988.

Andersson D. I. (2006) The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr Opin Microbiol*; 9: 461-465.

Andersson D. I. and Hughes D. (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol*; 8: 260-271.

Andersson D. I. and Hughes, D. (2012) Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resist Updat*; 15: 162-172.

Andersson D. I. and Hughes D. (2014) Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol*; 12: 465-478.

Anjum R., Grohmann E. and Malik A. (2011) Molecular characterization of conjugative plasmids in pesticide tolerant and multi-resistant bacterial isolates from contaminated alluvial soil. *Chemosphere*; 84: 175-181.

Anjum R., Grohmann E. and Malik A. (2012) Exogenous isolation of conjugative plasmids from pesticide contaminated soil. *World J Microb Biot*; 28: 567-574.

Ansari M. I., Grohmann E. and Malik A. (2008) Conjugative plasmids in multi-resistant bacterial isolates from Indian soil. *J Appl Microbiol*; 104: 1774-1781.

Arias C. A., Contreras G. A. and Murray B. E. (2010) Management of multidrug-resistant enterococcal infections. *Clin Microbiol Infect*; 16: 555-562.

Arienzo M., Adamo P. and Cozzolino V. (2004) The potential of *Lolium perenne* for revegetation of contaminated soils from a metallurgical site. *Sci Total Environ*; 319: 13-25.

Ash R. J., Mauck B. and Morgan M. (2002) Antibiotic resistance of Gram-negative bacteria in rivers, United States. *Emerg Infect Dis*; 8: 713-716.

Austin D. J., Kakehashi M. and Anderson R. M. (1997) The transmission dynamics of antibiotic–resistant bacteria: the relationship between resistance in commensal organisms and antibiotic consumption. *Proc R Soc Lond (Biol)*; 264: 1629-1638.

Ayala A., Muñoz M. F. and Arguëlles S. (2014) Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev*; 2014: 1-31.

Bahl M. I., Burmølle M., Meisner A., Hansen L. H., Sørensen S. J. (2009) All IncP-1 plasmid subgroups, including the novel ε subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid*; 62: 134-139.

Bais H. P., Weir T. L., Perry L. G., Gilroy S. and Vivanco J. M. (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol*; 57: 233-266.

Baker-Austin C., Wright M. S., Stepanauskas R. and McArthur J. V. (2006) Co-selection of antibiotic and metal resistance. *Trends Microbiol*; 14: 176-182.

Baltrus D. A. (2013) Exploring the costs of horizontal gene transfer. *Trends Ecol Evol*; 28: 489-495.

Bamborough L. and Cummings S. (2009) The impact of increasing heavy metal stress on the diversity and structure of the bacterial and actinobacterial communities of metallophytic grassland soil. *Biol Fertil Soils*; 45: 273-280.

Baquero F., Negri M. C., Morosini M. I. and Blázquez J. (1993) Effect of selective antibiotic concentrations on the evolution of antimicrobial resistance. *APUA Newsletter*; 11: 4-5.

Baquero F. (2001) Low-level antibacterial resistance: a gateway to clinical resistance. *Drug Resist Updat*; 4: 93-105.

Baquero M. R., Nilsson A. I., Turrientes M. del C., Sandvang D., Galan J. C., Martinez J. L., Frimodt-Moller N., Baquero F. and Andersson D. I. (2004) Polymorphic mutation frequencies in *Escherichia coli*: emergence of weak mutators in clinical isolates. *J Bacteriol*; 186: 5538-5542.

Baquero F., Martínez J. L. and Cantón R. (2008) Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol*; 19: 260-265.

Baquero F., Alvarez-Ortega C. and Martinez J. L. (2009) Ecology and evolution of antibiotic resistance. *Environ Microbiol Rep*; 1: 469-476.

Barber M. and Rozwadowska-Dowzenko M. (1948) Infection by penicillin-resistant staphylococci. *Lancet*; 2: 641-644.

Barlow R. S., Fegan N. and Gobius K. S. (2009) Integron-containing bacteria in faeces of cattle from different production systems at slaughter. *J Appl Microbiol*; 107: 540-545.

Barns S. M., Cain E. C., Sommerville L. and Kuske C. R. (2007) Acidobactetia phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. *Appl Environ Microbiol*; 73: 3113-3116.

Barraud O., Baclet M. C., Denis F. and Ploy M. C. (2010) Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. *J Antimicrob Chemother*; 65: 1642-1645.

Barraud O., Badell E., Denis F., Guiso N. and Ploy, M. C. (2011) Antimicrobial drug resistance in *Corynebacterium diphtheriae mitis*. *Emerging Infect Dis*; 17: 2078-2080.

Barrutia O., Artetxe U., Hernández A., Olano J. M., García-Plazaola J. I., Garbisu C. and Becerril J. M. (2011a) Native plant communities in an abandoned Pb-Zn mining area of northern Spain: implications for phytoremediation and germplasm preservation. *Int J Phytoremediation*; 13: 256-270.

Bass L., Liebert C. A., Lee M. D., Summers A. O., White D. G., Thayer S. G. and Maurer J. J. (1999) Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in *avian Escherichia coli*. *Antimicrob Agents Chemother*; 43: 2925-2929.

Battaglia A., Calace N., Nardo E., Petronio B. M. and Pietroletti M. (2007) Reduction of Pb and Zn bioavailable forms in metal polluted soils due to paper mill sludge addition. Effects on Pb and Zn transferability to barley. *Bioresour Technol*; 98: 2993-2999.

Becerra-Castro C., Monterroso C., Prieto-Fernández A., Rodríguez-Lamas L., Loureiro-Viñas M., Acea M. J. and Kidd P. S. (2012) Pseudometallophytes colonising Pb/Zn mine tailings: A description of the plant-microorganism-rhizosphere soil system and isolation of metal-tolerant bacteria. *J Hazard Mater*; 217-218: 350-359.

Béjà O., Suzuki M. T., Heidelberg J. F., Nelson W. C., Preston C. M., Hamada T., Eisen J. A., Fraser C. M. and DeLong E. F. (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature*; 415: 630-633.

Berg J., Tom-Petersen A. and Nybroe O. (2005) Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. *Lett Appl Microbiol*; 40:146-151.

Berg J., Thorsen M. K., Holm P. E., Jensen J., Nybroe O. and Brandt K. K. (2010) Cu exposure under field conditions co-selects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environ Sci Technol*; 44: 8724-8728.

Bergstrom C. T., Lipsitch M. and Levin B. R. (2000) Natural selection, infectious transfer and the existence conditions for bacterial plasmids. *Genetics*; 155: 1505-1519.

Bertels F. and Rainey P. B. (2011). Within-genome evolution of REPINs: a new family of miniature mobile DNA in bacteria. *PLoS Genet*; 7: e1002132.

Besser J. M., Brumbaugh W. G., Allert A. L., Schmitt C. J. and Ingersoll C. G. (2009) Ecological impacts of lead mining on Ozark streams-toxicity of sediment and pore water. *Ecotoxicol Environ Saf*; 72: 516-526.

Bhullar K., Waglechner N., Pawlowski A., Koteva K., Banks E. D., Johnston M. D., Barton H. A. and Wright G. D. (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS ONE*; 7: e34953.

Binh C. T. T., Heuer H., Kaupenjohann M. and Smalla K. (2008) Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol Ecol*; 66: 25-37.

Binh C. T. T., Heuer H., Kaupenjohann M. and Smalla K. (2009) Diverse *aadA* gene cassettes on class 1 integrons introduced into soil via spread manure. *Res Microbiol*; 160: 427-433.

Björnsson L., Hugenholtz P., Tyson G. W. and Blackall L. L. (2002) Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. *Microbiology*; 148: 2309-2318.

Böckelmann U., Szewzyk U and Grohmann E. (2003) A new enzymatic method for the detachment of particle associated soil bacteria. *J Microbiol Methods*; 55: 201-211.

Bolan N., Adriano D. and Mahimairaja S. (2004) Distribution and bioavailability of trace elements in livestock and poultry manure by-products. *Crit Rev Environ Sci Technol*; 34: 291-338.

Bolan N. S., Park J. H., Robinson B., Naidu R. and Huh K. Y. (2011) Phytostabilization: a green approach to contaminant containment. *Adv Agron*; 112: 145-204.

BOPV (2005) Prevención y corrección de la contaminación del suelo. Ley 1/2005 de 4 de febrero. *Boletín Oficial del País Vasco*; nº32, 16 de febrero de 2005.

Boucher Y., Labbate M., Koenig J. E. and Stokes H. W. (2007) Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends Microbiol*; 15: 301-309.

Bouma J. E. and Lenski R. E. (1988) Evolution of a bacteria/plasmid association. *Nature*; 335: 351-352.

Bouskill N., Barker-Finkel J., Galloway T. S., Handy R. D. and Ford T. E. (2010) Temporal bacterial diversity associated with metal-contaminated river sediments. *Ecotoxicology*; 19: 317-328.

Bragg J. G. and Wagner A. (2009) Protein material costs: single atoms can make an evolutionary difference. *Trends Genet*; 25: 5-8.

Brevik E. C. and Sauer T. J. (2015) The past, present, and future of soils and human health studies. *Soil*; 1: 35-46.

Brown J. R. (2003) Ancient horizontal gene transfer. *Nat Rev Genet*; 4: 121-132.

Bruins M. R., Kapil S. and Oehme F. W. (2000) Microbial resistance to metals in the environment. *Ecotox Environ Safe*; 45: 198-207.

Buckley U. (1984) RNA-DNA ratio: an index of larval fish growth in the sea. *Mar Biol*; 80: 291-298.

Bulow F. J. (1970) RNA-DNA ratios as indicators of recent growth rates of a fish. *J Fish Res Board Can*; 27: 2343-2349.

Burmølle M., Webb J. S., Rao D., Hansen L. H., Sørensen S. J. and Kjelleberg S. (2006) Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol*; 72: 3916-3923.

Butler E., Whelan M. J., Ritz K., Sakrabani R. and van Egmond R. (2011) Effects of triclosan on soil microbial respiration. *Environ Toxicol Chem*; 30: 360-366.

Byrne-Bailey K. G., Gaze W. H., Kay P., Boxall A. B., Hawkey P. M. and Wellington E. M. (2009) Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob Agents Chemother*; 53: 696-702.

Byrne-Bailey K. G., Gaze W. H., Zhang L., Kay P., Boxall A. B., Hawkey P. M. and Wellington E. M. H. (2010) Integron prevalence and diversity in manured soil. *Appl Environ Microbiol*; 77: 684-687.

