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Characterization of microbial communities in Technosols constructed for industrial wastelands restoration

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I dedicate this dissertation to my family,
who always motivated and encouraged me to fulfill my dreams,
and especially….
to my mother for her unconditional love
to my Father who is no more with us,
but still live and walk beside us everyday,
‘unseen, unheard, but always near, still loved, still missed….

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Résumé

L'augmentation de la dégradation des sols et ses conséquences sur les services écosystémiques nécessite le développement de stratégies de restauration de ces sols. La constitution de Technosols, résultant de l'assemblage de sols pollués et de déchet industriels, est une approche innovatrice pouvant à la fois permettre de restaurer les sols et de recycler des sous-produits industriels. Des études récentes ont mis en évidence que les Technosols pouvaient assurer des services écosystémiques tels que la production primaire. Toutefois, notre connaissances des autres services écosystémiques rendus par les Technosols tels que les cycles biogéochimiques est limitée. En raison de la contribution significative des communautés microbiennes aux cycles biogéochimiques dans les sols, l'objectif de ce travail de thèse était l'effet du type de Technosol sur les communautés microbiennes et plus particulièrement les communautés fonctionnelles impliquées dans le cycle de l'azote. Dans ce contexte, (i) la densité et la diversité de la communauté bactérienne totale, (ii) la densité de la communauté crenarchéenne ainsi que (iii) la densité et les activités des communautés nitrifiante et dénitrifiante ont été étudiées dans deux types de Technosols différents. Les résultats obtenus montrent que la diversité et la composition de la communauté bactérienne des deux Technosols n'étaient pas significativement différentes entre elles, et similaires à celles de 'sols naturels', les *Proteobacteria* étant le phylum dominant (50-80%). La densité de la communauté bactérienne oxydant l'ammonium était non seulement plus importante que celle des crenarcheae qui oxyde l'ammonium, mais est également corrélée avec l'activité potentielle de nitrification suggérant ainsi que les bactéries sont responsables de l'oxydation de l'ammonium dans les Technosols. La densité des dénitrifiants et de la communauté oxydant l'ammonium sont du même ordre de grandeur que celles observées dans les sols agricoles. L'analyse de la distribution de l'activité et de la densité des communautés nitrifiante et dénitrifiante dans les différents horizons des Technosols montre un effet négatif de la profondeur, cet effet étant plus marqué que l'effet du type de Technosol étudié. Les propriétés physico-chimiques des Technosols et la densité de la communauté bactérienne oxydant l'ammonium sont corrélés à l'activité de nitrification alors que l'activité de dénitrification était contrôlée principalement par les propriétés physico-chimiques des Technosols, et dans une moindre mesure par la densité de la communauté dénitrifiant possédant le gène *nirS*. L'estimation de la stabilité fonctionnelle du processus de dénitrification vis-à-vis de périodes stress hydrique et thermique a montré que les Technosols présentaient une plus haute résistance et une meilleure résilience que des sols remédiés par traitement thermique uniquement. Ce travail souligne le potentiel des Technosols à assurer les services écosystémiques tels que le cycle de l'azote, ainsi que leur forte capacité à résister et à se remettre de stress environnementaux. Tout ceci semble donc indiquer que la construction de Technosols est une technologie prometteuse qui pourrait permettre la restauration de friches industrielles et le recyclage des déchets industriels.

Mots-clés: sols pollués, cycle de l'azote, communauté microbienne, services écosystémiques, Technosols

Abstract

Increasing soil degradation and its consequences on overall ecosystem services urge for restoration strategies. Construction of Technosols through assemblage of treated soil and industrial wastes is an innovative technology for the restoration of polluted land and re-use of industrial by-products. Recent studies have evidenced that Technosols could support ecosystemic services such as primary production but the knowledge about other soil functions, such as biogeochemical cycling, is limited. Due to the significant contribution of microbial communities to soil functioning, this PhD work was carried out to study the effect of the type of Technosol on microbial communities with a focus on functional guilds involved in N cycling. For this purpose, the abundance and diversity of the total bacterial community and the abundance of crenarchaeal community together with the abundance and activities of the nitrifying and denitrifying communities were investigated in two types of Technosols. Results demonstrated that diversity and composition of the bacterial community were similar to 'natural soils' and were not significantly different between the two Technosols with *Proteobacteria* being the dominant phylum (50-80%). The bacterial ammonia oxidizers were greater in number than crenarchaeal ammonia oxidizers but also correlated to the potential nitrification activity suggesting that bacteria are the dominant ammonia oxidizers in Technosols. The abundance of both the ammonia oxidizers and the denitrifiers were in the same range than that observed in other soil systems. Analyses of the vertical distribution of the activity and abundance of N-cycling communities in the Technosols showed a significant depth-effect, which was more important than the Technosol type-effect. Technosols physicochemical properties and the abundance of the bacterial ammonia oxidizers were the main drivers of the nitrification activity whereas the denitrification activity was controlled mainly by the Technosols physicochemical properties and, to a minor extent, by the abundances of the *nirS* denitrifiers. The estimation of the functional stability of the denitrification process against the heat-drought stresses revealed that Technosol exhibited the high resistance and resilience in comparison to the thermally treated soil. This work highlighted the potential of constructed Technosols to ensure the N cycling ecosystem services, along with a high capacity to resist and recover from environmental stresses, suggesting that construction of Technosols is a promising technology and a solution for the restoration of industrial wastelands and waste recycling.

Key-words : Contaminated soils, Nitrogen cycling, Microbial community, Ecosystem services, Technosol

List of abbreviations

EC	European Commission
UNCCD	United Nations Convention to Combat Desertification
WMO	World Meteorological Organization
UNEP	United Nations Environment Programme
OECD	Organisation for Economic Cooperation and Development
IUSS	International Union of Soil Sciences
WRB	World reference base
C	Carbon
N	Nitrogen
GISFI	Groupement d'Intérêt Scientifique sur les Friches Industrielles
FAO	Food and Agriculture Organization of the United Nations
EU	European Union
SCAPE	Soil Conservation and Protection in Europe
MEA	Millenium Ecosystem Assessment
EEA	European Environment Agency
NRC	National Research Council
PAHs	Polyaromatic hydrocarbons
PCBs	Polychlorinated biphenyls
SER	Society for Ecological Restoration International
IPCC	Intergovernmental Panel on Climate Change
UNESCAP	United Nations Economic and Social Commission for Asia and the Pacific
WHO	World Health Organization
DNA	Deoxyribose Nucleic Acid
RNA	Ribonucleic Acid
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
AMO	Ammonia monooxygenase
HAO	Hydroxylamine oxydoreductase
NOR	Nitrite oxydoreductase

Bacterial *amoA* Gene encoding subunit A of ammonia monooxygenase in bacteria

Archaeal *amoA* Gene encoding subunit A of ammonia monooxygenase in archaea

DNRA	Dissimilatory Nitrate Reduction to Ammonium
NAR	Nitrate reductase
NIR	Nitrite reductase
napA	Gene encoding the periplasmic nitrate reductase
narG	Gene encoding the membrane bounded nitrate reductase
nirK	Gene encoding the copper containing subunit of nitrite reductase
nirS	Gene encoding the cytochrome cd1-containing subunit of nitrite reductase
norB	Gene encoding the nitric oxide reductase
norC	Gene encoding the nitric oxide reductase
nosZ	Gene encoding the multicopper homodimeric nitrous oxide reductase
N_2O	Nitrous oxide
NO	Nitric oxide
A-RISA	Automated Ribosomal Intergenic Spacer Analysis
IGS	Intergenic spacer region
PVPP	Polyvinyl polypyrrolidone
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
qPCR	Real-time quantitative PCR
OTU	Operational Taxonomic Units
ANOVA	Analysis of variance
r	Correlation coefficient
PNA	Potential nitrification activity
PDA	Potential denitrification activity

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Soil is a non-renewable natural resource at least at human life span. It is essential for life on earth providing a wide range of ecosystem services including food production, nutrient cycling, water regulation, climate regulation and detoxification and decomposition of wastes (Andrews et al. 2004, Costanza et al. 1997, EC 2010). Although it is a vital resource, it has been increasingly under pressure due to human activities. Since the industrial revolution, soil resources have tremendously been affected by human activities, including industrialization, urbanization and intensive agriculture, yielding the changes of land usage. In industrial as well as emerging countries, soils are increasingly degraded and global change might contribute to exacerbate this situation ultimately disrupting its quality and functioning. Today, one could estimate that about 40-50% of soil resources had been transformed or degraded by human activities (Vitousek et al. 1997). According to UNCCD 1.5 billion people are suffering from soil degradation which cannot function anymore to provide soil basic services (UNCCD 2012).

Land degradation led to the decrease of the primary production as well as the soil biodiversity and consequently it has severe environmental and socio-economic issues (FAO 2010, UNEP 1997). The restoration of degraded lands to recover soil functioning to get back to sustainable soil ecosystem services is a challenging task. Several traditional approaches (addition of various amendments and ameliorants, acid washing, physical drainage etc.) are used to reclaim the soils which have been degraded through various human or natural hazards (salinization, acidification, intensive agriculture, industrial contamination etc.). For the restoration of industrially degraded lands, the construction of Technosols is an innovative approach consisting in the assemblage of treated soils and industrials wastes and byproducts (Sere et al. 2008). Technosols which have recently been classified by the IUSS (IUSS Working Group WRB 2006) are in expansion but knowledge about their biological components especially of the microbial life is scarce.

Soil microorganisms are key players of several soil ecosystemic services including primary production, filter function (decomposition of toxic and waste materials) and regulation of the climate (biogeochemical cycling) (Bodelier 2011, de Bello et al. 2010, Vinas et al. 2005). Being abundant in soil environment representing about 1-3 tones of C per hectares (Chaussod et al. 1986, Wild 1993), soil microbes are highly diverse representing one of the highest reservoir of biodiversity in terrestrial ecosystems (Bodelier 2011, Torsvik &

Øvreås 2002). In this context, microbial communities can have a central role in successful soil reclamation aiming at restoring soil physicochemical and biological characteristics to ensure the provision of soil ecosystemic services. In this scenario, the investigation of the microbial communities in constructed Technosols will help in estimating the extent of restoration success, in special regard to the restoration of soil basic ecosystem services.

This PhD work aims at characterizing soil microbial community abundance, diversity and activity in constructed Technosols. We focused on functional communities involved in N cycling to address Technosols functioning. N-cycling microbial communities involved in N cycle were selected as model microbial communities because of their role in the availability of nutrients for plants colonizing lands under restoration. The PhD work was carried out on a 1 ha experimental Technosols comprising two different Technosols differing by their water diffusion capabilities, built on an old industrial wasteland in 2007 at Homécourt, France (Groupement d'Intérêt Scientifique sur les Friches Industrielles, GISFI). Over a three year period, different parameters were estimated to monitor the abundance, diversity and activity of soil microbial communities involved in N cycle. This PhD work is part of a larger program piloted by Jérôme Cortet (ENSAIA Nancy) under the framework of GESSOL IV funded by the Ministry of Environment and Ecology to address the consequences of soil restoration through the construction of Technosols on soil biological quality.

The thesis is comprised of five chapters. The **first** chapter discusses the scientific context, the interest and the objectives of PhD work. It includes soil degradation phenomenon, major reasons of soil degradation, its socio-economic impacts and degraded soils restoration strategies along with construction of Technosols, the main focus of our work. It describes the importance of soil ecosystemic services and their significance for all form of life on earth. Importance of soil microbial communities in driving major soil ecosystemic services such as N cycling are also discussed in details to give an overview of the entire work.

The following three chapters present the results of the experimental works carried out during the PhD. The second and third chapters are describing global and N cycling microbial communities in the two constructed Technosols. The **second** chapter describes the structure, abundance and diversity of total bacterial communities and the abundances of the total crenarchaeal communities and of the functional guilds involved in N cycling. Bacterial community structure and the community composition were investigated in the two different Technosols over a two year-period. The **third** chapter describes the distribution of N cycling communities' abundance and activity along depth profiles in the two different Technosols with the aim of elucidating the influence of original parent materials on Technosol

functioning. The **fourth** chapter, reports a study aiming at evaluating the functional stability of the microbial activities in Technosols by comparison with adjacent contaminated site and with arable soils to estimate the level of recovery of Biotechnosols functioning after disturbance. To perform this study, the functional stability of N cycling microbial communities was addressed by measuring their resistance and resilience to heat/draught stresses applied experimentally under laboratory conditions. These results are discussed in light of the ability of the Biotechnosols designed to restore contaminated industrial sites to cope with environmental stresses.

Finally, the **fifth** chapter discusses the original results obtained from the experimental work and their possible outcomes on overall work. This general discussion is aiming at synthesizing our advances in understanding the functioning of Biotechnosols through the detailed study of N-cycling soil microbial communities which are key players in soil fertility ecosystemic services. These results are placed in the context of giving the interest of constructed Technosols, an innovative technology for restoration of degraded lands.

CHAPTER 1 LITERATURE REVIEW

LITERATURE REVIEW

1. Soil and ecosystem

1.1. Soil nonrenewable resource

Soil is a vital and non-renewable resource at human life-span, a natural capital and a central pillar in terrestrial ecosystem functioning (Robinson & Lebron 2010). Soil is providing multiple services for humans and ecosystems (Louwagie et al. 2011) among which its importance for the primary production has for long been identified. All forms of life on earth are directly or indirectly dependent on soil functioning and these characteristics make soil an integral part and irreplaceable natural resource. The growth of human population and of its activity exerted pressures on land usage and in this context, conversion of land usage with the demand for different soil ecosystemic services is an increasing issue with key environmental, social and economic issues. FAO clearly identified that growth of human population will increase the need for food augmenting the pressure on the environment and causing an environmental crisis (FAO 2010). Several economists recently suggest that we recently shift from the economy of the abundance to the economy of restriction of environmental resources and point out that this shift most likely plays an important role in the economic crisis of developed countries still relying on a model of constant growth (EU 2004). Thus in this context the sustainability of soil resources is a key factor for the current and future human needs. The European Community recently tackled this question by preparing reports aiming at establishing a European Directive for soil protection (COM 2006). In this context, soil rehabilitation was identified as a key element of soil protection strategy as well as the estimation of rehabilitation on pressures, states and impact of the pollution on soil resources. Driving forces, Pressures, State, Impact, Response (DPSIR) framework (Blum 2001) has been proposed that facilitated the assessment of the various factors exerting pressure on the soil resources (SCAPE 2004) (Fig. 1.1).

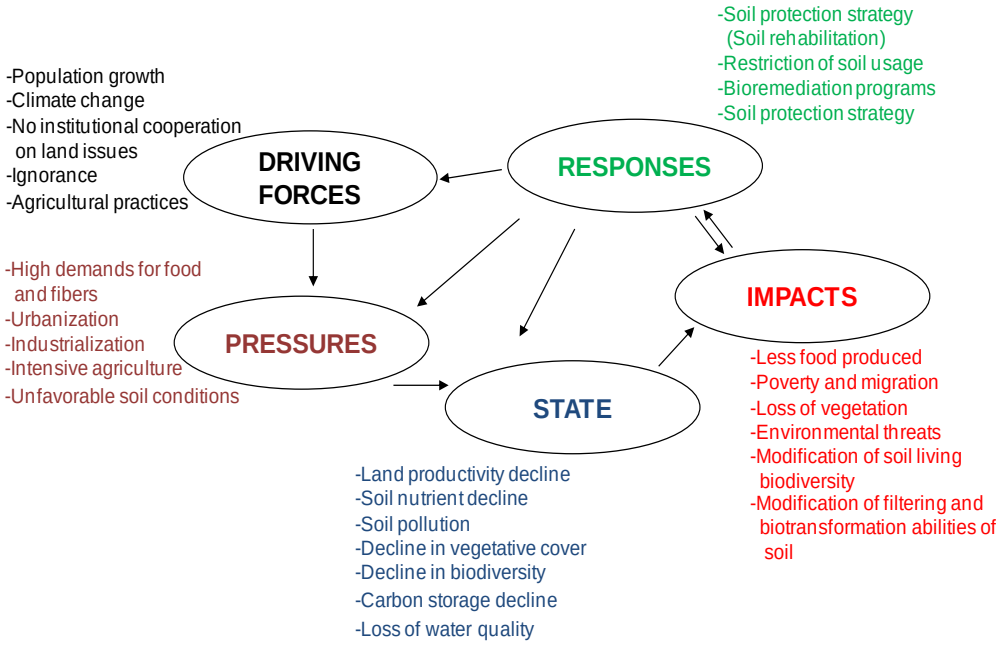

Fig. 1.1. Factors exerting pressure on soil resources

Combination of various tools to assess the factors exerting pressure on soil resources (adapted from SCAPE (2004)).

1.2. Soil ecosystemic services

Costanza et al. (1997) identified and gave economic value to 'the services of ecological systems that are critical to the functioning of the Earth's life-support systems'. The major point they make was to record the ecosystemic services and to estimate their value which they estimated in the range of US$ 16-54 trillion (10^{12}) per year. Despite of their recognized importance, ecosystem services are not fully implemented in commercial markets and, as a consequence, they are not considered in policy decisions. Consequently, this may compromise the sustainability of humans in the Biosphere. The importance of soil ecosystem services have been proposed at various international forums and the different aspects of these services were debated for a long time (Daily et al. 1997, OECD 2004, UNEP 2000). For this reason, the Millenium Ecosystem Assessment addressed this issue by identifying ecosystemic services, among which those depending on soil resources (MEA 2005a). MEA defines ecosystemic services as the interaction of various components of the ecosystem work together in maintaining a balance between ongoing processes important for sustaining life on this planet. Such processes whether directly or indirectly are involved in improving the overall human well-being (Daily et al. 1997). The major benefits provided by ecosystemic services are classified into provisioning, regulating, cultural and supporting services (MEA 2005b),

which have recently been described with special reference to soil ecosystem by Dominati et al. (2010) and are presented in detail in Table 1.1.

Soil being the interface between air and water resources as well as the place where the most of human activities are performed, has been proposed as a dynamic regulatory center in majority of the processes occurring in the ecosystem (Barrios 2007). It is heterogeneous and a complex system responsible for fulfilling human needs such as primary production, decomposition of waste materials, regulating environmental interactions, habitat for a vast range of biological life, filtering and buffering of water and storage and cycling of the nutrients (Andrews et al. 2004, Bardgett RD et al. 2011, Dominati et al. 2010, EC 2010, EU 2010, Lal 2008, UNEP 2000). Despite of their recognized interest in accomplishing several ecosystemic services soil importance still remains underappreciated (NRC 2009) and this natural resource remains under increasing pressure due to human activities, and nowadays soil degradation has become a serious problem that crosses national borders and socio-economic levels (FAO 2010).

Table 1.1. Provision of ecosystem services from soil natural capital (adapted from Dominati et al. (2010).

Soil ecosystemic services	
Provisioning Services	Provision of physical support
	Provision of food, wood and fibre
	Provision of raw materials
Supporting Processes	Nutrient cycling
	Water cycling
	Soil biological activity
Regulating Services	Flood mitigation
	Filtering of nutrients
	Biological control of pests and diseases
	Recycling of wastes and detoxification
	Carbon storage and regulation of N_2O and CH_4
Cultural Services	Spirituality
	Knowledge
	Sense of place
	Aesthetics

2. Soil degradation

2.1. What is soil degradation?

A site is classified as 'a degraded land' when as a result of human activities, it cannot support anymore vegetation development (Burelle et al. 2005). Human activities such as deforestation, intensive agriculture, industrialization and urbanization can lead to soil degradation. It causes the loss of arable land susceptible to be used for primary production and consequently causes significant loss in productive capacity (UNEP 2006).

Soil degradation has recently become an important issue because of considerable losses of the soil resources. It has been estimated that about 40-50% of the soil area has been transformed or degraded through human activities (Vitousek et al. 1997). According to the UNCCD, land degradation is directly affecting 1.5 billion people (UNCCD 2012). It has also become a major issue in Europe (COM 2006) where European Commission asks each member state to identify, list and prioritize polluted sites. Consequently, the French Ministry of Ecology and Environment recorded no less than 4365 contaminated sites in the Basol database (http://basol.ecologie.gouv.fr/). A large majority of these sites are contaminated with hydrocarbons or heavy metals, and will need remediation (Basol 2011). Similar actions have been carried out in Europe, and the European Environment Agency (EEA) in a recent report entitled 'the state of soil in Europe' estimates that 250 000 sites in the EEA member countries are requiring clean up (EEA 2007). EEA concluded that (i) soil degradation will still increase further if no safety measures are taken and (ii) degraded soils presenting identified risk for human health and for the sustainability of the environment must urgently be restored.

2.2. Causes of soil degradation

Soil degradation has multiple causes originating mainly from human activities and global changes. It has drastic impacts on terrestrial ecosystems (Hudson & Alcantara-Ayala 2006, Jie et al. 2002) and about 2000 million ha of soil (representing up to 15% of the total earth) have been degraded so far (UNEP 2006). Physical, chemical and biological processes are responsible for soil degradation (Lal 1993, Shrestha 2011). Physical degradation involves a decline in soil structure, with compaction, sealing and crusting leading to reduction in water infiltration and increase in runoff (Lal 2001). Chemical degradation of soils includes acidification, salinization, mineral fertilization, pollution with organic compounds (pesticides, PAHs, PCBs) and/or heavy metals and loss of organic matter with incidence on soil fertility

(Shrestha 2011). Biological degradation results from physical and chemical degradations of soils impacting on soil living organisms causing a decline of soil biodiversity and of related ecosystemic services. In many cases, these three processes are acting synergistically as a result of human activities such as changes in land usage, intensification of crop production, deforestation, industrialization and urbanization.

Recently, the intergovernmental panel on climate change (IPCC) underlines the impact of human activities on destocking carbon contributing to greenhouse gas emission leading to global change observed on earth (IPCC 2000). Climate changes induced environmental hazards are such as floods, drought, water and wind erosions, surface run-off and salinization, which are also detrimental to soil (FAO 1994, Shrestha 2011).

Since the past two centuries, industrialization, urbanization and agriculture activities have rapidly and deeply impacted natural resources available on earth leading notably to soil degradation (Jie et al. 2002) (Fig. 1.2). In addition, loss of vegetation due to deforestation, agriculture intensification to offset the deficit in food requirement and overgrazing are considered as the important factors in human induced degradation (UNEP 2006). Other factors coming under human intervention include the unbalanced fertilizer use, over-pumping of ground water and non-use of soil conservation management strategies (UNESCAP 2000).

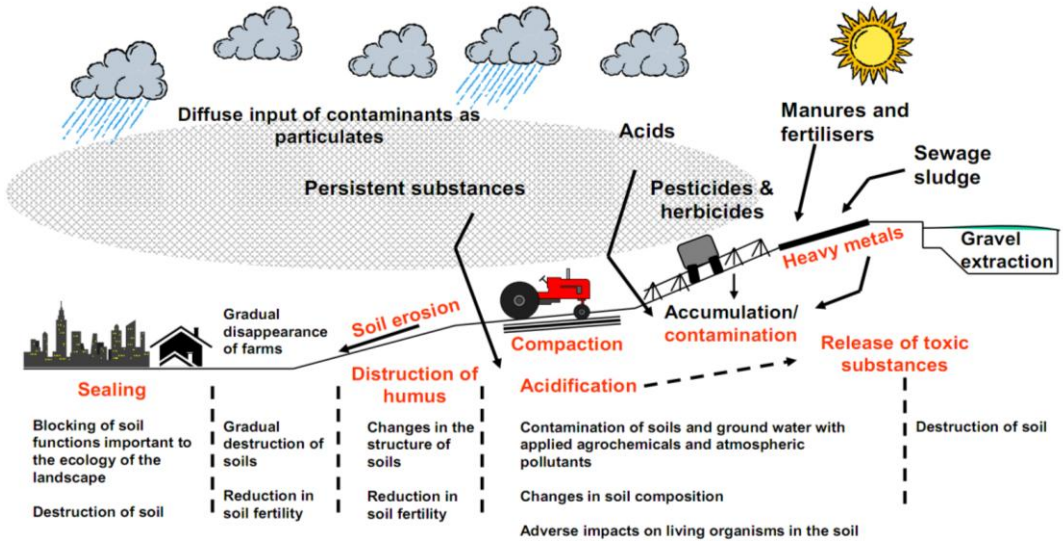

Fig. 1.2. Impacts of human activities on soil resources (Adapted from JRC 2008).

Overall, since the 18th century (industrial revolution) and increasingly during the 20th century's (urbanization and green revolution), human activities have dramatically affected

natural resources available on earth including soil (Avidano et al. 2005). While talking on part of industries, chemistry (synthesis of pesticides, fertilizers, dumping of industrial wastes, release of PAHs etc.) was a consistent tool for ecosystem intensification. Irresponsible disposal of industrial wastes and discharge of considerable amounts of their contaminants has adversely affected the environment having strong impacts on the nature and properties of the soils. Many of the pollutants are recalcitrant to degradation and may change the constitutive materials of the soil and disrupt soil nutrient cycling. Old industrial sites are the examples of such soils, which are no more useful in terms of soil basic functioning. They lack the ability to support plant growth and show high level of contamination dangerous for human health but also for the environment since they can act as pollution sources for water and air resources. Taking into account this scenario, there is a dire need to restore the industrially contaminated soils.

3. Soil restoration

3.1. What is restoration?

Ecological restoration of degraded land is defined as the processes applied to degraded land to recover soil ecosystemic services (SER 2002, 2004) with the aim to return to an ecosystem in proximity to its condition prior to disturbance (Bradshaw 2002). The focus is to recreate the structure and the function, two important attributes of the degraded soils and recreating only one of these two does not constitute the restoration (Bradshaw 2002).

