이학박사 학위논문

미생물 생태학적인 시각으로 바라본 인간에 의한 교란이 환경에 미치는 영향

Effects of Human Disturbance on the Environment: A Microbial Ecology Perspective

2015년 02월

서울대학교 대학원

화학생물공학부

Dorsaf kerfahi

Effects of Human Disturbance on the Environment: A Microbial Ecology Perspective

Dorsaf Kerfahi

Advisor: Professor Junghoon Lee, Ph.D.

A Thesis Submitted in Partial Fulfillment of the Degree of Doctor of Philosophy

February 2015

School of Chemical and Biological Engineering

Seoul National University

ABSTRACT

The effects of human disturbance are increasing rapidly and can be observed in ecosystems in terms of effects on larger organisms. This thesis analyzes three case studies to explore how human disturbances affect the environment at the microbial scale.

The first study examined how land use change affects the structure and diversity of fungal communities in Malaysian tropical forests in Borneo. It was clear that α and β-diversity, as well as community composition differed across different logging histories (unlogged, once-logged and twice-logged), and oil palm plantations.

The second study examined how increasing atmospheric CO_2 on the ocean impacts the sediment bacterial communities off Vulcano, Italy in the Mediterranean. Bacterial community composition changed with increasing CO_2, and bacterial diversity increased with higher CO_2. The globally increasing ocean CO_2 can be associated with shifts in sediment bacterial community composition, but most of these organisms are resilient.

The third study compared the effect of both raw and acid treated (functionalized) multi-walled carbon nanotubes (MWCNTs) on soil bacterial communities, applying different concentrations of MWCNTs (0 µg/g, 50 µg/g, 500 µg/g and 5000 µg/g) to a soil microcosm system. Bacterial diversity was not affected by either type of MWCNT. However, overall soil bacterial community composition for functionalized carbon nanotubes at high concentrations were affected. This last study suggests that fMWCNTs may alter microbial community composition on the timescale of at least weeks to months. In contrast, it appears that raw MWCNTs do not affect soil microbial community composition.

These different case studies are examples of the ways in which metagenetic studies can help recognize the impact of human disturbance on the environment, ultimately enabling us to allow effective management decisions in order to protect the environment.

Keywords: carbon nanotubes; environment; human disturbance; logging; metagenetic; microbial community; ocean acidification.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GENERAL INTRODUCTION

Humans have long had a diverse range of impacts on nature. Some human activities, such as agriculture, have influenced ecosystem processes for thousands of years (Kalis et al. 2003). Humans seem to have caused widespread extinctions of large animals from as long as 50,000 years ago, through over-hunting. However, in recent centuries the human impact has become far more pervasive and profound, resulting from an exponential increase in human population and vastly intensified industrial and technological activity (Garbuio et al. 2012).

Many of the impacts of humans on ecological systems can be regarded as 'disturbances' – perturbations whose effects may last over time, sometimes persisting for hundreds or even thousands of years (Rykiel 1985 and Zak 1992). Disturbance is in fact also a natural feature of all ecosystems, and occurs on a wide range of spatial and time scales (Walker 1999). It is becoming increasingly clear that natural disturbance is key to understanding many aspects of the structure and function of ecosystems, including nutrient cycling, community composition and species diversity.

Human disturbances have added to the frequency, scales and types of disturbances that affect ecosystems. They are as diverse as climate change, fundamental changes in atmospheric and ocean composition, pollution by novel man-made chemicals, fires, logging and forest clearance for agriculture.

In order to understand what is happening to the natural world, and what may happen in the future, it is necessary to study these anthropogenic disturbances. In this way, we may avoid losing more of the world's diversity and risking collapse of its renewable natural resources.

The scale is now so great that humans are now noticeably altering fundamental global processes. Human activities now dominate natural global nutrient and elemental fluxes, for example, sulfur, nitrogen and carbon. Atmospheric CO_2 concentrations have been increased to about 40% above preindustrial levels (Schlesinger et al. 1997), and there are strong indications of climate change as a result of this. Soil calcium levels are declining over large areas because of high rates of leaching caused by acidic deposition (Likens et al. 1998). Through both active logging and land use change to agriculture, humans have altered land ecosystems over huge areas. Moreover, the range and intensity of human environmental effects are only likely to intensify over the coming decades (Tilman et al. 2001).

Understanding how to live with anthropogenic disturbances, and how to moderate their consequences, is an imperative area of research that must take its place alongside technological research and development (Botkin et al. 1989).

In this thesis, I would like to present the results and conclusions of several studies I have undertaken to understand certain types of human-induced disturbances on the environment. So far, many published studies have focused on the human impacts on larger organisms, from the scale of insects to large mammals and trees (Madsen and Fox 1995, Sakio 1997, Carney and Sydeman 1999, Verhulst et al. 2000, Rodgers and Parker 2003, Stankowich 2008, French et al. 2010). In the present work, I studied small organisms using the latest molecular techniques. These organisms, including fungi and bacteria, play a vital role in ecosystem processes around the world, including the recycling of dead organic material and release as available nutrients. They represent a major part of the living biomass and certainly the greater part of the diversity of life.

The approach used here was based on the new metagenetic techniques, which use massively parallel DNA sequencing to characterize and categorize living communities (Dupré and O'Malley 2007). Three different cases were studied to explore the response to human disturbance actions on the environment. First, the impact of selective logging and the clearance of tropical forest for different land uses on fungal communities. Logging and the conversion of tropical forests to agriculture seem to be two of the most pervasive threats to global biodiversity. In the second study, I investigated the impact of increasing atmospheric CO_2 on the diversity and composition of marine sediment bacterial communities. A shift on bacterial community composition and an increase of the diversity with increasing CO_2 were observed. Finally, I studied the environmental response to the exposure to multi-walled carbon nanotubes on soil bacterial community composition, where soil bacteria were influenced by the functionalized carbon nanotubes.

OBJECTIVES OF THE STUDY

The objective of the present study is to investigate the effects of human disturbance on microbial communities in the environment by studying three cases of human disturbances.

The following questions will be approached:

1) Do the diversity and composition of microbial communities vary with respect to human disturbance, among habitats receiving different levels of human use?

2) Does the microbial community vary with soil proprieties such as soil pH, and soil organic matter content?

3) Do habitat types characterized by different degrees of environmental stress and human disturbance action interact in their influence on the abundance and composition of microbial communities?

CHAPTER 1: WHAT IS DISTURBANCE?

1.1 Introduction

In current ecology, disturbance is considered as a worldwide theme (Sousa 1985, Pickett & White 1985). Almost every area in the Earth is affect by anthropogenic or natural disturbances, regardless to their geographic or climatic characteristics. These disturbances can affect the structure or the function of populations and communities in their natural ecosystems. Many studies have shown that disturbances are one of the most important ecological factors to maintain the heterogeneity and species diversity in the ecosystem (Kolasa and Pickett 1991).

It has been also, demonstrated that human disturbance is an important and widespread factor to drive environmental change, from local scale such as pollution, land use change… to global scale such as climate change (Acevedo-Whitehouse and Duffus 2009). Most of these environmental shifts affect the long-term persistence of natural communities. However, the impact of anthropogenic disturbances on individual physiological responses and the resulting effects on long-term persistence, and fitness of a species still remains poorly understood (Wikelski and Cooke 2006). Understanding these effects is a critical and important to efficiently evaluate the decline of a population's risk.

1.2 Definition

Disturbance is a common phenomenon of many ecosystems that happens at all ecological organization levels and at various temporal and spatial scales (Zak 1992). It is often used as synonym to stress and perturbation when applied to ecosystem dynamics. It has been observed as uncommon, irregular events that cause abrupt structural variations in natural communities and move them away from static, near equilibrium conditions. It is a relatively discrete happening in time and space, that alter the communities structure and composition, and the ecosystem functioning. It can alter the density, the biomass, or the spatial distribution of the populations by affecting the availability of resources and substrate, or by changing the physical environment (Walker 1999).

Disturbances are often characterized by the central tendency, the availability and distribution

of three aspects: frequency, extend and magnitude. Frequency measures the number of events happening per unit of time or the probability of happening. Extent means the physical area affected by the disturbance in a given time period. Magnitude includes the intensity which is the strength of the disturbing force of an event (e.g. wind speed for hurricanes) and the severity which is the consequence of the event (e.g. the number of trees destroyed by passage of a hurricane) (Sousa 1985).