Caille O., Rossier C. and Perron K. (2007) A copper-activated two-component system interacts with zinc and imipenem resistance in *Pseudomonas aeruginosa*. *J Bacteriol*; 183: 4561-4568.

Campbell V., Murphy G. and Romanuk T. N. (2011) Experimental design and the outcome and interpretation of diversity-stability relations. *Oikos*; 120: 399-408.

Caporaso J. G., Lauber C. L., Walters W. A., Berg-Lyons D., Lozupone C. A., Turnbaugh P. J., Fierer N. and Knight R. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA;* 108; 4516-4522.

Caporaso J. G., Lauber C. L., Walters W. A., Berg-Lyons D., Huntley J., Fierer N., Owens S. M., Betley J., Fraser L., Bauer M., Gormley N., Gilbert J. A., Smith G. and Knightcs R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*; 6: 1621-1624.

Canali S. and Benedetti A. (2005) Soil Nitrogen Mineralization. In: Bloem J., Hopkins D. H. and Anna B. (Eds.) *Microbiological Methods for Assesing Soil Quality*. CABI Publishing, Cambridge, USA: 127-135.

Castaneda-Garcia A., Do T. T. and Blazquez J. (2011) The K+ uptake regulator TrkA controls membrane potential, pH homeostasis and multidrug susceptibility in *Mycobacterium smegmatis*. *J Antimicrob Chemother*; 66: 1489-1498.

Chang X. S., Meyer M. T., Liu X. Y., Zhao Q., Chen H., Chen J. A., Qiu Z., Yang L., Cao J. and Shu W. (2010) Determination of antibiotics in sewage from hospitals, nursery and

slaughterhouse, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. *Environ Pollut*; 158: 1444-1450.

Chapman J. S. (2003) Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int Biodeterior Biodegradation*; 51: 271-276.

Chapman P. M. (2007) Determining when contamination is pollution-weight of evidence determinations for sediments and effluents. *Environ Int*; 33: 492-501.

Chee-Sanford J. C., Mackie R. I., Koike S., Krapac I. G., Lin Y. F., Yannarell A. C., Maxwell S. and Aminov R. I. (2009) Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *J Environ Qual*: 38: 1086-1108.

Chou H. H., Chiu H. C., Delaney N. F., Segrè D. and Marx C. J. (2011) Diminishing returns epistasis among beneficial mutations decelerates adaptation. *Science*; 332: 1190-1192.

Christensen G. D., Simpson W. A., Younger J. J., Baddour L. M., Barrett F. F., Melton D. M. and Beachey E. H. (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol*; 22: 996-1006.

Christie P. J., Atmakuri K., Krishnamoorthy V., Jakubowski S. and Cascales E. (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu Rev Microbiol*; 59: 451-485.

Christie P. J., Whitaker N. and González-Rivera C. (2014) Mechanism and structure of the bacterial type IV secretion systems. *Biochim Biophys Acta*; 1843: 1578-1591.

Clatworthy A. E., Pierson E. and Hung D. T. (2007) Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol*; 3: 541-548.

Clemmesen *C.*, Bühler V., Carvalho G., Case R., Evans G., Hauser L., Hutchinson W. F., Kjesbu O. S., Mempel H., Moksness E., Otteraa H., Paulsen H., Thorsen A. and Svaasand T. (2003) Variability in condition and growth of Atlantic cod larvae and juveniles reared in mesocosms: environmental and maternal effects. *J Fish Biol*; 62: 706-723.

Cleven B. E. E., Palka-Santini M., Gielen J., Meembor S., Krönke M. and Krut O. (2006) Identification and Characterization of Bacterial Pathogens Causing Bloodstream Infections by DNA Microarray. *J Clin Microbiol*; 44: 2389-2397.

Collis C. M. and Hall R. M. (2004) Comparison of the structure- activity relationships of the integron-associated recombination sites attI3 and attI1 reveals common features. *Microbiology*; 150: 1591-1601.

Colpaert J. V. (1998) Biological Interactions: The Significance of Root-Microbial Symbioses for Phytorestoration of Metal-Contaminated Soils. In: Vangronsveld J. and Cunningham S. C. (Eds.) *Metal-Contaminated Soils: In situ Inactivation and Phytorestoration*. Springer-Verlag, Berlin, Heidelberg: 75-84.

Corsaro D. and Greub G. (2006) Pathogenic potential of novel *Chlamydiae* and diagnostic approaches to infections due to these obligate intracellular bacteria. *Clin Microbiol Rev*; 19: 283-297.

Costello E. K. and Schmidt S. K. (2006) Microbial diversity in alpine tundra wet meadow soil: novel *Chloroflexi* from a cold, water-saturated environment. *Env Microbiol*; 8: 1471-1486.

Couturier M., Bahassi E. M. and Van Melderen L. (1998) Bacterial death by DNA gyrase poisoning. *Trends Microbiol*; 6: 269-275.

Creamer R. E., Brennan F., Fenton O., Healy M. G., Lalor S. T. J., Lanigan G. J., Regan J. T. and Griffiths B. S. (2010) Implications of the proposed Soil Framework Directive on agricultural systems in Atlantic Europe-a review. *Soil Use Manage*; 26: 198-211.

Dahlberg C. and Chao L. (2003) Amelioration of the cost of conjugative plasmid carriage in *Escherichia coli* K12. *Genetics*; 165: 1641-1649.

Daikos G. L., Kosmidis C., Tassios P. T., Petrikkos G., Vasilakopoulou A., Psychogiou M., Stefanou I., Avlami A. and Katsilambros N. (2007) Enterobacteriaceae Blood- stream infections: presence of integrons, risk factors, and outcome. *Antimicrob Agents Chemother*; 51: 2366-2372.

Dalkmann P., Broszat M., Siebe C., Willaschek E., Sakinc T., Huebner J., Amelung W., Grohmann E. and Siemens J. (2012) Accumulation of pharmaceuticals, Enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. *PLoS ONE*; 7: e45397.

Dang H., Ren J., Song L., Sun S. and An L. (2008) Dominant chloramphenicol-resistant bacteria and resistance genes in coastal marine waters of Jiaozhou Bay, China. *World J Microbiol Biotechnol*; 24: 209-217.

Datta S. and Wattal C. (2010) Carbapenemase producing Gram-negative bacteria in tertiary health care setting: therapeutic challenges. *JIMSA*; 23: 17-20.

Davies J., Spiegelman G. B. and Yim G. (2006) The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol*; 9: 445-453.

Davies J. and Davies D. (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev*; 74: 417-433.

Davies J. (2013) Specialized microbial metabolites: functions and origins. *J Antibiot*; 66: 361-364.

Davis K. E. R., Sangwan P. and Janssen P. H. (2011) *Acidobacteria*, *Rubrobacteridae* and *Chloroflexi* are abundant among very slow-growing and mini-colony-forming soil bacteria. *Environ Microbiol*; 13: 798-805.

Dawson W., Rohr R. P., van Kleunen M. and Fischer M. (2012) Alien plant species with a wider global distribution are better able to capitalize on increased resource availability. *New Phytol*; 194: 859-867.

D'Costa V. M., McGrann K. M., Hughes D. W. and Wright G. D. (2006) Sampling the antibiotic resistome. *Science*; 311: 374-377.

D'Costa V. M., Kin C. E., Kalan L., Morar M., Sung W. W., Schwarz C., Froese D., Zazula G., Calmels F., Debruyne R., Golding G. B., Poinar H. N. and Wright G. D. (2011) Antibiotic resistance is ancient. *Nature*; 477: 457-461.

Dealtry S., Ding G. C., Weichelt V., Dunon V., Schlüter A., Martini M. C., Del Papa M. F., Lagares A., Amos G. C., Wellington E. M., Gaze W. H., Sipkema D., Sjöling S., Springael D., Heuer H., van Elsas J. D., Thomas C. and Smalla K. (2014) Cultivation-independent screening revealed hot spots of IncP-1, IncP-7 and IncP-9 plasmid occurrence in different environmental habitats. *PLoS One*; 2: e89922.

DeBruyn J. M., Nixon L. T., Fawaz M. N., Johnson A. M. and Radosevich M. (2011) Global biogeography and quantitative seasonal dynamics of *Gemmatimonadetes* in soil. *Appl Environ Microb*; 77: 6295-6300.

De Lipthay J. R., Rasmussen L. D., Oregaard G., Simonsen K., Bahl M. I., Kroer N. and Sorensen S. J. (2008) Acclimation of subsurface microbial communities to mercury. *FEMS Microbiol Ecol*; 65: 145-155.

Demanèche S., Sanguin H., Poté J., Navarro E., Bernillon D., Mavingui P., Wildi W., Vogel T. M. and Simonet P. (2008) Antibiotic-resistant soil bacteria in transgenic plant fields. *Proc Natl Acad Sci USA*; 105: 3957-3962.

Deshpande L. M., Fritsche T. R., Moet G. J., Biedenbach D. J. and Jones R. N. (2007) Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. *Diagn Microbiol Infect Dis*; 58: 163-170.

Diaz-Ricci J. C. and Hernández M. E. (2000) Plasmid effects on *Escherichia coli* metabolism. *Crit Rev Biotechnol*; 20: 79-108.

Dick R. P., Breakwell D. P. and Turco R. F. (1996) Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran, J. W. and Jones, A. J. (Eds.) *Methods for Assessing Soil Quality.* SSSA Special Publ. 49. SSSA, Madison, WI: 247-271.

Ding G. C., Radl V., Schloter-Hai B., Jechalke S., Heuer H., Smalla K. and Schloter M. (2014) Dynamics of soil bacterial Communities in response to repeated application of manure containg sulfadiazine. *PLoS One*; 9: e92958.

Dionisio F., Conceição I. C., Marques A. C., Fernandes L. and Gordo I. (2005) The evolution of a conjugative plasmid and its ability to increase bacterial fitness. *Biol Lett*; 1: 250-252.

Di Rosa R., Creti R., Venditti W., Amelio R. D., Arciola C. R., Montanaro L. and Baldassari L. (2006) Relationship between biofilm formation, the enterococcal surface protein and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. *FEMS Microbiol Lett*; 256: 145-150.

Djordjevic S. P., Stokes H. W. and Roy Chowdhury, P. (2013) Mobile elements, zoonotic pathogens and commensal bacteria: conduits for the delivery of resistance genes into humans, production animals and soil microbiota. *Front Microbiol*; 4: 86.

Doelman P., Jansen E., Michels M. and van Til M. (1994) Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. *Biol Fertil Soils*; 17: 177-1784.