In the context of rarefaction of environment resources due to increasing impact of human activities, restoration ecology represents a challenging issue to diminish the impact of degradation onto human populations and to recover good environmental status of degraded land. Numerous studies underline the crucial importance of land restoration for its ecological balance with expected outcome in facilitating interactions among the living organisms (Belisle 2005, Cramer et al. 2008, Murphy & Lovett-Doust 2004, Reis et al. 2010, Turner 2005). As stated by (Bradshaw 1987, Bradshaw 2002) restoration strategies should consider all the components associated with a considered environment, which for soil means all living organisms (Ormerod 2003). Within this framework, restoration researches and related industrial activities has been developed worldwide during the last two decades (Ormerod 2003). However, although a business has been developed for restoration of wasted lands, one could observe that the mitigation of the ecological damages of degraded land resulting from their land usage remains poorly studied and up to now, only few companies tackle this issue.

Soil restoration aims at re-establishing physical (structure, density, composition etc.) but also chemical (organic matter, C:N ratio, heavy metal contents etc.) characteristics of soil to offer convenient properties for soil living organisms to prosper. This will result in improvement of soil physico-chemical properties but will also promote the colonization of restored land with soil-living organisms, among which are soil microorganisms which are recognized key players of a number of ecosystemic services such as nutrient cycling. Indeed, restoration of soil ecosystemic services is of prime interest to reach an acceptable quality to achieve change in land usage. It is noteworthy that in most of the cases the initial properties of restored land cannot be recovered and that consequently, land owner, politics and the players of the society have to discuss to reach a consensus to attribute new land usage. Therefore, one of the key items of restoration is the consultation of all the actors involved in land rehabilitation in order to get a consensual decision of strategies adopted to reach a shared and achievable objective. Among achievable goals, rehabilitation can be conducted with the objective of restoring soil basic services including the primary production, nutrient cycling and or soil living-organisms diversity accounting for soil ecosystemic services achievement.

Also of prime interest, restored lands should be sustainable to be able to reach the objectives they should fulfill. Therefore, restored land should be functionally stable in order to tackle different environmental changes. Despite its obvious interest, the functional stability of restored soil is poorly studied. Conversely, numerous studies describe the functional stability of arable soils by estimating its ability to withstand a wide range of stresses (resistance) and to recover (resilience) (Greenland & Szabolcs 1994, Griffiths et al. 2000, Pimm 1984, Seybold et al. 1999). Resistant and resilience of soil microbial communities have been shown to be the main drivers of soil adaptation in response to environmental stresses (Griffiths et al. 2005). Functional stability of soil ecosystemic functions is considered as an integrated parameter reflecting soil health and quality (Griffiths et al. 2001). One could therefore conclude that within the framework of rehabilitation of degraded lands, shared objectives resulting from the consultation of all partners involved in this action must be defined to achieve multiple targets scheduled to happen at different time points but keeping in mind the question of the sustainability of the solution proposed. In Europe, guidelines describing key steps to rehabilitate degraded land have recently been proposed (COM 2006).

3.2. Restoration of degraded soils

Since it can take more than 500 years to form just two centimeters of topsoil (EU 2010), one could agree that at human lifespan, soil degradation cannot be recovered without curing treatment (Bai ZG et al. 2008). To tackle soil degradation, different remediation techniques have been developed and the ultimate purpose is to re-establish the functions that have been lost.

3.3. Traditional approaches

Traditional approaches for the reclamation of the contaminated lands include the total removal and reconstitution of the upper soil layer with natural arable soil (Dick et al. 2006, Dickinson 2000). Different amendments have also been used for the reclamation of degraded lands like fertilizers on soils particularly poor in nutrients, gypsum for reclamation of salt affected soils, and organic waste application in mineral soils. Other technologies include physical treatments such as drainage coupled with the use of different amendments such as lime, organic mulch and phosphates (Bradshaw 1983). Metal contaminated soils have been reported to be eliminated by using acid washing techniques (Mulligan et al. 2001a, Mulligan et al. 2001b, Tokunaga & Hakuta 2002). However, acid washing can affect the soil physical and chemical structure thereby decreasing soil productivity (Reed et al. 1996). Heavy metal contaminated soils can also be decontaminated by chemical extraction using several chelating agents (EDTA, DTPA) (Papassiopi et al. 1999, Wasay et al. 1998). Alternatively, heavy metals can be stabilized in soil by adding complex chelating agents. However, it has been suggested that this approach was not promising because they do not form stable chelate complex and therefore become less effective (Tokunaga & Hakuta 2002). Moreover many chelating agents are poorly degradable and can persist in the environment for longer periods with putative toxic effects (Bucheli-Witschel & Egli 2001, Nowack 2002).

These classical technologies are relying on the availability of arable soils which are already endangered due to increasing human activities. Therefore, although being supposed to be environmentally friendly, restoration approaches can themselves cause losses of arable soils (Bradshaw 1997). To overcome this paradox, recently the construction of Technosols has been proposed to restore degraded lands. Its principle is the construction of an artificial soil on the contaminated site by mixing treated soil with industrial wastes and green byproducts.

4. Technosols

4.1. Introduction

Technosols results from the assemblage of treated soil, recycled wastes materials and industrial by-products (IUSS Working Group WRB 2006). These compounds are formulated and layered to build typical profiles (Sere et al. 2008). Being made of technogenic materials, Technosols may undergo pedogenic development (Sere et al. 2010). Technosols present the triple benefit (i) to treat a polluted site, (ii) to offer a solution for industrial by-products and wastes and (iii) to not use arable land for bioremediation purposes (Sere et al. 2008). Construction of Technosols is therefore a reclamation strategy entering in the scope of ecological engineering (Sere et al. 2008).

4.2. Global context

In the context of the worldwide increase of urban and industrial waste production (Van-Camp et al. 2004), construction of Technosols is offering an alternative market for their recycling. The interest of these waste products and also the green waste compost for ameliorating soil properties such as physical, chemical and biological characteristics has been reported (Chantigny et al. 1999, Chantigny et al. 2000a, Mugnai et al. 2012, Semple et al. 2001, Trepanier et al. 1998). It is noteworthy that the application of organic residues such as compost or farmyard manure to agricultural field can contribute to improve crop production and to reduce the use of chemicals (Adegbidi et al. 2003). Numerous studies report that organic amendments of soil generally increase the size of the microbial C biomass as well as the overall microbial activity thereby improving soil ecosystem services (Bastida et al. 2009, Perez-de-Mora et al. 2006, Ros et al. 2003).

Industrial byproducts produced by the pulp and paper industries, which generate about 31 million dry tons of the paper sludges (Cathie K & Guest D 1991), are a cheap source of technogenic materials rich in organic matter more recalcitrant to degradation than compost. The amendment of soil with these compounds improves soil organic matter content (MEF 1997) being slowly decomposed in soil (Chantigny et al. 2000b, Fierro et al. 2000). For these reasons, these compounds are presenting both economical and environmental interest for soil bioremediation.

4.3. GISFI: Technosols

The Technosols studied in this thesis were constructed as model to assess the potential of this innovative technology for restoration of soil functioning. It is the Soil and Environmental Laboratory (UMR 1120 INRA, Equipe Pédogenèse et fonctionnement des sols très anthropisés) which has been at the origin of the creation of the 'Groupement d'Intérêt Scientifique sur les Friches Industrielles' (GISFI; www.gisfi.fr) aiming at offering research facilities and business units built in collaboration with private companies to develop new technologies to restore degraded lands. Being located in the Eastern part of France where charcoal industry was operating for more than two hundred years several heavily polluted sites with heavy metals and PAHs are available. The GISFI was built on one of those sites in Homécourt. In September 2007, over an area of 1 ha two types of Technosols were constructed in collaboration with TVD (Groupe Traitement Valorization Décontamination) society. Each Technosol was established on 0.5 ha plot (Fig. 1.3 (B)) (Technosols treatments/types in next section).

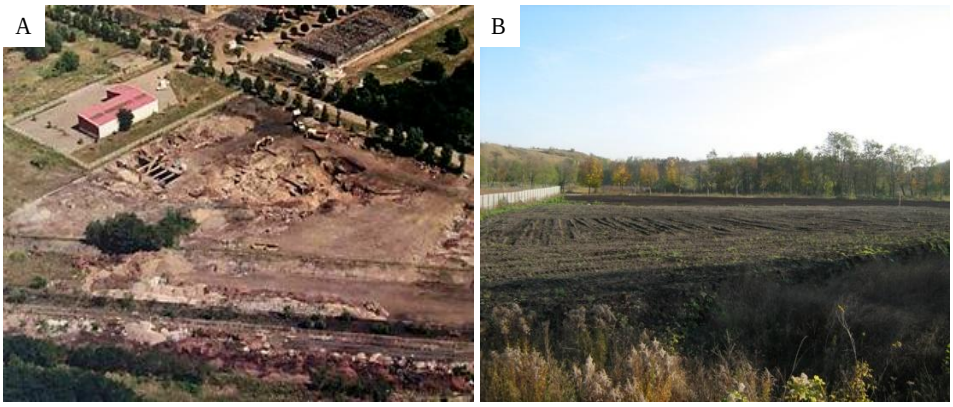

Fig. 1.3. Technosols site of 1 ha

A) shows the site before restoration; B) shows the site after the restoration (Photography gratefully provided by Geoffroy Séré and Christophe Schwartz, LSE UMR 1120 INRA).

4.3.1. Technosols parent materials

4.3.1.1. Green waste compost

Compost is derived from the composting of green waste (e.g. pruning of a tree, grass cuttings). Composting results from the aerobic fermentation of organic waste, followed by its maturation. The finished product is a stabilized material, sanitized and rich in organic matter.

The green waste compost used here was NFU 44-051 standard product from 'Communauté Urbaine de Strasbourg'.

4.3.1.2. Paper by-products

It is a by-product of sewage produced from recycled paper. Its composition is also very close to the paper, comprising of an organic phase composed mainly of cellulose and lignin, and a mineral phase made of sizing agents such as resin and mineral fillers to improve the qualities of paper, including calcium carbonate etc. Also known as paper mill sludge, the paper by-products are classified as the wastes from the production and processing of paper, paperboard and pulp (ADEME 2002) and do not belong to the category of hazardous waste. The paper by-products used here came from Novacare factory (Laval-sur-Vologne, 88) and paper manufacturing industry of Raon (Raon-l'Etape, 88).

4.3.1.3. Treated industrial soil

The soil from a former coke industrial-plant located in Homécourt heavily contaminated with polycyclic aromatic hydrocarbons (PAHs), was excavated, sieved (<5-10 cm) and then treated by a process of low temperature thermal desorption. The process involves heating to a temperature between 400 and 600 °C, which allows degradation of organic pollutants; smoke is then filtered. The treated soil is classified in the category of waste from decontamination of soil and groundwater (no°19 13) by act no°2002-540, 18/04/2002 of European Union (ADEME 2002).

4.3.2. Technosols construction

Two types of Technosols were made ((i) T1 and (ii) T2) (Fig. 1.4). They mainly differ by their water diffusion properties

4.3.2.1. Technosol T1

It consists of;

i. 'Compost horizon' (approximately 15 cm)
ii. Mixture of paper by-products and treated industrial soil (volumetric ratio; 1:1) (approximately 60 cm) which is the 'horizon under developmental processes' and
iii. A layer composed of paper by-products (approximately 30 cm).

4.3.2.2. Technosol T2

It consists of;

i. 'Compost horizon' (approximately 15 cm),
ii. Mixture of paper by-products and treated industrial soil (volumetric ratio; 1:1) (approximately 60 cm) which is the 'horizon under developmental processes' and
iii. An impermeable layer composed of limed-paper by-products (approximately 25 cm).

The first two horizons of this T2 Technosol are similar to the T1 while the third one is described as a confinement horizon (about 0.25 m), which is composed of limed paper by-products (5% by volume,) having specific property of compaction and impermeability. The interest of this impermeable layer is to avoid leaching of pollutants from the restored soil into the aquifers.

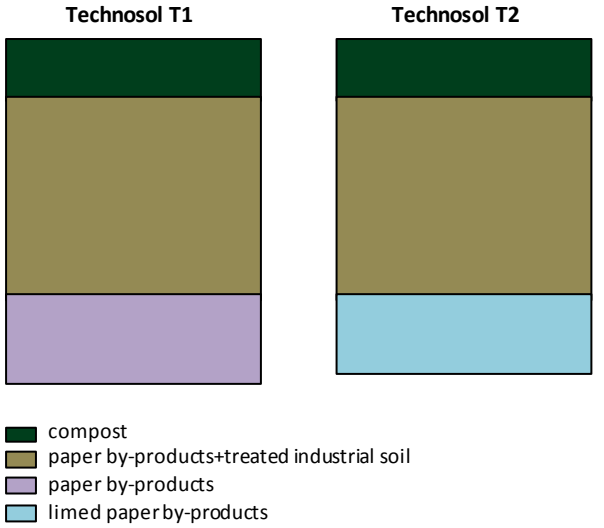

Fig. 1.4. Composition of T1 and T2 Technosols established at GISFI

Various steps in the construction of T1 and T2 are shown in the Fig. 1.5. After completion of construction of Technosols, sowing of a grass cover made of 40 % of Fescue (*Festuca L.*), 30 % of rye grass (*Lolium perenne*) and 30 % of orchard grass (*Dactylis*) was done in March 2007.

Fig. 1.5. Construction of T1 and T2 Technosols

(Photography gratefully provided by Geoffroy Séré and Christophe Schwartz, LSE UMR 1120 INRA).

5. Microbial communities in soil ecosystem

The biodiversity of microorganisms in the soil is the highest on earth, with estimations ranging from several thousand to several million different genomes per gram of soil (Torsvik & Øvreås 2002, Whitman et al. 1998). These microorganisms are responsible for organic matter turnover, soil aggregation, plant nutrition, degradation of toxic compounds and nutrient transformations (Bell et al. 2005, Bodelier 2011, de Bello et al. 2010, Falkowski et al. 2008, Kennedy 1999, Kirk et al. 2004, Smith & Goodman 1999, Vinas et al. 2005, Wall & Virginia 1999, Yao et al. 2000). Soil microorganisms are therefore considered as major contributors to soil ecosystem services (Table 1.2). For example, soil microbial communities drive nutrient cycling (Bodelier 2011), thereby controlling crucial aspects of soil ecosystem services such as productivity, leaching (nitrate losses down the soil profile and subsequent pollution of freshwater and estuaries), and release of active greenhouse gases (e.g. N_2O, CH_4, CO_2).

Table 1.2. Microbes and their role in ecosystemic services (Bodelier 2011)

Microbes	Processes	Ecosystemic services	Ecosystemic services category
Heterotrophic bacteria/archaea	Organic matter decomposition	Decomposition, nutrient cycling, climate regulation, water purification	Supporting, regulating
Photoautotrophic bacteria	Photosynthesis	Primary production, carbon sequestration	Supporting, regulating
Chemo(litho)-autotrophic	Specific elemental transformations	Nutrient cycling, climate regulation, water purification	Supporting, regulating
Unicellular phytoplankton	Photosynthesis	Primary production, carbon sequestration	Supporting, regulating
Archaea	Specific elemental transformations	Nutrient cycling, climate regulation, carbon sequestration	Supporting, regulating
Protozoa	Mineralization of other microbes	Decomposition, Nutrient cycling, Soil formation	Supporting
Viruses	Lysis of hosts	Nutrient cycling	Supporting
All	Production of metabolites, xenobiotic degradation	Production of precursors to industrial and pharmaceutical products	Provisional
All	Huge diversity, versatility, environmental and biotechnological applications	Educational purposes, getting students interested in science	Cultural

The present work focuses on the microbial community in recently constructed man-made soils, Technosols. These soils are young with soil formation processes in progress, where microbial diversity can be crucial for processes such as soil formation and plant establishment (Heijden et al. 2008, Rillig & Mummey 2006). The functional potential of the microbial communities in early ecosystem development has been reported (Banning et al. 2011, Chapin et al. 1994) and an increase in soil microbial biomass, diversity and activity has been observed during the restoration of the disturbed sites (Banning et al. 2008). Investigating multiple indicators of microbial communities in Technosols, including their structure, abundance, activity and functional stability, will provide an insight into the extent of ecosystem recovery in this soil system. Here we focused on the microbial communities involved in N cycling.

6. The nitrogen cycle

6.1. Nitrogen and its cycling

Nitrogen (N) is an essential component of the living world, included in key organic molecules such as proteins and nucleic acids (DNA and RNA) two polymers of life (Canfield et al. 2010). It ranks fourth behind oxygen, carbon, and hydrogen as the most common chemical element in living tissues and all organisms require N in order to grow. It is one of the elements most often limiting in plant nutrition (Hofstra & Bouwman 2005), therefore of high relevance for the agroecosystem productivity (Hai et al. 2009, Mosier et al. 2002, Niboyet et al. 2011, Reich et al. 1997). Despite its abundance in earth's atmosphere, most living organisms are limited by its availability (Galloway et al. 2003). Although the majority of air we breathe is composed of N_2 (78% of the atmosphere), most of the N in the atmosphere is unavailable for metabolic processes in living organisms because of the strong triple bond between N atoms in N_2 molecules, which makes it relatively inert. N can be fixed through natural processes, can be bonded to hydrogen or oxygen to form inorganic compounds, mainly ammonium (NH_4^+) and nitrate (NO_3^-) or converted into organic N (e.g. urea - $(NH_2)_2CO$). Once such compounds are present in the soil, microorganisms may metabolize them, and drive the belowground cycling of N, which includes N mineralization, nitrification and denitrification processes, converting one form of nitrogen into another until it is released to the atmosphere, leached or taken up by plants (Fig. 1.6).

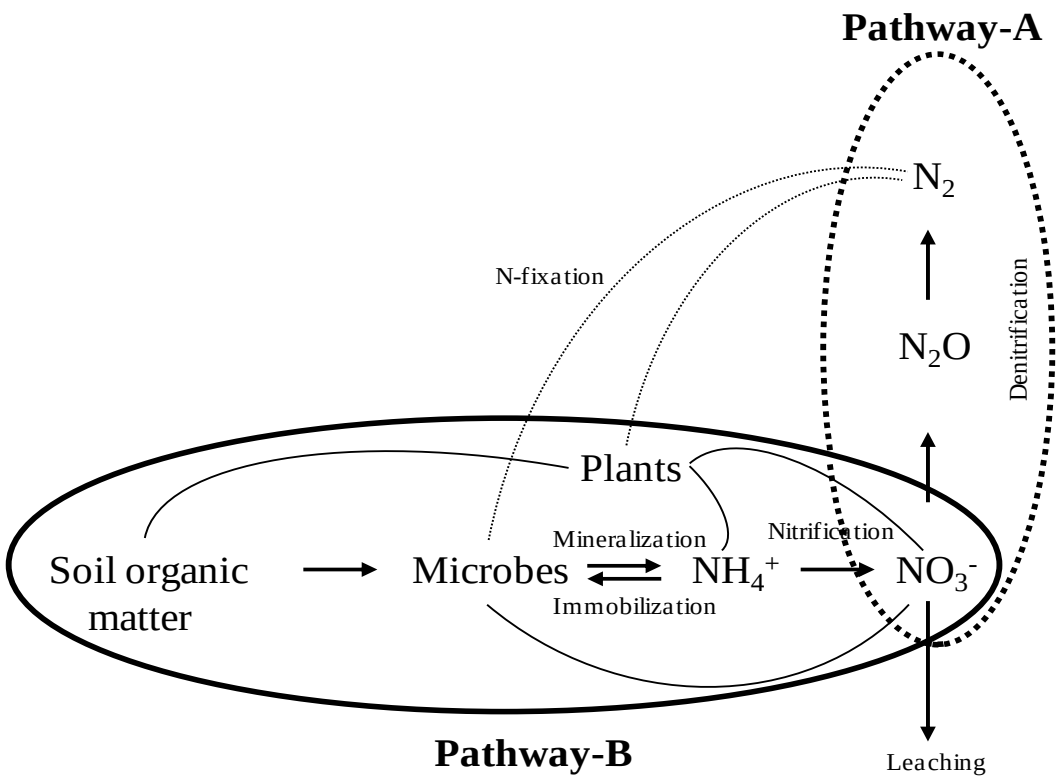

Fig. 1.6. Simplified soil N cycle (adapted from Schimel et al. (2005)). Pathway A indicates the ways that add or remove N from the soil, while pathway B shows the ways that make N available to plant or microbes.

N cycling is centrally driven by the microbial communities and has been extensively focused as a model study in the field of microbial ecology (Colloff et al. 2008, Kowalchuk & Stephen 2001, Philippot & Hallin 2005, Prosser 1989). The N cycle can be divided into two main domains. The first one is comprised of the processes that are responsible for adding or removing N from the soil, such as N fertilization, N fixation, denitrification, volatilization and leaching (Aubert et al. 2005). The second domain is comprised of the processes that make N available for plants and microorganisms, and includes decomposition, mineralization, nitrification, microbial immobilization and plant assimilation (Stevenson & Cole 1999).

Over the last decades, our knowledge on the microorganisms and the enzymes involved in N cycling has considerably increased. We now have acquired a decent picture of how soil microorganisms control the various N cycling steps (De Boer & Kowalchuk 2001, Falkowski 1997, Francis et al. 2007, Hayatsu et al. 2008). In our work, we have focused on nitrification (the conversion of ammonium into nitrate) and the denitrification (returning of soluble N back to atmosphere), two major processes of the soil N cycle that are responsible

for N losses from the soil. Both these processes have strong ecological and economical significance. For example, the agricultural systems receive annually 25% of global fertilizer N in ammonium form that ultimately needs to be converted to nitrate through nitrification (Gruber & Galloway 2008, Xia et al. 2011). It can also lead to net loss of nitrogen (Kowalchuk & Stephen 2001) affecting the agricultural production. The annual losses of N fertilizer are estimated to $15.9 billion (Raun & Johnson 1999) that include the greenhouse gases (nitric oxide (NO) and nitrous oxide (N_2O)) from both nitrification and denitrification (Conrad 1996, Godde & Conrad 2000). N_2O is one of the greenhouse gases that is considered in the KYOTO protocol. It contributes approximately 6% to the total radiative forcing of the earth's atmosphere (IPCC 2001) and is involved in ozone depletion (Bange 2000, Dickinson & Cicerone 1986). Global warming effect of this gas is estimated to be 300 times greater than CO_2 (Forster et al. 2007) and the atmospheric concentration of N_2O has increased from 275 to about 310 ppbv since the last hundred years (Beauchamp 1997, IPCC 1995).

6.2. Nitrification

6.2.1. Nitrification process

Nitrification is the biological oxidation of ammonia (NH_4^+, originating from ammonium added to soils or produced by the decomposition of organic matter) to nitrate (NO_3^-) via nitrite (NO_2^-) (Fig. 1.7). It is thought to be the main process connecting the oxidative and reductive parts of the N cycle. Nitrification produces nitrous and nitric oxides, thereby contributing directly (with N_2O) or indirectly (with NO) to greenhouse gas emissions. The nitrate produced may be either taken up by plants or immobilized by soil microorganisms. Since both soil particles and nitrate are negatively charged, and that nitrate is a small molecule, nitrate in the soil can easily be leached to groundwater.

6.2.2. Nitrification steps

The first step of nitrification is nitritation, the conversion of ammonia to nitrite (NO_2^-), via hydroxylamine (NH_2OH), catalyzed successively by the ammonia monooxygenase (AMO, encoded by the *amoA* gene) and the hydroxylamine oxidoreductase (HAO) enzymes. Nitratation, the second step of nitrification, is the conversion of nitrite to nitrate, catalyzed by the nitrite oxidoreductase (NOR) enzyme. This step is carried out by various genera and widely known as a rapid process (Gelfand & Yakir 2008). It is thought to be performed by

Nitrobacter, an Alphaproteobacteria but some other genera such as *Nitrospina*, *Nitrococcus* are also able to oxidize nitrite into nitrate (Watson et al. 1981).

Ammonia-oxidation, the first step of nitrification, is the rate-limiting step of nitrification in a variety of environments (Kowalchuk & Stephen 2001, Phillips et al. 2000), as nitrite is rarely found to accumulate in the environment (Eldemerdash & Ottow 1983, Prosser 1989).

Both of the nitrification steps are aerobic and autotrophic: the communities involved in the process use oxygen as the terminal electron acceptor while the N and the CO_2 are utilized as the energy and carbon source (Konneke et al. 2005, Prosser 1989). Besides autotrophic nitrification, other processes have been shown to be involved in ammonia transformations. They include heterotrophic bacteria and fungi which can perform nitrification, but their process rate is slower than autotrophic communities (Verstraete & Alexander 1973, Watson et al. 1981). Such processes have not been shown to yield energy; hence they are not expected to contribute to cell growth: their physiological role as well as the phylogenetic diversity of heterotrophic nitrification still remains unclear (Hayatsu et al. 2008).