The impact of any disturbance on the ecosystem depends on several factors: the soil, the vegetation cover, and the climate characteristics. It depends also on the frequency, the magnitude, the extend, and the type of the disturbance. The magnitude of most of disturbances varies extensively, although they have almost similar impacts. Intensifying physical impacts such as high-intensity may harmfully affect the species richness and soil biodiversity (Johansen 1993).

1.3 Types of disturbances

There are two types of disturbances: it may be natural (non-human) driven by natural elements (e.g. earthquake, volcano, erosion, flood, hurricane) or anthropogenic, caused by human (e.g. agriculture, logging, transportation, industry). The latter disturbances are usually greater in extent than natural disturbances; this might be explained by the broad distribution of human. They are also somewhat more severe than natural disturbances, but almost similar in frequency (Walker 1999).

1.4 Importance of studying disturbance

It is important to know about dramatic natural disturbances because they cause destruction of the ecosystem and injury to human and damage to the other living organisms. Disturbances can also have positive and beneficial effects to organisms such as nutrient recycling, and maintenance of species diversity. As a consequence of the exponentially increase of human population density, the rate of disturbance arises. Some anthropogenic disturbances are broadcasted (e.g. spills of oil, bomb explosions). Yet, more important disturbances that may have greater consequences, are receiving less attention for example urbanization, industrialization, toxic waste, forests logging. Both natural and anthropogenic disturbances

are threatening the globe (Walker, 1999).

1.5 Agents of disturbance

Both physical and biological processes represent agents of disturbance. The physical processes are generally associated with the term of disturbance. For example fires, storms, floods, drought, high winds, and landslides. The biological agents of disturbance (White 1979) include everything from predation to non-predatory behaviors that accidentally destroy other organisms (e.g. digging by mammals and ants in grasslands). In general, the biological and physical agents of disturbance have same similar impact in the ecosystem. The timing of biological disturbance is generally, associated with more complex set of controls. For example, the predation depends on several factors such as the functional, numerical, and developmental responses of the predator (Murdoch and Oaten 1975). Consecutively, these responses are, also influenced by the physical environment such as the habitat complexity, the presence of alternative prey, the availability of prey refuges, and the impact of higher level predators and parasites (Sousa 1985).

1.6 Conclusion

Most of the communities are expected to experience disturbance at some spatial and temporal scale. However, its role in the dynamics of population remains mainly ignored, except in some temperate regions. This overlooking is due to many reasons; in fact, one of the most important causes may be that main disturbances often are repeated at time intervals longer that the duration of a research project (Oliver 1981). Therefore, it is always, difficult to directly observe the effects of disturbance on the ecosystem. Yet, a long interval repetition of a disturbance on the community does not necessary show that the impact is not significant. When the organisms that are affected by disturbance have long life, the impact of disturbance may persist over time even for thousands of years. In fact, it was demonstrated that disturbance may play as an important role as do the biological interactions in the dynamics of organisms such as competition and predation, which have got more attention from ecologists. The relationship between disturbance and biological processes seems to have a major effect on the distribution and organization of communities in their environment (Sousa 1985).

CHAPTER 2: THE METAGENOMICS APPROACH

2.1 The basic concepts of metagenomics

Understanding how microorganisms and/or their genes interact in various environmental niches is a major challenge for the environmental microbiology. Nevertheless, in many environments, less than 1% of the microorganisms can be cultured with the existing technologies. The list of cultured microorganisms may skew microbial diversity assessment in nature. Even with the recent success of novel culture methods (Sait et al. 2002, Zengler et al. 2002, Stevenson et al. 2004, Binnerup et al. 2008, Nichols et al. 2010), it is still difficult to culture most of pertinent microbes in the suitable conditions for their growth. The development of culture-independent techniques, which bypass the need for isolation and laboratory cultivation of different species, has deeply enhanced environmental microbiological studies. Now, these new techniques allow the handling of the bulk genomic DNA retrieved from natural microbial communities. This is referred to as metagenomics (Handelsman et al. 1998).

Metagenomics is a technology that allows us to obtain various genes or pathway components from the whole microbial community, and has led to the accumulating number of DNA data sets. Currently, these genome sequences are being exploited for many new biotechnological applications (Streit and Schmitz 2004) and for increasing our knowledge of microbial ecology (Tyson et al. 2004, Suenaga 2012). Metagenomics is also called environmental genomics, community genomics, ecogenomics or microbial population genomics; it consists of the genome-based analysis of whole communities of complexly interacting organisms in diverse ecological environments. The term ‘metagenome’ was first defined by Jo Handelsman and colleagues in 1998 (Handelsman et al. 1998) as the combined genome of the entire microbiota of a specific environment (Dupré and O’Malley 2007). Metagenomics approaches have been used to understand the structure (gene/species richness and distribution) and the functional (metabolic) potential of environmental microbial communities (Suenaga 2012).

Metagenomics consists of two approaches: sequence-driven and function-driven analysis of

uncultured microbiota (Gabor et al. 2007). Functional metagenomics includes screening metagenomic libraries for a particular phenotype, e.g. antibiotic production or enzyme activity, and then identifying the phylogenetic origin of the cloned DNA (Dinsdale et al. 2008). On the other hand, sequence-driven approaches include screening clones for the highly conserved 16S rRNA genes for identification purposes and then sequencing the whole clone to identify other genes of interest, or large-scale sequencing of the complete metagenome to examine the phylogenetic anchors in the reconstructed genomes (Riesenfeld et al. 2004, Hoff et al. 2008, Sleator et al, 2008). PCR (Polymerase chain reaction) is most commonly used for sequence-driven screening of soil DNA (Zhou et al. 2002, Courtois et al. 2003). Both approaches require suitable primers and probes that are derived from conserved regions of known genes and gene products. This approach has been used to identify phylogenetic anchors such as 16S rRNA genes and genes encoding enzymes with highly conserved domains such as polyketide synthases, nitrile Hydratases, etc… (Daniel 2005).

2.2 Historical overview of metagenomics

Because microbes possessed the same genetic material that multi-cellular organisms did, also many similar biological processes (including reproductive ones), microbes, especially viruses and bacteria were used as tools to understand genetic inheritance. As single-gene studies gave way to whole-genome studies in the 1990s, the focus on microbes and microbial knowledge to molecular biology was further reinforced, especially as the sequencing of prokaryotes and viruses speedily developed.

Despite genomic studies of individual isolated microbes provided huge amounts of new information and understanding to comparative evolutionary biology in particular (Ward and Fraser 2005), has so far provided only limited information about uncultured microbial species and function (Nelson 2003). A broadening of molecular microbiology's environmental scope has been resulted to remedy this insufficiency. Ecological studies of microbes have historically been marginal to both general ecology because of more general tendencies in biology and to ordinary microbiology because of the predominance of the pure culture model (Costerton 2004). Microbial ecology gains its real disciplinary recognition in the late 1960s. This discipline consists in understanding microorganisms in their ecological contexts – whatever the method is used – rather than as isolated individuals in artificial environments

(Brock 1987). Most of microorganisms live within complex communities (e.g. biofilms) and around 99% of prokaryotes are still unculturable under laboratory conditions. Taking an environmental approach allowed microbial genomics to extend beyond the sequencing of cultured isolated microorganisms to the sequencing of DNA extracted directly from their environments (Amann et al. 1995). This move out to sequencing in the natural environments largely extended the range of the data collected as well as understandings the species biodiversity and their evolution.

Instead of individual genomes or single gene markers, metagenomics allow us to work with big amounts of the DNA collected directly from microbial communities in their natural environments in order to investigate biodiversity, functional interactions and evolutionary relationships (Dupré et al. 2007).

2.3 The need for metagenomics in studying microbial communities

Microbiology has traditionally been based on growing microorganisms in pure cultures in the laboratory. However, metagenomic information now shows that most microorganisms cannot be cultured, and were formerly unknown. This cultivation bottleneck has skewed the assessment of microbial diversity and limited our knowledge of the world's microbial communities in the world. Metagenomics provides a relatively unbiased view of microbial community structure and of its potential function. Currently, metagenomics is becoming a basic technology for understanding the ecology and evolution of microbial ecosystems (Hugenholtz and Tyson 2008).