Dong J., Mao W. H., Zhang G. P., Wu F. B. and Cai Y. (2007) Root excretion and plant tolerance to cadmium toxicity-a review. *Plant Soil Environ*; 53: 193-200.

Doran J. W. and Zeiss M. R. (2000) Soil health and sustainability: managing the biotic component of soil quality. *Appl Soil Ecol*; 15: 3-11.

Dortch Q. F., Roberts T. L., Clayton J. R. Jr. and Ahmed S. I. (1983) RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Mar Ecol Prog Ser*; 13: 67-71.

Dröge M., Pühler A. and Selbitschka W. (2000) Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol Gen Genet*; 3: 1-9.

Edgar R. C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods*; 10: 996-998.

Elser J. J., Watts T., Bitler B. and Markow T. A. (2006) Ontogenetic coupling of growth rate with RNA and P contents in five species of*Drosophila*. *Ecology*; 2: 846-856.

Elser J. J., Kyle M. M., Smith M. S. and Nagy J. D. (2007) Biological stoichiometry in human cancer. *PLoS ONE*; 2: e1028.

Epelde L., Becerril J. M., Mijangos I. and Garbisu C. (2009a) Evaluation of the efficiency of a phytostabilization process with biological indicators of soil health. *J Environ Qual*; 38: 2041-2049.

Epelde L., Becerril J. M., Alkorta I. and Garbisu C. (2009b) Heavy metal phytoremediation: microbial indicators of soil health for the assessment of remediation efficiency. In: Singh A., Kuhad R. C. and Ward O. P. (Eds.) *Advances in Applied Bioremediation*. Springer-Verlag, Berlin: 299-313.

Epelde L., Becerril J. M., Barrutia O., González-Oreja J. A. and Garbisu C. (2010) Interactions between plant and rhizosphere microbial communities in a metalliferous soil. *Environ Pollut*; 158: 1576-1583.

Epelde L., Burges A., Mijangos I. and Garbisu C. (2014a) Microbial properties and attributes of ecological relevance for soil quality monitoring during a chemical stabilization field study. *Appl Soil Ecol*; 75: 1-12.

Epelde L., Becerril J. M., Alkorta I. and Garbisu, C. (2014b) Adaptive long-term monitoring of soil health in metal phytostabilization: ecological attributes and ecosystem services based on soil microbial parameters. *Int J Phytorem*; 16: 971-981.

Epelde L., Lanzén A., Blanco F., Urich T. and Garbisu C. (2015) Adaptation of soil microbial community structure and function to chronic metal contamination at an abandoned Pb-Zn mine. *FEMS Microbiol Ecol*; 91: 1-11.

Ernst W. H. O. (2005) Phytoextraction of mine wastes - options and impossibilities. *Chem Erde*; 65: 29-42.

Fajardo C., Ortíz L. T., Rodríguez-Membibre M. L., Nande M., Lobo M. C. and Martin M. (2012) Assessing the impact of zero-valent iron (ZVI) nanotechnology on soil microbial structure and functionality: A molecular approach. *Chemosphere*; 86: 802-808.

Fernández-Calviño D., Arias-Estévez M., Díaz-Raviña M. and Bååth E. (2011) Bacterial pollution induced community tolerance (PICT) to Cu and interactions with pH in long-term polluted vineyard soils. *Soil Biol Biochem*; 43: 2324-2331.

Fleming A. (1929) On the antibacterial action of cultures of a Penicillium, with a special reference to their use in the isolation of *B. influenze*. *Brit J Exp Path*; 10: 226-236.

Fleming A. (1932) On the specific antibacterial properties of penicillin and potassium tellurite. Incorporating a method of demonstrating some bacterial antagonisms. *J Pathol*; 35: 831-842.

Forsberg K. J., Reyes A., Wang B., Selleck E. M., Sommer M. O. A. and Dantas G. (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science*; 337: 1107-1111.

Foyer C. H. and Noctor G. (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid Redox Signal*; 11: 861-905.

Furuya E. Y. and Lowy F. D. (2006) Antimicrobial-resistant bacteria in the community setting. *Nat Rev Microbiol*; 4: 36-45.

Gadd G. M. (2009) Biosorption: critical review of scientific rationale, environmental importance and significance for pollution treatment. *J Chem Technol Biotechnol*; 84: 3-28.

Gaillard M., Pernet N., Vogne C., Hagenbüchle O. and van der Meer J. R. (2008) Host and invader impact of transfer of the clc genomic island into *Pseudomonas aeruginosa* PAO1. *Proc Natl Acad Sci USA*; 105: 7058-7063.

Galan A., Garcia-Bermejo L., Troyano A., Vilaboa N. E., Fernandez C., de Blas E. and Aller P. (2001) The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays). *Europ J Cell Biol*; 80: 312-320.

Galende M. A., Becerril J. M., Barrutia O., Artetxe U., Garbisu C. and Hernandez A. (2014a) Field assessment of the effectiveness of organic amendmendts for aided phytostabilization of a Pb-Zn contaminated mine soil. *J Geochem Explor*; 145: 181-189.

Galende M. A., Becerril J. M., Gómez-Sagasti M. T., Barrutia O., Garbisu C. and Hernandez A. (2014b) Agro-industrial wastes as effective amendments for ecotoxicity reduction and soil health improvement in aided phytostabilization. *Environ Sci Pollut Res*; 21: 10036-10044.

Garbisu C., Hernández-Allica J., Barrutia O., Alkorta I. and Becerril J. M. (2002) Phytoremediation: a technology using green plants to remove contaminants from polluted areas. *Rev Environ Health*; 17: 173-188.

Garbisu C., Alkorta I. and Epelde L. (2011) Assessment of soil quality using microbial properties and attributes of ecological relevance. *Appl Soil Ecol*; 49: 1-4.

García-Sánchez A., Moyano A. and Muñez C. (1999) Forms of cadmium, lead, and zinc in polluted mining soils and uptake by plants (Soria Province, Spain). *Commun Soil Sci Plant Anal*; 30: 1385-1402.

Gaze W. H., Zhang L., Abdouslam N. A., Hawkey P. M., Calvo-Bado L., Royle J., Brown H., Davis S., Kay P., Boxall A. B. A. and Wellington E. M. (2011) Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J*; 5: 1253-1261.

Geier H., Mostowy S., Cangelosi G. A., Behr M. A. and Ford T. E. (2008) Autoinducer-2 triggers the oxidative stress response in *Mycobacterium avium*, leading to biofilm formation. *Appl Environ Microbiol*; 74: 1798-1804.

Geslin C., Llanos J., Prieur D. and Jeanthon C. (2001) The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. *Res Microbiol*; 152: 901-905.

Ghosh S. and LaPara T. M. (2007) The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J*; 1: 191-203.

Giedraitienè A., Vitkauskienè A., Naginienè R. and Pavilonis A. (2011) Antibiotic resistance mechanisms of clinically important bacteria. *Medicina (Kaunas);* 47: 137-146.

Giller K. E., Witter E. and McGrath S. P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biol Biochem*; 30: 1389-1414.

Gillings M., Boucher Y., Labbate M., Holmes A., Krishnan S., Holley M. and Stokes H. W. (2008a) The evolution of class 1 integrons and the rise of antibiotic resistance. *J Bacteriol*; 190: 5095-5100.

Gillings M. R., Krishnan S., Worden P. J. and Hardwick S. A. (2008b) Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples. *FEMS Microbiol Lett*; 287: 56-62.

Gillings M. R., Gaze W. H., Pruden A., Smalla K., Tiedje J. M. and Zhu Y. G. (2015) Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J*; 9: 1269-1279.

Girlich D., Poirel L. and Nordmann P. (2010) First isolation of the blaOXA 23 carbapenemase gene from an environmental *Acinetobacter baumannii* isolate. *Antimicrob Agents Chemother*; 54: 578-579.

Gold H. S. (2001) Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clin Infect Dis*; 33: 210-219.

Gómez M. J. and Neyfakh A. A. (2006) Genes Involved in Intrinsic Antibiotic Resistance of *Acinetobacter baylyi*. *Antimicrob Agents Chemother*; 50: 3562-3567.

Gómez-Sagasti M. T., Becerril J. M., Epelde L., Alkorta I. and Garbisu C. (2015) Early gene expression in *Pseudomonas fluorescens* exposed to a polymetallic solution. *Cell Biol Toxicol*; 31: 39-81.

Götz A., Pukall R., Smit E., Tietze E., Prager R., Tschäpe H., van Elsas J. D. and Smalla K. (1996) Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl Environ Microbiol*; 62: 2621-2628.

Götz A. and Smalla K. (1997) Manure enhances plasmid mobilization and survival of Pseudomonas putida introduced into field soil. *Appl Environ Microbiol*; 63: 1980-1986.

Grayston S. J., Wang S., Campbell C. D. and Edwards A. C. (1998) Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem*; 30: 369-378.

Gregory A. S., Watts C. W., Griffiths B. S., Hallett P. D., Kuan H. L. and Whitmore A. P. (2009) The effect of long-term soil management on the physical and biological resilience of a range of arable and grassland soils in England. *Geoderma*; 153: 172-185.

Gremion F., Chatzinotas A. and Harms H. (2003) Comparative 16S rDNA and 16S rRNA sequence analysis indicates that *Actinobacteria* might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. *Environ Microbiol*; 5: 896-907.

Griffin J. N., O'Gorman E. J., Emmerson M. C., Jenkins S. R., Klein A. M. and Loreau M. (2009) Biodiversity and the stability of ecosystem functioning. In: Naeem S., Bunker D., Hector A., Loreau M. and Perrings C. (Eds.) Biodiversity, Ecosystem Functioning, and Human Wellbeing: An Ecological and Economic Perspective. *Oxford University Press*, Oxford: 78-93.

Griffiths B. S., Liu Q., Wang H., Zhang B., Kuan H. L., McKenzie B. M., Hallett P. D., Neilson R. and Daniell T. J. (2008) Restoration of soil physical and biological stability are not coupled in response to plants and earthworms. *Ecol Restor*; 26: 102-104.

Griffiths B. and Philippot L. (2013) Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiol Rev*; 37: 112-129.

Gstalder M. E., Faelen M., Mine N., Top E. M., Mergeay M. and Couturier M. (2003) Replication functions of new broad host range plasmids isolated from polluted soils. *Res Microbiol*; 154: 499-509.

Gullberg E., Cao S., Berg O. G., Ilback C., Sandegren L., Hughes D., Andersson D. I. (2011) Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog*; 7: e1002158.

Guo G., Zhou Q. and Ma L. Q. (2006) Availability and assessment of fixing additives for the in situ remediation of heavy metal contaminated soils: a review. *Environ Monit Assess*; 116: 513-528.