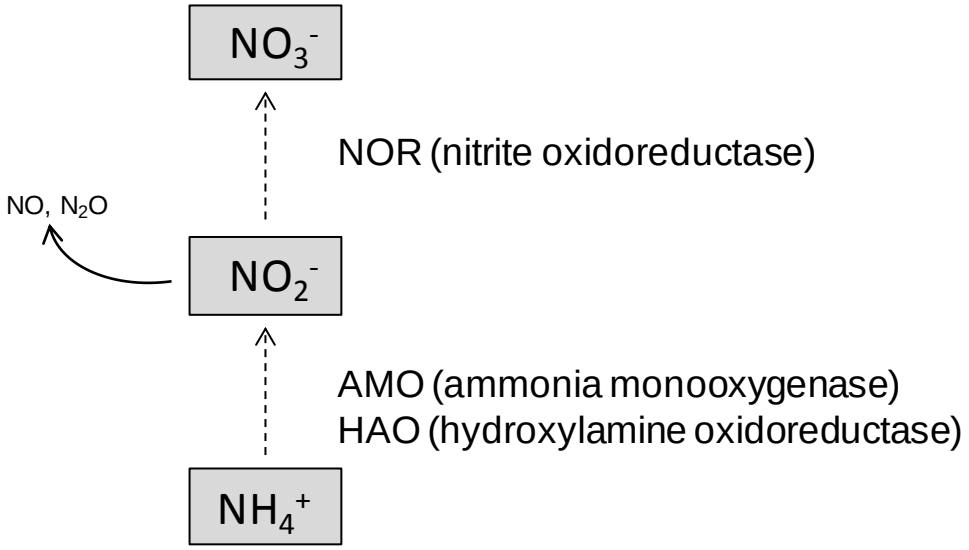

Fig. 1.7. Nitrification pathway including the ammonia monooxygenase enzyme and the gene encoding that enzyme

6.2.3. Ecology of ammonia-oxidizers

While autotrophic ammonia-oxidizing bacteria of the beta- and gamma-subgroups of proteobacteria (Bock & Wagner 2006, Kowalchuk & Stephen 2001, Purkhold et al. 2000) were long thought to be the only microorganisms to oxidize ammonium, it was recently

demonstrated that some archaea could also perform this process (Konneke et al. 2005, Treusch et al. 2005, Venter et al. 2004). Ammonia oxidizing bacteria (AOB) and archaea (AOA) of the phylum Proteobacteria and Crenarchaeota/Thaumarchaeota, respectively, are the now widely studied actors of oxidation of ammonia to nitrite (Jia & Conrad 2009, Offre et al. 2009).

Both AOA and AOB are ubiquitous and diverse. The distribution of AOA (Beman & Francis 2006, Chen et al. 2008, Francis et al. 2005, Leininger et al. 2006, Prosser & Nicol 2008) and AOB (Beman & Francis 2006, Kowalchuk & Stephen 2001) has been studied across a wide range of environments. Leininger et al. (2006) reported that the abundance of the AOA were 1.5 to 232 fold higher than that of the AOB in the upper horizons from the 12 different soils. The AOA/AOB ratio increased with soil depth to up to 3000 (Leininger et al. 2006). Similarly He et al. (2007) and Chen et al. (2008) also found greater abundance of the AOA in different soils with the AOA/AOB ratios ranging from 1.02 to 12.4 and 1.2 to 69.3 respectively. These results together with some recent observations suggested that the AOA are predominant across a range of environments (Cao et al. 2011, Isobe et al. 2012). In contrast, abundance of the AOB was found to be significantly higher than AOA in an acid soil with and without amendment of montmorillonite (Jiang et al. 2011). More recently Fortuna et al. (2012) also reported that AOB were one order of magnitude higher than the AOA across six soils of variable texture and mineralogy. These results are supported by a few other studies (Fan et al. 2011, Li et al. 2011, Mosier & Francis 2008, Wells et al. 2009).

Given the significance of ammonia oxidation in global N cycle, understanding the ecology of the ammonia oxidizing groups catalyzing this process and their relative contribution to nitrification can be critical for overall N availability and losses from soil ecosystem. Using stable isotope probing, Zhang et al. (2010, 2012), demonstrated autotrophic growth of AOA in soil and their contribution to soil ammonia oxidation. Moreover, by using acetylene inhibition of ammonia oxidation it was shown that growth of archaeal but not the bacterial ammonia oxidizers occurred in the samples with active nitrification suggesting AOA as the main contributor in ammonia oxidation (Offre et al. 2009). However ammonia oxidation stimulation by the ammonium and inhibition by the acetylene also revealed that the change in ammonia oxidation activity was related to the AOB abundance but not the AOA in agricultural soils indicating the functional dominance of the AOB (Jia & Conrad 2009). Di et al. (2009) also found a significant relationship between the abundance of the AOB and the nitrification rates suggesting that nitrification is driven by bacteria rather than archaea in

nitrogen rich grassland soils. Moreover, Mertens et al. (2009) demonstrated that bacteria and not the crenarchaea drove nitrification in Zn contaminated soils.

Soil fertility, pH and temperature can influence the ecology of ammonia oxidizers and their function (Avrahami & Conrad 2003, Erguder et al. 2009, He et al. 2007, Phillips et al. 2000). For example, the AOA are reported to play greater role under low nutritional state whereas the AOB are considered as the copiotrophic that are associated with the environments of high nutrition (Bouskill et al. 2012). Accordingly Di et al. (2010) related the greater AOB populations to the higher fertility and higher N inputs in topsoils of three grasslands. pH is another important factor affecting ammonia oxidizers and Nicol et al. (2008) suggested that the autotrophic ammonia oxidation is mainly related to the archaea in acidic soils.

6.3. Denitrification

6.3.1. Denitrification process

Denitrification is a heterotrophic dissimilation process in the N cycle. Under anaerobic conditions, nitrate (NO_3^-) is converted to nitrite (NO_2^-) then to gaseous nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2). Denitrification is the main biological process that removes N from ecosystems and involves a wide variety of microorganisms that use oxidized N forms as electron acceptors, resulting in production of energy.

Note that dissimilatory nitrate reduction to ammonium (DNRA) may also take place, resulting in the formation of reduced N products (NH_4^+, Fig. 1.8). However, soil conditions are generally more favorable for denitrification than DNRA (Tiedje 1988). Like denitrification, DNRA utilizes the N oxides as terminal electron acceptor and can lead to the production of gaseous N compounds including N_2O (Fazzolari et al. 1998). DNRA may account for up to 14.9% of total reduction of the ^{14}N-labelled nitrate added to the soil, with NH_4^+ being the major product (Yin et al. 2002). This process can contribute to alleviate N losses through leaching and thereby reduce underground water pollution.

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NH_4^+$$

Fig. 1.8. Dissimilatory nitrate reduction pathway (DNRA)

6.3.2. Denitrification Steps

Denitrification is a multiple-step process (Fig. 1.9). First, NO_3^- is reduced to NO_2^- by the nitrate reductases, be it the membrane bound nitrate reductase (Nar) or the periplasmic nitrate reductase (Nap) those catalytic subunits are encoded by the genes *narG* and *napA*, respectively (Potter et al. 2001, Zumft 1997). Nitrate reduction is performed by taxonomically diverse bacteria that possess one or both of the nitrate reductases (Philippot & Hojberg 1999).

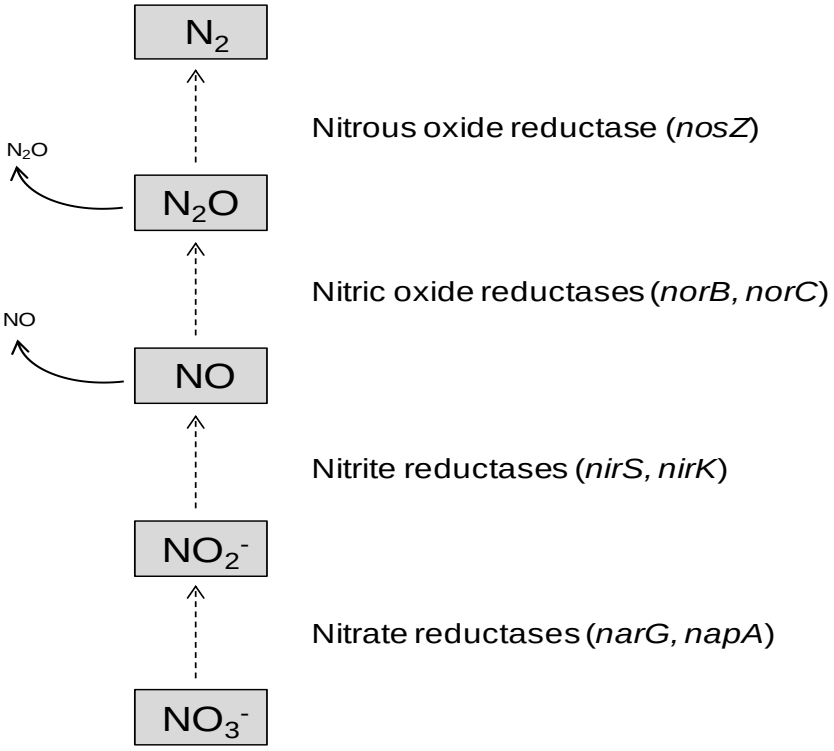

Fig. 1.9. Denitrification pathway including the different enzymes involved
The genes coding for the catalytic subunits of these enzymes are indicated in parenthesis.

The second step of the denitrification is the reduction of soluble nitrite (NO_2^-) into to the gazeous form nitric oxide (NO). This reaction is catalyzed by two different enzymes, the copper nitrite reductase and the cytochrome cd_1 nitrite reductase, encoded by the genes *nirK* and *nirS*, respectively (Zumft 1997). In the third step, nitric oxide reductase reduces NO to N_2O, a complex of two subunits encoded by the genes *norB* and *norC*. Gene homologues to nitric oxide reductase have been found in non-denitrifying bacteria (Mahne & Tiedje 1995) indicating that it is likely not denitrification-specific.

Finally, nitrous oxide (N_2O) is reduced to dinitrogen (N_2) by a multicopper homodimeric nitrous oxide reductase encoded by the *nosZ* gene (Knowles 1982). Recently it has been evidenced that some denitrifying bacteria lack the *nosZ* gene (Jones et al. 2008).

This suggests that the lack of *nosZ* gene among the denitrifying organisms can influence the overall N_2O budget, because the end product of the denitrification is N_2O instead N_2, as demonstrated experimentally (Philippot et al. 2011). Moreover, it has been reported a lower abundance of *nosZ* gene as compared to other denitrifying genes (Hallin et al. 2009, Henry et al. 2006) and the lack of *nosZ* can further result in the increased N_2O emissions thus influencing the overall global climate as N_2O is a greenhouse gas. Both the production as well the consumption of the N_2O makes the last step of denitrification process crucial in soil N cycle. The better understanding of the enzymology of the denitrification will certainly help in management of N_2O emissions (Richardson et al. 2009).

6.3.3. Ecology of denitrifiers

Denitrifiers exist in a wide range of environments including soil, sewage, marine and fresh water (Philippot & Hallin 2006, Tavares et al. 2006). The diversity of the denitrifiers is high with more than 60 genera including bacteria, archaea and fungi capable of denitrification (Philippot et al. 2007). In soils, denitrifiers represent up to 5% of the soil microbial community (Bru et al. 2007, Cheneby et al. 2004, Djigal et al. 2010, Henry et al. 2006, Tiedje 1988) and according to the different estimates they range from 10^5 to 10^8 denitrifiers per gram of soil (Dandie et al. 2008, Hallin et al. 2009, Henry et al. 2004).

The major environmental factors regulating denitrification are C and N availability and the oxygen partial pressure (Hochstein et al. 1984, Philippot et al. 2007, Tiedje 1988). Under anaerobic condition the key factor regulating denitrification is the organic matter availability since C is utilized as electron donor during reduction of the nitrogen oxides (Bremner 1997). Overall increase in soil water content stimulates the denitrification because high soil moisture restricts the oxygen diffusion (Vinther 1984). In fact, at low oxygen concentration (below 5%) when nitrate is available, denitrification can become the main microbial respiratory process (Hochstein et al. 1984).

7. Objectives

This PhD work is part of a larger program 'BIOTECHNOSOL' piloted by Jérôme Cortet (ENSAIA Nancy) under the framework of GESSOL IV funded by the Ministry of Environment and Ecology to address the consequences of soil restoration through the construction of Technosols on soil biological quality. The main focus of the project was to investigate the ability of the Technosols to perform the essential functioning of the natural soil

such as plant growth and the diversity and functioning of the soil organisms involved in many soil physical (aggregation, bioturbation) and chemical processes (nutrient cycling). A group of scientist comprised of different domains of soil biology and ecology (micrororganismes, microfauna, mesofauna, macrofauna), agronomy and biophysics studied the Technosols through various approaches. In this program, our aim was to investigate the total bacterial community but also the functional communities involved in N cycling, a soil process crucial for the nutrition of plants colonizing lands under restoration.

The present work was carried out to improve our knowledge on the restorative effect of constructed Technosols on various ecological aspects of N cycling. The objective of this thesis was to assess whether the constructed Technosols are capable to deliver soil ecosystem services using N cycling as model service. For this purpose, we focused on the functional guilds of ammonia oxidizers and denitrifiers, which are key players in the N-cycle. Abundance and activity of these functional communities were studied in two different Technosols in relation to both horizontal and vertical distributions. In addition, the abundance and diversity of the total bacterial community and the abundance of the crenarchaeal community were also investigated. Finally we estimated the functional stability of the N cycling activities to assess the ability of Technosols to withstand an environmental perturbation. Overall, the following lines of direction were set to achieve the defined objective;

- Characterize the microbial communities in two different constructed Technosols.
- Understand the vertical distribution of N cycling microbial communities along depth profiles in two different constructed Technosols.
- Estimate the functional stability (resistance and resilience) of N-cycling processes in constructed Technosols.

CHAPTER 2 TAXONOMIC AND FUNCTIONAL CHARACTERIZATION OF MICROBIAL COMMUNITIES IN TECHNOSOLS CONSTRUCTED FOR REMEDIATION OF A CONTAMINATED INDUSTRIAL WASTELAND

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Taxonomic and functional characterization of microbial communities in Technosols constructed for remediation of a contaminated industrial wasteland

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Abstract

Purpose: The construction of Technosols is an emergent technology based on the assemblage of technogenic materials for ecological reclamation of polluted land and waste recycling. Although this technology is in expansion, knowledge about the microbial communities in Technosols is limited, despite their central role in ecosystem functioning. In this two year study, the diversity and the abundance of total and functional microbial communities were characterized in two types of Technosols constructed to reclaim contaminated sites.

Materials and methods: The structure of the microbial community was analyzed by Automated Ribosomal Intergenic Spacer Analysis fingerprinting in both types of Technosols and the taxonomic diversity was further assessed by 16S rRNA clone library sequencing. Real time PCR was used to quantify the abundance of the total bacterial and crenarchaeal community and of the functional guilds involved in N-cycling.

Results and discussion: 16S rRNA sequencing showed that *Proteobacteria* was the main phylum in the Technosols (50-80%). The other significant phyla identified were *Bacteroidetes, Firmicutes, Choloroflexi* and *Actinobacteria*. Real time PCR quantification of the abundance of ammonia oxidizers, nitrate-reducing and denitrifying microbial communities involved in nitrogen cycling revealed that bacterial ammonia-oxidizers were more abundant than crenarchaeal ammonia-oxidizers. A high spatial variability of the microbial community, which decreased with time, was also observed.

Conclusions: At the phyla and class levels, the composition of the microbial community in constructed Technosols were similar to 'natural' soils. Both the total bacteria and microbial guilds involved in N-cycling were abundant but in contrast to most 'natural' soils, bacteria and not crenarchaea were the numerically dominant ammonia oxidizers in both types of Technosols. The decrease with time of the variability in microbial community structure support early pedogenic evolution of recently constructed Technosols.

Keywords Constructed soils • Contaminated soils • Microbial community • Nitrogen cycling • Technosols

1. Introduction

Soil is a non-renewable resource that carries out essential functions for terrestrial ecosystem services such as plant production, nutrient cycling and filtering (Heijden et al. 2008; Nannipieri et al. 2003; Wardle and Giller 1996). However, soil is under increasing pressure from human activities and soil degradation has become a major issue in Europe (COM 2006). Intensive agriculture, urbanization and industrialization are the major causes of soil degradation. Irresponsible disposal of industrial waste, careless use of chemicals and heavy metals and discharge of considerable amounts of contaminants have degraded the capacity of soils to provide essential services. For example, in France no less than 4365 sites, most contaminated with hydrocarbons or heavy metals, need remediation (Basol 2011).

Traditional approaches for polluted soil remediation consist in excavation and replacement of the upper soil layer or treatment of the polluted soil *in* or *off* site. Soil construction is an innovative soil remediation process based on the assemblage of treated soil, recycled waste and industrial by-products, which are formulated and layered to build a new soil profile, Technosol, at the polluted site (Sere et al. 2008; IUSS Working Group WRB 2006). The in-place restoration of the contaminated sites by soil construction is a solution for both ecological reclamation of polluted lands and waste recycling. Recent works showed that constructed Technosols can support sown and indigenous plants without any deficiency symptoms, suggesting that they could be an ecological means of reclaiming derelict land (Sere et al. 2008). In addition to plant biomass production, hosting biodiversity and nutrient cycling are essential functions of a natural soil. The biodiversity of microorganisms in the soil is the highest on earth and it is estimated that there are several thousand to several million different genomes per gram of soil (Torsvik and Øvreås 2002; Whitman et al. 1998). Microbial communities are key players in several soil functions including nutrient cycling (Bodelier 2011). For example, they are responsible for the nitrogen fixation, mineralization, nitrification and denitrification processes mediating the conversion between the different forms of nitrogen, which is the nutrient that most often limits plant growth. Microorganisms are also responsible for the degradation of toxic compounds, such as hydrocarbons, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Haritash and Kaushik 2009; Petrić et al. 2011; Vinas et al. 2005). However, despite their central role in the services provided by soil, little is known about the development of microbial communities in constructed Technosols.

This study was undertaken (i) to examine and characterize the diversity of the dominant bacterial taxa in Technosols constructed on industrial wasteland that had previously been contaminated by both PAHs and metals and (ii) to quantify the abundance of total bacterial and crenarchaeal communities and of microbial communities involved in the nitrogen cycling processes ammonia-oxidation and denitrification. Ammonia oxidation is the first and rate limiting step in the nitrification process, the oxidation of ammonium via nitrite to nitrate, which is of high environmental and agronomical interest. Indeed, during nitrification the relatively immobile nitrogen form ammonium is converted into the highly mobile form nitrate, which can either be assimilated by the plants or leached from soil leading to N losses. Denitrification, the microbial reduction of nitrate into gas, is the main biological process responsible for the return of fixed nitrogen to the atmosphere, thus completing the N cycle. It is also responsible for significant N-losses from the soil ecosystem and together with nitrification, it contributes to the emissions of the greenhouse gas N_2O (Conrad 1996).

2. Material and methods

2.1. Experimental site and sampling procedure

The study was carried out on a 1 ha experimental site built in 2007 on industrial wasteland in Homécourt, France (Groupement d'Intérêt Scientifique sur les Friches Industrielles, GISFI, http://www.gisfi.fr/index_fr.htm). The climate is continental with a mean rainfall of 760 mm $year^{-1}$ and a mean temperature of 10°C (extreme values: -21.6°C to 37.6°C). Three different types of waste and by-products were used as parent materials for the construction of Technosols (i) green waste compost from urban trees and grass cuttings, licensed under NF U 44-051, (ii) paper mill sludge, a by-product of sewage produced from recycled paper, and (iii) industrial soil excavated from a former coke plant, initially contaminated with PAHs, which was thermally desorbed before being used for soil construction. These different types of parent materials were staked and layered to build the Technosols at the contaminated site. Two types of Technosols were constructed (Spolic Garbic Technosol (Calcaric)) (Fig. 2.1). The first type (T1) consisted of (i) compost horizon (upper layer with a depth of approximately 15 cm), (ii) mixture of paper by-products and treated industrial soil (layer below the compost horizon with a depth of approximately 60 cm) and (iii) paper by-products (bottom layer with a depth of approximately 30 cm). The second type (T2) had two horizons similar to those of T1 and the third confinement horizon (0.25 m) at the bottom composed of

limed paper by-products (5% by volume) with specific compaction properties. This Technosol is intended to limit the infiltration of water carrying residues of the polluted industrial materials into the aquifers.

Fig. 2.1. Technosols during and after construction

Samples were collected according to a sampling grid dividing the T1 Technosol into 13 20 x 20 m plots and the T2 Technosol into 11 20 x 20 m plots. Three sub-samples were taken from the top soil layer (0-20 cm) from each plot and then mixed to form one composite sample for each of the 24 plots. Soil samples were collected in spring 2008 and 2009.

2.2. Physico-chemical properties of soil

Physico-chemical properties determined from the two Technosols are described in Table 2.1. The methods used for these analysis: pH water (ratio soil/solution = 1/5) (NF ISO 10390), organic matter and organic carbon (oxidation by heating at 900°C under O_2 flow), total nitrogen (Kjeldahl mineralization-NF ISO 11261), total calcium carbonate (reaction with HCl and measurement of the volume of carbon dioxide evolution with a Scheibler device-NF ISO 10693), cation exchange capacity (exchanged ammonium ions-NF ISO 11260), P_{Olsen} ($NaHCO_3$ extraction and then proportioning of phosphorus complexes by spectrometric methods-NF ISO 11263) (AFNOR 2004). All these analysis were carried out by the certified laboratory of the French agronomic research institute (Laboratoire d'Analyse des Sols, INRA, Arras).

Table 2.1. Physico-chemical properties of the constructed Technosols (T1 and T2)

Each value is the average of 6 samples (n=6) with the standard deviation given in parenthesis.

Parameter	T1	T2
Soil moisture (%)	29.6 (4.88)	32.0 (2.04)
Gravels (>0.5 cm) (g. kg^{-1})	195 (45.8)	161 (98.4)
Gravels (0.2-0.5 cm) (g. kg^{-1})	57.2 (26.1)	78.7 (34.4)
Clay (< 2 µm) (g. kg^{-1})	141 (18.0)	186 (44.8)
Fine silt (2-20 µm) (g. kg^{-1})	109 (8.80)	111 (20.2)
Coarse silt (20-50 µm) (g. kg^{-1})	56.3 (4.59)	78.2 (27.9)
Fine sand (50-100 µm) (g. kg^{-1})	145 (18.2)	131 (16.4)
Coarse sand (200-2000 µm) (g. kg^{-1})	131 (29.1)	161 (46.9)
Total Nitrogen (g. kg^{-1})	2.42 (0.48)	6.61 (5.02)
Organic Carbon (g. kg^{-1})	77.7 (6.75)	117 (44.9)
Organic matter (g. kg^{-1})	134 (11.5)	203 (77.5)
C/N	32.8 (4.98)	24.57 (11.2)
pH	8.36 (0.21)	8.18 (0.27)
Total $CaCO_3$ (g. kg^{-1})	408 (24.1)	325 (13)
Olsen Phosphorus (P_2O_5) (g. kg^{-1})	0.07 (0.03)	0.15 (0.12)

2.3. DNA extraction and purification

The soil samples were sieved to 2 mm and sub-sampled for DNA extraction. Three independent DNA extractions were performed for each plot, according to ISO 11053 "Soil quality-Method to extract DNA from soil" (Petric et al. 2011; Philippot et al. 2010), as described by Martin-Laurent et al. (2001). Briefly, 1 g of soil was homogenized in 4 ml of extraction buffer for 30 s at 1,600 rpm in a mini-bead beater cell disruptor (Mikro-Dismembrator S; B. Braun Biotech International). Soil and cell debris were removed by centrifugation. After sodium acetate precipitation, proteins were removed. Nucleic acids were then precipitated with ice-cold isopropanol and washed with 70% ethanol.

The soil DNA extracts were purified using polyvinyl polypyrrolidone (PVPP) and Sepharose 4B spin columns (Sigma-Aldrich, USA) as described by Martin-Laurent et al. (2001). A Nucleo Spin Extract-II" purification kit was used for the final purification step, according to the manufacturer's instructions (Macherey-Nagel, Germany).

The quality and size of the soil DNA were checked by electrophoresis on 1% (w/v) agarose gels. The DNA was quantified on gel after staining with ethidium bromide (200 µg/l) using ImageQuaNT (Molecular Dynamics, Ca, USA). A calibration curve performed with calf thymus DNA (Biorad) (5, 10, 20 and 200 ng of DNA) was used to estimate the amount of DNA extracted from the Technosols.

2.4. Automated Ribosomal Intergenic Spacer Analysis (A-RISA) fingerprinting of the microbial community structure

A-RISA was used to study the genetic structure of microbial community in the constructed Technosols. The 16S–23S intergenic spacer region of the bacterial rRNA was amplified in a final volume of 25 µl with 0.5 µM of universal primers 1552_f (5'-TCG GGC TGG ATG ACC TCC TT-3') and 132_r (5'-CCG GGT TTC CCC ATT CGC-3'), 2.5 U of Taq DNA polymerase (Appligene Oncor, France) and 0.25 ng of template DNA. The primer 1552_f was labeled with IRDye 800 fluorochrome in 5' (MWG SA Biotech, Ebersberg, Germany). PCR amplification was carried out in a gradient thermocycler PTC 200 (MJ Research, Waltham, Mass) under the following conditions: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; plus an additional cycle for 15 min at 72°C.

The quality of the PCR products was checked after electrophoresis on 2% agarose gel and the quantity estimated using a Smart Ladder DNA marker (Eurogentec, Belgium) and ImageQuaNT (Molecular Dynamics, Evry, France). After the PCR products had been homogenized to the same concentration (0.5 $ng.\mu L^{-1}$), 1.5 µl of the diluted PCR products, denatured at 92°C for 2 minutes, was loaded onto a 66 cm 3.7% polyacrylamide gel and run on a LiCor 4200 DNA Analyzer (Biosciences, USA) for 15 h at 1500 V/80 W. The gel was further analyzed using One-Dscan (ScienceTec) to convert fluorescence data into electrophoregrams where the peaks represent different PCR fragments. The height of the peaks was estimated using the Gaussian integration in One-Dscan to give the relative proportion of the fragments in the total products. The size of the DNA bands (in base pairs) was estimated using a DNA ladder of 15 bands ranging from 200 to 1200 bp.