2.4 Soil metagenomics

Soil is regarded as the most challenging of all natural environments for microbiologists (Daniel 2005). In fact, one gram of forest soil is estimated to contain around 4×10^7 prokaryotic cells (Richter and Markewitz 1995), and one gram of cultivated soils and grasslands is expected to contain approximately 2×10^9 prokaryotic cells (Paul and Clark 1989). The number of distinct prokaryotic genomes has been estimated to range from 2,000 to 18,000 genomes per gram of soil (Torsvik et al. 1998, Doolittle 1999, Torsvik et al. 2000). This number might well be underestimated because of rare and unrecovered species that

might have not been included in these analyses (Torsvik et al. 2002). Therefore, the diversity of prokaryotic cells present in one gram of soil may exceed that of the known catalogue of prokaryotes (16,177 species were listed in the statistics of the taxonomy browser of the National Center for Biotechnology Information on January 25th 2005). The substantial spatial heterogeneity and the complex chemical and biological properties of soil environments may contribute to the microbial diversity present in soil samples.

Soil contains mineral particles of different sizes, shapes and chemical characteristics. It also contains a variety of soil biota and organic compounds in different stages of decomposition. Prokaryotes represent the most abundant organisms in soil, and can form the largest component of the soil biomass (Hassink et al. 1993). Soil microorganisms often strongly adhere to or adsorb onto soil particles (e.g. sand grains, clay, etc). The metabolism and the survival of soil microorganisms strongly depend upon the availability of water and nutrients (Daniel 2005).

To investigate soil microbial diversity and its corresponding gene pool, it is important to extend the scale of soil surveys. Direct cultivation or indirect molecular approaches can be used to study soil microbial diversity. Cultivation and isolation of microorganisms is the traditional method, but as only 1% of the soil bacteria can be cultured using standard cultivation methods (Amann et al. 1995, Torsvik et al. 2002), the diversity of microbial communities in soil has been mostly unexplored. Recently, new technologies have been developed for the DNA sequence analysis of soil microorganisms (Kaeberlein et al. 2002, Joseph et al. 2003). Of these new technologies, we cite 454-pyrosequencing and Illumina (Daniel 2005).

2.5 Next-generation DNA sequencing methods

Over the past several years, massively parallel DNA sequencing platforms have become fairly widespread, reducing the cost of DNA sequencing, and making the sequencing capacity of a major genome center available to any investigator. Next-generation DNA sequencing is rapidly developing and has the potential to greatly accelerate biological research, by permitting the broad analysis of genomes, which become less expensive and widespread (Shendure and Ji 2008). New methods for more efficient and lower cost preparation of

sequencing sequence libraries have been developed (Head et al. 2011). Among these technologies, we used 454-pyrosequencing and Illumina in the present study.

2.5.1 454-Pyroseqencing

Pyrosequencing is a technology implemented by Roche's 454 platform. It was introduced in 2005 as the first next-generation system on the market, and was successfully commercialized (Quince et al. 2009, Liu 2012). It generates a large number of intermediate length DNA reads through a massively parallel sequencing-by-synthesis approach (Figure 1). The GS FLX implementation generates ~400,000 reads of ~250 base pairs in a single run. In many environmental genomics applications of pyrosequencing, DNA is extracted from an entire microbial community, and a particular target region bordered by conserved primers is amplified by PCR before sequencing. This generates an amplicon dataset, in which every read stems from a homologous region, and the sequence variation between the reads reflects the phylogenetic diversity in the community (Quince et al. 2009).

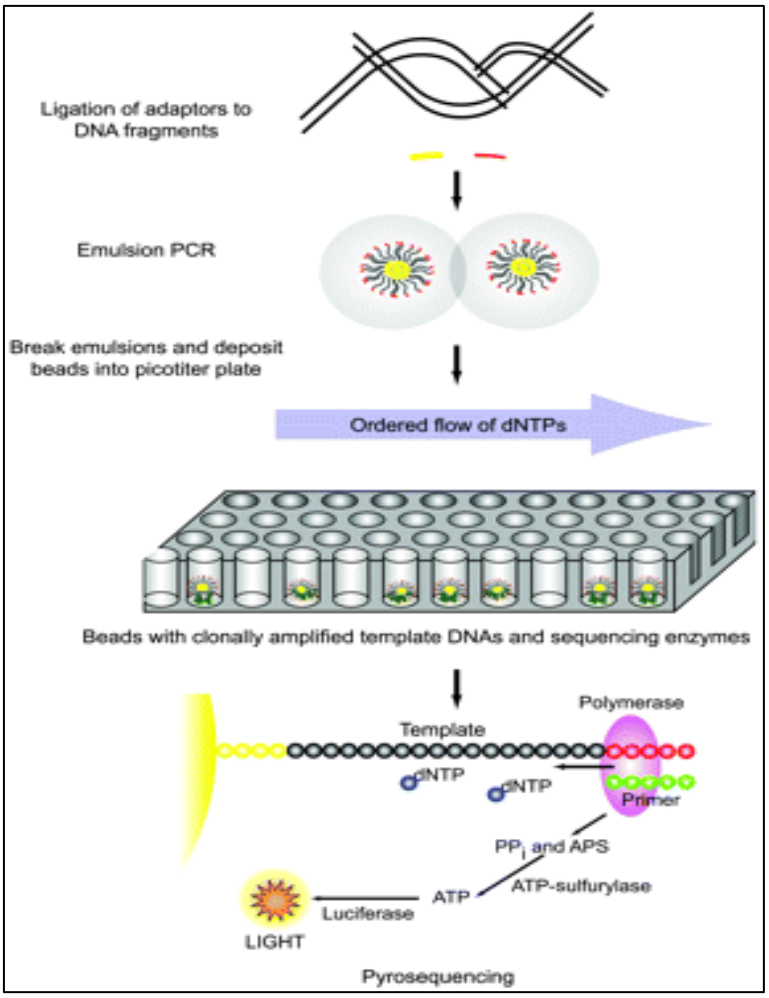

Figure 1: Roche 454 pyrosequencing (Voelkerding et al. 2009)

2.5.2 Illumina (Solexa)

The Solexa sequencing platform was commercialized in 2006, and in early 2007, the company was purchased by Illumina (Liu 2012). This technology is based on sequencing by synthesis (SBS) (Ansorge 2009, Liu 2012). It is the most successful and widely adopted next-generation sequencing technology worldwide. The Illumina sequencing process consists firstly of preparation of the library on the lab bench, followed by the cluster amplification, sequencing-by-synthesis and image analysis on proprietary instruments. Then, the processing of post-sequencing data (Figure 2). A certain amount of bias can be introduced at all three stages, which can to some extent distort the apparent community (Aird et al. 2011).

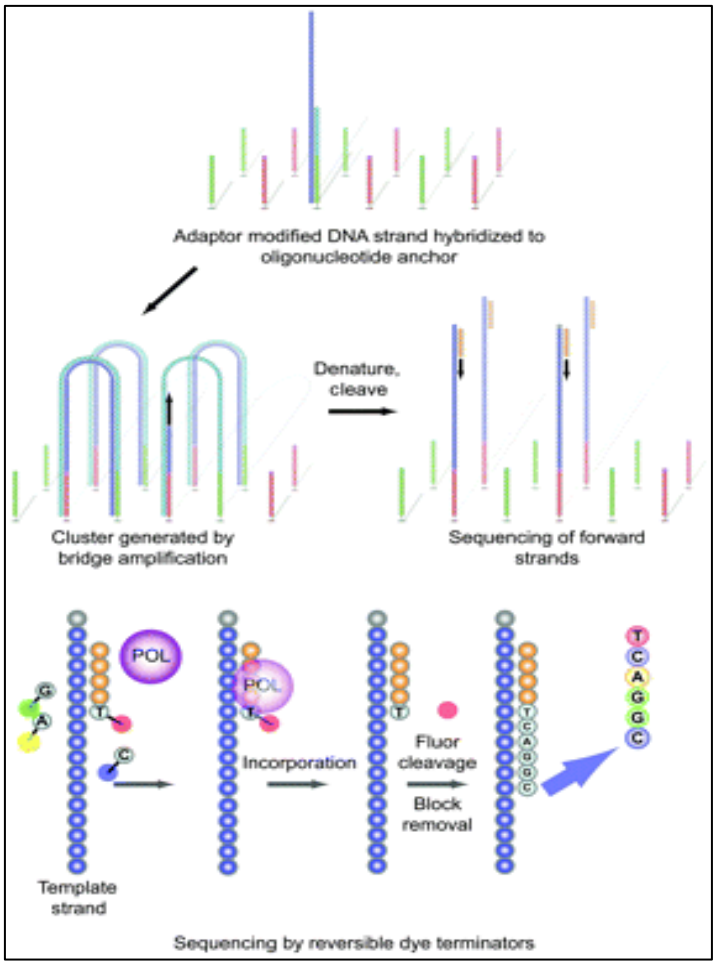

Figure 2: Illumina Solexa sequencing (Voelkerding et al. 2009).