Guo Z., Megharaj M., Beer M., Ming H., Mahmudur Rahman M., Wu W. and Naidu R. (2009) Heavy metal impact on bacterial biomass based on DNA analyses and uptake by wild plants in the abandoned copper mine soils. *Bioresource Technol*; 100: 3831-3836.

Hall J. P., Harrison E., Lilley A. K., Paterson S., Spiers A. J. and Brockhurst M. A. (2015) Environmentally co-occurring mercury resistance plasmids are genetically and phenotypically diverse and confer variable context-dependent fitness effects. *Environ Microbiol*; 25: 2034-2039.

Hamilton-Miller J. M. (2004) Antibiotic resistance from two perspectives: man and microbe. *Int J Antimicrob Ag*; 23: 209-212.

Handelsman J. (2004) Metagenomics: application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev*; 68: 669-685.

Harada K. M., Aso Y., Hashimoto W., Mikami B. and Murata K. (2006) Sequence and analysis of the 46.6-kb plasmid pA1 from *Sphingomonas* sp. A1 that corresponds to the typical IncP- 1beta plasmid backbone without any accessory gene. *Plasmid*; 56: 11-23.

Harrison E. and Brockhurst M. A. (2012) Plasmid-mediated horizontal gene transfer is a coevolutionary process. *Trends Microbiol*; 20: 262-267.

Hayashi S., Abe M., Kimoto M., Furukawa S. and Nakazawa T. (2000) The DsbA-DsbB disulfide bond formation system of *Burkholderia cepacia* is involved in the production of

protease and alkaline phosphatase, motility, metal resistance, and multi-drug resistance. *Microbiol Immunol*; 44: 41-50.

Head I. M., Saunders J. R. and Pickup R. W. (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb Ecol*; 35: 1-21.

Hermisson J. and Pennings P. S. (2005) Soft sweeps: molecular population genetics of adaptation from standing genetic variation. *Genetics*; 169: 2335-2352.

Heuer H., Krsek M., Baker P., Smalla K. and Wellington M. H. (1997) Analysis of Actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-eletrophoretic separation in denaturing gradients. *Appl Environ Microbiol*; 63: 3233-3241.

Heuer H., Krögerrecklenfort E., Wellington E. M. H., Egan S., van Elsas J. D., van Overbeek L., Collard J. M., Guillaume G., Karagouni A. D., Nikolakopoulou T. L. and Smalla K. (2002) Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol Ecol*; 42: 289-302.

Heuer H. and Smalla K. (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol*; 9: 657-666.

Heuer H., Focks A., Lamshöft M., Smalla K., Matthies M. and Spiteller M. (2008) Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol Biochem*; 40: 1892-1900.

Heuer H., Kopmann C., Bihn C., Top E. and Smalla K. (2009) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. *Environ Microbiol*; 11: 937-949.

Heuer H., Schmitt H. and Smalla K. (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr Opin Microbiol*; 14: 236-243.

Heuer H. and Smalla K. (2012) Plasmids foster diversification and adaptation of bacterial populations in soil. *FEMS Microbiol Rev*; 36: 1083-1104.

Høiby N., Bjarnsholt T., Givskov M., Molin S. and Ciofu O. (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents*; 35: 322-332.

Houba V. J. G., Temminghoff E. J. M., Gailkhorst G. A. and van Vark W. (2000) Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. *Commun Soil Sci Plant Anal*; 31: 1299-1396.

Hoyle D. V., Davison H. C., Knight H. I., Yates C. M., Dobay O., Gunn G. J., Amyes S. G. B. and Woolhouse M. E. J. (2006) Molecular characterisation of bovine faecal *Escherichia coli* shows persistence of defined ampicillin resistant strains and the presence of class 1 integrons on an organic beef farm. *Vet Microbiol*; 115: 250-257.

Hu P., Brodie E. L., Suzuki Y., McAdams H. H. and Andersen G. L. (2005) Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. *J Bacteriol*; 187: 8437-8449.

Hu X., Zhou Q. and Luo Y. (2010) Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environ Pollut*; 158: 2992-2998.

Hughes V. C. and Datta N. (1983) Conjugative plasmids in bacteria of the “pre-antibiotic” era. *Nature*; 302: 725-726.

IHOBE (1998) Calidad del suelo. Valores Indicativos de Evaluación (VIE-A, VIE-B, VIE-C). Investigación del suelo. Gobierno Vasco, Departamento de urbanismo, Vivienda y Medio Ambiente, Victoria-Gasteiz, p. 272.

Islam M. S. and Tanaka M. (2005) Nutritional condition, starvation status and growth of early juvenile Japanese sea bass (*Lateolabrax japonicus*) related to prey distribution and feeding in the nursery ground. *J Exp Mar Biol Ecol*; 323: 172-183.

Jaglic Z. and Červinková D. (2012) Genetic basis of resistance to quaternary ammonium compounds - the qac genes and their role: a review. *Vet Med*; 57: 275-281.

Janssen P. H. (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol*; 72: 1719-1728.

Janssen P., Van Houdt R., Moors H., Monsieurs P., Morin N., Michaux A., Benotmane M. A., Leys N., Vallaeys T., Lapidus A., Monchy S., Médigue C., Taghavi S., McCorkle S., Dunn

J., van der Lelie D. and Mergeay M. (2010) The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. *PLoS ONE*; 5: e10433.

Jechalke S., Kopmann C., Richter M., Moenickes S., Heuer H. and Smalla K. (2013) Plasmid-mediated fitness advantage of *Acinetobacter baylyi* in sulfadiazine-polluted soil. *FEMS Microbiol Lett*; 348: 127-132.

Jechalke S., Schreiter S., Wolters B., Dealtry S., Heuer H. and Smalla K. (2014) Widespread dissemination of class 1 integron components in soils and related ecosystems as revealed by cultivation-independent analysis. *Front Microbiol*; 4: 420-429.

Jefferson K. K. (2004) What drives bacteria to produce a biofilm? *FEMS Microbiol Lett*; 236: 163-173.

Jefferson K. K., Goldmann D. A. and Pier G. B. (2005) The use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother*; 49: 2467-2473.

Jevons M. P., Coe A. W. and Parker M. T. (1963) Methicillin resistance in *staphylococci*. *Lancet;* 1: 904-907.

Jiang L. and Pu Z. (2009) Different effects of species diversity on temporal stability in single-trophic and multitrophic communities. *Am Nat*; 174: 651-659.

Jiang L., Hu X., Xu T., Zhang H., Sheng D. and Yin D. (2013) Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. *Sci Total Environ*; 458: 267-272.

Jimenez B. and Chávez A. (2004) Quality assessment of an aquifer recharged with wastewater for its potential use as drinking source: "El Mezquital Valley" case. *Water Sci Technol*; 50: 269-276.

Jones R. N., Hare R. S., Sabatelli F. J. and the Ziracin Susceptibility Testing Group (2001) *In vitro* Gram-positive antimicrobial activity of evernimicin (SCH 27899), a novel oligosaccharide, compared with other antimicrobials: a multicentre international trial. *J Antimicrob Chemother*; 47: 15-25.

Jones-Dias D., Manageiro V. and Caniça M. (2016) Influence of agricultural practice on mobile *bla* genes: IncI1-bearing CTX-M, SHV, CMY and TEM in *Escherichia coli* from intensive farming soils. *Environ Microbiol*; 18: 260-272.

Jorquera M. A., Hernandez M., Martinez O., Marschner P. and Mora M. D. L. L. (2010) Detection of aluminium tolerance plasmids and microbial diversity in the rhizosphere of plants grown in acidic volcanic soil*. Eur J Soil Biol*; 46: 255-263.

Juwarkar A. A., Misra R. R. and Sharma J. K. (2014) Recent trends in bioremediation. In: Parmar N. and Singh A. (Eds.) *Geomicrobiology and Biogeochemistry.* Springer, Berlin Heidelberg: 81-100.

Kabas S., Faz A., Acosta J. A., Zornoza R., Martínez-Martínez S., Carmona D. M. and Bech J. (2012) Effect of marble waste and pig slurry on the growth of native vegetation and heavy metal mobility in a mine tailing pond. *J Geochem Explor*; 123: 69-76.

Kallifidas D., Kang H. S. and Brady S. F. (2012) Tetarimycin A, an MRSA-Active antibiotic identified through induced expression of environmental DNA gene clusters. *J Am Chem Soc*; 134: 19552-19555.

Kämpfer P. T. (2010) Order *Sphingobacteriales*. In: Krieg N. R., Ludwig W, Whitman W. B., Hedlund B. P., Paster B. J., Staley J. T., Ward N., Brown D. (Eds.) *Bergey's Manual of Systematic Bacteriology: Volume 4: The Bacteroidetes*, Springer: 330-351.

Kandeler E. and Gerber H. (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol Fertil Soils*; 6: 68-72.

Kavamura V. N. and Esposito E. (2010) Biotechnological strategies applied to the decontamination of soils polluted with heavy metals. *Biotechnol Adv*; 28: 61-69.

Kennell D. and Magasanik B. (1962) The relation of ribosome content to the rate of enzyme synthesis in *Aerobacter aerogenes*. *Biochim Biophys Acta*; 55: 139.

Kimura T. and Nishioka H. (1997) Intracellular generation of superoxide by copper sulphate in *Escherichia coli*. *Mutat Res*; 389: 237-242.

Knapp C. W., Zhang W., Sturm B. S. M. and Graham D. W. (2010) Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions. *Environ Pollut*; 158: 1506-1512.

Koditschek L. K. and Guyre P. (1974) Antimicrobial resistant coliforms in New York Bight. *Mar Pollut Bull*; 5: 71-74.

Köhler W. (2007) The present state of species within the genera Streptococcus and Enterococcus. *Int J Med Microbiol*; 297: 133-150.

Kolar C. S. and Lodge D. M. (2001) Progress in invasion biology: predicting invaders. *Trends Ecol Evol*; 16: 199-204.

Kopmann C., Jechalke S., Rosendahl I., Groeneweg J., Krögerrecklenfort E., Zimmerling U., Weichelt V., Siemens J., Amelung W., Heuer H. and Smalla K. (2013) Abundance and transferability of antibiotic resistance as related to the fate of sulfadiazine in maize rhizosphere and bulk soil. *FEMS Microbiol Ecol*; 83: 125-134.

Kozdrój J. and van Elsas J.D. (2000) Response of the bacterial community to root exudates in soil polluted with heavy metal assessed by molecular and cultural approaches. *Soil Biol Biochem*; 32: 1405-1417.