2.5. 16S rRNA gene clone libraries and sequence analysis

On the basis of the A-RISA results, DNA from three plots from both types of Technosols (plots 14, 18, 21 for T1 and plots 3, 7, 16 for T2) was selected to construct six 16S rRNA clone libraries. 16S rRNA was amplified using the primers 341_f and 926_r (Watanabe et al. 2001). The amplification reaction was carried out in a final 25 µl volume containing 2.5 µl of 10x Taq polymerase buffer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of each primer and 0.625 U of Taq polymerase. PCR was performed in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) with one cycle of 4 min at 94°C; 39 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and one final elongation step of 5 min at 72°C.

16S rRNA PCR products were purified from the gel using the Qiaex II kit as described by the manufacturer (QIAGEN, France). The purified PCR products were ligated into the pGEM-T Easy Vector System (Promega, France) and transformed into *Escherichia coli* JM 109 according to the manufacturer's instructions (Promega, France). Ninety-six 16S rRNA recombinant clones from each of the six libraries were PCR-amplified using the universal primers SP6 and T7 under the following conditions: 94°C for 4 min, 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 90 s and a final extension step at 72°C for 7 min. The 16S rRNA PCR products were sequenced by Beckman Coulter Genomics, UK. The 16S rRNA gene sequences obtained were checked for chimera using Bellerophon (Huber et al. 2004), and taxonomic affiliation was performed using Greengene (*http://greengenes.lbl.gov/cgi-bin/nph-index.cgi*).

16S rRNA sequences obtained from clone libraries were aligned using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and the alignment obtained was uploaded to UniFrac (http://bmf2.colorado.edu/unifrac/index.psp). Pairwise UniFrac significance tests were then conducted to determine the differences in the composition of the bacterial communities in the Technosol samples using nucleotide sequences (Lozupone and Knight 2005). Distance matrix files were generated using PHYLIP (http: //evolution.genetics.washington.edu/phylip.html) and imported to MOTHUR (Schloss et al. 2009) to group the sequences into operational taxonomic units (OTU) defined at a threshold of 95% similarity. Diversity indices such as Shannon, Simpson and Chao were then calculated using MOTHUR.

2.6. Quantification of total and N-cycling microbial communities

Before running quantitative PCR (qPCR) assays, the potential presence of qPCR inhibitors was tested by mixing a known amount of pGEM-T plasmid with the soil DNA extracts or water with the plasmid-specific T7 and SP6 primers. This inhibition test showed that the measured cycle threshold (Ct) values were not significantly different between the DNA extracts and the controls with water, indicating that no inhibition occurred.

The bacterial (AOB) and crenarchaeal (AOA) ammonia-oxidizers were quantified as described by Leininger et al. (2006) and Tourna et al. (2008), respectively and the nitrate reducers and denitrifiers were quantified as described by Bru et al. (2007), Henry et al. (2006) and Henry et al. (2004). The genes encoding catalytic enzymes responsible for ammonia-oxidation (bacterial and crenarchaeal *amoA*), nitrate reduction (*narG* and *napA*), nitrite reduction (*nirK, nirS)* and nitrous oxide reduction (*nosZ*) were used as molecular markers. The total bacterial community was quantified using 16S rRNA primer-based qPCR assays as described previously (Lopez-Gutierrez et al. 2004). We also quantified the total crenarchaeal community, which includes the archaeal ammonia-oxidizers using the primers described by Ochsenreiter et al. (2003). The primer used for qPCR assays of the different genes are given in Table 2.2. qPCR assays were carried out with an ABI Prism 7900HT (Applied Biosystems, USA). SYBR green dye was used for detection in a reaction mixture of 15 µl containing 7.5 µl of the SYBR green® (2x) PCR master mix (Thermo: Absolute qPCR SYBR green®, Ref: AB-1162B), 2 µM of each specific primers, T4gp32 (250 ng/reaction; Q-BIOgene, France), and 0.1 ng of DNA sample. Independent qPCR assays were performed on three replicates of each of the 24 soil samples for each of the genes (n_{tot}=72). Standard curves were obtained with serial plasmid dilutions of a known amount of the plasmid DNA containing a fragment of the 16S rRNA or the *amoA, narG, napA, nirK, nirS,* or *nosZ* genes.

Table 2.2. Primers used for the quantitative PCR

Genes	Primers	Sequences (5'->3')	References
16S rRNA (Bacteria)	341F	CCTACGGGAGGCAGCAG	(Lopez-Gutierrez et al. 2004)
	534R	ATTACCGCGGCTGCTGGCA	
16S rRNA (Crenarchaea)	771F	ACGGTGAGGGATGAAAGCT	(Ochsenreiter et al. 2003)
	957R	CGGCGTTGACTCCAATTG	
***amoA* (Bacteria)**	*AmoA*1F	GGGGTTTCTACTGGTGGT)	(Leininger et al. 2006)
	*AmoA*2R	CCCCTCKGSAAAGCCTTCTTC	
***amoA* (Archaea)**	crenamoA23F	ATGGTCTGGCTWAGACG	(Tourna et al. 2008)
	crenamoA616R	GCCATCCATCTGTATGTCCA	
narG	*narGG*-F	TCGCCSATYCCGGCSATGTC	(Bru et al. 2007)
	narGG-R	GAGTTGTACCAGTCRGCSGAYTCSG	
napA	*napA*3F	TGGACVATGGGYTTYAAYC	(Bru et al. 2007)
	*napA*4R	ACYTCRCGHGCVGTRCCRCA	
nirS	*nirS*4QF	GTWAACGSWAAGGARACWGG	(Throbäck et al. 2004)
	*nirS*6QR	GAWTTCGGRTGWGTCTTWAYGAA	
nirK	*nirK* 876F	ATSGGCGGVCASGGCGA	(Henry et al. 2004)
	nirK 1040R	GCCTCGATCAGRTTRTGGTT	
nosZ	*nosZ* 1840F	CGCRACGGCAAWAAGGTWMWWGT	(Henry et al. 2006)
	nosZ 2090R	CAKRTGCAKWGCRTGGCAGAA	

2.7. Statistical analysis

Where the qPCR data was not normally distributed, a log transformation was applied and further analysis was carried out using the Student *t*-test ($P<0.05$) or an ANOVA test with XLSTAT (Addinsoft 2009). The results of A-RISA fingerprints were analyzed by converting data from OneD-scan into a matrix summarizing the band presence (peaks) and intensity (height of peaks) using PrepRISA (*http://pbil.univ-lyon1.fr/ADE-4/microb/*). Data matrices (presence and intensity of bands) were then used to perform a principal component analysis (PCA) using ADE-4 (*http://pbil.univ-lyon1.fr/ADE-4/home.php*).

3. Results and discussion

3.1. Genetic structure of the bacterial community in the constructed Technosols

Fig. 2.2. shows the fingerprinting analysis of the genetic structure of the soil bacterial community in the various Technosol plots in 2008 and 2009. A-RISA fingerprints indicated a high diversity with more than 100 bands ranging from 200 to 900 bp and good reproducibility between the three replicates from the same plot.

Principal component analysis (PCA) of the A-RISA fingerprint matrix was carried out to determine the similarity of the bacterial community between plots (Fig. 2.3). It revealed that differences in the genetic structure of the microbial community were greater between plots than between the two types of constructed Technosols. Accordingly, several physico-chemical properties exhibited high variability such as the organic matter or total N (see Table 2.1). This high spatial variability is probably due to heterogeneous nature of the Technosols. Thus, the studied Technosols are a mixture of paper by-products and treated industrial soil together with green waste compost, which contains a high concentration of organic matter, derived from composting urban tree and grass cuttings. Accordingly, Ranjard et al. (2000) suggested that the heterogeneous distribution of bacteria was related to the level of organic carbon. However, in this experiment the high spatial variability of the bacterial community structure decreased in 2009 along the first PCA axis which explained 56% and 21% of the variance in the data in 2008 and 2009, respectively. This sharp decrease in variance explained by the first PCA axis may be attributed to ongoing physical and chemical transformation of the raw materials used to construct the Technosols, which led to rapid homogenization of the microbial habitat. This hypothesis is supported by Sere et al. (2010), who reported early pedogenic evolution in recently constructed Technosols similar to those studied in this work.

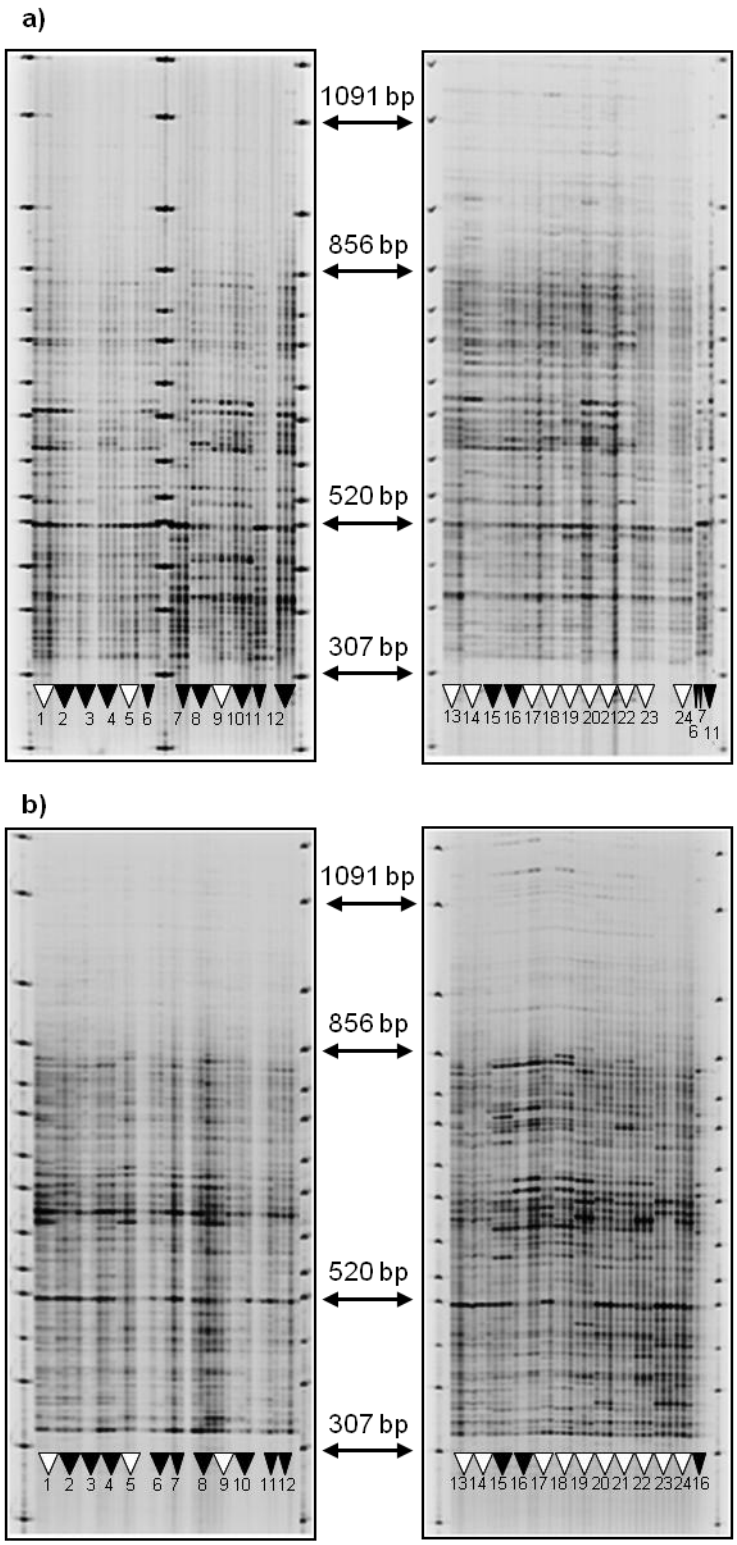

Fig. 2.2. A-RISA fingerprinting from two different Technosols

A-RISA profiles from T1 (▽) and T2 (▼) Technosols, sampled in 2008 (a) and 2009 (b). Plot numbers are indicated at the bottom of the gels (three replicates per plot).

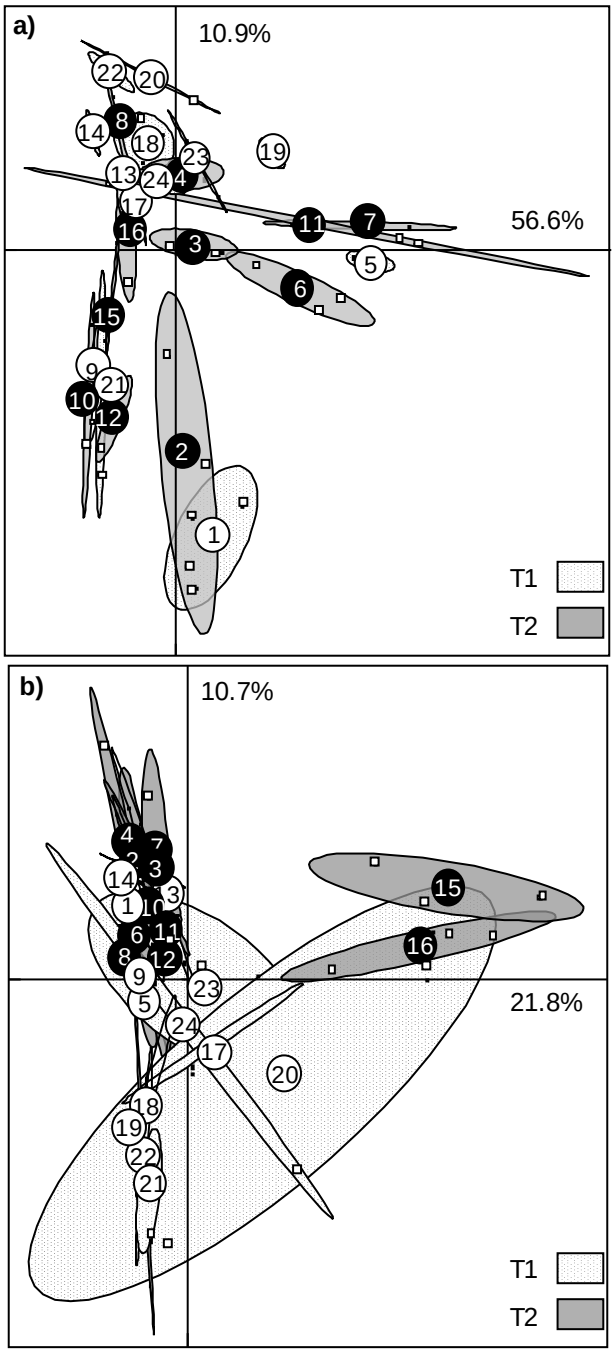

Fig. 2.3. Principal component analysis of the A-RISA fingerprinting profiles

Principal component analysis of the A-RISA profiles of the total bacterial community structure from the 24 plots from the two types of Technosols (T1 and T2) for 2008 (a) and 2009 (b).

3.2. Identification of the dominant bacterial taxa

For further analysis of the composition of the bacterial community present in the constructed Technosols, six 16S rRNA clone libraries were constructed and sequenced from three replicated plots for both Technosols. For this purpose, three different plots based on the 2009 A-RISA fingerprinting were selected from each type of Technosols for clone library analyses. The results of the taxonomic affiliation of about 550 sequences using Greengene are shown in Fig. 2.4. In accordance to the A-RISA data and soil properties, large differences in the proportion of the identified taxa were observed between plots. For example, the proportion of *Acidobacteria* varied from 3% to 20% and that of *Firmicutes* from 2% to 17%. In all plots, *Proteobacteria* were the dominant phylum accounting for up to 50-80% of bacteria in the constructed Technosols. Of the *Proteobacteria*, *Gammaproteobacteria* dominated in 4 of the 6 plots. The other significant phyla identified were *Bacteroidetes*, *Firmicutes*, *Choloroflexi* and *Actinobacteria*. An analysis of thirty-two libraries of 16S rRNA genes of members of the domain Bacteria prepared from a variety of soils showed that members of the phylum *Proteobacteria* accounted for an average of 39% (range from 10 to 77%) of the libraries (Janssen 2006). Most *Proteobacteria* members were classified as *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, the other dominant phyla being *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes* and *Choloroflexi* (Janssen 2006). These results suggested that, at phyla or class level, the composition of the bacterial community in the constructed Technosols was very similar to that in “natural” soils.

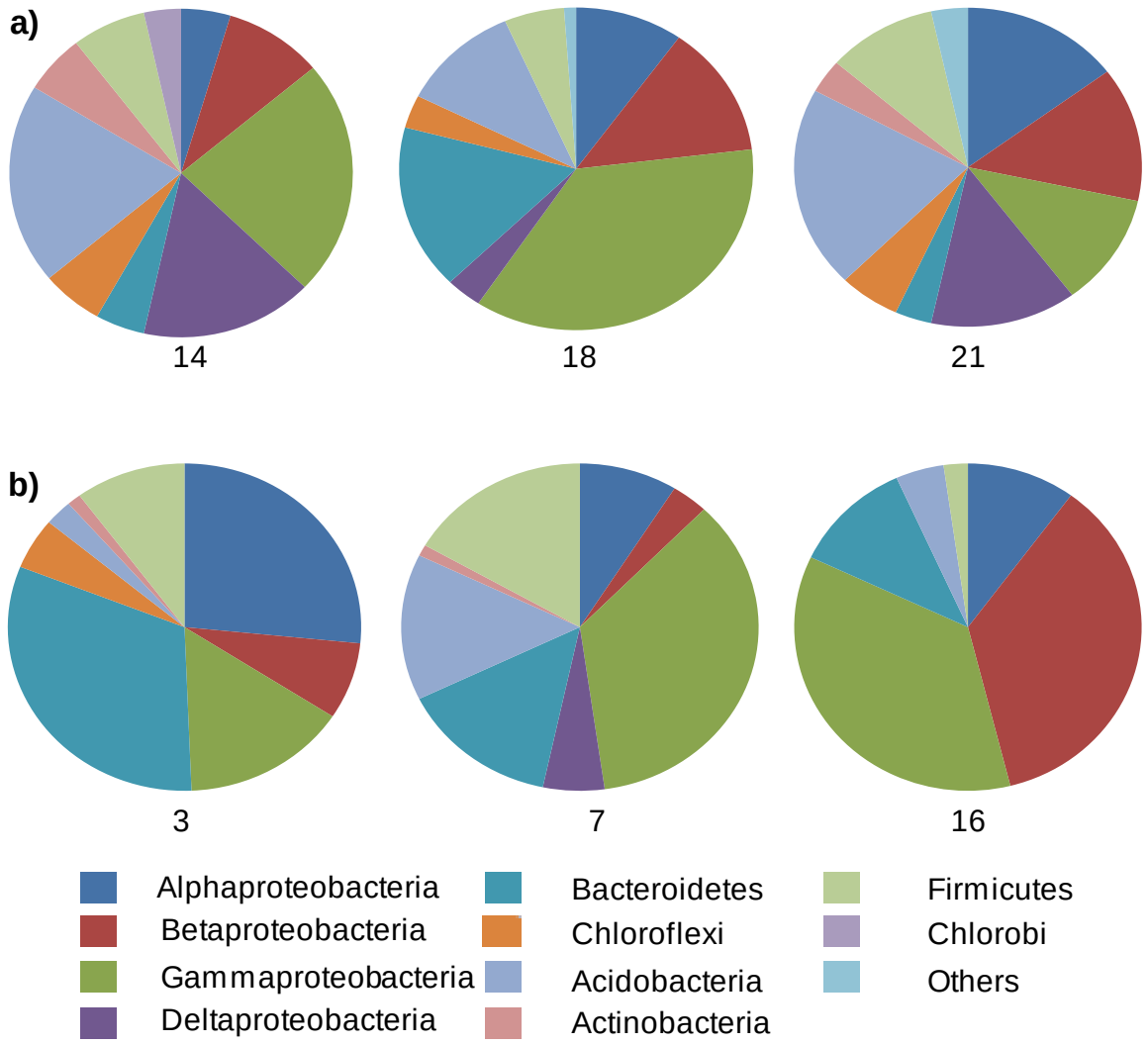

Fig. 2.4. Bacterial community composition analyses in two different Technosols Relative abundance of dominant bacterial groups from T1 and T2 Technosols, determined by sequencing 16S rRNA clone libraries. a) plots 14, 18, 21 (T1) b) plots 3, 7, 16 (T2).

The test for phylogenetic differences in the clone libraries from the different plots using UniFrac showed that bacterial communities in the two Technosols were similar (data not shown). For further analysis of the bacterial diversity in the samples, the number of operational taxonomic units (OTU) and diversity indices were calculated using MOTHUR (Table 2.3). The number of OTUs varied between 20 and 44 without any difference between the two types of constructed Technosols (see Table 2.3) and with the exception of plot 16, all the diversity indices calculated were similar for all plots. Despite differences in water diffusion properties between T1 and T2, our data indicate that the type of Technosol did not

influence the diversity of the microbial community (see Table 2.3). Indeed, the main difference between the two types of Technosols was the confinement horizon, 0.75 m below the surface, but, in this study, samples were collected from the upper horizon to study the bacterial community at plant root system level.

Table 2.3. Comparison of OTUs and diversity indices for 16S rRNA gene libraries

Analyses include 16S rRNA gene libraries from 3 different plots for the two types of constructed Technosols T1 and T2.

	T1 Plots			**T2 plots**		
	14	**18**	**21**	**3**	**7**	**16**
# of Seq.	86	91	88	79	90	89
# of OTU (0.05)	40	39	38	32	44	20
Shannon (H)	3.37	3.36	3.43	3.18	3.46	2.51
Simpson (1/D)	27.03	27.78	35.71	23.26	30.30	8.33
Chao1	70.00	52.91	55.14	41.43	87.88	29.00
95% lci	50.17	43.52	43.20	34.48	60.42	22.00
95% hci	128.47	81.83	94.47	67.91	161.27	60.45

3.3. Abundance of total bacterial and crenarchael communities and of microbial guilds involved in N-cycling

The sizes of the total bacterial and crenarchaeal communities in the Technosols were estimated by qPCR targeting the 16S rRNA genes. Average values for bacterial 16S rRNA genes ranged from 9.1×10^5 to 1.1×10^7 copies per ng of soil DNA (Fig. 2.5), which is in the same range as that observed in previous studies quantifying the size of the bacterial community in "natural" soils (Bru et al. 2007; Nemergut et al. 2010). Interestingly, neither the

crenarchaeal 16S rRNA genes nor the crenarchaeal *amoA* genes were detected in the majority of samples (data not shown) whereas the abundance of bacterial *amoA* genes was up to 3.8 x 10^4 copies per ng of soil DNA. However, previous studies reported that crenarchaea predominate among ammonia-oxidizers in soils with *amoA* gene copies of crenarchaea up to 3,000-times more abundant than bacterial *amoA* genes (Bru et al. 2011; Leininger et al. 2006; Schleper and Nicol 2010). Understanding the relative contribution of AOA and AOB to ammonia-oxidation and soil functioning is of great interest since the recent discovery that crenarchaea, and not only bacteria, are capable of ammonia oxidation (Erguder et al. 2009; Prosser and Nicol 2008). Using ^{13}C-enriched carbon dioxide, Zhang et al. (2010) recently provided evidence for the role played by crenarchaea in soil ammonia-oxidation. This is supported by Wessen et al. (2011) who suggested that AOA were responsible for nitrate leaching through conversion of ammonia to nitrate *via* nitrite in an arable soil. However, our results showed a very low abundance of AOA in comparison to the AOB, suggesting that ammonia-oxidation is driven by bacteria and not crenarchaea in the constructed Technosols. Interestingly, it has been reported that nitrification was also driven by AOB and not AOA in a zinc-contaminated soil (Mertens et al. 2009).