2.5.3 454-pyroseqencing vs Illumina

Until recently, EST (Expressed Sequence Tag) sequences were obtained using traditional Sanger technology (Wlaschin et al. 2005). Currently, most research groups active in this area employ next-generation sequencing technologies including 454 and Illumina (Birzele et al. 2010, Jacob et al. 2010). 454-pyrosequencing can generate up to 1 Gb of data in a single run, creating an average read length of 330 bp and an average error percentage of 4%. A major limitation of this technology consists in the resolution of homopolymer regions (Shendure and Ji 2008), as well as the high cost, which is around $10 per million bases (Liu 2012). Nevertheless, Illumina sequencing can produce up to 90 Gb of data in a single run, producing read lengths up to 100 bp, with an average error percentage of 1-1.5% (Shendure and Ji 2008,

Hammond et al. 2011) and it costs only $0.07 per million bases (Liu 2012).

In conclusion, from the next generation sequencing systems described above, the Illumina HiSeq 2000 features the biggest output and lowest reagent cost, and the Roche 454 system has the longest read length.

2.6 Targeted genes

2.6.1 16S rRNA gene

The 16S ribosomal RNA (rRNA) genes (Figure 3) are vital to cellular function and at least one copy is found in each prokaryotic genome (Acinas et al. 2004). They are also present in all mitochondrial genomes, which have lost most of their ancestral gene content during the symbiosis evolutionary history (Gray et al. 1999). Because this is a universal gene, it represents an ideal target for phylogenetic studies and taxonomic classification (Woese 1987). The ribosomal products of the rRNA genes can fold into a complex, stable secondary structure, consisting of stems and loops (Noller 1985). The sequences of some of the loops are conservative among almost all bacterial species because of the essential functions involved, whereas the features of the supporting structural parts are highly variable and specific to one or more classes (Jonasson et al. 2002). Since the invention of the polymerase chain reaction (PCR) technique (Bartlett and Stirling 2003), the variable regions, V1–V9, of the 16S rRNA genes (rDNAs) have been used for species identification (Wang and Qian 2009).

Combined with cloning and sequencing, the application of universal primers for direct PCR amplification of diverse 16S genes from total community DNA has generated a huge quantity of data, and completely redefined the picture of the diversity of prokaryotic life. As a result of direct 16S rRNA gene analysis, soil - which is generally shown to contain a low diversity of microorganisms when analyzed using cultivation based techniques - is now, known to be one of the most diverse environments for prokaryotic organisms (Jonasson et al. 2002). 16S rRNA gene analysis can provide valuable information about microbial community diversity and evolution (Steele and Streit 2005).

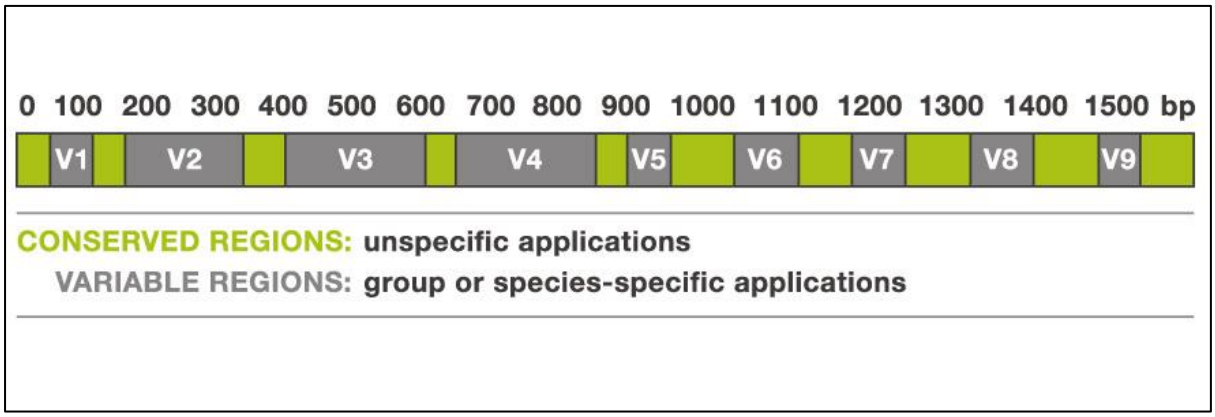

Figure 3: Conserved and hypervariable regions of 16S rRNA gene.

2.6.2 ITS region of the fungal ribosomal repeat

The internally transcribed spacer (ITS) region of the nuclear ribosomal repeat unit is considered so far the most commonly sequenced region for queries of fungal systematics and taxonomy at and below the genus level (Nilsson et al. 2009). It has been widely used as a molecular marker for genus- and species-level identification in ecological and taxonomic studies of fungi (Hibbett et al. 2011). It offers high information content and easy amplification, and it was recently designated as the official barcode for fungi (Schoch et al. 2012).

The internally transcribed spacers (ITS) consist of two highly variable spacers, called ITS1 and ITS2 which are located between the 18S and the 5.8S subunits and between the 5.8S and the 28S subunits respectively (Figure 4). They have been widely used in fungal studies because ITS sequences are significantly more variable than the subunit sequences themselves (Viaud et al 2000). This rDNA operon exists as multiple copies in genomes, delivering a large number of ITS copies per cell (upwards of 250; Nilsson et al. 2009). ITS analysis has been used with success to describe soil fungal community composition and species diversity (Buchan et al. 2002).

Figure 4: Schematic overview of the fungal ITS region

CHAPTER 3: THE IMPACT OF LOGGING AND FOREST CONVERSION TO OIL PALM ON FUNGAL COMMUNITIES IN BORNEO, MALAYSIA

3.1 Introduction

Tropical forests are one of the world's most important reservoirs for biodiversity (Whitmore 1990). They contain an exceptional concentration of the world's species, but are being reduced in area faster than any other ecosystem (Asner et al. 2009). Roughly half of the world's natural extent of tropical forest has been logged or converted to different land uses (Wright 2005). Some 403 million hectares of tropical rainforests have been included in timber estates and slated for selective logging (Blaser 2011), and between 2000 and 2010, approximately 13 million hectares of forest within the tropics were cleared for agricultural activities (FAO 2010, Hansen et al. 2008), including oil palm plantations (Morris 2010).

Anthropogenic disturbances in tropical forests are causing a dramatic decline in global biodiversity, and in associated biological processes that maintain the productivity and sustainability of ecosystems (Achard et al. 2002). Several studies have shown that selective logging does not drastically impact the overall species richness and diversity of tropical forest (Berry et al. 2010, Edwards et al. 2011, Gibson et al. 2011, Woodcock et al. 2013), however, it has been shown that the impact of selective logging could be anything between fairly mild and severe depending on the intensity of logging (Burivalova et al. 2014) with changes in the composition of species, as forest-interior specialists decline and edge-tolerant, gap specialists increase in abundance (Hamer et al. 2003, Cleary et al. 2007). In contrast, the conversion of both primary and logged forest to agricultural land uses has been to shown to have a far greater negative impact on biodiversity than does logging. Conversion to agriculture results in a major reduction of biodiversity, again across a host of animal and plant taxa (Edwards et al. 2010). The conversion of primary and logged forest to agricultural plantations also results in a substantial decrease of the functional diversity of tropical ecosystems, with implications for the provision of ecosystem functions, whereas logging has lesser impacts on these metrics (Baraloto et al. 2012, Edwards et al. 2013). Land use change not only affects plants and larger animals, but soil biota as well. Land use change alters soil pH, carbon and nutrient content

(McGrath et al. 2001, Murty et al. 2002), causing shifts in soil microbial communities (Cornejo et al. 1994, Lauber et al. 2008, Tripathi et al. 2012).