Kragelund C., Levantesi C., Borger A., Thelen K., Eikelboom D., Tandoi V., Kong Y., van der Waarde J., Krooneman J., Rossetti S., Thomsen T. R. and Nielsen P. H. (2007) Identity, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. *FEMS Microbiol Ecol*; 59: 671-682.

Krauland M. (2010) Novel integron gene cassette arrays identified in a global collection of multi-drug resistant non-typhoidal *Salmonella enterica*. *Curr Microbiol*; 60: 217-223.

Kristiansson E., Fick J., Janzon A., Grabic R., Rutgersson C., Weijdegård B., Söderström H. and Larsson D. G. (2011) Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS ONE*; 6: e17038.

Kropf S., Heuer H., Grüning M. and Smalla K. (2004) Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. *J Microbiol Methods*; 57: 187-195.

Krupenikov I. A., Boincean B. P. and Dent D. (2011) The Black Earth: Ecological Principles for Sustainable Agriculture on Chernozem Soils. (Eds.) Springer, Dordrecht, The Netherlands: 146.

Kuan H. L., Fenwick C., Glover L. A., Griffiths B. S. and Ritz K. (2006) Functional resilience of microbial communities from perturbed upland grassland soils to further persistent or transient stresses. *Soil Biol Biochem*; 38: 2300-2306.

Kümmerer K. and Henninger A. (2003) Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect*; 9: 1203-1214.

Kümmerer K. (2009) Antibiotics in the aquatic environment - a review - part I. *Chemosphere;* 75: 417-434.

Kumpiene J., Guerri G., Landi L., Pietramellara G., Nannipieri P. and Renella G. (2009) Microbial biomass, respiration and enzyme activities after in situ aided phytostabilization of a Pb- and Cu-contaminated soil. *Ecotox Environ Saf*; 72: 115-119.

Lanzén A., Jørgensen S. L., Huson D. H., Gorfer M., Grindhaug S. H., Jonassen I., Øvreås L. and Urich T. (2012) CREST - Classification Resources for Environmental Sequence Tags. *PLoS ONE*; 7: e49334.

Loreau M. and de Mazancourt C. (2013) Biodiversity and ecosystem stability: a synthesis of underlying mechanisms. *Ecol Lett*; 16: 106-115.

Laroche E., Pawlak B., Berthe T., Skurnik D. and Petit F. (2009) Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in Escherichia coli isolated from a densely populated estuary (Seine, France). *FEMS Microbiol Ecol*; 68: 118-130.

Leiby N. and Marx C. J. (2014) Metabolic erosion primarily through mutation accumulation, and not tradeoffs, drives limited evolution of substrate specificity in *Escherichia coli*. *PLoS Biol*; 12: e1001789.

Lemire J. A., Harrison J. J. and Turner R. J. (2013) Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nat Rev* Microbiol; 11: 371-384.

Leyval C., Turnau H. and Haselwandter K. (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza*; 7: 139-153.

Li J., Shao B., Shen J., Wang S. and Wu Y. (2013) Occurrence of chloramphenicol-resistance genes as environmental pollutants from swine feedlots. *Environ Sci Technol*; 47: 2892-2897.

Liao X. P., Tang W., Zhou R. Q. and Shi B. (2008) Adsorption of metal anions of vanadium (V) and chromium (VI) on Zr (IV)-impregnated collagen fiber. *Adsorption*; 14: 55-64.

Liu A., Fong A., Becket E., Yuan J., Tamae C., Medrano L., Maiz M., Wahba C., Lee C., Lee K., Tran K. P., Yang H., Hoffman R. M. Salih A. and Miller J. H. (2011) Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. *Antimicrob Agents Chemother*; 55: 1204-1210.

Ling L. L., Schneider T., Peoples A. J., Spoering A. L., Engels I., Conlon B. P., Mueller A., Schäberle T. F., Hughes D. E., Epstein S., Jones M., Lazarides L., Steadman V. A., Cohen D. R., Felix C. R., Fetterman K. A., Millett W. P., Nitti A. G., Zullo A. M., Chen C. and Lewis K. (2015) A new antibiotic kills pathogens without detectable resistance. *Nature*; 517: 455-459.

Lorenz N., Hintemann T., Kramarewa T., Katayama A., Yasuta T., Marschner P. and Kandeler E. (2006) Response of microbial activity and microbial community composition in soils to long-term arsenic and cadmium exposure. *Soil Biol Biochem*; 38: 1430-1437.

Lubelski J., Konings W. N. and Driessen A. J. (2007) Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. *Microbiol Mol Biol R*; 71: 463-476.

Ludwig D. F. and Iannuzzi T. J. (2005) Incremental ecological exposure risks from contaminated sediments in an urban estuarine sediment. *Integr Environ Assess Manage*; 1: 374-390.

Luo Y., Mao D., Rysz M., Zhou Q., Zhang H., Xu L. and Alvarez P. J. J. (2010) Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ Sci Technol*; 44: 7220-7225.

Mächler E. and Altermatt F. (2012) Interaction of species traits and environmental disturbance predicts invasion success of aquatic microorganisms. *PLoS One*; 7: e45400.

Madsen J. S., Burmølle M., Hansen L. H. and Sørensen S. J. (2012) The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunol Med Microbiol; 65: 183-195.

Malik A., Ertugrul-Kaan Ç., Bohn C., Böckelmann U., Knobel K. and Grohmann E. (2008) Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbial Lett*; 279: 207-216.

MAPA, 1994. Métodos oficiales de análisis de suelos y aguas para riego. In: Ministerio de Agricultura, Pesca y Alimentación, vol. 3. Métodos Oficiales de Análisis, Madrid.

Marcato C. E., Pinelli E., Cecchi M., Winterton P. and Guiresse M. (2009) Bioavailability of Cu and Zn in raw and anaerobically digested pig slurry. *Ecotoxicol Environ Saf*; 72: 1538-1544.

Marshall B. M., Ochieng D. J. and Levy S. B. (2009) Commensals: Underappreciated reservoir of antibiotic resistance. *Microbe*; 4: 231-238.

Martin B., Humbert O., Camara M., Guenzi E., Walker J., Mitchell T., Andrew P., Prudhomme M., Alloing G., Hakenbeck R., Morrison D. A., Boulnois G. J. and Claverys J. P. (1992) A highly conserved repeated DNA element located in the chromosome of streptococcus pneumonia. *Nucleic Acids Res*; 20: 3479-3483.

Martin C., Timm J., Rauzier J., Gomez-Lus R., Davies J. and Gicquel B. (1990) Transposition of an antibiotic resistance element in mycobacteria. *Nature*; 345: 739-743.

Martin M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*; 17: 10-12.

Martinez J. L. and Baquero F. (2002) Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clin Microbiol Rev*; 15: 647-679.

Martinez J. L., Baquero F. and Andersson D. I. (2007) Predicting antibiotic resistance. *Nat Rev Microbiol*; 5: 958-965.

Martinez J. L. (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science*; 321: 365-367.

Martinez J. L., Sánchez M. B., Martínez-Solano L., Hernandez A., Garmendia L., Fajardo A. and Alvarez-Ortega C. (2009) Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev*; 33: 430-449.

Massé D. I., Cata Saady N. M. and Gilbert Y. (2014) Potential of biological processes to eliminate antibiotics in livestock manure: an overview. *Animals*; 4: 146-163.

Mata M. T., Baquero F. and Perez-Diaz J. C. (2000) A multidrug efflux transporter *in Listeria monocytogenes*. *FEMS Microbiol Lett*; 187: 185-188.

Matz C. and Kjelleberg S. (2005) Off the hook- how bacteria survive protozoan grazing. *Trends microbiol*; 13: 302-307.

McGrath S. P. and Cunliffe C. H. (1985) A simplified method for the extraction of the metals Fe, Zn, Cu, Ni, Cd, Pb, Cr, Co and Mn from soils and sewage sludges. *J Sci Food Agric*; 36: 794-798.

McMurray C. T. and Tainer J. A. (2003) Cancer, cadmium and genome integrity. *Nature Gen*; 34: 239-241.

McNaughton S. J. (1994) Biodiversity and function of grazing ecosystems. In: Schulze E. D. and Mooney H. A. (Eds.) *Biodiversity and Ecosystem Function.* Springer-Verlag, London: 361-383.

Meeinkuirt W., Pokethitiyook P., Kruatrachue M., Tanhan P. and Chaiyarat R. (2012) Phytostabilization of a Pb- contaminated mine tailing by various tree species in pot and field experiments. *Int J Phytorem*; 14: 925-938.

Mergeay M., Monchy S., Vallaeys T., Auquier V., Benotmane A., Bertin P., Taghavi S., Dunn J., Van der Lelie D. and Wattiez R. (2003) Ralstonia metallidurans, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol Rev*; 27: 385-410.

Meroueh S. O., Minasov G., Lee W., Shoichet B. K. and Mobashery S. (2003) Structural aspects for evolution of beta-lactamases from penicillin-binding proteins. *J Am Chem Soc*; 125: 9612-9618.

Meyer J. S. (2002) The utility of the terms “bioavailability” and “bioavailable fraction” for metals. *Mar Environ Res*; 53: 417-423.

Mijangos I., Albizu I., Epelde L., Amezaga I., Mendarte S. and Garbisu C. (2010) Effect of liming on soil properties and plant performance of temperate mountainous grasslands. *J Environ Manage;* 91: 2066-2074.

Modi R. I., Wilke C. M., Rosenzweig R. F. and Adams J. (1991) Plasmid macro evolution: selection of deletions during adaptation in a nutrient-limited environment. *Genetica*; 84: 195-202.

Modi R. I., Castilla L. H., Puskas-Rozsa S., Helling R. B. and Adams J. (1992) Genetic changes accompanying increased fitness in evolving populations of *Escherichic coli*. *Genetics*; 130: 241-249.

Monier J. M., Demanèche S., Delmont T. O., Mathieu A., Vogel T. M. and Simonet P. (2011) Metagenomic exploration of antibiotic resistance in soil. *Curr Opin Microbiol*; 14: 229-235.

Moreno J. L., Hernández T., Pérez A. and García C. (2002) Toxicity of cadmium to soil microbial activity: effect of sewage sludge addition to soil on the ecological dose. *Appl Soil Ecol*; 21: 149-158.

Morosini M. I., Ayala J. A., Baquero F., Martinez J. L. and Blazquez J. (2000) Biological cost of AmpC production for *Salmonella enterica* serotype Typhimurium. *Antimicrob Agents Chemother*; 44: 3137-3143.

Moura A., Henriques I., Ribeiro R. and Correia A. (2007) Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J Antimicrob Chemother*; 60: 1243-1250.