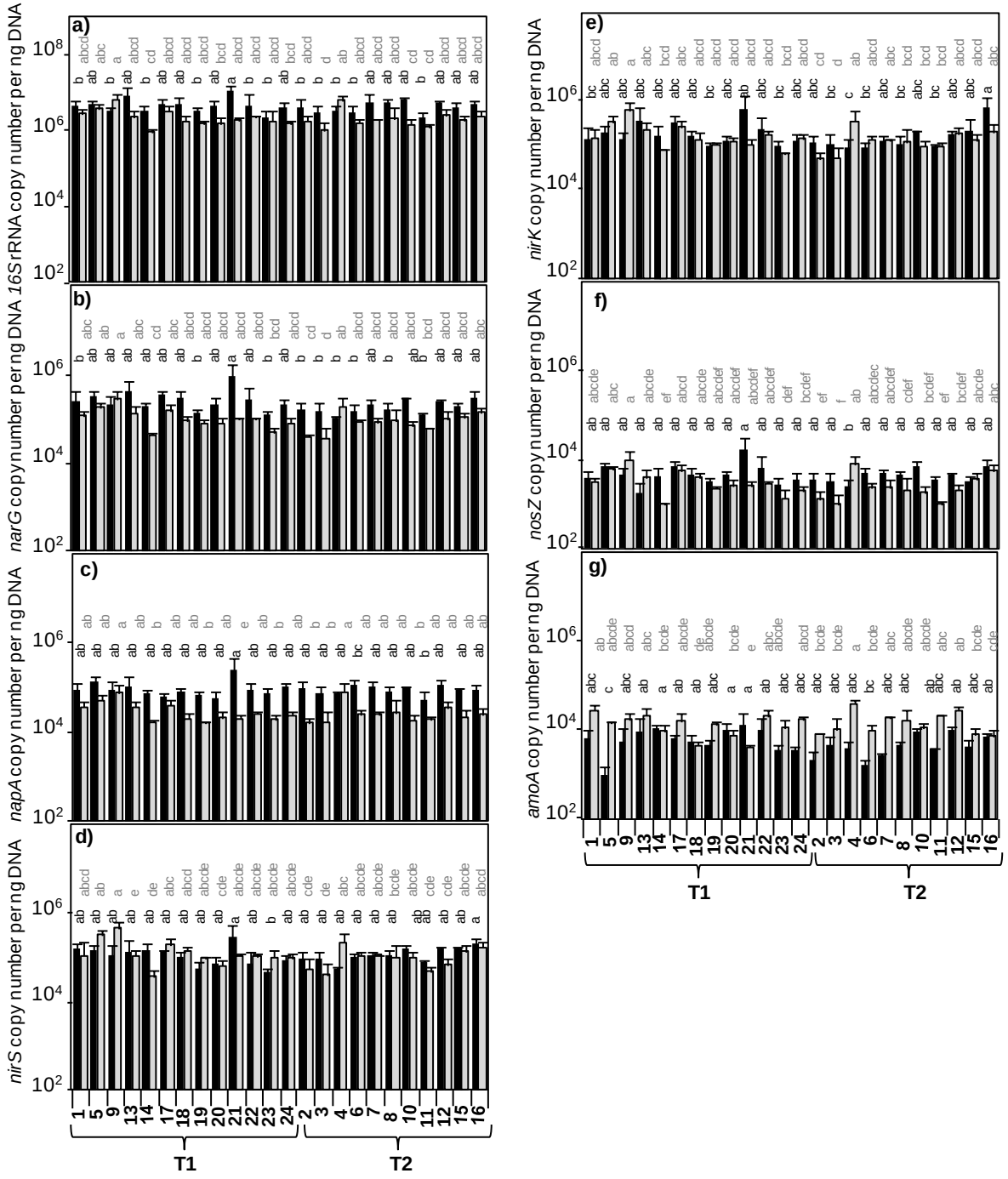

Fig. 2.5. Abundance of the total bacterial and the N cycling communities

Abundance of the different bacterial communities quantified in Technosols (T1 and T2) plots in 2008 (▬) and 2009 (▬), estimated by qPCR of 16S rRNA, *amoA*, *napA*, *narG*, *nirS*, *nirK* and *nosZ* genes. Bars indicate gene copy numbers expressed per ng extracted DNA. For the same year, identical letters above the bars (mean ± standard deviation, n=3) indicate that plots are not significantly different.

The *narG*, *napA*, *nirK* and *nirS* gene copy numbers, used as proxies for the abundances of the nitrate reducing and denitrifying bacteria, were on average 10 to 20 times lower than the bacterial 16S rRNA gene copy numbers (see Fig. 2.5). Similar abundances were observed in "natural" soils (Bru et al. 2011; Bru et al. 2007; Henry et al. 2006; Philippot et al. 2009). The proportion of nitrate reducers within the total bacterial communities was about 2 to 5% and that of denitrifiers 0.4 to 6%, which is in agreement with previous studies (Bru et al. 2007, Henry et al. 2006). The abundance of the *nosZ* gene encoding the nitrous oxide reductase catalyzing the last step of the denitrification was 20 times lower than the abundance of the other denitrification genes in all plots (see Fig. 2.5). An analysis of the complete genomes of sequenced denitrifying bacteria has shown that approximately 1/3 have a truncated denitrification pathway and lack the *nosZ* gene (Jones et al. 2008). Accordingly, lower abundances of *nosZ* gene compared to the *nirK* and *nirS* genes are commonly reported in soil (Henry et al. 2006) and the same trend was observed in the studied Technosols. This can be of environmental importance since recent work showed that shifts in the proportion of bacteria with *nosZ* could influence the emissions of the greenhouse gas N_2O (Philippot et al. 2011). Together with the diversity data, the quantification of total bacterial and crenarchaeal communities and of microbial guilds involved in N-cycling showed that differences between plots were more significant than between the two types of constructed Technosols with no clear trend between the two sampling dates.

4. Conclusions

In conclusion, the results indicate that, at the phyla and class levels, the composition and the diversity of the microbial community in constructed Technosols were very similar to 'natural' soils. Both the total bacteria and microbial guilds involved in N-cycling were abundant but in contrast to most 'natural' soils, bacteria and not crenarchaea were the numerically dominant ammonia oxidizers in the two types of Technosols. Both diversity and abundance of the microbial communities had a high spatial variability, which was due to the heterogeneous nature of the constructed Technosols. This variability in microbial community structure decreased with time suggesting early pedogenic evolution of recently constructed Technosols leading to homogenization of the microbial habitat. Further studies focusing on microbial activities are now required for confirming the potential of Technosols constructed

using treated industrial soil in mixture with wastes or by-products at contaminated sites to fulfil soil functions.

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CHAPTER 3 DISTRIBUTION OF BACTERIA AND NITROGEN-CYCLING MICROBIAL COMMUNITIES ALONG CONSTRUCTED TECHNOSOL DEPTH-PROFILES

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Distribution of bacteria and nitrogen-cycling microbial communities along constructed Technosol depth-profiles

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Abstract

Technosol construction through assemblage of treated soil and recycled wastes is an innovative option for the restoration of degraded lands and re-use of industrial wastes. Recent studies have evidenced that Technosols could support soil functions such as primary production but the knowledge about other ecosystemic services, such as nutrient cycling, is limited. We investigated how the total bacterial community and key functional microbial communities involved in nitrogen cycling were influenced by the depth and type of Technosol. We found that despite being artificially constructed, Technosols exhibited a gradual change in microbial activity and abundance along the soil profile. Both nitrification and denitrification rates decreased with increasing depth as previously observed in other soil systems. Potential denitrification and nitrification were correlated with Technosol physicochemical properties but also with abundances of *nirS* denitrifiers and bacterial *amoA* gene, respectively. The correlation between nitrification rates and bacterial ammonia oxidizers suggests that bacteria are driving nitrification in Technosols. A-RISA fingerprints showed a distinct community structure along different Technosol layers. Technosol properties affected denitrification strongly than nitrification underlining the importance of better understanding the microbial communities in Technosols to maximize their potential for nutrient cycling, an essential ecosystem function.

Key words: soil depth; contaminated soils; denitrification; nitrification; ammonia-oxidation; quantitative PCR

1. Introduction

Soil degradation has significantly affected the world's soil resources over the past century and has become one of the greatest socioeconomic and environmental problems facing mankind (FAO 2010; GEF 2006). Indeed, soil is a non-renewable resource and the alteration of soil quality can result in the decrease of its capacity to perform ecosystem services such as plant production, nutrient cycling, and filtering, which has tremendous implications for both the agriculture as well as the environment (Andrews et al. 2004; Daily et al. 1997; Doran and Zeiss 2000; EC 2010; Jie et al. 2002; Nannipieri et al. 2003; Scherr 1999; Wardle and Giller 1996). Degradation of soil arises from erosion by wind or water, physical deterioration but also from chemical deterioration by pollutants. Human activities such as agriculture, urbanization and industrialization are the major causes of soil contamination by pollutants.

Among the different strategies used to restore the contaminated lands, construction of Technosols over the polluted sites is an innovative technology for ecological reclamation of the contaminated lands. This in-place restoration of the polluted lands is based on the assemblage of treated soil, recycled waste and industrial by-products, which are formulated and staked to build a new soil profile at the contaminated site (Sere et al. 2008; IUSS Working Group WRB 2006). Along with soil restoration, construction of Technosols can also be an efficient technique to recycle and better utilize the wastes and by-products in a way that is more economical and environmentally friendly. In order to assess the potential of Technosols to fulfill soil ecosystem services, one hectare of Technosols were built at an experimental site in France for reclamation of a former industrial wasteland that had previously been contaminated by both polycyclic aromatic hydrocarbons (PAHs) and heavy metals. Treated industrial soil and industrial by-products were differentially assembled in layers to obtain two types of Technosols differing in their water diffusion properties.

Previous work has shown that spontaneous vegetation or sown alfalfa and rye-grass grew well without any deficiency symptoms on both Technosols but with some differences in the diversity and dry biomass production (Sere et al. 2008). Analysis of the microbial communities indicated that *Proteobacteria*, *Bacteroides*, *Firmicutes* and *Acidobacteria* were the dominant bacterial phyla in the top horizon of both Technosols (Unpublished data). Sere et al. (2010) also showed that these Technosols were subjected to a dynamic evolution resulting in the rapid development of new horizons. However, such changes in the soil structure and in the water status along the Technosol profiles in addition to the initial

superposition of different parent materials can have a strong influence on the vertical distribution of microorganisms and their activities. Therefore, it is of importance for Technosol construction to understand how these structural changes and modifications of the water status along the Technosol depth profile will affect the ecosystem services provided by microorganisms such as nutrient cycling.

Because of their role in the cycling of the nutrient nitrogen, we focused on the ammonia-oxidizers performing the first step of the nitrification process and on the denitrifiers. These guilds are often used as model communities in microbial ecology since they are responsible for N-losses through leaching and gaseous N emissions (Philippot et al. 2009; Phillips et al. 2000). The main objective of this work was therefore to study how the distribution of (i) the abundance and the structure of the total bacterial community and of (ii) the abundances and the activities of these key functional microbial communities were influenced by the depth and type of Technosol.

2. Material and methods

2.1. Experimental site and soil sampling

The study was set up on a 1 ha experimental site French Scientific Interest Group – Industrial Wasteland (GISFI; http://www.gisfi.fr), Homécourt, France. Two different Technosols (Spolic Garbic Technosol (Calcaric)) were constructed (T1 and T2) for the restoration of the contaminated lands. The Technosol T1 consisted of (i) compost horizon (upper layer; approximately 15 cm), (ii) mixture of paper by-products and treated industrial soil (layer below the compost horizon; approximately 60 cm) and (iii) paper by-products (bottom layer; approximately 30 cm). The compost used for the upper horizon derived from the composting of green waste (e.g. pruning of a tree, grass cuttings). The treated soil from the second horizon is the soil from a former coke industrial-plant, excavated, sieved (<5-10 cm) and then treated by a process of low temperature thermal desorption. The process involves heating to a temperature between 400 and 600 °C. It was a NFU 44-051 standard product from 'Communauté Urbaine de Strasbourg'. The paper by-products also known as paper mill sludge are the wastes from the production and processing of paper, paperboard and pulp. The second type (T2) had two horizons similar to those of T1 and the third confinement horizon (25 cm) at the bottom composed of limed paper by-products (5% by volume) with specific compaction properties. T2 is intended to limit the infiltration of water carrying

residues of the polluted industrial materials into the aquifers. The vegetation cover includes spontaneous plants and sown perennial grasses (alfalfa and rye-grass).

In April 2010, three pits (1 m^2) for each type of Technosols were dug at the site. According to the pedological observations, three horizons were defined in each of the profile, which varied with an approximate depth of 0-15; 15-35; 35-85 cm. From the middle portion of each horizon three soil samples were collected and sieved at 2 mm. The soil samples were then stored at -20°C for further analysis.

2.2. Physico-chemical properties of Technosols

The different methods used to analyze the physico-chemical properties of the Technosols were: pH in water (ratio soil/solution = 1/5) (NF ISO 10390), organic matter and organic carbon by oxidation by heating at 900°C under O_2 flow, total nitrogen by Kjeldahl mineralization (ISO 11261), total calcium carbonate by addition of HCl and measurement of the volume of carbon dioxide evolution with a Scheibler device (ISO 10693), cation exchange capacity by exchanged ammonium ions (ISO 11260), P_{Olsen} by $NaHCO_3$ extraction and then proportioning of phosphorus complexes by spectrometric methods (ISO 11263). All the above analyses were carried out by the certified INRA laboratory of soil analysis located in Arras.

2.3. DNA extraction

Soil stored at -20°C was used for carrying out DNA extractions. Independent DNA extractions were performed for three replicate samples of each horizon from the six pits of the two types of Technosols (54 samples in total) according to ISO 11063 "Soil quality-Method to extract DNA from soil" (Petric et al. 2011) as described by Martin-Laurent et al. (2001). Briefly, 1 g of soil was homogenized in 4 ml of extraction buffer for 30 s at 1,600 rpm in a mini-bead beater cell disruptor (Mikro-Dismembrator S; B. Braun Biotech International) followed by centrifugation at 14000 x g for 1 min to eliminate soil and cell debris. For protein precipitation, supernatant was incubated on ice for 10 min with 1/10 volume of 3 M sodium acetate and centrifuged (14000 x g, 5 min, 4°C). After precipitation with ice-cold isopropanol, nucleic acids were purified using polyvinylpolypyrrolidone and Sepharose 4B spin columns (Sigma-Aldrich, USA). An additional purification step was performed using a Nucleo Spin Extract-II" purification kit as described by the manufacturer (Macherey-Nagel, Germany).

The quality and size of the soil DNA was determined by gel quantification. In brief, purified DNAs were run in 1% (w/v) agarose gels followed by staining with ethidium bromide (200 µg/l). Known concentrations of calf thymus DNA (Biorad) (5, 10, 20 and 200 ng of DNA) were included in the gel. At last quantification was performed using ImageQuaNT (Molecular Dynamics, Ca, USA) which related the intensity of DNA bands to the intensity of the calf thymus DNA to estimate the amount of DNA extracted. The average soil DNA yield in the different Technosol layers was 1.64, 1.59 and 0.94 µg g^{-1} soil and 1.21, 1.18 and 1.01 µg g^{-1} soil for the first, second and third horizons of T1 and T2 respectively.

2.4. Automated Ribosomal Intergenic Spacer Analysis (A-RISA) fingerprinting of the microbial community structure

Microbial community fingerprinting was performed using A-RISA to characterize the genetic structure of bacterial community at different depths along Technosol profiles. The 16S-23S rRNA intergenic spacer region (IGS) of the ribosomal operon was amplified in a final volume of 25 µl with 0.5 µM of universal primers 1552_f (5'-TCG GGC TGG ATG ACC TCC TT-3') labeled in 5'with IRDye 800 fluorochrome (MWG SA Biotech, Ebersberg, Germany)and 132_r (5'-CCG GGT TTC CCC ATT CGC-3'), 2.5 U of Taq DNA polymerase (Appligene Oncor, France) and 0.25 ng of template DNA. PCR amplification was carried out in a gradient thermocycler PTC 200 (MJ Research, Waltham, Mass) under the following conditions: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; plus an additional cycle for 15 min at 72°C.

The concentration of A-RISA PCR products was estimated and 1.5 µl of the product, denatured at 92°C for 2 min, was loaded onto a 66 cm 3.7% polyacrylamide gel and run on a LiCor 4200 DNA Analyzer (Biosciences, USA) for 15 h at 1500 V/80 W. The data was analyzed using One-Dscan (ScienceTec). This software converted fluorescence data into electrophoregrams where the peaks represented PCR fragments. Lengths (in base pairs) were estimated using a DNA ladder of 15 bands ranging from 200 to 1200 bp. The surface of the peak was estimated using the Gaussian integration in One-Dscan.

2.5. Quantification of total and N-cycling microbial communities

The abundances of the total bacterial and crenarchaeal communities were estimated in all Technosols DNA extracts using a 16S rRNA primer-based quantitative PCR (qPCR) assays as described in previous studies (Lopez-Gutierrez et al. 2004; Ochsenreiter et al. 2003), while genes encoding the catalytic enzymes responsible for ammonia oxidation (*amoA*), and denitrification (*narG*, *napA*, *nirK*, *nirS*, *nosZ*) were used as molecular markers to assess the abundances of bacterial (AOB) and crenarchaeal (AOA) ammonia oxidizers and denitrifiers respectively (Bru et al. 2007; Henry et al. 2004; Henry et al. 2006; Leininger et al. 2006; Tourna et al. 2008). Quantification of all microbial communities was carried out with an ABI Prism 7900HT (Applied Biosystems, USA) using SYBR green dye as the detection system in a reaction mixture of 15 µl containing 7.5 µl of the SYBR green® (2x) PCR master mix (Thermo: Absolute qPCR SYBR green®), 2 µM of each specific primers, T4gp32 (250 ng/reaction; Q-BIOgene, France), and 0.1 ng of DNA sample. The thermocycler conditions and the primers used for qPCR assays of AOA, AOB, *napA*, *narG*, *nirS*, *nirK*, *nosZ* were as described previously (Bru et al. 2007; Henry et al. 2004; Henry et al. 2006; Leininger et al. 2006; Tourna et al. 2008). At least two independent qPCR assays were performed on three replicate samples from each of the three horizons from the two types of Technosols. Standard curves were obtained by performing qPCR assay on serial dilutions of known amount of plasmid DNA containing the targeted gene (from 10^7 to 10^1 copies).

Possible inhibitory effect of the coextracted substances on qPCR amplifications was tested by mixing a known amount of pGEM-T plasmid (Promega, USA) with the soil DNA extracts or water with the plasmid-specific T7 and SP6 primers, before running qPCR assays. In all cases, no inhibition was detected as the measured cycle threshold (Ct) values were not significantly different between the Biotechnosol DNA extracts and the controls with water. The qPCR amplification efficiencies calculated for the genes 16S rRNA (bacteria), 16S rRNA (crenarchaea), *amoA* (bacteria and archaea), *napA*, *narG*, *nirK*, *nirS* and *nosZ* were always more than 87%.

2.6. Nitrogen cycling process measurements

Measurements of potential ammonia-oxidation rates were used as proxies of potential nitrification activity (PNA). The assays were carried out using the ISO method (ISO 15685 (2004)) described by Hoffmann et al. (2007). Briefly, 12-14 g fresh soil (equivalent to 10 g dry soil) was incubated in a flask with 40 ml of the reagent solution containing 1 mM K-

phosphate buffer, 5 mM Na-chlorate, and 1.5 mM $(NH_4)_2SO_4$. The flasks were incubated over an orbital shaker (175 rpm; 25°C). After 1, 5, 8, 24, 29, and 48h, ammonium oxidation was stopped by adding 2 ml K-chloride (2 M), followed by a centrifugation step (3,000 x for 2 min). Supernatant was recovered to measure the nitrite concentration at λ= 530 nm on a Beckman Coulter D4 800 spectrophotometer. The PN rates were calculated as μg NO_2-N h^{-1}.

Potential denitrification activity (PDA) were determined using the acetylene inhibition technique, which blocks the last step in the denitrification pathway resulting in the accumulation of N_2O (Yoshinari et al. 1977). Therefore, in this work the potential denitrification activity is refered to the rate of production of N_2O, which was calculated using non-linear model approach as described by Pell et al. (1996). Briefly, 20 mL distilled water and 3 mM KNO_3, 1.5 mM succinate, 1 mM glucose and 3 mM acetate were added to gas-tight flasks containing 10 g fresh soil (equivalent to 7.5-8.5 g dry soil). The flasks were then sealed and purged five times, by evacuating the ambient air and filling with N_2. 10 ml of acetylene was injected in flasks to reach 0.1 atm partial pressure and the flasks were incubated at 25 °C with agitation (175 rpm) for 30 min. Gas samples were collected every 30 min for 3h and N_2O was analyzed using a gas chromatograph (Perkin Elmer Clarus500, Norwalk, CT, USA) equipped with an EC-detector.

2.7. Statistical analysis

Community structure data obtained from the OneD-scan software was converted into a matrix summarizing the band presence (peaks) and intensity (height of peaks), using a computer program PrepRISA (http://pbil.univ-lyon1.fr/ADE-4/microb/). Principal component analysis (PCA) on covariance matrix was performed on the data matrix (size and intensity of bands), using ADE-4 (http://pbil.univ-lyon1.fr/ADE-4/home.php).

The factors accounting for the variation of each variable between Technosols, replicates and horizon depths were analyzed with the following analysis of variance model:

$$Z_{ijkl} = \mu + Technosols_i + replicate(Technosols)_{ij} + horizon\ depth_k + Technosols * horizon\ depth_{ik} + replicate(Technosols) * horizon\ depth_{ijk} + \varepsilon_{ijkl}$$

Where Z is the quantitative variable of interest (abundances of bacterial and crenarchaeal 16S rRNA and bacterial and crenarchaeal *amoA*, *nap*, *narG*, *nirS*, *nirK*, *nosZ* and potential nitrification and denitrification activities), Technosol explains the Technosol type

main effect (i = 1, 2), replicate(Technosols) is the biological replicate effect nested within each Technosol type (j = 1, 2, 3), horizon depth explains the horizon main effect (k = 1, 2, 3), Technosols*horizon depth and replicate(Technosols)*horizon depth are interaction effects and ε is the residual error. For each trait, the normality and homogeneity of the residual distribution were studied. For all microbial abundance variables (except for the abundances of *nirK*, *nosZ* and crenarchaeal 16S rRNA), a logarithmic transformation proved to be necessary to have normally and homogeneously distributed residues. For the abundances of the *nirK*, *nosZ*, crenarchaeal 16S rRNA and the potential nitrification/denitrification activities, the same model was fitted in a generalized linear framework (quasi-Poisson distribution, logarithmic link function). Significance of the effects was assessed with F-tests. Multiple comparisons between levels of factors were performed using Tukey's HSD tests and Student test corrected for multiple comparisons (Bonferroni correction). Correlations between traits (abundances, activities and physicochemical variables) were assessed using Pearson product-moment correlation coefficients. Note that because for each physicochemical variable, we had only one measurement per Technosol type, per horizon and per biological replicate (no technical replicate), we correlated those measurements to the fitted values (for the microbial abundances and activities) of the analysis of variance model. A False Discovery Rate (FDR) procedure was used to correct for multiple tests. A FDR of 0.05 was chosen as the significance threshold for detecting a correlation. The analysis was performed with the *fdrtool* function in the fdrtool package for R (Strimmer et al. 2008).

3. Results

3.1. Soil Physico-chemical properties

Table 3.1 provides the main physico-chemical properties of the different horizons of two Technosols. Several soil properties including the parameters controlling the soil nutrient contents and fertility status (such as total N, organic carbon and organic matter) were found to be different between the two Technosols (Table 3.1). However, we noticed that in overall, the differences in most of the soil properties were due to the upper horizon of Technosol T2. This horizon contains more nitrogen, organic carbon, organic matter and phosphorus but less total $CaCO_3$ and has a lower pH as well (Table 3.1). In contrast, many properties related to the soil texture were not significantly influenced by either the Technosol type or the horizon depth.

Table 3.1. Physico-chemical properties of the Technosols depth profiles

Physico-chemical properties of the constructed Technosols (T1 and T2). Each value is the average of 3 samples (n=3; standard deviations indicated underneath between parenthesis). Significant differences between averages are indicated by different letters (Tukey HSD test, $p < 0.05$). No letters were assigned when no difference was found between any two pairs.

Parameter	T1 horizons (depth in cm)			T2 horizons (depth in cm)		
	0-15	15-35	35-85	0-15	15-35	35-85
Soil moisture (%)	27.1 (4.95)	26.3 (2.16)	29.0 (1.35)	36.0 (5.61)	28.3 (1.24)	31.2 (4.84)
Water holding capacity (g. g^{-1})	1.10 (0.03)	0.92 (0.07)	0.90 (0.03)	0.99 (0.01)	0.94 (0.06)	0.96 (0.04)
Gravels (>0.5 cm) (g. kg^{-1})	202^{ab} (31.5)	187^{ab} (63.9)	190^{ab} (46.2)	78.9^{b} (39.4)	244^{a} (47.0)	262^{a} (42.3)
Gravels (0.2-0.5 cm) (g. kg^{-1})	45.6 (12.1)	68.7 (34.0)	86.9 (49.6)	66.37 (37.0)	90.9 (33.6)	88.5 (8.56)
Clay (< 2 µm) (g. kg^{-1})	151^{b} (13.6)	130^{b} (16.5)	175^{ab} (28.2)	224^{a} (17.2)	146^{b} (11.0)	153^{b} (2.83)
Fine silt (2-20 µm) (g. kg^{-1})	104^{b} (10.0)	115^{ab} (1.53)	123^{a} (12.2)	125^{a} (11.3)	97.0^{b} (17.0)	116^{ab} (9.90)
Coarse silt (20-50 µm) (g. kg^{-1})	57.6^{b} (4.04)	55.0^{b} (5.57)	64.7^{b} (4.04)	102^{a} (15.3)	54.3^{b} (4.16)	61.5^{b} (14.9)
Fine sand (50-200 µm) (g. kg^{-1})	132 (16.0)	158 (6.11)	113 (31.2)	129 (23.0)	131 (11.9)	117 (11.3)
Coarse sand (0.2-2 mm) (g. kg^{-1})	139 (30.3)	123 (31.5)	115 (42.4)	200 (25.7)	120 (7.57)	135 (33.9)
Total Nitrogen (g. kg^{-1})	2.71^{b} (0.47)	2.13^{b} (0.32)	2.46^{b} (0.51)	10.9^{a} (2.52)	2.27^{b} (0.29)	2.23^{b} (0.39)
Organic Carbon (g. kg^{-1})	79.47^{b} (8.21)	75.9^{b} (6.05)	87.8^{b} (14.2)	156^{a} (20.0)	78.8^{b} (13.0)	77.3^{b} (4.03)
Organic matter (g. kg^{-1})	137^{b} (14.2)	131^{b} (10.0)	152^{b} (25.2)	269^{a} (34.7)	136^{b} (22.6)	134^{b} (7.07)
C/N	29.77^{b} (5.00)	35.8^{a} (3.05)	36.4^{ab} (6.58)	14.5^{c} (1.43)	34.7^{ab} (1.97)	35.1^{ab} (4.38)
pH	8.42^{a} (0.17)	8.31^{a} (0.27)	8.17^{ab} (0.12)	7.95^{b} (0.12)	8.42^{a} (0.05)	8.24^{ab} (0.19)
Total $CaCO_3$ (g. kg^{-1})	406^{a} (28.7)	410^{a} (24.6)	401^{a} (62.6)	208^{b} (30.0)	442^{a} (34.6)	409^{a} (14.1)
Olsen Phosphorus (P_2O_5) (g. kg^{-1})	0.07^{b} (0.03)	0.06^{b} (0.04)	0.10^{ab} (0.05)	0.25^{a} (0.06)	0.06^{b} (0.01)	0.07^{ab} (0.02)

3.2. Bacterial community structure along Technosols profiles

Fig. 3.1. shows the A-RISA fingerprints of the bacterial community structure obtained at different depths from the three pits opened for the each type of Technosol. Between 80 and 100 bands were detected, the majority of them ranging between 300 and 850bp in each fingerprint not matter the Technosols considered. Differences were observed between the A-RISA fingerprints obtained from soil samples collected at different depths. Thus, each studied layer displayed a specific bacterial community characterized by differences in the intensity and number of bands. In most cases, the complexity of the banding patterns decreased with the horizon depths suggesting less diverse communities in deeper horizons.