Fungi constitute as one of the most diverse and dominant groups of organisms in soil, and they play important ecological roles in the ecosystem as decomposers, pathogens and plant mutualists (Wu et al. 2007, Orgiazzi et al. 2012). Understanding the structure and diversity of soil fungal communities is fundamental in identifying their function in the ecosystem and their impact on plant communities (Martin et al. 2011). However, minimal work has been done in the tropics to assess the effect of land use changes on soil bacterial communities (Tripathi et al. 2012, Lee-Cruz et al. 2013), until recently relatively little was known about the impacts of tropical land use change on soil fungal communities. Various studies have suggested that forest clearance to tree plantations or agricultural crops shifts soil fungal communities, which is linked to strong changes in soil properties (Lauber et al. 2008, Lupatini 2013). However, previous studies of forest clearance to other forms of agriculture on forest fungal communities have mostly been limited to techniques that give relatively low taxonomic resolution (i.e. T-RFLP and PCR-DGGE). Nevertheless, a recent study by McGuire et al. (2014), which used high throughput sequencing to analyze soil fungal communities in Southeast Asian tropical forests in west Malaysia, showed that conversion of primary forest to oil palm plantations alters fungal community composition and function, whereas primary and logged forests were more similar in composition and nutrient cycling potential. However, further studies are needed to understand the impacts of logging cycles on soil fungal communities and of the conversion of logged forest to agriculture, since logged forests now dominate the tropics (Wright 2005) and are much more likely to be converted to agriculture than primary forests (Hansen et al. 2008, Asner et al. 2009).

In this study, we also focused on the rainforests of the Sundaland region, of Southeast Asia, about 1,800 km away in east Malaysia (Borneo). Across the Sundaland region, the primary forest has been subject to differing degrees of logging intensity. Much of the region's forest (about 50%) has never been logged, while many areas have been subject to one or two logging cycles (Wilcove et al. 2013). Also, oil palm is one of the most rapidly expanding crops in this region. This provided an opportunity to study the effect of different intensities of logging on the soil fungal community and also to evaluate if conversion of forest to oil palm

plantation has a stronger impact on soil fungi than logging, as is the case for numerous macroscopic taxa. Our objective here was to understand whether land use change has an impact on the structure and diversity of fungal communities in the Yayasan Sabah (YS) logging concession in Malaysian, Borneo. We compared the fungal communities in forests with different logging histories (unlogged, once-logged and twice-logged), and oil palm plantations. We examined whether there are differences in α and β-diversity, as well as community composition. These results may provide important information for soil management policies, and estimation of ecological impact of land use change in this region.

3.2 Materials and Methods

3.2.1 Study area

The study area is located within the Yayasan Sabah (YS) logging concession and contiguous oil palm plantation areas, in Sabah, Malaysian Borneo (4°58' N, 117°48' E) (Figure 5). The forests in this area are naturally dominated by valuable timber tree species belonging to the family Dipterocarpaceae (Fisher et al. 2011). Due to logging for the wood industry and clearance for palm oil plantations, the area of forest in Borneo - as elsewhere in the tropics - has been dramatically reduced in recent decades (Gibbs et al. 2010).

Fieldwork was conducted in the Ulu Segama-Malua Forest Reserve (US-MFR) and adjacent oil palm estates in Sabah, Borneo. Some areas of forest were logged between 1970 and 1990, and some of these were then re-logged between 2000 and 2007. During the first logging rotation, approximately 113 m^3 per hectare (range 73 m^3 to 166 m^3) of commercially valuable trees > 0.6 m diameter were extracted. During the second logging rotation, an additional 31 m^3 per hectare (range 15 m^3 to 72 m^3) of timber were removed (Edwards et al. 2011, Fisher et al. 2011). Selectively logged forest in the US-MFR is adjacent to the 45,200 ha Danum Valley Conservation Area (DVCA) and Palum Tambun Watershed Reserve, containing large areas of unlogged forest (Edwards et al. 2010, Edwards et al. 2011). Oil palm plantations are situated to the north and south of the US-MFR, with mature palms of 20 to 30 years old, planted at a density of 100 trees per hectare.

Figure 5: Location of the sample site in Sabah, Malaysian Borneo.

3.2.2 Soil sampling and DNA extraction

From September to October 2012, twenty-four transects each of 200 m in length were located across four different land uses: unlogged (primary) forests, once-logged and twice-logged forests, and oil palm plantations, with six transects per habitat. Within each habitat, distances between transects ranged from 500 m to 65 km, whereas across habitats, distances between transects ranged from 1 km to 67 km. From each transect, at 50 m intervals, approximately 50 g from the top 5 cm of soil (excluding the leaf litter layer) was taken in a sterile plastic bag using a trowel, giving five samples of soil per transect. The trowels were thoroughly cleaned with ethanol between successive transect sampling. All soil samples were then sieved (2 mm) in laboratory to homogenize the sample and stored at –20℃ until DNA extraction (Edwards et al. 2011). Twenty-four soil DNA extractions (one for each transect) were performed using 0.3 g of soil, with the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the directions described by the manufacturer.

3.2.3 PCR amplification and pyrosequencing

Fungal DNA was amplified using ITS primers targeting the internal transcribed spacer (ITS) region 1 and 2. Forward primers comprised the 454 Fusion Primer A-adaptor, a specific multiplex identifier (MID) barcode, and the ITS1F primer (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993), while the reverse primer was composed of the B-adapter and ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). Polymerase chain reactions (PCR) were performed in 50 µl reactions using the following temperature programs: 95°C for 10 min s; 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 7 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and quantified using PicoGreen (Invitrogen) spectrofluorometrically (TBS 380, Turner Biosystems, Inc. Sunnyvale, CA, USA). 50 ng of purified PCR product for each sample were combined in a single tube and sent to Macrogen Inc. (Seoul, Korea) for sequencing using 454/Roche GS FLX Titanium Instrument (Roche, NJ, USA).

3.2.4 Sequence processing

Initial quality filtering and denoising were performed following the 454 SOP in the mothur pipeline (Schloss et al. 2011). The ITS1 region was verified and extracted using the ITS1 extractor for fungal ITS sequences (Nilsson et al. 2010). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mother (Edgar et al. 2011). Operational taxonomic units (OTUs) were assigned using the QIIME implementation of UCLUST (Edgar 2010), with a threshold of 97% pairwise identity. OTUs were classified taxonomically using the classify command in mothur at 80% Naïve Bayesian bootstrap cutoff with 1000 iterations against the UNITE database (Abarenkov et al. 2010). Ectomycorrhizal (EcM) fungi were determined by matching taxonomy assignments with established EcM lineages as determined by recent phylogenetic and stable isotope data (Tedersoo et al. 2010).

3.2.5 Soil analyses

Total nitrogen, total carbon, available phosphorous, and pH were measured at the National Instrumentation Center for Environmental Management (NICEM, South Korea) based on the standard protocol of the SSSA (Soil Science Society of America).

3.2.6 Statistical analysis

To correct for differences in number of reads, which can bias diversity estimates, all samples were rarified to 3,347 reads per sample. To test for effects of land use types on the OTU richness and diversity indices, we used a linear model (LM) for normal data or generalized linear model (GLM) for non-normal data, considering land use as the major factor. We used the same procedure to test whether relative abundance of the most abundant phyla differed among different land use types. We also assessed the effect of land use on the relative abundance at the order and genus levels within those phyla that showed significant differences due to land use. Post-hoc Tukey tests were used for pairwise comparisons. When neither a linear nor a generalized linear model fitted the data, we used a Kruskall-Wallis test to assess the effect of land use on the relative abundance of fungal taxa, with the Bonferroni correction to assess pairwise comparisons.

To test whether species composition results may have been influenced by pseudoreplication within study sites, we used a Mantel test (Mantel Nonparametric Test Calculator 2.0) (Liedloff 1999) to compare transect matrices of fungal compositional to geographic distance between pairs of transects within a site and between pairs of transects across the entire dataset (Ghazoul 2002, Ramage et al. 2013).

The OTU-based community similarity was calculated by using the Bray-Curtis index (Magurran 2004). Non-metric multidimensional scaling (NMDS), to visualize the change in species composition across the land use types, was conducted in Primer-E software (Version 6, Plymouth, UK). Then, we tested the difference among different land use types using an analysis of similarity (ANOSIM).

We measured β-diversity amongst land use types following Anderson et al. (2011), which is defined as the variation in community structure without defining a particular gradient or

direction. Therefore, we estimated true β-diversity following Whittaker (1960) *in* Koleff et al. 2003 for every land use type. In addition, we calculated β-diversity as the average distance from each site to the group centroid (Anderson et al. 2011). The betadisper function in R was used to test if β-diversity showed any difference between land use types. True β-diversity (i.e. $S/\bar{\alpha}$) for each pair of samples within each of the four land uses was estimated by the following equation:

$$\frac{S}{\bar{\alpha}} = \frac{a+b+c}{(2a+b+c)/2}$$

Where *S* is the total number of OTUs in two samples, $\bar{\alpha}$ is the average number of OTUs for both samples, *a* is the shared OTUs between both samples, *b* is the OTUs found only in sample 1 and *c* are the OTUs found only in sample 2. To compare true β-diversity among land uses, we used a linear model using land use as factor, and sample as random factor to control for pseudoreplication, as every sample is used in more than one comparison within each land use. Post-hoc Tukey tests were used for pairwise comparisons among different land uses.