Mulvaney, R. L. (1996) Nitrogen-Inorganic forms. Methods of Soil Analysis. Part 3. (Eds.) *Chemical Methods*. SSSA, Madison, WI: 1123-1184.

Myers R. M., Fischer S. G., Lerman L. S. and Maniatis T. (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res*; 13: 3131-3145.

Myers J. A., Curtis B. S. and Curtis W. R. (2013) Improving accuracy of cell and chromophore concentration measurements using optical density. *BMC Biophys*; 6:4.

NCCLS (2004) Performance standards for antimicrobial susceptibility testing; fourteenth informational supplement. *The National Committee for Clinical Laboratory Standards*: Wayne, Pennsylvania, 24: 96-130.

Nesme J., Cécillon S., Delmont T. O., Monier J. M., Vogel T. M. and Simonet P. (2014) Large-scale metagenomic based study of antibiotic resistance in the environment. *Curr Biol*; 10: 1096-1100.

Nesvera J., Hochmannová J. and Pátek M. (1998) An integron of class 1 is present on the plasmid pCG4 from Gram-positive bacterium *Corynebacterium glutamicum*. *FEMS Microbiol Lett*; 169: 391-395.

Neu H. C. (1992) The crisis in antibiotic resistance. *Science*; 257: 1064-1073.

Nichols D., Cahoon N., Trakhtenberg E. M., Pham L., Mehta A., Belange A., Kanigan T., Lewis K. and Epsteins S. S. (2010) Use of Ichip for high-throughput *in situ* cultivation of "uncultivable" microbial species. *Appl Environ Microbiol*; 76: 2445-2450.

Nies D. H. (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev*; 27: 313-339.

Nies D. H. (2004) Metals and their compounds in the environment. Part II. In: Anke K., Ihnat M. and Stoeppler M. (Eds.) *The elements: Essential and toxic effects on microorganisms*. Weinheim, VCH.

Nijssen S., Florijn A., Top J., Willems R., Fluit A. and Bonten M. (2005) Unnoticed spread of integron- carrying *Enterobacteriaceae* in intensive care units. *Clin Infect Dis*; 41: 1-9.

Norberg P., Bergström M., Jethava V., Dubhashi D. and Hermansson M. (2011) The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. *Nat Commun*; 2: 1-11.

Nordmann P., Naas T., Fortineau N. and Poirel L. (2007) Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus spp*. and *Pseudomonas aeruginosa* in 2010. *Curr Opin Microbiol*; 10: 436-440.

Norman A., Hansen L. H. and Sorensen S. J. (2009) Conjugative plasmids: vessels of the communal gene pool. *Philos Trans R Soc Lond B Biol Sci*; 364: 2275-2289.

Norström T., Lannergård J. and Hughes D. (2007) Genetic and phenotypic identification of fusidic acid-resistant mutants with the small-colony-variant phenotype in *Staphylococcus aureus*. *Antimicrob Agents Chemother*; 51: 4438-4446.

Novick R. P. (1987) Plasmid incompatibility. *Microbiol Rev*; 51: 381-395.

Nübel U., Engelen B., Felske A., Snaldr J., Wieshuber A., Amann R. I., Ludwig W. and Backhaus H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol*; 178: 5636-5643.

Ochs M. M., McCusker M. P., Bains M. and Hancock R. E. (1999) Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. *Antimicrob Agents Chemother*; 43: 1085-1090.

Ohm H., Marschner B. and Broos K. (2011) Respiration and priming effects after fructose and alanine additions in two copper- and zinc-contaminated Australian soils. *Biol Fert Soils*; 47: 523-532.

Oksanen J., Blanchet F. G., Kindt R., Legendre P., Minchin P. R., O'Hara R. B., Simpson G. L., Solymos P., Stevens M. H. H. and Wagner H. (2013) Vegan: Community Ecology Package. R package version 2.0-10 (online).

Øvreås L., Forney L., Daae F. L. and Torsvik V. (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol*; 63: 3367-3373.

Palmer K. L., Kos V. N. and Gilmore M. S. (2010) Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr Opin Microbiol*; 13: 632-639.

Papp-Wallace K. M., Endimiani A., Taracila M. A. and Bonomo R. A. (2011) Carbapenems: past, present, and future. *Antimicrob Agents Chemother*; 55: 4943-4960.

Park S. and Ely R. L. (2008) Candidate stress genes of *Nitrosomonas europaea* for monitoring inhibition of nitrification by heavy metals. *Appl Environ Microb*; 74: 5475-5482.

Park C. and Zhang J. (2012) High expression hampers horizontal gene transfer. *Genome Biol Evol*; 4: 523-532.

Parkhill J., Dougan G., James K. D., Thomson N. R., Pickard, D, Wain J, Churcher C., Mungall K. L., Bentley S. D., Holden M. T. G., Sebaihia M., Baker S., Basham D., Brooks K., Chillingworth T., Connerton P., Cronin A., Davis P., Davies R. M., Dowd L., White N.,

Farrar J., Feltwell T., Hamlin N., Haque A., Hien T. T., Holroyd S., Jagels K., Krogh A., Larsen, T. S., Leather S., Moule, S., O'Gaora P., Parry C., Quail M., Rutherford K., Simmonds M., Skelton J., Stevens K., Whitehead S. and Barrell B. G. (2001) Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature*; 413: 848-852.

Peak N., Knapp C. W., Yang R. K., Hanfelt M. M., Smith M. S., Aga D. S. and Graham D. W. (2007) Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ Microbiol*; 9: 143-151.

Pérez-Esteban J., Escolástico C., Ruiz-Fernández J., Masaguer A. and Moliner A. (2013) Bioavailability and extraction of heavy metals from contaminated soil by *Atriplex halimus*. *Environ Exp Bot*; 88: 53-59.

Perni S., Andrew P. W. and Shama G. (2005) Estimating the maximum growth rate from microbial growth curves: definition is everything. *Food Microbiol*; 22: 491-495.

Perron K., Caille O., Rossier C., van Delden C., Dumas J. L. and Köhler T. (2004) CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. *J Biol Chem*; 279: 8761-8768.

Popowska M., Rzeczycka M., Miernik A., Krawczyk-Balska A., Walsh F. and Duffy B. (2011) Influence of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes. *Antimicrob Agents Chemother*; 56: 1434-1443.

Potters G., Horemans N. and Jansen M. A. K. (2010) The cellular redox state in plant stress biology- a charging concept. *Plant Physiol Biochem*; 5: 292-300.

Powers R. F. (1980) Mineralizable soil nitrogen as an index of nitrogen availability to forest trees. *Soil Sci Soc Am J*; 44: 1314-1320.

Powlson D. S., Brookes P. C. and Christensen B. T. (1987) Measurement of soil microbial biomass provides an early indication of changes in the total soil organic matter due to straw incorporation. *Soil Biol Biochem*; 19: 159-164.

Pukall R., Tschäpe H. and Smalla K. (1996) Monitoring the spread of broad host and narrow host range plasmids in soils microcosms. *FEMS Microbiol Ecol*; 20: 53-66.

Queenan A. M. and Bush K. (2007) Carbapenemases: The versatile β-lactamases. *Clin Microbiol Rev*; 20: 440-458.

Quinn J. P. (1998) Clinical problems posed by multiresistant nonfermenting Gram-negative pathogens. *Clin Infect Dis*; 27: 117-124.

Rademaker J. L. W. and De Bruijn F. J. (2004) Computer-assisted analysis of molecular fingerprint profiles and database construction. *Molecular Microbial Ecology Manual*; 1397-1446.

Rahube T. O. and Yost C. K. (2012) Characterization of a mobile and multiple resistance plasmid isolated from swine manure and its detection in soil after manure application. *J Appl Microbiol*; 112: 1123-1133.

Rappe M. S. and Giovannoni S. J. (2003) The uncultured microbial majority. *Annu Rev Microbiol*; 57: 369-394.

Rastogi G., Barua S., Sani R. K. and Peyton B. M. (2011) Investigation of microbial populations in the extremely metal-contaminated Coeur d'Alene River sediments. *Microb Ecol*; 62: 1-13.

Reysenbach A. L., Giver L. J., Wickham G. S and Pace N. R. (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Appl Environ Microbiol*; 58: 3417-3418.

Reis M. P., Barbosa F. A. R., Chartone-Souza E. and Nascimento A. M. A. (2013) The prokaryotic community of a historically mining-impacted tropical stream sediment is as diverse as that from a pristine stream sediment. Extremophiles; 17: 301-309.

Rhodes G., Huys G., Swings J., McGann P., Hiney M., Smith P. and Pickup R. W. (2000) Distribution of oxytetracycline resistance plasmids between Aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant Tet A. *Appl Environ Microbiol*; 66: 3883-3890.

Riley M. A. and Wertz J. E. (2002) Bacteriocins: evolutions, ecology, and application. *Annu Rev Microbiol*; 56: 117-137.

Rocha E. R. and Smith C. J. (1999) Role of the alkyl hydroperoxide reductase (*ahpCF*) gene in oxidative stress defense of the obligate Anaerobe *bacteroides fragilis*. *J Bacteriol*; 181: 5701-5710.

Rognes T., Flouri T., Ijaz U. Z., Mahé F., Nichols B. and Quince C. (2015) VSEARCH (v1.0.10_linux_x86_64) (online).

Rosenfeld M., Ramsey B. W. and Gibson R. L. (2003) *Pseudomonas* acquisition in young patients with cystic fibrosis: pathophysiology, diagnosis, and management. *Curr Opin Pulm Med*; 9: 492-497.

Rosewarne C. P., Pettigrove V., Stokes H. W. and Parsons Y. M. (2010) Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for co-selection with heavy-metal resistance. *FEMS Microbiol Ecol*; 72: 35-46.

Rosner J. L. and Storz G. (1994) Effects of peroxides on susceptibilities of *Escherichia coli* and *Mycobacterium smegmatis* to isoniazid. *Antimicrob Agents Chemother*; 38: 1829-1833.

Rossolini G., Condemi M. A., Pantanella F., Docquier J. D., Amicosante G. and Thaller M. C. (2001) Metallo-β-lactamase producers in environmental microbiota: new molecular Class B enzyme in *Janthinobacterium lividum*. *Antimicrob Agents Chemother*; 45: 837-844.

Salt D. E, Smith R. D. and Raskin I. (1998) Phytoremediation. *Annu Rev Plant Physiol*; 49: 643-668.

San Millan A., Peña-Miller R., Toll-Riera M., Halbert Z. V., McLean A. R., Cooper B. S. and MacLean R. C. (2014) Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. *Nat Commun*; 5: 5208.