A separate Principal Component Analysis (PCA) was carried out for each Technosol bacterial community profile to investigate the differences between bacterial community structures at different horizon depths. It revealed significant discrimination at all depths in each Technosol type (Fig. S3.1, supplementary data). Thus, for all of the six profiles, the 3 horizons are well separated and the first axis of the PCA explained more than 50% of the variance (Fig. S3.1, supplementary data). However, when combining the two types of Technosols, no differences were found between T1 and T2 (data not shown).

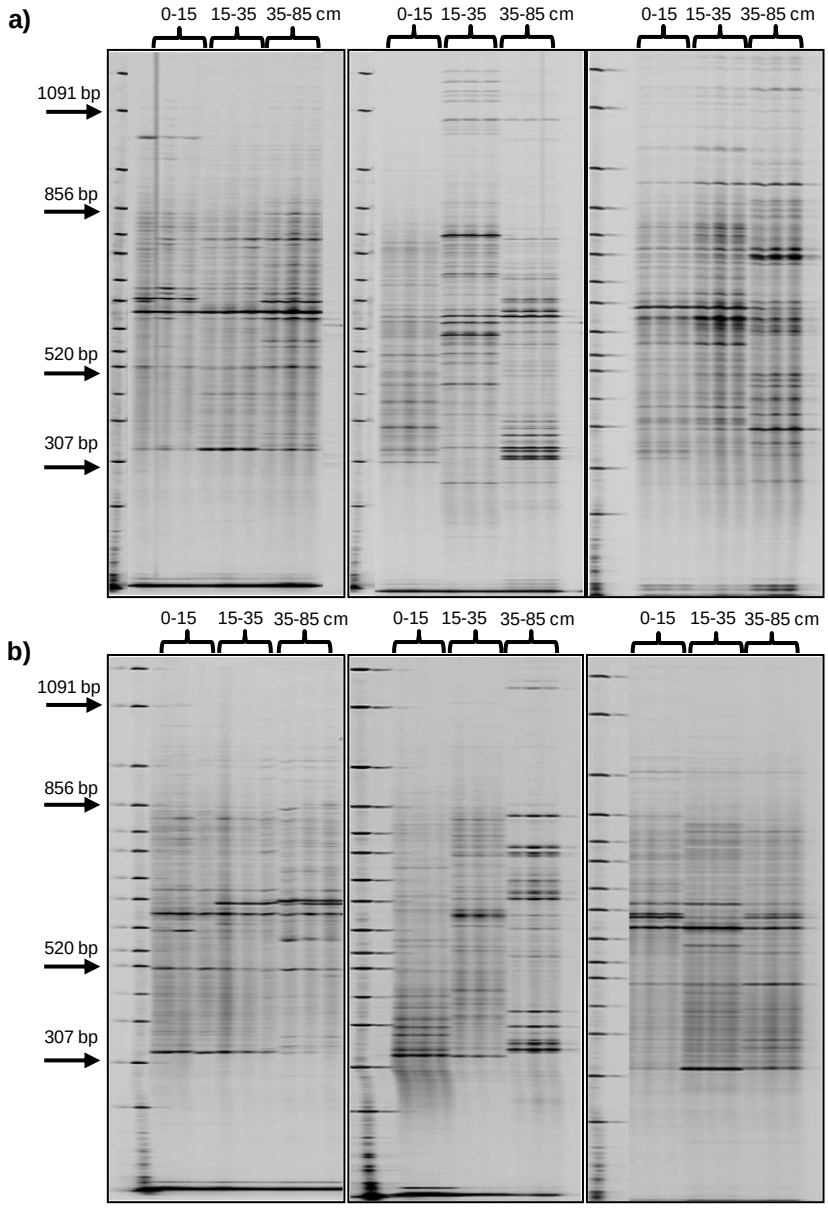

Fig. 3.1. A-RISA fingerprinting of Technosols depth profiles

A-RISA profiles from T1 (a) and T2 (b) Technosols, sampled at different horizons. Soil depth (in cm) is indicated above the gel pictures (three replicates per horizon).

3.3. Abundance of the total and N-cycling microbial communities

The abundances of the total bacterial and crenarchaeal communities and of the functional guilds involved in N-cycling were estimated at three depths within the six profiles of two constructed Technosols through qPCR assays. To minimize the bias related to possible differences in the DNA extraction yield between samples, we calculated the gene copy number per ng of DNA rather than per gram of soil. The 16S rRNA gene copy numbers of

bacteria and crenarchaea in the two Technosols ranged from 3.0 x 10^5 to 5.8 x 10^6 and 9.7 x 10^2 to 8.9 x 10^3 copies per ng of soil DNA respectively (Fig. 3.2a). In all soil samples, bacteria dominated over the crenarchaea but both the bacterial as well as the crenarchaeal 16S rRNA abundances significantly decreased with depth (Table S3.1, supplementary data) in the two Technosols (Fig. 3.2a).

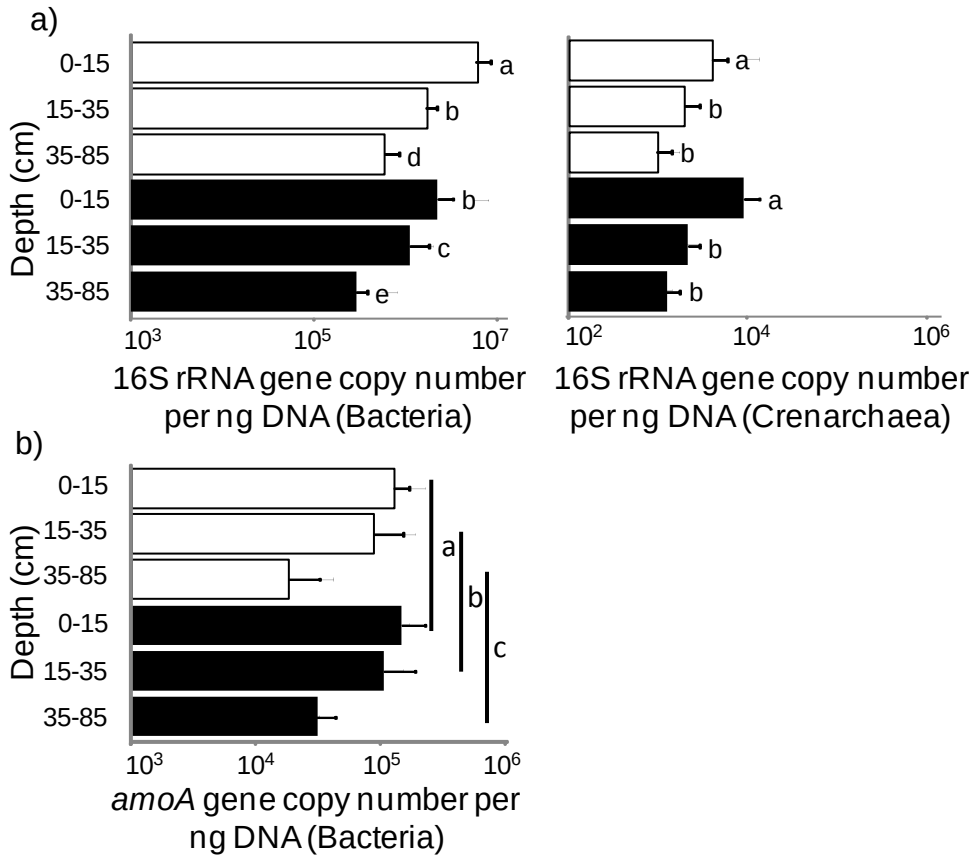

Fig. 3.2. Abundance of the total bacterial and crenarchaeal communities and of ammonia oxidizers

Abundance of the total bacterial and crenarchaeal communities (a) and of the ammonia oxidizing community (b) in the two types of Technosols (T1 ☐ and T2 ■) at different horizons estimated by qPCR of 16S rRNA genes and *amoA* genes, respectively. Bars indicate mean (± standard deviation) gene copy numbers per horizon depth and per Technosol (n = 9) expressed per ng extracted DNA. Letters adjacent to the bars indicate the significance of the differences between means. Since the Technosol*horizon depth interaction effect was not significant for bacterial *amoA* gene, we indicated the significance of the differences between horizon depths, averaged over the two Technosols. One should thus read $amoA_{0\text{-}15}$ > $amoA_{15\text{-}35}$ > $amoA_{35\text{-}85}$.

The abundance of the ammonia-oxidizing community was assessed by measuring AOA and AOB *amoA* gene copy number by real time PCR. AOB were more abundant in the upper horizon (up to 1.5×10^5 copies per ng of soil DNA) and decreased with a lowest value of 1.8×10^4 copies per ng of soil DNA in the third horizon (Fig. 3.2b). In contrast, the amplification of the AOA *amoA* genes was successful only in a few samples with numbers just above the detection limit (data not shown) and consequently the AOA were not included in the subsequent analyses.

Similarly to the ammonia-oxidizers, the denitrifiers abundance was significantly higher in the upper horizons than in the deeper ones. Among the genes encoding the first step of the denitrification pathway, *narG* predominated over *napA*, with values ranging from 6.4×10^4 to 1.4×10^6 and 4.3×10^3 to 7.5×10^4 respectively (Fig. 3.3). The abundances of the *nirK* and *nirS* genes encoding the nitrite-reductase denitrifcation enzymes were similar with values ranging from 1.8×10^4 to 8.4×10^5, 1.8×10^4 to 2.6×10^5, respectively while the *nosZ* gene copy number was slightly lower (Fig. 3.3).

Analyses of the community abundance data revealed significant differences according to both the horizon depth and the Technosol type. The strength of the differences in the abundance of denitrifying genes were dependent on the Technosol type (Technosols*Horizon depth effect, Table S3.2, supplementary data). In contrast differences in the abundance of the nitrifying AOB *amoA* gene along the depth gradient are independent of the Technosol type.

3.4. N-cycling process rates

For the measurement of potential nitrification activity (PNA), the rates of ammonia-oxidation, which is the limiting step in the nitrification process, were monitored. Fig. 3.4a shows the PNA along the different horizons of the two Technosol profiles. Overall, PNA was higher in Technosol T2 than in T1 (Fig. 3.4). A significant effect of the horizon depth was also observed with highest PNA of 1.10 and 2.30 ng NO_2-$N.g^{-1}$ $soil.min^{-1}$ in Technosols T1 and T2, respectively in the upper horizons. However, the interaction between Technosol type and depth was not significant. Similarly to PNA, potential denitrification activity (PDA) was significantly influenced by the Technosol type and depth but also by the interaction of these two variables (Table S3.3, supplementary data). PDA ranged from 4.82 to 26.8 ng N_2O-$N.g^{-1}$ $soil.min^{-1}$ in the lower and upper horizons, respectively.

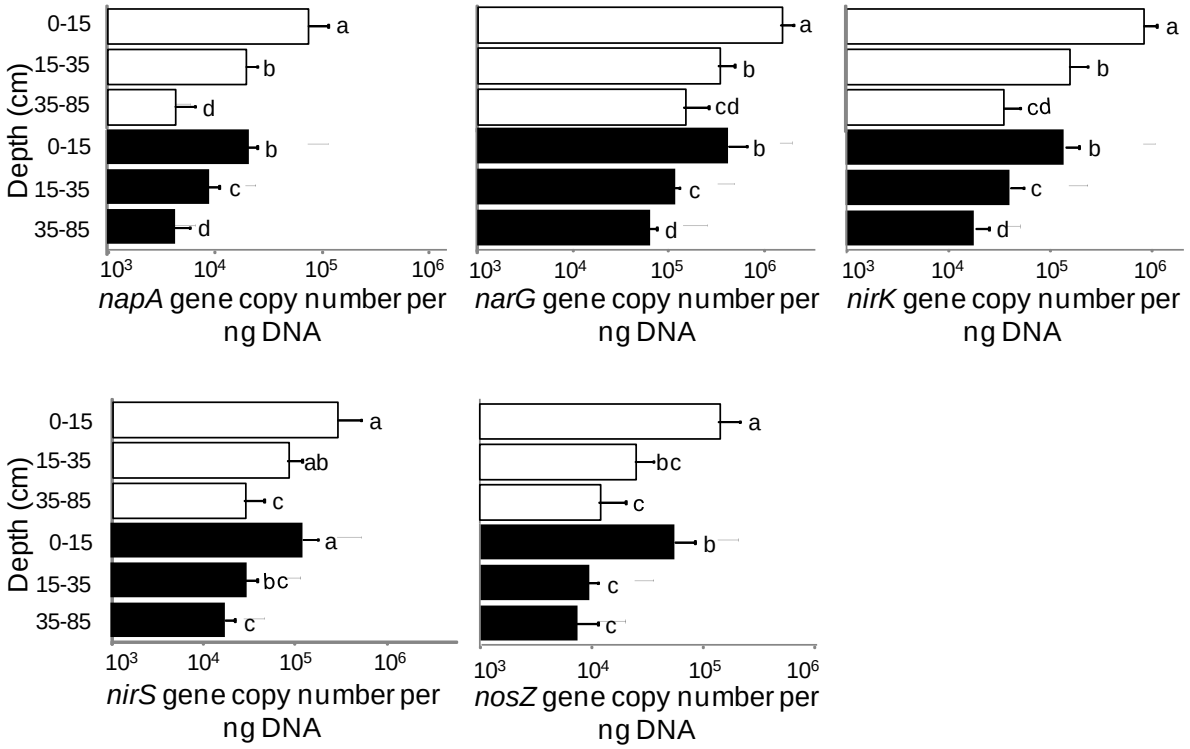

Fig. 3.3. Abundance of the denitrifier community in the two types of Technosols Abundance of the denitrifier community in the two types of Technosols (T1 □ and T2 ■) at different horizons estimated by qPCR of *napA*, *narG*, *nirK*, *nirS* and *nosZ* genes. Bars indicate mean (± standard deviation) gene copy numbers per horizon depth and per Technosol (n = 9) expressed per ng extracted DNA. Letters adjacent to the bars indicate the significance of the differences between means.

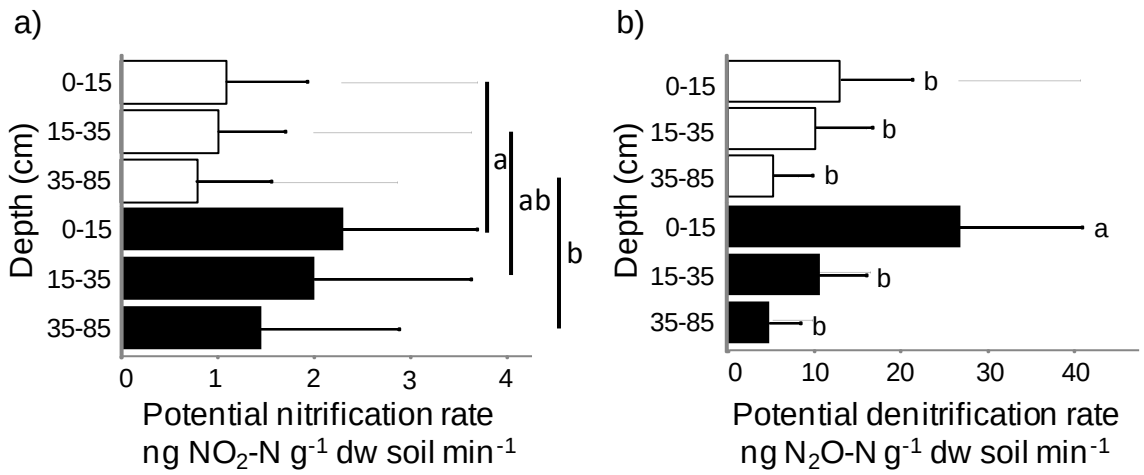

Fig. 3.4. N cycling activities along Technosol depth profiles

Potential nitrification (a) and denitrification (b) rates measured at different horizons of two types of Technosols (T1 ☐ and T2 ■). Means (± standard deviation) per horizon depths and per Technosol are shown (n = 9). Letters adjacent to the bars indicate the significance of the differences between means. Since the Technosol*horizon depth interaction effect being was not significant for the potential nitrification activity, we indicated the significance of the differences between horizon depths, averaged over the two Technosols. One should thus read $PNA_{0\text{-}15} \geq PNA_{15\text{-}35} \geq PNA_{35\text{-}85}$.

3.5. Relationships between Technosols physico-chemical properties along the soil profiles and the abundances and activities of the N-cycling communities

We found that the changes in soil physico-chemical properties across the Technosol profiles significantly influenced nitrogen-cycling in both Technosols. Thus, PNA and PDA were positively correlated to total nitrogen, organic carbon and organic matter (r=0.57, r=0.54 and r=0.54 for PNA and r=0.91, r=0.89 and r=0.88 for PDA respectively; FDR=0.05 Fig. 3.5). Technosol physical properties such as clay, coarse silt and coarse sand contents were significantly positively correlated to PDA (r=0.72, r=0.8 and r=0.76 respectively; FDR=0.05) but not to PNA. Accordingly, soil moisture, which is related to the soil physical properties, was also a strong driver of PDA only (r=0.89, FDR=0.05). Strong negative correlation were mostly observed for PDA, which was affected by the gravel content, the C:N ratio, the pH and the total $CaCO_3$ content (r=-0.76, r=-0.86, r=-0.66 and r=-0.84 respectively; FDR=0.05). No significant correlation was observed between the Technosol physico-chemical properties and the abundance of the different microbial communities except for the *nirS* denitrifiers that were

slightly affected by the coarse sand and the total $CaCO_3$ contents of the Technosols (r=0.62 and r=-0.57 respectively; FDR=0.05) (Fig. 3.5). However we found that PDA was overall correlated to the abundance of the *nirS* denitrifiers (r=0.61; FDR=0.05) while PNA to the abundance of the AOB (r=0.56; FDR=0.05). Interestingly, when looking at the two Technosols separately, we found that PDA was also positively correlated to *nirK* (r=0.58; P<0.05) and *napA* (r=0.62; P<0.05) but only in the Technosol T2 (Fig.S3.2, supplementary data).

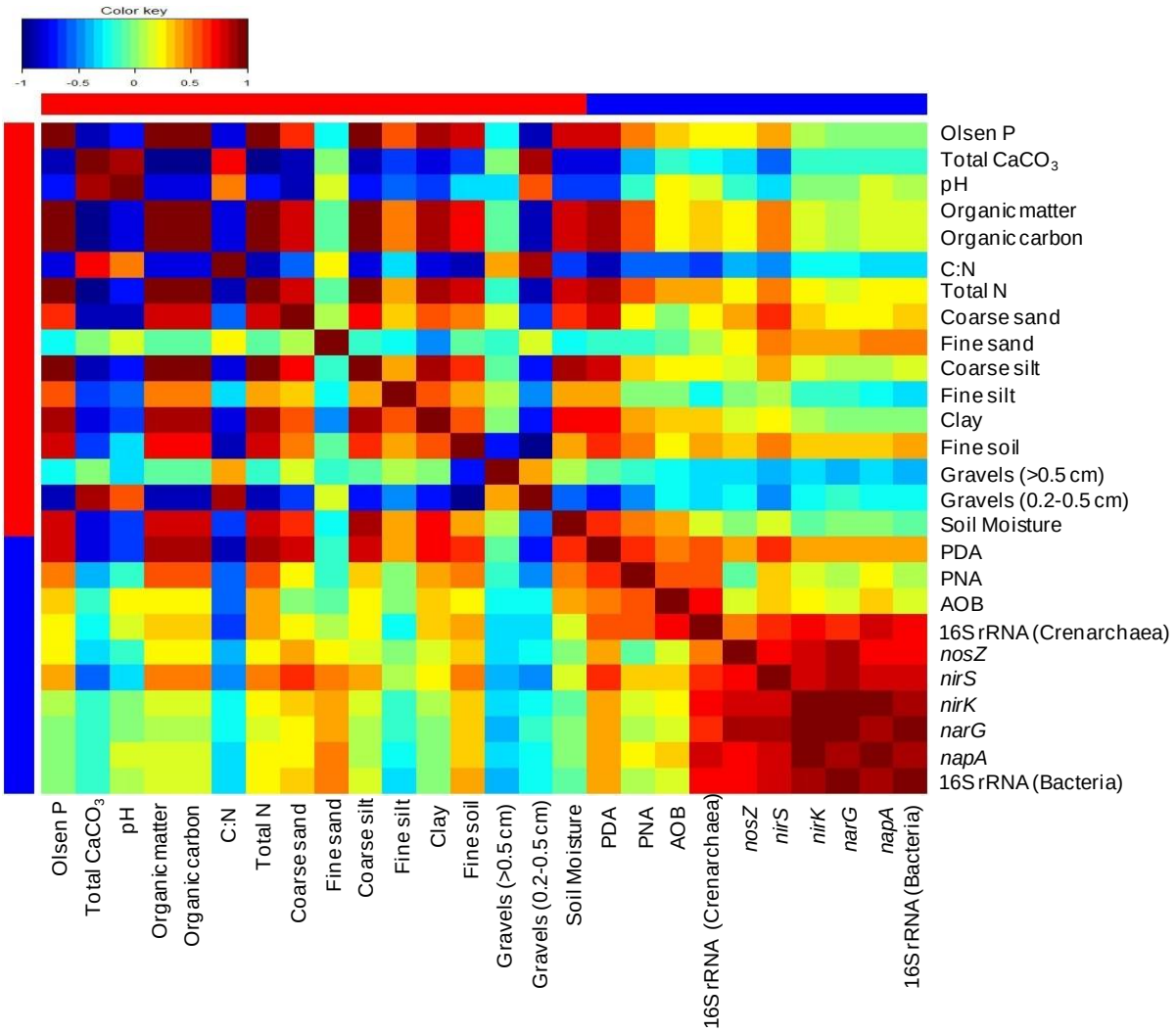

Fig. 3.5. Correlation matrix between the community abundance and activity

Correlation matrix between the abundance and activity of the nitrifier/denitrifier community and the physicochemical properties of the two types of Technosols. The colour scale above the figure indicates the intensity and the direction of the correlation.

4. Discussion

The aim of our study was to monitor the distribution of key microbial guilds involved in the N-cycle and the corresponding processes in two types of Technosols and to analyze how they were influenced by changes in Technosol properties along the depth profile.

As previously observed at the same site, we found a large spatial variability in the genetic structure of the bacterial community (unpublished data), which prevents drawing robust conclusions on the effect of the Technosol type on the bacterial diversity while pattern of variations of the bacterial diversity along the Technosol depth showed a similar trend. Measurements of N-cycling processes showed that PNA was slightly higher in the T2 than in T1-type Technosol ($P<0.01$) but still in the lower range of the rates previously reported in other soil systems (Enwall et al. 2005; Wheatley et al. 2001; Wilke et al. 2005). The Technosol type also affected PDA, which was in average one order of magnitude higher than PNA with rates up to 26.8 ng N_2O-N.g^{-1} soil min^{-1}. Similar rates were observed in arable, forest and grassland soils (Enwall et al. 2005; Barton et al. 2000; Rochette et al. 2000), which indicates that the overall activity of N-cycling microorganisms in the constructed Technosols is in the same range than that of the other types of soils.