3.3 Results

A total of 114,744 quality sequences were obtained from the 24 soil samples, with coverage ranging from 3,347-6,456 sequences. After rarifying to 3,347 reads per sample, we obtained a total of 80,328 sequences, and of these around 84% sequences were classified up to phylum level with a total of 5,327 OTUs (defined at ≥ 97% sequence similarity level). Fungal OTU richness (i.e. number of OTUs) was marginally significantly different across land use types ($F_{3,24}$ = 2.98, P = 0.05; Figure 6a), with lowest levels of OTU richness observed in oil palm plantations compared to primary and logged forests. Predicted OTU richness calculated using the Chao1 estimator was significantly higher in logged forests than oil palm plantations ($F_{3,24}$ = 4.74, P = 0.01; Figure 6b), whereas Shannon index did not show any variation among land uses ($F_{3,24}$ = 1.93, P = 0.15).

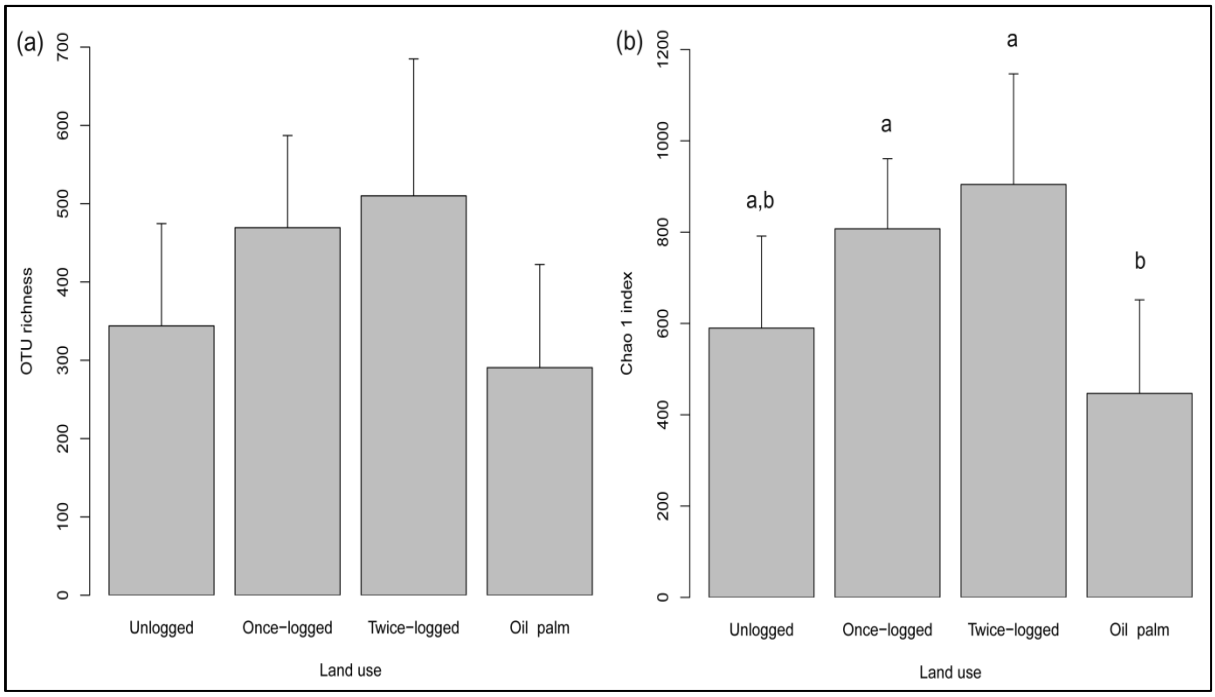

Figure 6: Diversity indices of the fungal community across different land uses in Sabah, Malaysian Borneo. (a) OTU richness and (b) Chao1 index. Pairwise comparisons are shown; different letters denote significant differences between groups at $P < 0.05$.

Soils in different land use types showed changes in physico-chemical properties whereby levels of soil total carbon (C), total nitrogen (N) and available phosphorous (P) tended to decrease at oil palm plantations (Figure 7). By most measures, oil palm soils had significantly lower soil C and N contents compared to forest soils (For C: $F_{3,24} = 5.48$, $P = 0.006$; N: $F_{3,24} = 4.36$, $P = 0.01$). pH did not show any statistical variations among different land uses. Forest soils had lower pH (4.23 ± 0.37) compared to oil palm plantation soils (4.75 ± 0.59).

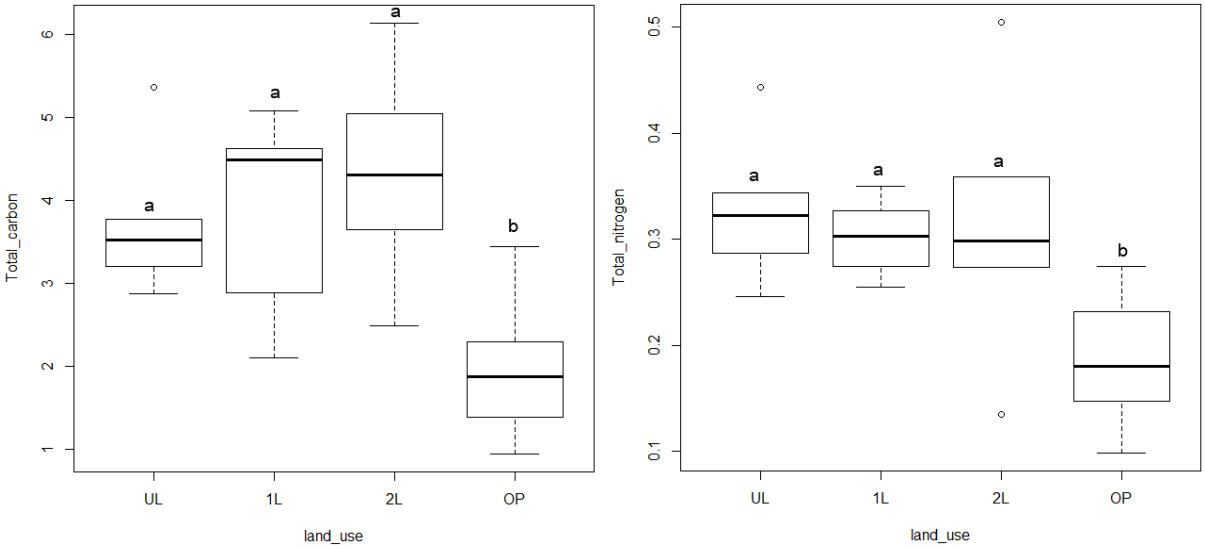

Figure 7: Variation of total carbon and total nitrogen among the soil of different land use types in Malaysian, Borneo.

The NMDS plots of pairwise Bray–Curtis dissimilarities showed that fungal communities were clustered significantly across land use types (ANOSIM: R = 0.51, P < 0.001; Figure 8). Mantel tests showed no effect of distance on the composition of fungal communities across different land use types in Borneo (all P > 0.07). The majority of fungal sequences recovered in our study belonged to the Basidiomycota and Ascomycota, with relative abundances of 52% and 29%, respectively (Figure 9). The basal fungal lineages represented 2%, followed by Glomeromycota and Chytridiomycota with less than 1%, and 15% of the detected sequences were unclassified (Figure 9). We found a significant change in the relative abundance of the two most dominant phyla: Basidiomycota ($F_{3,24}$ = 4.23, P = 0.01) and Ascomycota ($F_{3,24}$ = 4.81, P = 0.01) along different land uses. The abundance of Basidiomycota was greater in the unlogged forest, intermediate in the logged forest, and least in the oil palm. Conversely, the oil palm plantations had greater abundance of Ascomycota compared to forest soils (Figure 9). At the order level, there were significant differences in the relative abundances of the most dominant orders (Table 1). The results revealed that the relative abundance of *Agaricales*, *Russulales*, *Thelephorales*, *Trichosporonales*, *Sebacinales* and *Helotiales* were significantly higher in the forest than oil palm plantations (P < 0.05; Table 1). However, *Hypocreales*, *Sporidiobolales* and *Pleosporales*, were more abundant in oil palm plantations than unlogged and logged forests (P < 0.05; Table 1).