Sandaa R. A., Enger O. and Torsvik V. (1999) Abundance and diversity of archaea in heavy-metal-contaminated soil. *Appl Environ Microbiol*; 65: 3293-3297.

Sandvang D., Aarestrup F. M. and Jensen L. B. (1997) Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica Typhimurium* DT104. *FEMS Microbial Lett*; 157: 177-181.

Sastre I., Vicente M. A. and Lobo M. C. (1996) Influence of the application of sewage sludges on soil microbial activity. *Bioresource Technol*; 57: 19-23.

Sato T. and Kuramitsu H. (1998) Plasmid maintenance renders bacteria more susceptible to heat stress. *Microbiol Immunol*; 42: 467-469.

Scallan E., Hoekstra R. M., Angulo F. J., Tauxe R. V., Widdowson M. A., Roy S. L., Jones J. L. and Griffin P. M. (2011) Foodborne illness acquired in the United States- major pathogens. *Emerg Infect Dis*; 17: 7-15.

Schirmer M., Ijaz U. Z., D'Amore R., Hall N., Sloan W. T. and Quince C. (2015) Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. *Nucleic Acids Res*; 43: e37.

Schlüter A., Szczepanowski R., Pühler A. and Top E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol Rev*; 31: 449-477.

Schmidt A. S., Bruun M. S., Dalsgaard I. and Larsen J. L. (2001) Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Appl Environ Microbiol*; 67: 5675-5682.

Schmieder R. and Edwards R. (2012) Insights into antibiotic resistance through metagenomic approaches. *Future Microbiol*; 7: 73-89.

Schröder U. C., Beleites C., Assmann C., Glaser U., Hübner U., Pfister W., Fritzsche W., Popp J. and Neugebauer U. (2015) Detection of vancomycin resistances in enterococci within 3 ½ hours. *Sci Rep*; 3: 8217.

Seaver L. C. and Imlay J. A. (2001) Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J Bacteriol*; 183: 7182-7189.

Selenska-Pobell S. (2002) Diversity and activity of bacteria in uranium waste piles. In: Keith-Roach M. and Livens F. (Eds.) *Interactions of Microorganisms with Radiomuclides.* Oxford, Elsevier Sciences: 225-253.

Sentchilo V., Mayer A. P., Guy L., Miyazaki R., Tringe S. G., Barry K., Malfatti S., Goessmann A., Robinson-Rechavi M. and van der Meer J. R. (2013) Community-wide plasmid gene mobilization and selection. *ISME J*; 7: 1173-1186.

Seybold C. A., Herrick J. E. and Brejda J. J. (1999) Soil resilience: a fundamental component of soil quality. *Soil Sci*; 164: 224-234.

Shachrai I., Zaslaver A., Alon U. and Dekel E. (2010) Cost of unneeded proteins in *E. coli* is reduced after several generations in exponential growth. *Mol Cell*; 38: 758-767.

Shi L., Zheng M., Xiao Z., Asakura M., Su J., Li L. and Yamasaki S. (2006) Unnoticed spread of class 1 integrons in Gram-positive clinical strains isolated in Guangzhou, China. *Microbiol Immunol*; 50: 463-467.

Siemens J., Ilg K., Pagel H. and Kaupenjohann M. (2008) Is colloid-facilitated phosphorus leaching triggered by phosphorus accumulation in sandy soils? *J Environ Qual*; 37: 2100-2107.

Silver S. and Phung L. T. (2005) A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *J Ind Microbiol Biotechnol*; 32: 587-605.

Singh B. K., Quince C., Macdonald C. A., Khachane A., Thomas N., Al-Soud W. A., Sørensen S. J., He Z., White D., Sinclair A., Crooks B., Zhou J. and Campbell C. D. (2014) Loss of microbial diversity in soils is coincident with reductions in some specialized functions. *Environ Microbiol*; 16: 2408-2420.

Skurnik D., Le Menac'h A., Zurakowski D., Mazel D., Courvalin P., Denamur E., Andremont A. and Ruimy R. (2005) Integron-associated antibiotic resistance and phylogenetic grouping of *Escherichia coli* isolates from healthy subjects free of recent antibiotic exposure. *Antimicrob Agents Chemother*; 49: 3062-3065.

Smalla K., Heuer H., Götz A., Niemeyer D., Krögerrecklenfort E. and Tietze E. (2000) Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl Environ Microbiol*; 66: 4854-4862.

Smalla K., Wieland G., Buchner A., Zock A., Parzy J., Kaiser S., Roskot N., Heuer H. and Berg G. (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol*; 67: 4742-4751.

Smalla K., Haines A. S., Jones K., Krogerrecklenfort E., Heuer H., Schloter M. and Thomas C. M. (2006) Increased abundance of IncP-1 (beta) plasmids and mercury resistance genes in polluted river sediments-first discovery of IncP-1 (beta) plasmids with a complex *mer* transposon as sole accessory element. *Appl Environ Microbiol*; 72: 7253-7259.

Smalla K. and Heuer H. (2006) How to assess the abundance and diversity of mobile genetic elements (MGE) in soil communities? In Nannipieri P. and Smalla K. (Eds.) *Nucleic Acids and Proteins in Soil*. Edited Springer-Verlag, Berlin: 313-330.

Smillie C., Garcillán-Barcia M. P., Francia M. V., Rocha E. P. and de la Cruz F. (2010) Mobility of plasmids. *Microbiol Mol Biol Rev*; 74: 434-452.

Sørensen S. J., Bailey M., Hansen L. H., Kroer N. and Wuertz S. (2005) Studying plasmid horizontal transfer in situ: a critical review. *Nat Rev Microbiol*; 3: 700-710.

Speir T. W. and Ross D. J. (2002) Hydrolytic enzyme activities to assess soil degradation and recovery. In: Burns R. G. and Dick R.P. (Eds.) *Enzymes in the Environment*. Marcel Dekker, New York: 407-431.

Starikova I., Al-Haroni M., Werner G., Roberts A. P., Sørum V., Nielsen K. M. and Johnsen P. J. (2013) Fitness costs of various mobile genetic elements in *Enterococcus faecium* and *Enterococcus faecalis*. *J Antimicrob Chemother*; 68: 2755-2765.

Stasiak G., Mazur A., Wielbo J., Marczak M., Zebracki K., Koper P. and Skorupska A. (2014) Functional relationships between plasmids and their significance for metabolism and symbiotic performance of *Rhizobium leguminosarum* bv. *trifolii*. *J Appl Genet*; 55: 515-527.

Stecher B., Maier L. and Hardt W. D. (2013) Blooming' in the gut: how dysbiosis might contribute to pathogen evolution. *Nat Rev Microbiol*; 11: 277-284.

Stepanauskas R., Glenn T. C., Jagoe C. H., Tuckfield C., Lindell A. H., King C. J. and McArthur J. V. (2006) Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ Microbiol*; 8: 1510-1514.

Stokes H. W., Nesbø C. L., Holley M., Bahl M. I., Gillings M. R. and Boucher Y. (2006) Class 1 integrons potentially predating the association with tn402-like transposition genes are present in a sediment microbial community. *J Bacteriol*; 188: 5722-5730.

Stokes H. W. and Gillings M. R. (2011) Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol Rev*; 35: 790-819.

Stres B., Philippot L., Faganeli J. and Tiedje J. M. (2010) Frequent freeze-thaw cycles yield diminished yet resistant and responsive microbial communities in two temperate soils: a laboratory experiment. *FEMS Microbiol Ecol*; 74: 323-335.

Summers A. O., Wireman J., Vimy M. J., Lorscheider F. L., Marshall B., Levy S. B., Bennet S., and Billard L. (1993) Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. *Antimicrob Agents Chemother*; 37: 825-834.

Suzuki M. T., Taylor L. T. and Delong E. F. (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5' -nuclease assays. *Appl Environ Microbiol*; 66: 4605-4614.

Svara F. and Rankin D. J. (2011) The evolution of plasmid-carried antibiotic resistance. *BMC Evol Biol*; 11: 130.

Szczepanowski R., Braun S., Riedel V., Schneiker S., Krahn I., Pühler A. and Schlüter A. (2005) The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. *Microbiology*; 151: 1095-111.

Szczepanowski R., Bekel T., Goesmann A., Krause L., Kromeke H., Kaiser O., Eichler W., Puhler A. and Schluter A. (2008) Insight into the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. *J. Biotechnol*; 136: 54-64.

Tahlan K., Ahn S. K., Sing A., Bodnaruk T. D., Willems A. R., Davidson A. R. and Nodwell J. R. (2007) Initiation of actinorhodin export in *Streptomyces coelicolor*. *Mol Microbiol*; 63: 951-961.

Tarai B., Das P. and Kumar D. (2013) Recurrent challenges for clinicians: emergence of methicillin-resistant *Staphylococcus aureus*, vancomycin resistance, and current treatment options. *J Lab Physicians*; 5: 71-78.

Tate R. L. (2002) Microbiology and enzymology of carbon and nitrogen cycling. In: Burns, R. G. and Dick, R. P. (Eds.) *Enzymes in the Environment*. Marcel Dekker, New York: 227-248.

Taubes G. (2008) The bacteria fight back. *Science*; 321: 356-361.

Taylor J., Wilson B., Mills M. and Burns R. (2002) Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol Biochem*; 34: 387-401.

Teitzel G. M. and Parsek M. R. (2003) Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl Environ Microbiol*; 69: 2313-2320.

Teitzel G. M., Geddie A., De Long S. K., Kirisits M. J., Whiteley M. and Parsek M. R. (2006) Survival and Growth in the presence of elevated copper: transcriptional profiling of copper stressed *Pseudomonas aeruginosa*. *J Bacteriol*; 188: 7242-7256.

Tejada M., Moreno J. L., Hernandez M. T. and Garcia C. (2008) Soil amendments with organic wastes reduce the toxicity of nickel to soil enzyme activities. *Eur J Soil Biol*; 44: 129-140.

Ten Brink B. J. E., Hosper S. H and Colijn F. (1991) A quantitative method for description and assessment of ecosystems: the AMOEBA- approach. *Mar Pollut Bull*; 23: 265-270.

Tennstedt T., Szczepanowski R., Braun S., Pühler A. and Schlüter A. (2003) Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol Ecol*; 45: 239-252.