Both PNA and PDA decreased with increasing depth in the two types of Technosols but the interaction between these two factors was significant only for the latter. Indeed, PDA was at least two times higher in the upper horizon from the T2 Technosol than in any other horizons (Figure 3.4). Accordingly, analysis of the Technosol physico-chemical properties revealed that the T2 upper horizon differ the most with, for example, a much lower gravel content, C/N ratio and total $CaCO_3$ concentration (Table 3.1). A decrease in potential denitrification with soil depth was reported in previous studies (Gift et al. 2010; Groffman et al. 2002; Pintar and Lobnik 2005; Vilain et al. 2012) and has been attributed to changes in organic carbon and soil texture, which in turn influences the oxygen partial pressure (pO_2) (Cosandey et al. 2003). Indeed it is well known that the activity of the denitrifiers is controlled by the pO_2, the availability of organic carbon and nitrogen oxides (Knowles 1982; Tiedje 1988). Accordingly, we found that changes in texture (coarse gravel, sand, clay and silt content), organic matter and organic carbon, total N but also in soil moisture between Technosol types and depths had a strong influence on PDA (Fig. 3.5). As observed in an agricultural soil by Jia et al. (2009), PNA was lower in the deeper horizons but the changes in the measured Technosol properties across depths were not as strong drivers of PNA as for PDA (Fig. 3.5). Therefore our results demonstrate that the nitrogen cycling processes,

nitrification and denitrification, are influenced by the Technosol depth and to a minor extent, by the Technosol type. Altogether our results suggest that despite being artificially constructed, Technosols exhibit a gradual change in N-cycling activities along the soil profile with decreasing rates as depth increases as previously observed for other microbial activities in soil systems (Ajwa et al. 1998; Fierer et al. 2003; Lavahun et al. 1996; Taylor et al. 2002).

The depth also clearly affected the abundance of the total bacterial community and of the N-cycling microbial guilds in both types of Technosol (Figs. 3.2 and 3.3). Quantification of the ammonia-oxidizers, nitrate reducers and denitrifiers in the upper horizon at the same site but at different sampling occasions resulted in comparable ranges (Unpublished data). Similar to the previous studies investigating the vertical distribution of abundance of microorganisms in arable, grassland and forest soils (Fierer et al. 2003; Mergel et al. 2001; Regan et al. 2011), all targeted groups were more abundant in the upper horizons of the Technosols. It is unclear which soil factors were responsible for this distribution since no significant correlation were found between the gene quantification data and the measured Technosol properties except for *nirS*. In contrast, Enwall et al. (2010) found that both *nirK* and *nirS* gene copy numbers were correlated with some soil physical parameters, suggesting a relationship between denitrifiers abundance and soil structure. In a recent work, Fierer at al. (2003) reported that carbon availability was the main factor affecting microbial community through the soil profile. While denitrifiers are mostly heterotrophs (Tiedje 1988), their abundances were not related to the variation in total carbon across depths or between Technosols. The fact that carbon content might not be a strong driver of the denitrifier abundance has previously been suggested in studies showing that neither the short-term addition of artificial root exudates, sugar nor plant residues in soil microcosms resulted in a significant increase of denitrifiers (Henderson et al. 2010; Henry et al. 2008; Miller et al. 2008). On the other hand, the decline in carbon availability with the soil depth is not only due to decreasing carbon concentration but also to a reduction of carbon quality (Ajwa et al. 1998; Richter and Markewitz 1995), which was not assessed in this work. Vertical changes in soil microbial community structure were also demonstrated in forest and arable lands (Fierer et al. 2003; Agnelli et al. 2004; Watanabe et al. 2001). Accordingly, the decrease in microbial community abundances with depth was concomitant to shifts in the bacterial community structure showing distinct communities in the different Technosol layers (Fig.3.1). Changes in N-cycling processes rates with depth could therefore be due not only to the decrease in abundance of the corresponding microbial guilds but also to changes in the total bacterial community structure with the possible development of populations having a lower specific

activity in the deeper horizon. Interestingly, we found differences in the microbial community within the same constructed horizon. Thus the abundance and the structure of the bacterial community differed between 15-35 and 35-85 cm within the paper by-products and treated industrial soil horizon. The same was observed for the abundances of *narG* and *nirK* denitrifiers. This supports previous work suggesting early pedogenesis of Technosols (Sere et al. 2010). Altogether our results underline the importance of the variations of soil properties across depth in driving the abundance, the diversity and the activity of microbial communities in the constructed Technosols.

Analyses of the relationships between the abundance and the activity of the nitrifier and denitrifier communities indicated that PDA was significantly correlated with the abundances of the *nirS* denitrifiers while PNA with that of the AOB. Interestingly, the spatial variation of PDA also correlated to the distribution of the *nirS* denitrifiers at the field scale in previous studies (Philippot et al. 2009; Enwall et al. 2010). As underlined by Röling (2007), these results do not suggest that counting the number of microorganisms performing a particular process is sufficient for estimating the corresponding fluxes but rather than the differences in abundances can be of importance for microbial process rates when the environmental conditions controlling these processes are favorable (Cheneby et al.2009; Hallin et al. 2009; Petersen et al.2012). Despite a rapidly increasing body of literature in the field, it remains unclear whether AOA or AOB are the main contributors to nitrification (Martens-Habbena et al. 2009; Schleper 2010; Schleper and Nicol 2010; Wessen et al. 2011; Zhang et al. 2012). In this study, we couldn't detect the AOA in most samples. Even though the primers we used for the amplification of AOA have been successfully applied to assess their abundance in diverse environments and across a range of land uses (Wessen et al. 2010; Bru et al. 2011; Fan et al. 2011; Trias et al. 2012; Long et al. 2012), we can not rule out the possibility that an unknown group of AOA is present in the Technosol. However, our results showing that PNA was related to the abundance of the AOB and that the AOA were not even detected suggest that nitrification is driven by bacteria and not crenarchaea in the studied Technosols. Similar evidence for the AOB rather than AOA being the main contributors to nitrification has recently been reported in nitrogen-rich grassland soils (Di et al. 2009).

5. Conclusions

Our results indicate that N-cycling process rates and the abundance of the corresponding microbial guilds in Technosols are in the same range as in the other terrestrial ecosystems. This suggests that Technosols, which are constructed for reclamation of contaminated soils, can successfully perform essential ecosystem services such as those involved in nutrient cycling. The correlation between the AOB abundance and potential nitrification suggests that bacteria and not archaea are driving nitrification in Technosols. Despite being man-made soils, we have also shown that the vertical distribution of microorganisms in Technosols is similar to that observed in other soils with a decrease of both the activity and the abundance of the ammonia-oxidizers and denitrifiers with increasing depth. The type of Technosols also had an impact on the N-cycling communities, especially in the upper horizon. However, Technosol depth was a more important driver of the studied microbial communities than Technosol type. These results will improve the understanding of the biological functioning of Technosol, which is a promising technology for the restoration of degraded lands and recycling of industrial wastes.

Acknowledgements

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Supplementary data

Table S3.1. Analysis of Variance and Deviance table for qPCR of 16S rRNA

		*q*16S Bacteria		*q*16S Crenarchaea	
Source of variation	*df*	*MS*	*F*	*Dev*	*F*
Technosols	1	4.66	58.27***	$0.01x10^3$	0.04
Horizon depth	2	20.72	259.41***	$27.79x10^3$	50.02***
Replicate(Technosols)	4	0.92	11.57***	$1.90x10^3$	1.71
Technosols*Horizon depth	2	0.34	4.28*	$0.26\ x10^3$	0.47
Replicate(Technosols)* Horizon depth	8	0.18	2.26*	$5.84\ x10^3$	3.01*

df : degrees of freedom, MS : Mean Square, Dev : Deviance, F : Fisher's F.
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Table S3.2. Analysis of Variance and Deviance table for qPCR of ammonia oxidizers and denitrifiers

		***q*AOB**		***qnap***		***qnarG***		***qnirK***		***qnirS***		***qnosZ***	
Source of variation	*df*	*MS*	*F*	*MS*	*F*	*MS*	*F*	*MS*	*F*	*Dev*	*F*	*Dev*	*F*
Technosols	1	10.65	62.29***	3.26	24.55***	11.05	61.89***	8.09	70.08***	$1.09x10^4$	1.31	$1.69x10^5$	20.46***
Horizon depth	2	11.76	68.78***	19.41	146.15***	19.95	111.68***	21.31	184.59***	$117.71 x10^4$	69.83***	$13.31 x10^5$	80.62***
Replicate(Technosols)	4	14.02	82.06***	0.05	0.34	0.68	3.82*	0.79	6.86***	$24.69 x10^4$	7.36***	$0.34x10^5$	1.04
Technosols*Horizon depth	2	0.09	0.53	1.33	10.03***	0.97	5.44**	0.88	7.65**	$1.30 x10^4$	7.75**	$0.85 x10^5$	5.15*
Replicate(Technosols) *Horizon depth	8	0.82	4.82***	0.56	4.22**	0.66	3.68^{**}	0.66	5.73***	$1.14 x10^4$	1.95	$3.99 x10^5$	6.05***

df : degrees of freedom, MS : Mean Square, Dev : Deviance, F : Fisher's F.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Table S3.3. Analysis of Deviance table for N cycling activities

		Nitrification Rate		Denitrification Rate	
Source of variation	*df*	*Dev*	*F*	*Dev*	*F*
Technosols	1	0.27	7.89**	2.58	11.19**
Horizon depth	2	0.55	8.04**	12.24	26.58***
Replicate(Technosols)	4	0.52	3.79*	2.79	3.04*
Technosols*Horizon depth	2	0.04	0.51	1.73	3.77*
Replicate(Technosols)* Horizon depth	8	1.57	5.68***	4.77	2.59*

df : degrees of freedom, Dev : Deviance, F : Fisher's F.
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

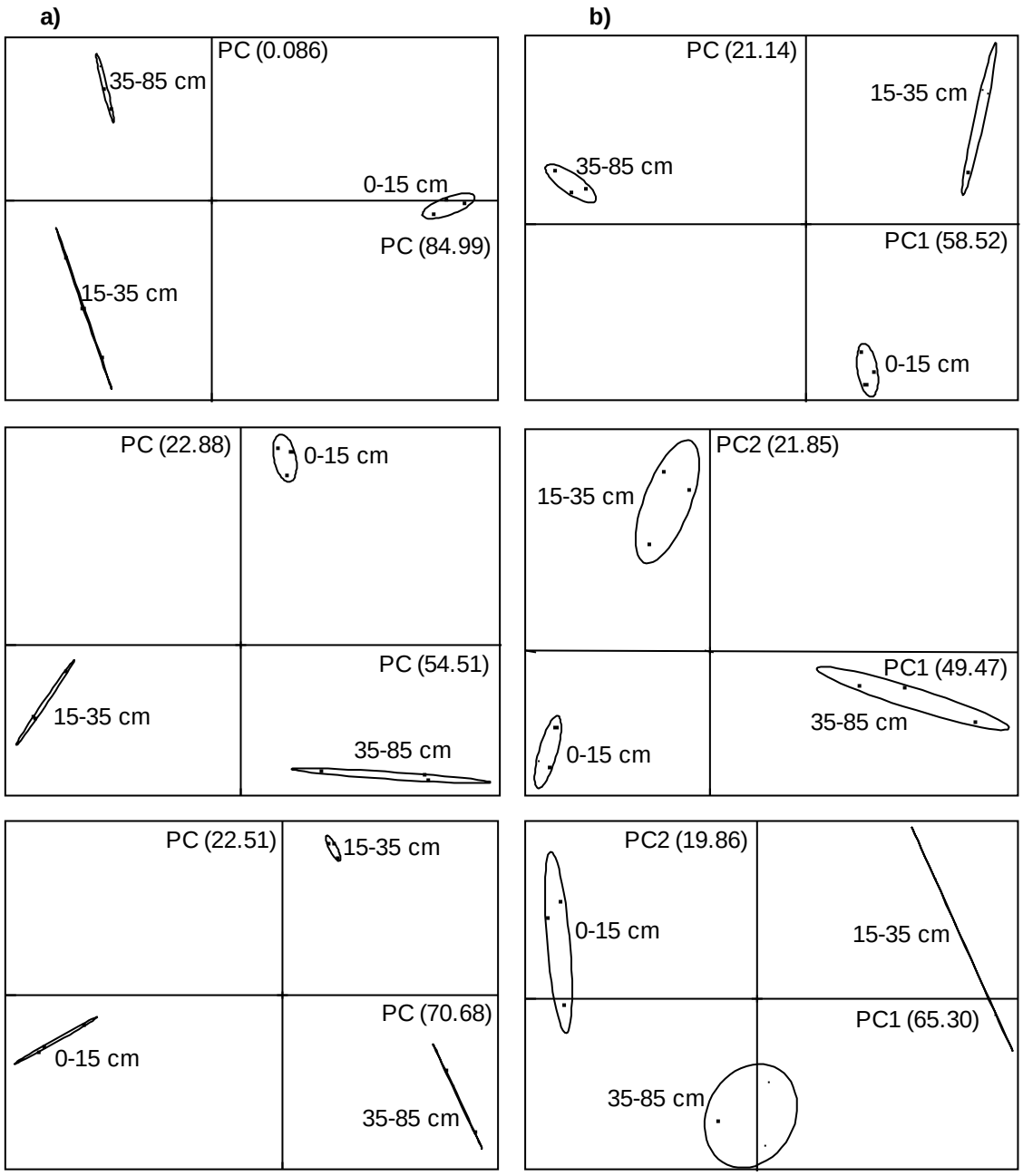

Fig. S3.1. Principal Component Analysis of the A-RISA depth profiles

Principal Component Analysis of the A-RISA profiles of the total bacterial community structure from three different horizons of the six pits sampled from the two types of Technosols (T1 (a) and T2 (b)).

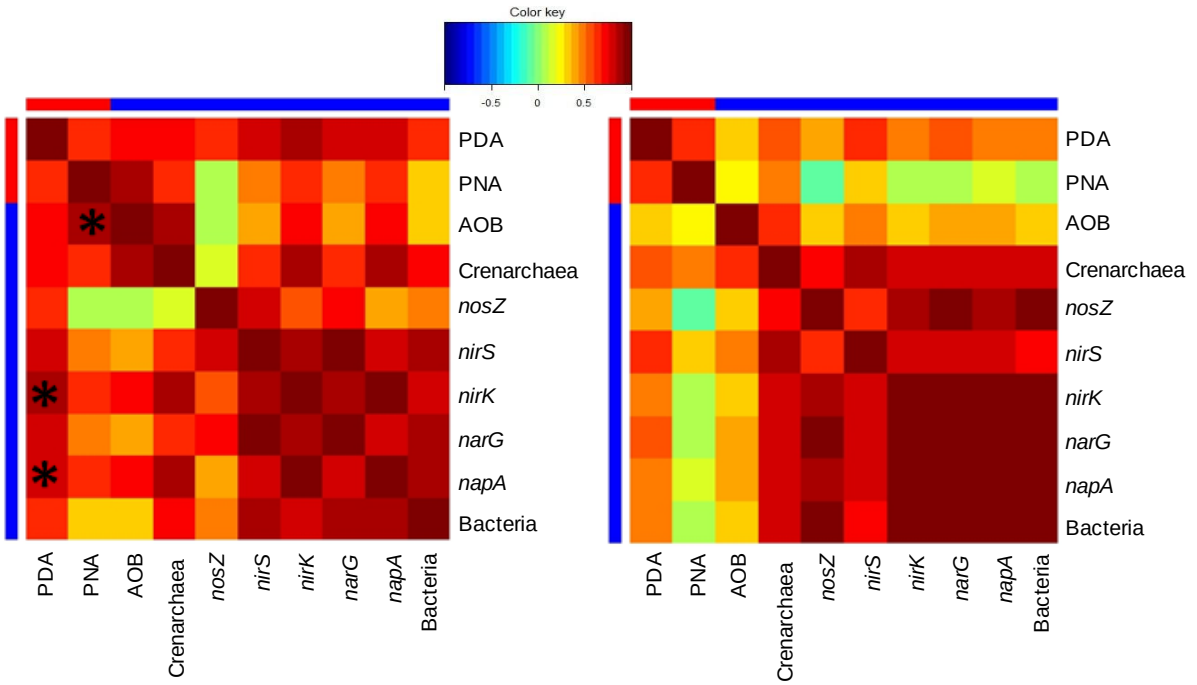

Fig. S3.2. Correlation matrix between community abundance and activities separately in two Technosols

Correlation matrix explaining the relation of the abundance and activity data for each Technosol individually. Colour scale above of the figure gives the intensity of the correlation (* shows the significant positive correlations).

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CHAPTER 4 ASSESSMENT OF THE RESILIENCE AND RESISTANCE OF REMEDIATED SOILS USING DENITRIFICATION AS MODEL PROCESS

Assessment of the resilience and resistance of remediated soils using denitrification as model process (Article to be submitted)

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Running title: Resilience and resistance of remediated soils

Key word: Soil remediation, stability, denitrification, polycyclic aromatic hydrocarbons

Abstract

Soil degradation is increasing urging for remediation strategies. We showed that thermal remediation of a contaminated soil leads to a low resilience of denitrification activity after heat stresses. However, addition of compost improved its stability. This study underlines the relevance of resistance and resilience ecological concepts for assessing remediation strategies.

Soil carries out functions that are crucial for the environment and life on earth and is therefore an essential nonrenewable natural resource for mankind. However, the intensification of human activities is responsible for the degradation of soil through the disposal of industrial waste, careless use and discharge of considerable various chemicals and heavy metals. For ecological reclamation of the heavily contaminated soils, various strategies consisting mostly in treating the polluted soil *in* or *off* site have been developed (Mulligan et al. 2001; Salt et al. 1995; Sere et al. 2008; Tokunaga et al. 2002).

The success of the soil remediation processes is most often evaluated by investigating whether the capacity to fulfill the essential soil functions and services such as biogeochemical cycling or primary production has been restored or not. Thus, Sere et al. (2008) showed that thermally treated soil can support sown and indigenous plants without any deficiency symptoms. Microbial activity, which often varies with increasing contamination, was also

used as an indicator of soil restoration (Kelly and Tate 1998). However, little is known about the capacity of the restored soil to resist (resistance) and recover from (resilience) environmental stresses even though resistance and resilience are the two components of ecosystem stability as described by Pimm (1984) and Loreau et al. (2002).

Here we assessed the functional stability of an industrial soil excavated from a former coking plant site initially polluted with polycyclic aromatic hydrocarbons which was thermally treated for organic pollutant degradation (Decontaminated soil) or thermally treated and staked with compost and paper by products (Technosol) and of an adjacent arable soil. For this purpose, we measured resistance and resilience of a nitrogen cycling process, denitrification, to heat-drought stresses in the three soils during one month.

1. Evaluation of the effect of heat-drought stresses on the potential denitrification activity in the different soils

Triplicate samples were randomly collected in 2011 in Homécourt, France, on a 1 ha experimental site built in 2007 on industrial wasteland using the decontaminated soil (D) and the Technosol (T) and on an adjacent arable field (A). For each of the three soils, 45 plasma flasks (n=3), containing 25 g of dry soil were incubated at 25°C during 28 days out of 30 flasks were exposed to 40°C for either 24h (low stress) or 48h (high stress) at day 0. Following the stresses, a constant water holding capacity of 70% was maintained in the microcosms. Measurements of potential denitrification activity (PDA) using the acetylene inhibition technique (Pell et al. 1996; Yoshinari et al. 1977) showed at the beginning of the incubation that in the non-stressed soils (control), T exhibited rates of 107 N_2O-N g^{-1} dry soil min^{-1}, which was in the same range as the A soil (Fig. 4.1, Tukey HSD test, $P > 0.05$). Measurements of PDA in another arable field located near the experimental site also showed similar rates (data not shown). In contrast, lower rates of 34 N_2O-N g^{-1} dry soil min^{-1} were observed in the D soil (Tukey HSD test, $P < 0.05$), indicating that as expected, thermal treatment of the contaminated soil greatly affected the soil microbial processes. However, the three time increase in PDA rates in T compared to D suggests that addition of paper-mill sludge and green waste compost stimulated the soil microbial processes, therefore improving functioning of the remediated soil to a level which was identical to those of the adjacent arable soils. This increase of denitrification activity after addition of organic carbon has been

described in a large body of literature (Dambreville et al. 2006; Miller et al. 2008; Myrold and Tiedje 1985; Sutton-Grier et al. 2009).

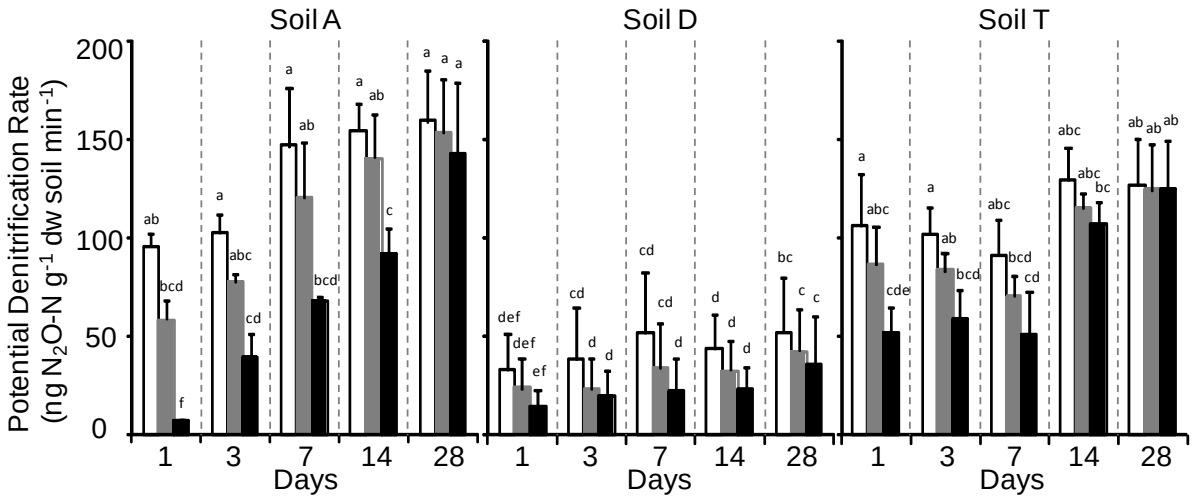

Fig. 4.1. Potential denitrification rates in different soils

Potential denitrification rates in unstressed control (□) soil samples and in the samples subjected to the low (▒) and high (■) levels of stresses from three different soils measured during 28 days after the stresses. Soil A is the arable soil, soil D is the decontaminated soil and soil T is the Technosol. Bars indicate mean (± standard deviation) in ng N_2O-N g^{-1} dry soil min^{-1} (n = 3). Significant differences between each soil type-by-stress level combination within each day are indicated by different letters (Repeated measures ANOVA followed by Tukey HSD tests, $p < 0.05$ was chosen as the significance thresold).

2. Resistance and resilience of the remediated soils

To monitor the resistance and the resilience of PDA in the different soils, the ratio of the rates measured in the stressed soil to the control soil was calculated 1, 3, 7, 14 and 28 days after the end of the stress. After 1 day, the investigated soils differ in their resistance to the low stress compared to the control treatment with a decrease of the activity ranging between 25 and 40% (Fig. 4.2). A significantly higher resistance was observed in the T soil compared to the A soil, while the resistance of the D soil was not different to that from the A or T soil. Significant differences (Tukey test, $P < 0.05$) were also observed in the high stress to control soil PDA ratios with values of 7%, 46% and 49 % in A, D and T, respectively, indicating a

much lower resistance of the arable soil compared to the remediated soils. This could be explained, for example, by differences in soil structure since the highest clay and lowest sand content were observed in the A soil (Table 4.1). Accordingly, the role of soil properties in determining soil stability has been previously reported (Griffiths et al. 2008; Zhang et al. 2010). In this work, only the activity of the denitrifying community was monitored and therefore we cannot rule out that the lower resistance of the A soil was also due to differences in the diversity or abundance of the corresponding microbial community as previously suggested (Griffiths and Philippot (*in press*); Wittebolle et al. 2009). However, the objectives of this work were not to disentangle the biotic and abiotic factors driving resistance and resilience of the studied microbial process in the remediated soils but to assess the success of the remediation strategy using soil stability as an indicator.

Table 4.1 Physico-chemical properties of three different soils

Arable soil (soil A), Decontaminated soil (soil D), constructed Technosol (soil T).

Parameter	Soil moisture (%)	Clay (< 2 µm) (g. kg^{-1})	Fine silt (2-20 µm) (g. kg^{-1})	Coarse silt (20-50 µm) (g. kg^{-1})	Fine sand (50-200 µm) (g. kg^{-1})	Coarse sand (200-2000 µm) (g. kg^{-1})	Total Nitrogen (g. kg^{-1})	Organic Carbon (g. kg^{-1})	C/N	Organic matter (g. kg^{-1})	pH	CEC (cmol+/kg)
Soil A	22.2	380	311	249	53	7	1.73	21.9	12.7	37.9	7.61	18.1
Soil D	21.4	115	182	116	180	407	3.34	109	32.6	188	8.54	15.3
Soil T	27.9	178	262	117	158	285	5.75	112	19.4	193	8.34	20.7

The A and T soils showed a good recovery following both the low and the high stresses with no differences in PDA observed after 28 days between the stressed soils and the control treatment (Figs 4.1 and 4.2). Similar resilience of microbial activities such as respiration to heat and drought was previously reported (Pesaro et al. 2004; Tobor-Kaplon et al. 2006). In contrast, the higher impact of heat on the D soil was confirmed by the lack of any recovery of PDA following the two stresses (Fig. 4.2). To summarize, the Technosol showed a strong resilience and a better resistance than the adjacent arable soil, which was used as a pristine soil in this study. This result suggests that the remediation process through Technosol construction can not only restore the capacity of soil to fulfill the essential functions and services but also improve its stability.