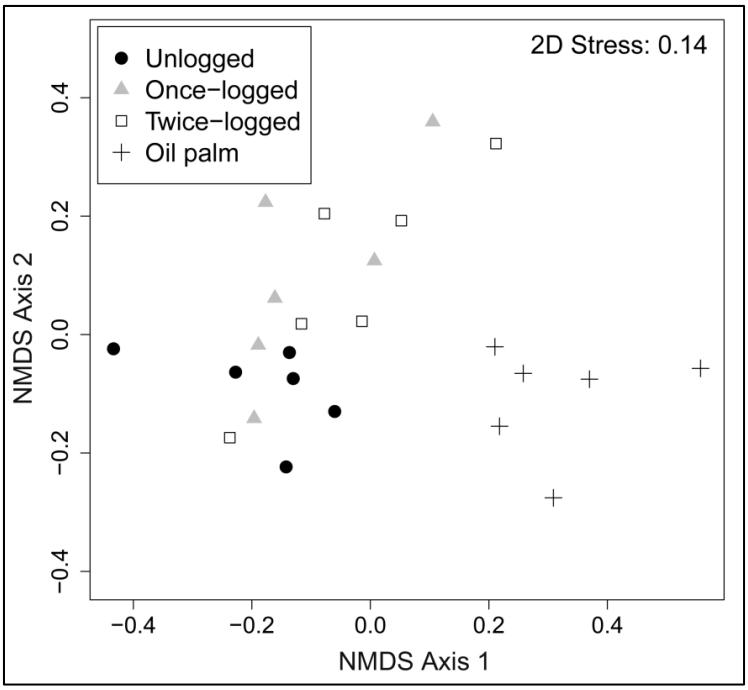

Figure 8: Non-metric multidimensional scaling (NMDS) ordination showing clustering of fungal communities among different land uses in Sabah, Malaysian Borneo.

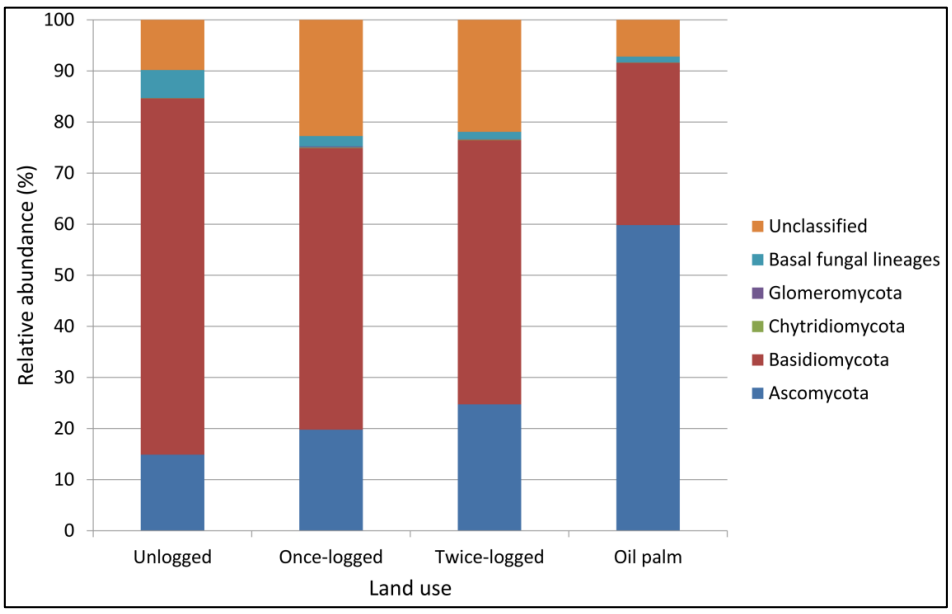

Figure 9: Relative abundance of dominant fungal phyla among different land uses in Sabah, Malaysian Borneo.

Table 1: Comparison of relative abundance of the dominant orders within the phyla Ascomycota and Basidiomycota among land uses[a].

Organism	**F or χ^{2}[b]**	**df**	**P**	**Pairwise comparisons[c]**
Ascomycota				
Helotiales	3.67	3, 24	0.02	Once-logged > oil palm
Hypocreales	5.54	3, 24	0.006	Once-logged/twice-logged/unlogged < oil palm
Pleosporales	6.42	3, 24	0.003	Once-logged/unlogged < oil palm
Basidiomycota				
Agaricales	10.9	3, 24	0.0001	Once-logged/twice-logged/unlogged > oil palm
Russulales	11.3	3, 24	0.0001	Unlogged > Once-logged/twice-logged/oil palm
Sebacinales	11.1*	3	0.01	Unlogged/twice-logged > oil palm
Sporidiobolales	15.1*	3	0.001	Once-logged/twice-logged/unlogged < oil palm
Thelephorales	10.0*	3	0.01	Unlogged/twice-logged > oil palm
Trichosporonales	13.3	3, 24	0.0001	Once-logged/twice-logged/unlogged > oil palm

[a] Only orders for which significant differences were found are shown.

[b] Effect of land use on relative abundance evaluated by linear or generalized linear model or by the Kruskal-Wallis test (*).

[c] Pairwise comparisons by *post hoc* Tukey test for linear/generalized linear models or *P* values Bonferroni-corrected for Kruskal-Wallis. Differences were considered significant at a P value of <0.05.

A total of 11,421 sequences belonged to known groups of ectomycorrhizal (EcM) fungi, with the EcM fungi representing around 10% (11,421 sequences) of the total detected fungal sequences, with 180 OTUs. The relative abundance of EcM sequences was significantly different across land use types, with highest and lowest relative abundances observed in primary forest (mean relative abundance = 26%) and oil palm plantations (mean relative abundance = 0.5%), respectively (X^2 = 18.04, P < 0.001; Figure 10). From our soil samples, we identified 14 genera belonging to EcM fungi with *Russula* as the most dominant genus (65% of total EcM sequences), followed by *Tomentella*, *Sebacina* and *Lactarius*. The relative abundance of these four dominant EcM genera combined were significantly higher in forest soils compared to oil palm plantations (P < 0.05). For *Russula* alone, we also found a difference in abundance between unlogged (greatest abundance), once-logged (less abundant), and twice-logged forest (lowest abundance of *Russula*) (P < 0.05 in each case).

The β-diversity, measured as the average distance of all samples to the centroid in each land use type, did not show significant difference among land uses ($F_{3,24}$ = 2.77, P = 0.06). However, there was a significant effect of forest conversion to oil palm plantations on fungal true β-diversity (i.e. $S/\bar{\alpha}$; $F_{3,24}$ = 3.85, P = 0.01), with oil palm having the lowest true β-diversity compared to unlogged and logged forests (Figure 11). Logging did not produce any significant change in true β-diversity.

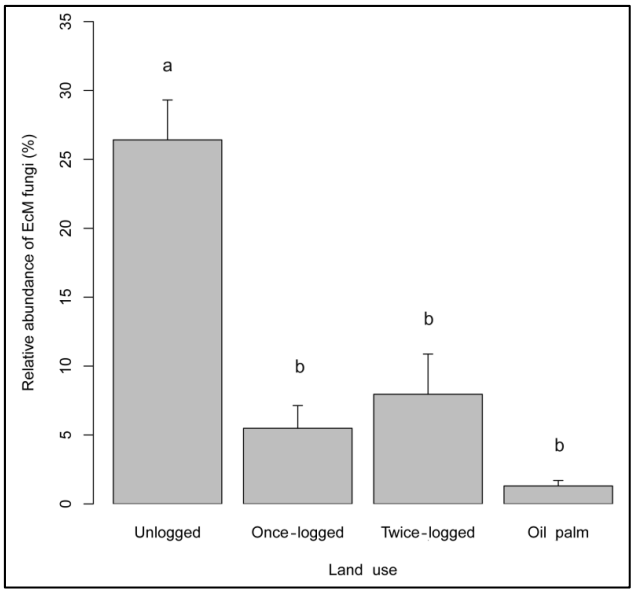

Figure 10: Relative abundance (means ± SD) of ectomycorrhizal (EcM) fungal sequences among different land uses in Sabah, Malaysian Borneo. Pairwise comparisons are shown; different letters denote significant differences between groups at $P < 0.05$.