Tett A., Spiers A. J., Crossman L. C., Ager D., Ciric L., Dow J. M., Fry J. C., Harris D., Lilley A., Oliver A., Parkhill J., Quail M. A., Rainey P. B., Saunders N. J., Seeger K., Snyder L. A., Squares R., Thomas C. M., Turner S. L., Zhang X. X., Field D. and Bailey M. J. (2007) Sequence-based analysis of pQBR103; a representative of a unique, transfer-proficient mega plasmid resident in the microbial community of sugar beet. *ISME J*; 1: 331-340.

Thiele-Bruhn S. (2003) Pharmaceutical antibiotic compounds in soil - a review. *J Plant Nutr Soil Sci*; 166: 145-167.

Thomas C. M. (2000) The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread. Amsterdam: Harwood Academic Publishers.

Thomas C. M. and Nielsen K. M. (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol*; 3: 711-721.

Tilman D., Reich P. B. and Knops J. M. H. (2006) Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature*; 441: 629-632.

Top E., De Smet I., Verstraete W., Dijkmans R. and Mergeay M. (1994) Exogenous isolation of mobilizing plasmids from polluted soils and sludges. *Appl Environ Microbiol*; 60: 831-839.

Top E. M., Van Elsas E. D., DeSmet S. N. and Forney L. J. (1998) Enhancement of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in soil by dissemination of catabolic plasmids. *Antonie Van Leuwenhoek*; 73: 87-94.

Turner P. E., Cooper V. S. and Lenski R. E. (1998) Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids. *Evolution*; 52: 315-329.

Udikovic-Kolic N., Wichmann F., Broderick N. A. and Handelsman J. (2014) Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc Natl Acad Sci U S A*; 111: 15202-15207.

Van Belkum A. and Hermans P. W. (2001) BOX PCR fingerprinting for molecular typing of streptococcus pneumoniae. *Methods Mol Med*; 48: 159-168.

Van Elsas J. D., Gardener B. B. M., Wolters A. C. and Smit E. (1998) Isolation, characterization, and transfer of cryptic gene-mobilizing plasmids in the wheat rhizosphere. *Appl Environ Microbiol*; 64: 880-889.

Van Hoek A. H., Mevius D., Guerra B., Mullany P., Roberts A. P. and Aarts H. J. (2011) Acquired antibiotic resistance genes: an overview. *Front Microbiol*; 2: 203.

Van Rensburg E., Den Haan R., Smith J., van Zyl W. H. and Gorgens J. F. (2012) The metabolic burden of cellulase expression by recombinant *Saccharomyces cerevisiae* Y294 in aerobic batch culture. *Appl Microbiol Biotechnol*; 96: 197-209.

Vance E. D., Brookes P. C. and Jenkinson D. S. (1987) An extraction method for measuring soil microbial biomass C. *Soil Biol Biochem*; 19: 703-707.

Vangronsveld J., Herzig R., Weyens N., Boulet J., Adriaensen K., Ruttens A., Thewys T., Vassilev A., Meers E., Nehnevajova E., van der Lelie D. and Mench M. (2009) Phytoremediation of contaminated soils and groundwater: Lessons from the field. *Environ Sci Pollut R*; 16: 765-794.

Vedler E., Vahter M. and Heinaru A. (2004). The completely sequenced plasmid pEST4011 contains a novel IncP1 backbone and a catabolic transposon harboring *tfd* genes for 2,4-dichlorophenoxyacetic acid degradation. *J Bacteriol*; 186: 7161-7174.

Vogan A. A. and Higgs P. G. (2011) The advantages and disadvantages of horizontal gene transfer and the emergence of the first species. *Biolo Direct*; 6:1.

Vogel C., Mueller C. W., Höschen C., Buegger F., Heister K., Schulz S., Schloter. M and Kögel-Knabner I. (2014) Submicron structures provide preferential spots for carbon and nitrogen sequestration in soils. *Nat Commun*; 5: 2947.

Vogwill T. and MacLean R. C. (2015) The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl*; 8: 284-295.

Volkmann H., Schwartz T., Bischoff P., Kirchen S., and Obst U. (2004) Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J Microbiol Methods*; 56: 277-286.

Walsh C. (2000) Molecular mechanisms that confer antibacterial drug resistance. *Nature*; 406: 775-781.

Walsh T. R. (2006) Combinatorial genetic evolution of multiresistance. *Curr Opin Microbiol*; 9: 476-482.

Walsh F., Ingenfeld A., Zampicolli M., Hilber-Bodmer M., Frey J. E. and Duffy B. (2011) Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. *J Microb Methods*; 86: 150-155.

Wang S., Deng K., Zaremba S., Deng X., Lin C., Wang Q., Tortorello M. L. and Zhang W. (2009) Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress. *Appl Environ Microbiol*; 75: 6110-6123.

Wang Y., Kern S. E. and Newman D. K. (2010) Endogenous phenazine antibiotics promote anaerobic survival of *Pseudomonas aeruginosa* via extracellular electron transfer. *J Bacteriol*; 192: 365-369.

Wang L., Oda Y., Grewal S., Morrison M., Michel F. C. and Yu Z. (2012) Persistence of resistance to erythromycin and tetracycline in swine manure during simulated composting and lagoon treatments. *Microb Ecol*; 63: 32-40.

Ward N. L., Challacombe J. F., Janssen P. H., Henrissat B., Coutinho P. M., Wu M., Xie G., Haft D. H., Sait M., Badger J., Barabote R. D., Bradley B., Brettin T. S., Brinkac L. M., Bruce D., Creasy T., Daugherty S. C., Davidsen T. M., DeBoy R. T., Detter J. C., Dodson R. J., Durkin A. S., Ganapathy A., Gwinn-Giglio M., Han C. S., Khouri H., Kiss H., Kothari S. P., Madupu R., Nelson K. E., Nelson W. C., Paulsen I., Penn K., Ren Q., Rosovitz M. J., Selengut J. D., Shrivastava S., Sullivan S. A., Tapia R., Thompson L. S., Watkins K. L., Yang Q., Yu C., Zafar N., Zhou L. and Kuske C. R. (2009) Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. *Appl Environ Microbiol*; 75: 2046-2056.

Wei G., Tingxing H., Jingyan W., Yuanbo G. and Hua R. (2008) Soil carbon pool and fertility under natural evergreen broadleaved forest and its artificial regeneration forests in southern Sichuan Province, China. *Acta Ecol Sin*; 28: 2536-2545.

Wei S., Zhou Q., Zhan J., Wu Z., Sun T., Lyubu Y. and Prasad M. N. V. (2010) Poultry manured *Bidens tripartite* L. extracting Cd from soil-potential for phytoremediating Cd contaminated soil. *Bioresource Technol*; 101: 8907-8910.

Weinert N., Meincke R., Gottwald C., Heuer H., Gomes N. C. M., Schloter M., Berg, G. and Smalla, K. (2009) Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their parent cultivar differ less than those of different potato cultivars. *Appl Environ Microbiol*; 75: 3859-3865.

Wegst-Uhrich S. R., Navarro D. A. G., Zimmerman L. and Aga D. S. (2014) Assessing antibiotic sorption in soil: a literature review and new case studies on sulfonamides and macrolides. *Chem Cent J*; 8: 5.

Whitman W. B., Coleman D. C. and Wiebe W. J. (1998) Prokaryotes: the unseen majority. *Proc Natl Acad Sci USA*; 95: 6578-6583.

Wickham S. A. and Lynn D. H. (1990) Relation between growth rate, cell size, and DNA content in colpodean ciliates (*Ciliophora: Colpodea*). *European J Protistol*; 25: 345-352.

Wickham H. (2009) ggplot2: elegant graphics for data analysis. Springer, New York.

Witte W. (1998) Medical consequences of antibiotic use in agriculture. *Science*; 279: 996-997.

Wong J. W. C., Ip C. M. and Wong M. H. (1998) Acid-forming capacity of Pb-Zn mine tailings and its implications for mine rehabilitation. *Environ Geochem Health*; 20: 149-155.

Wozniak R. A. and Waldor M. K. (2010) Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol*; 8: 552-563.

Wright G. D. (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Rev Microbiol*; 5: 175-186.

Wright M. S., Baker-Austin C., Lindell A. H., Stepanauskas R., Stokes H. W. and McArthur J. V. (2008) Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J*; 2: 417-428.

Wright G. D. (2010) Antibiotic resistance in the environment: a link to the clinic? *Curr Opin Microbiol*; 13: 589-594.

Wu J., Joergensen R. G., Pommerening B., Chaussod R. and Brookes P. C. (1990) Measurement of soil microbial biomass by fumigation-extraction: an automated procedure. *Soil Biol Biochem*; 22: 1167-1169.

Xiong J., Alexander D. C., Ma J. H., Deraspe M., Low D. E., Jamieson F. B. and Roy P. H. (2013) Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid encoding IMP-9-mediated carbapenem resistance, from outbreak isolate *pseudomonas aeruginosa* 96. *Antimicrob Agents Chemother*; 57: 3775-3782.

Xu Z., Li L., Shirtliff M. E., Peters B. M., Peng Y., Alam M. J., Yamasaki S. and Shi L. (2010) First report of class 2 integron in clinical *Enterococcus faecalis* and class 1 integron in *Enterococcus faecium* in South China. *Diagn Microbiol Infect Dis*; 68: 315-317.

Yang J., Wang C., Shu C., Liu L., Geng J., Hu S. and Feng J. (2013) Marine sediment bacteria harbor antibiotic resistance genes highly similar to those found in human pathogens. *Microb Ecol*; 65: 975-981.

Yim G., Wang H. H. and Davies J. (2007) Antibiotics as signalling molecules. *Philos Trans R Soc Lond B Biol Sci*; 362: 1195-1200.

You Y., Hilpert M. and Ward M. J. (2012) Detection of a common and persistent *tet(L)*-carrying plasmid in chicken-waste-impacted farm soil. *Appl Environ Microbiol*; 78: 3203-3213.

Young I. M. and Crawford J. W. (2004) Interactions and self-organization in the soil-microbe complex. *Science*; 304: 1634-1637.

Yusof M., Mei Yi D. C., Enhui J. L., Mohd Noor N., Wan Ab Razak W. and Saad Abdul Rahim A. (2011) An illustrated review on Penicillin and Cephalosporin: An instant study guide for pharmacy students. WebMedCentral; WMC002776.

Zhu Y. G., Johnson T. A., Su J. Q., Qiao M., Guo G. X., Stedtfeld R. D., Hashsham S. A. and Tiedje J. M. (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Natl Acad Sci USA*; 110: 3435-3440.

Zur Wiesch P. A, Kouyos R., Engelstädter J., Regoes R. R. and Bonhoeffer S. (2011) Population biological principles of drug-resistance evolution in infectious diseases. *Lancet Infect Dis*; 11: 236-247.