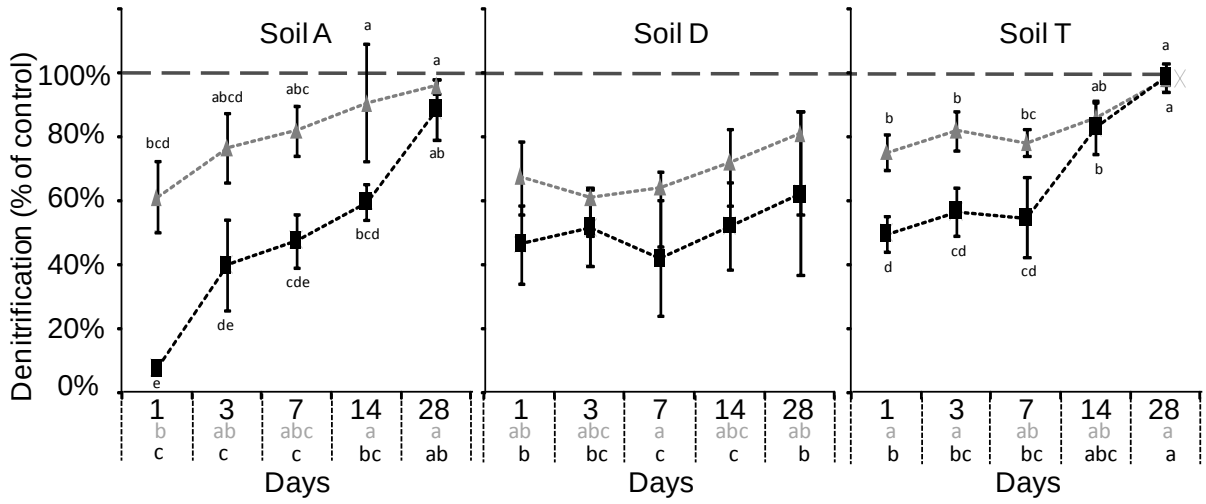

Fig. 4.2. Relative change in potential denitrification rates in different soils

Relative change in potential denitrification rates during 28 days after the low and high levels of heat stresses, compared to unstressed controls (Low stress: ▲ High stress ■). Soil A is the arable soil, soil D is the decontaminated soil and soil T is the Technosol. Significant differences between stress levels within each soil type are indicated by letters above or underneath the symbols (Repeated measures ANOVA on arcsin-transformed data followed by Tukey HSD tests). Note that for the D soil, no significant difference was found between any stress level-by-day combination pairs. Significant differences between soil types for each-stress level and for each day are indicated by grey and black letters respectively for low stress and high stress underneath the x-axis (Repeated measures ANOVA on arcsin-transformed

data followed by Tukey HSD tests). In both cases, $P < 0.05$ was chosen as the significance threshold.

Altogether these results showed that the thermal remediation of the contaminated soil lead to low process rates and stability compared to the arable field. However addition of compost and paper-mill sludge to the remediated soil greatly improved its resistance and resilience together with soil functions. This study also demonstrates that the ecological concepts of resistance and resilience can be of high policy relevance for assessing the success of soil remediation strategies in the context of climate change.

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CHAPTER 5 GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

General Discussion

The construction of Technosols is an emergent technology to restore degraded land (Sere et al. 2008). However, the ability of Technosols to perform soil ecosystem services such as nutrient cycling remains unknown. Microbial communities are key-players of soil ecosystemic services and therefore one could hypothesize that they might be of prime importance in restoring soil ecosystem services. Investigation of microbial community abundance, composition and activity can give insight in the status of restored systems (Harris 2009). In this PhD work, two types of Technosols constructed at the GISFI experimental station to restore an industrial wasteland were studied in details in the context of a collaborative program of research coordinated by Jérôme CORTET (UMR LSE, INRA/University of Lorraine) funded by the Ministry of Ecology in the framework of the national call GESSOL IV. My PhD work was part of the task of the program given to UMR Agroecology. The overall aim of this work was to study the functional diversity of microbial communities of Technosols. To perform this work, given the expertise of the laboratory hosting me for my PhD work, we have decided to characterize microbial communities involved in N cycling. The first objective was to characterize the bacterial community structure and abundances of N cycling communities (second chapter). The second objective was to investigate the distribution of the structure of total bacterial community and of the abundance and activity of N cycling communities along depth profiles (third chapter). The third objective was to address the functional stability of N cycling microbial communities. To reach this objective, the capacity of N cycling microbial communities of the Technosols to withstand an environmental stress was addressed by assessing the response of the denitrification process to heat/drought stresses in Technosols and arable and contaminated soils (fourth chapter). In this chapter, the different experimental results will be discussed together to draw an outcome of the entire PhD work.

Total bacterial community structure and diversity

In a first approach, in order to characterize the microbial diversity of the Technosols, we used molecular techniques based on direct extraction of nucleic acids from soil samples (Martin-Laurent et al. 2001) and their further characterization by PCR based approaches. For this purpose, abundance and diversity of the total bacterial community and the abundance of the crenarchaeal community was measured in Technosols by targeting the rRNA genes from

both bacteria and crenarchaea. The quantification of 16S rRNA genes showed a high abundance of the bacterial community in both types of Technosols whereas the crenarchaeal numbers were just above the detection limit. Neither the sampling date nor the Technosol type significantly affected the abundance of the total microbial community whereas within the same Technosol, a large spatial variability was observed. However, the crenarchaeal 16S rRNA genes were successfully quantified in both Technosols while analyzing the depth distribution of microbial communities. In both Technosols, the abundance of the bacterial 16S rRNA was always higher than the crenarchaeal 16S rRNA genes in agreement to the observations recorded in a wide range of environments (Long et al. 2012, Petersen et al. 2012, Wessen et al. 2010). Indeed bacteria largely dominate over archaea and, Bates et al. 2011 reported that the relative abundance of archaea ranged from 0.08 to 15.6 % with an average of 2% across 146 soils of a wide variety of ecosystems. The abundances of both communities followed similar trends across the Technosol profiles showing a gradual decrease with increasing depth. Overall, the abundance of the total bacterial community in Technosols was always 10 to 20 times higher than the N cycling communities. These observations are consistent with previous reports (Djigal et al. 2010, Kandeler et al. 2009, Kandeler et al. 2006, Marhan et al. 2011, Philippot et al. 2009).

A-RISA fingerprinting revealed no significant differences between the two types of Technosols. However, a high variability in the global structure of bacterial communities was also observed in both Technosols. Such variability could be attributed to the processes employed to build this 1 ha experimental site characterized by its heterogeneous nature. For example, the organic matter content or the nutrient availability highly differs within the same Technosol. The variation of these intrinsic parameters might explain the important heterogeneity observed all over the experimental site. However, this heterogeneity seems to decrease over the three years period studied. One could suggest that ongoing pedogenic processes will lead to the homogenization of Technosols physicochemical properties and consequently of the microbial habitat. In order to characterize in more details the extinct of the microbial diversity in the Technosols, three samples from each Technosol showing highly differing A-RISA profiles (2009) were selected for community composition analysis by applying a cloning/sequencing approach. This study reveals that *Proteobacteria* was the major bacterial group in both Technosols representing about 50-80% of the clones sequenced. Other dominant bacterial phyla were *Bacteroidetes*, *Firmicutes*, *Chlorofexi*, *Acidobacteria* and *Actinobacteria*. The community composition was similar to those of the natural soils. It was not significantly different between the two types of Technosols. Nonetheless in

accordance with spatial variability observed with A-RISA fingerprinting, relative abundances of bacterial phyla were highly variable within a Technosol type (2 to 20%).

In addition, we also investigated the variation in the structure of the bacterial community along depth profiles of the Technosols. Significant changes in bacterial community structure with depth were observed suggesting a vertical stratification of bacterial communities in accordance with pedogenic formation. This vertical distribution of microbial communities across the Technosols profiles could be related to pedogenic processes ongoing at different depths leading to changes in soil composition or to changes in organic matter composition altering carbon availability. These two parameters are known as the major drivers of microbial community size and structure (Agnelli et al. 2004, Fierer et al. 2003, Prevost-Boure et al. 2011, Will et al. 2010, Williamson & Wardle 2007). Accordingly, the impact of freshly available organic C delivered by organic matter on the structure of microbial communities has also been reported (Bastian et al. 2009, Hidri et al. 2010). Moreover, within the same constructed horizon (at approximately 15-75 cm depth) differences in the microbial community structure were observed suggesting that they may diverge under the influence of the different parent materials at the constructed horizons along Technosols depth profile.

N cycling microbial communities

In order to evaluate the ability of Technosols to fulfill soil ecosystemic services, we have chosen the microbial communities involved in the nitrogen cycling as a model. These microbial communities have been studied in a diverse range of environments and among these the nitrifiers and the denitrifiers are intensively studied and are the matter of great interest for the research in microbial ecology. Here we have used various approaches to address N cycling microbial communities' abundances and the activities in Technosols.

Ammonia oxidizers and potential nitrification activities

The first step of the nitrification process (ammonia oxidation) is a rate limiting step which can be carried out both by the bacteria and the crenarchaea (Prosser & Nicol 2008). Whether archaea or bacteria are driving ammonia oxidation is still unclear. In the first study (second chapter) we observed that the AOB dominated over the AOA, which were below the detection limit in both Technosols at different sampling dates, suggesting that the AOB might be the main contributor to ammonia oxidation. This was confirmed by investigating the depth distribution of AOB and AOA along Technosol profiles. The dominance of the AOB over the AOA in the Technosols is in contradiction with previous reports indicating that the AOA are

the dominant ammonia oxidizers in various environments (Adair & Schwartz 2008, Bru et al. 2011, Chen et al. 2008, He et al. 2007, Leininger et al. 2006, Shen et al. 2008). However, more recently other studies have showed that AOB could also be as abundant as AOA is (Fan et al. 2011, Li et al. 2011, Mosier & Francis 2008, Wells et al. 2009).

Environmental conditions affecting the balance between AOA and AOB are yet unclear. It has been suggested that AOA are the main players under stress conditions like severe temperature, low pH or low substrate availability such as low nutritional state (Di et al. 2010, Erguder et al. 2009, Roesch et al. 2007, Valentine 2007, Verhamme et al. 2011). On the contrary, the AOA communities were found to be decreasing both with decrease and increase in pH (Hallin et al. 2009, Nicol et al. 2008). It is believed that AOB have greater ecophysiological diversity and potential to cover a broader range of habitats (Nicol et al. 2008, Wessen et al. 2010) and they are predominant and functionally important ammonia oxidizers in the nutrients rich environments (Di et al. 2010, Wells et al. 2009, Zhang et al. 2010) where they can adapt and flourish under high nutrient availability (Mahmood & Prosser 2006). Technosols, containing compost and paper by products are rich in organic matter, carbon and nitrogen contents that might have favored the development of AOB. This is supported by several studies suggesting that AOB are copiotrophic and are associated with nutrient-rich environments (Bouskill et al. 2012, Urakawa et al. 2008, Wells et al. 2009). In contrast, the AOA are well adapted to the oligotrophic environments (Schauss et al. 2009, Valentine 2007) and the high nutrient inputs such as the long-term application of the mineral and manure fertilizers can even cause the reduction in AOA and increase in abundance of AOB (Fan et al. 2011). Although AOB dominated in Technosols, we were unable to find any significant correlation between the soil properties and abundance of AOB, most likely due to the heterogeneity of Technosols. Interestingly, it was recently reported that in mangrove ecosystems, combination of several physicochemical properties rather than any single factor is involved in shaping the AOA and AOB community dynamics (Cao et al. 2011). In this work, we can not rule out the possibility that an unknown group of AOA is present in Technosols due to primer bias resulting in the underestimation of the abundance of the crenarchaeal ammonia oxidizing populations. However the primers we used for the amplification of AOA have been successfully applied to assess their abundance in diverse environments and across a range of land uses (Bru et al. 2011, Fan et al. 2011, Long et al. 2012, Trias et al. 2012, Wessen et al. 2010).

The community abundances of AOA and AOB have been linked with the nitrification activity (Erguder et al. 2009, Herrmann et al. 2011, Offre et al. 2009, Wessen et al. 2010) and

can therefore reflect the soil nitrification potential. In this work, we also found a significant correlation between the abundance of the AOB and the PNA thus enforcing our assumption that the bacteria were mainly responsible for ammonia oxidation in Technosols. This was in accordance to the studies reporting that AOB are controlling nitrification across a wide range of environments (Di et al. 2010, Di et al. 2009, Fan et al. 2011, Fortuna et al. 2012, Hoefferle et al. 2010, Jia & Conrad 2009, Morimoto et al. 2011, Petersen et al. 2012). In contrast, the AOA were found abundant with a significant correlation between the archaeal *amoA* abundance and nitrification rates in acidified forest soils (Isobe et al. 2012). Interestingly, despite of the higher abundance of AOA in various environments, AOB are evidenced to be functionally more important than AOA (Di et al. 2010, Fan et al. 2011, Jia & Conrad 2009). The capability of microbial communities to adapt a certain environment or the selection by the environment itself is central to understand the microbial ecology as they can influence the related process rates (Ke & Lu 2012). For the ammonia oxidizing communities, their functioning is dependent on their ecophysiology and adaptation in different conditions (Schleper & Nicol 2010) and the environmental parameters are able to play their part in ecological niche differentiation of ammonia oxidizing archaea or bacteria. We find a significant correlation between the total nitrogen, organic carbon and organic matter and the potential nitrification rates. The addition of the compost and paper by-products in Technosols has positively influenced the AOB over AOA and thus they became the dominant drivers of ammonia oxidation. Similarly by analyzing a variety of soils, Fortuna et al. (2012) suggested that the AOA abundance tended to be lower and did not respond to the addition of manures and AOB were likely to be predominant nitrifiers in soils receiving dairy slurry.

The abundance of the AOB was independent of the Technosol type whereas the PNA were slightly higher in Technosol T2. Hence, the Technosol depth effect was prominent than the Technosol type and AOB abundance and the potential nitrification rates were higher in the surface horizons. A general decline in microbial biomass and activity with increasing soil depth has been reported in various soils (Fierer et al. 2003, Taylor et al. 2002) and the differences in population distribution and their activities along the soil layers could be the matter of different environments at different depths. Accordingly, greater abundance and activities of the ammonia oxidizers have been found in top soils with high fertility than in the low fertile subsoils (Di et al. 2010, Jia & Conrad 2009, Leininger et al. 2006).

Denitrifiers and potential denitrification activities

The 16S rRNA gene copy numbers per genome may vary from 1 up to 15 (Klappenbach et al. 2001) while 1 copy per genome is present for *napA*, *nirS*, *nirK*, *nosZ* and up to 3 copies for *narG* gene (Philippot et al. 2002), therefore the denitrifers' gene copy numbers are better estimates of cell numbers than the 16S rRNA gene copy numbers. Abundance of the denitrifiers was not different between the two Technosols whereas the differences were higher within the same Technosol plots in accordance with their reported heterogeneity (second chapter). However, the depth distribution of denitrifiers' abundance significantly varies according to Technosol type. It also showed that the Technosol depth clearly affected the abundances of the denitrifying communities with marked vertical distribution with higher values in surface horizons. Previous reports have shown the depth effect on density and number of total bacterial and denitrifier community (Kandeler et al. 2009, Mergel et al. 2001) and the abundances of the microbial communities have been found higher in surface horizons across a range of soil systems (Fierer et al. 2003, Mergel et al. 2001). Various environmental factors (Henderson et al. 2010, Kandeler et al. 2006, Yoshida et al. 2009, Zhou et al. 2011) and also the soil structure (Enwall et al. 2010) are shown to affect the abundance of different denitrifying genes. In contrast, we did not find such significant correlations in Technosols except *nirS* abudance that was slightly affected by the coarse sand and the total $CaCO_3$ contents.

We also calculated the relative abundance of the functional communities and found that the proportion of the denitrifying communities in Technosols ranged between 0.4 and 6% of the total bacterial community, in agreement to previous studies (Bru et al. 2007, Cheneby et al. 2004, Djigal et al. 2010, Henry et al. 2006). In accordance to forest and arable soils (Cheneby et al. 2010, Kandeler et al. 2009) *narG* predominated over *napA* though their function are similar. However, taking into account that 3 copies of *narG* can be found per genome and that *napA* is present at a single copy per genome, one could hypothesize that the cells harboring those two genes are equally represented. Moreover it has also been reported that the nitrate reducing bacteria possess one or both of the nitrate reductases (Philippot et al. 2005, Philippot et al. 2002). The abundances of the *nirK* and *nirS* genes were in the same ranges whereas the *nosZ* was less abundant. Recently, it has been suggested that not all the denitrifiers contain the *nosZ* gene and thus are unable to reduce the N_2O to N_2 which can influence the overall N_2O budget, a potent greenhouse gas. An analysis of the complete genomes of sequenced denitrifying bacteria has shown that approximately 1/3 have a truncated denitrification pathway and lack the *nosZ* gene (Jones et al. 2008). This can have

consequences for greenhouse gas emissions since it was shown that a decrease of the proportion of the *nosZ* denitrifiers can result in increased N_2O emissions (Philippot et al. 2011). Interestingly, our results revealed that the proportion of *nosZ* was 20 times lower than the other denitrifiers in Technosols suggesting that in Technosols not all the denitrifiers contained *nosZ* with implications for the global greenhouse budget of N_2O since the higher proportion of the denitrifiers lacking the *nosZ* gene can increase the overall N_2O emissions.

Denitrification rates were significantly influenced by the Technosol type and depth. Overall, PDA was at least two times higher in the upper horizon from the T2 Technosol. It has been reported that the soil moisture, temperature, oxygen partial pressure, nitrate concentration and soil pH are the factors that regulate denitrification activity (Cosandey et al. 2003, Hallin et al. 2009, Hochstein et al. 1984, Knowles 1982 , Philippot et al. 2007, Simek & Cooper 2002, Tiedje 1988, Vinther 1984, Zumft 1997). Accordingly, the denitrification rates were positively correlated to the total nitrogen, organic carbon and organic matter in Technosols and this correlation was stronger for PDA than PNA. The higher PDA recorded for the surface horizon of T2 might be due to the fact that most differences between the physicochemical properties of two Technosols were also observed in the upper horizon of Technosol T2. This horizon showed more nitrogen, organic carbon, organic matter and phosphorus but less total $CaCO_3$ and has a lower pH. In addition to soil physicochemical properties, the change in denitrification activites could also be associated with the change in community composition or abundance across the Technosol depth. Indeed, the studies have reported the links between denitrification activity and composition and abundance of different denitrifiers (Enwall et al. 2010, Hallin et al. 2009, Throback et al. 2007). In our work, PDA was significantly correlated with the *nirS* in both Technosols and with *nirK* and *napA* only in T2. However we did not find any significant correlation between the denitrification rates and the abundance of other denitrifiers. Attard et al. (2011) suggested that soil environmental conditions were more related to the PDA than the abundance of the denitrifiers. They also reported that the denitrifiers' abundance and PDA cannot be necessarily coupled since the synthesis of the enzymes involved in this processs depend on the environmental conditions (Zumft 1997). Furthermore, under a certain type of environmental conditions the different denitrifying strains can generate different levels of denitrification thus the importance of the community composition can not be overruled (Ka et al. 1997, Salles et al. 2009).

Resistance and resilience of Technosols

In this thesis we assessed the success of the restoration of Technosols constructed wastelands through investigating hosted microbial communities with special regard to N cycling ecosystem services (second and third chapters). Once the functional potential of the Technosols has been assessed, we evaluated whether or not these restored soils are functionally stable. To address this question stability of the denitrification activity in Technosol was measured in response to heat-drought stresses and compared to the responses of other soils collected in the vicinity of the Technosols (thermally treated industrial soil and adjacent arable soil).

Analyses of the potential denitrification rates in the beginning of the incubation in the non-stressed soils revealed a significantly higher denitrification activity in Technosol than in thermally treated soil. This suggests that the construction of Technosols by assembling the thermally treated soil with compost and paper by-products has led to the stimulation of the microbial activity. Similar observations have been reported in response to soil amendment with biosolids, which increased the size and overall microbial activity (Bastida et al. 2009, Perez-de-Mora et al. 2006, Ros et al. 2003). In accordance to previous studies, enzyme activity was strongly reduced on the first day after heat stresses (Chaer et al. 2009, Gregory et al. 2009). The Technosol resists better to the heat-stress than the two other soils. It has been suggested that when an ecosystem experiences disturbance, its functional stability depends on the level of its functions and of its diversity. For example, a higher level of diversity would strengthen the resistance and the resilience (Ives et al. 2000, McCann 2000). It has been shown that among specific functions, denitrification and methane oxidation are usually more sensitive to environmental stresses than broad scale function such as organic matter decomposition (Girvan et al. 2005, Griffiths et al. 2000). The reduction in denitrification activity in response to heat stress was associated to the physiological response due to denaturation and inactivation of denitrifying enzymes (Wertz et al. 2007). However, heat stress is also altering soil properties with obvious consequences on microbial habitats affecting microbial abundance and diversity (Griffiths et al. 2008, Wittebolle et al. 2009, Zhang et al. 2010). The evaluation of the functional stability of the constructed Technosol in comparison to another remediation strategy and the arable soil as control shows that the Technosol and arable soil were able to recover their initial denitrification activity no matter of the intensity of the stress. On the contrary the thermally treated soil did not recover its initial ability to denitrify indicating a low stability. This observation highlights the benefit provided

by the Technosol construction which have a good capacity to withstand environmental stresses and could therefore fulfill sustainable soil ecosystemic services with high stability.

Conclusions and Perspectives

Our results demonstrated that the microbial structure and composition of Technosols were similar to that of natural soils. They were highly variable in Technosol type with *Proteobacteria* being the dominant phylum that ranged from 50-80%. Variability of bacterial community structure seems to decrease over the three-year period suggesting that pedogenic processes ongoing in the constructed Technosols may *in fine* lead towards the homogenization of the microbial habitat.

We evidenced that the abundances of the total bacteria and the N-cycling microbial communities are in the same range as compared to that of arable soils. AOB were found in more abundance than AOA. The significant correlation between the AOB abundance and potential nitrification activity suggests that bacteria rather than AOA are driving ammonia oxidation in Technosols. Among the denitrifiers the gene copy numbers of *nosZ* denitrifiers were lower than the other denitrifying genes enforcing the recent observations that not all the denitrifiers have the nitrous oxide reduction capability.

N cycling activities with values comparable to those from agricultural soils were correlated with the physicochemical properties but this correlation was stronger for potential denitrification activity than for potential nitrification activity. Potential denitrification activity was also correlated with the abundance of the *nirS*-denitrifiers in both Technosols. Our findings showed that both soil properties and abundance of the AOB were the principle determinant of PNA whereas PDA was mainly controlled by soil properties and, to a minor extent, by the abundances of the *nirS* denitrifiers.

The results showed that both the selection and the organization (formulation) of the Technosols parent materials (green waste compost, treated industrial soil, paper by-products) were suitable to built an artificial soil matrix fulfilling N cycling ecosystemic service. Overall the Technosol type effect was more prominent for the upper horizon whereas the Technosol depth effect was pronounced in both Technosols for the total bacterial and crenarchaeal communities but also for the N cycling functional communities.

The assessment of the functional stability of the Technosols revealed that the denitrification process within the N-cycle was resistant and resilient to heat stresses tested here. 28 days after the heat stress, Technosols recovered its initial potential denitrification activity thereby demonstrating that Technosols seems to be as functionally stable as an arable field.

Although the abundance and activities of N cycling communities were evidenced, there is need to better understand different aspects of N cycling community dynamics. For example, no significant correlation observed between the community abundance and the Technosol physico-chemical properties (except *nirS* denitrifiers), which underlines the need to understand the factors regulating these communities. In this regard, the manipulation of environmental parameters can help us to monitor the response of the functional communities to different environmental conditions. The exploration of the community composition of ammonia oxidizing bacteria and their comparison with other soil systems can be useful to assess the functional adaptation of certain ammonia oxidizers in Technosols.

Since the main objective of the 'Biotechnosols program' was to investigate the capacity of the Technosols to perform the essential functioning of the natural soil such as plant growth and the diversity and functioning of the soil organisms involved in many soil physical (aggregation, bioturbation) and chemical processes (nutrient cycling). This PhD work was the first study on the ecology of total bacterial and N cycling microbial communities in constructed Technosols to describe their capability to perform N cycling functions. However, further research in various other aspects linking the microbial communities and the other soil ecosystem services are required. For example, it would be interesting to study fungi in Technosols in relation to the 'decomposition' and 'soil formation' ecosystem services since they are involved in the breakdown and mineralization of the organic matter and in soil aggregate stabilization (Bodelier 2011).

The present work showing the abundance and functioning of the hosted microbial diversity crucial in plant nutrition suggests that this soil habitat could be feasible for the cultivation of crops ultimately restoring the soil primary production services. However, the overall evaluation of the success of restoration strategy is a challenging task. It is important to keep in mind that organisms other than bacteria and crenarchaea are living in soil and a more comprehensive understanding of life in Technosols could be achieved by assessing, the diversity and functioning of all the different soil organisms (microorganisms, Fungi, microfauna, mesofauna, macrofauna).

Construction of Technosols can help to achieve C sequestration in terrestrial ecosystems since the incorporation of wastes can also be important in mitigating the CO_2 emissions. Soil construction processes and the choice of parent materials should be made according to the level of functions to be restored, and by prioritizing restoration attributes: soil decontamination, development of the soil physicochemical characteristics, soil biological functions and the kind of plant cover to be re-established (rangeland, agricultural land etc.);

and their environmental acceptability. For this purpose, the testing of a large variety of wastes and by-products as the parent materials can be of significant importance (Sere et al. 2008). Many traditional approaches for soil restoration are already in use; therefore the cost effectiveness of this approach should also be kept in future agenda, to use constructed Technosols as the promising technology for ecological reclamation of contaminated industrial wastelands.

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