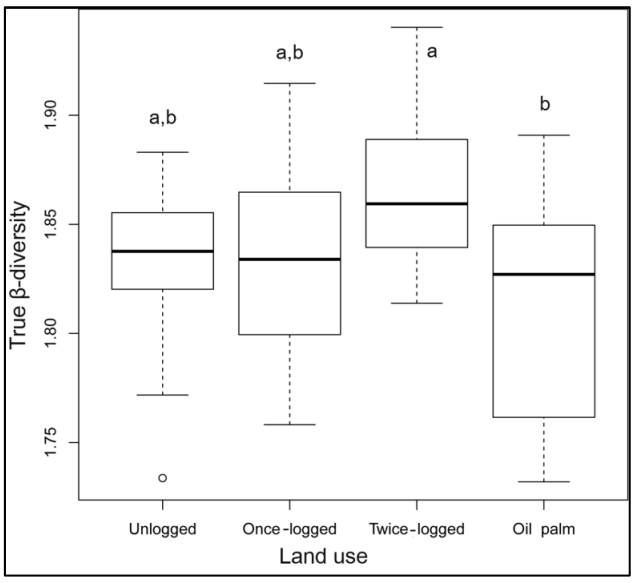

Figure 11: Fungal community true β-diversity (i.e. $S/\bar{\alpha}$) among the four land uses in Sabah, Malaysian Borneo. Boxes show the lower quartile, the median and the upper quartile. Pairwise comparisons are shown; different letters denote significant differences between groups at $P < 0.05$.

3.4 Discussion

Our results showed that fungal OTU richness and Chao1 index differed among land uses. Oil palm plantations had lower OTU richness, which can be attributed to the effects of anthropogenic intervention and forest conversion. This contrasts with the findings on bacterial communities of Lee-Cruz et al. (2013) in the same study site, where they found that OTU richness and diversity indices did not differ among land uses, and that α-diversity was similar in forests and oil palm plantations.

We found that the structure of fungal communities differed most fundamentally between forests and oil palm plantations. This finding mirrors that of McGuire et al. (2014), who found that the community of soil fungi collected across three different land uses in Malaysia differed between oil palm plantations and forests. Changes in fungal community composition in logged forest and former forest areas could directly impact the functioning of soil communities and their ability to provide key ecosystem services, such as decomposition and nutrient recycling (Lauber et al. 2008). Given that fertilizer prices are predicted to rise dramatically in the coming decades (Piesse and Thirtle 2009), these results suggest that improvements in agricultural methods by establishing diversified farms could be necessary for sustaining vital soil biodiversity and ecosystem-service values.

Soils from oil palm plantations showed significantly lower concentrations of total carbon and total nitrogen contents than from unlogged and logged forests. This confirms with the findings of Murty et al. (2002) and Waldrop et al. (2000) where they detected a decrease of carbon and nitrogen content in cultivated soils.

The abundance pattern of fungal taxa detected here is similar to the soils of the Neotropics, and elsewhere, where Basidiomycota and Ascomycota are also the most prevalent groups (Gomes et al. 2003, O'Brien et al. 2005, Oros-Sichler et al. 2006, Bridge and Newsham 2009, Wubet et al. 2012). The most abundant orders of Basidomycota in the sampled forest areas are *Russulales*, *Agaricales*, *Sporidiobolales*, *Telephorales*, *Trichosporonales* and *Sebacinales*. They all became less abundant in oil palm plantations compared to primary forest. These orders are generally reported to be the most varied and abundant groups of fungi in forests a round the world (Geml et al. 2009, Geml et al. 2010, Tedersoo et al. 2010), they have also been characterized as being lignicolous, saprobic or mycorrhizal, associated with litter

decomposition, and they are known to degrade plant-derived cellulose (Kuramae 2013). In our forest plots, the ectomycorrhizal orders *Russulales* and *Telephorales* were the most common. Likewise, this pattern has also been found in forests from the Neotropics and African tropics (Tedersoo et al. 2010, Smith et al. 2011, Ba et al. 2012).

The lower abundance of Basidiomycota in oil palm soils may be due to the lack of large quantities of coarse woody debris, often derived from roots or branches of forest trees, in oil palm plantations. However, it may be worth investigating whether the change in abundance of Basidiomycota - and change in their overall community structure - with conversion of forest to oil palm may have implications for long term nutrient processing in the oil palm soils. Since Basidiomycota, as a group, are often able to break down relatively recalcitrant substrates and changes in their abundance and community composition, they might impede the nutrient recycling in oil palm soils. Conversely, slower breakdown of recalcitrant organic substrates could actually lead to enhanced long term carbon storage in oil palm soils. The converse increase in Ascomycota may be seen in terms of the relatively lignin-poor and nutrient-rich character of most of the organic matter reaching the soil from roots and leaves of oil palm, and from the herbaceous weedy layer that grows under the palms.

We found that both a history of logging, and forest conversion to oil palm plantation, resulted in shifts in EcM fungal communities. The most drastic effects were with forest conversion to oil palm. However, logging history also had detectable impact on EcM fungi relative abundance. Ectomycorrhizas are one of the most important widespread types of mycorrhiza in forests of the cool temperate and boreal latitudes (Brearley 2012), and they also form an important group and often the dominant ecologically and economically important minority of Dipterocarpaceae family trees in tropical Asia (Moyersoen et al. 2001, Haug et al. 2005, Natarajan et al. 2005, Peay et al. 2010, Peay et al. 2012).

The lower abundance of EcM fungi in logged forests might be due to a thinner, more incomplete root mat following past logging disturbances. The much lower abundance of EcM fungi in oil palm plantation soils could be partly due to the much lower abundance of potential host roots (e.g. Dipterocarpaceae which are absent from the palm plantations). Such fungi have generally been found to recover slowly from disturbances even though potential host plants are present (Tedersoo et al. 2008). Among the detected genera in our study, we

found that *Russula, Sebacina, Lactarius* and *Tomentella* showed significant impact of land use change. *Russula* was relatively the most abundant EcM genus in our samples: this genus has been found to be common on roots of dipterocarp forests, tropical and southern hemisphere angiosperm forests (Moyersoen et al. 2001, Riviere et al. 2007, Rodrigues et al. 2013).

This study also investigated the impacts of land use change on the turnover of fungal communities across space (β-diversity), and the results suggest that there is a spatial homogenization of fungal communities in oil palm agriculture compared to forest: fungal β-diversity in oil palm plantations was lower than forest. Habitat conversion to agriculture also reduces β-diversity of soil bacteria in Amazonia (Rodrigues et al. 2013). In contrast, across the same land use system and sites in Borneo, where we studied, there was actually an increase in β-diversity of bacteria with both logging and conversion to oil palm (Lee-Cruz et al. 2013).

There are some caveats that accompany our findings. We worked in only one biogeographic region and on only one form of agriculture. It is thus important to replicate this work in other logging systems and in other key expanding crops, including soya, sugar cane, and cacao. We also did not explicitly demonstrate the impacts of changing fungal composition on soil ecosystem functions and services: this is a major knowledge gap, with critical importance to the development of sustainable logging and, in particular, agricultural systems in the tropics.

3.5 Conclusion

The conversion of both primary and logged forest to oil palm drives a change in the overall fungal community, including EcM fungi abundance, and an associated decrease in total fungal community beta-diversity. This finding invites further studies to investigate the l ong-term implications of such changes for agricultural sustainability. There was a more s ubtle long-term impact of logging on fungal communities. Most measurable features of the unlogged forest fungal community remained unchanged after logging. However, there were significant changes in EcM fungal abundance due to logging, which could have a pervasive impact since EcM fungi are thought to play a key role in tree growth and

community structure. Despite this, the lack of drastic changes in the overall forest fungal community structure following logging strengthens denotes that logged forest is not necessarily an irretrievably damaged and drastically altered system, and that protecting it from conversion to oil palm may still have considerable conservation benefits.

CHAPTER 4: RESPONSES OF BENTHIC BACTERIAL COMMUNITIES TO NATURAL CO_2 GRADIENT IN THE MEDITERRANEAN SEA OFF VULCANO, ITALY

4.1 Introduction

Covering around 70% of the Earth, marine sediments play a major role in ecosystem processes and underpin carbon and nutrient cycling (Widdicombe et al. 2011). They are colonized by a vast, but unknown, diversity of microorganisms living in a variety of habitats (Munn 2011). Anthropogenic CO_2 emissions have lowered the pH of surface waters of the world by about 0.1 pH unit and a further global reduction of 0.2 - 0.4 pH units is expected by 2100 (Caldeira and Wickett 2003). This rate of ocean acidification is, as far as we know, unprecedented and is rapidly exposing vast areas of seabed habitats to waters that are corrosive to carbonate (Olafsson et al. 2009).

Despite the importance of benthic microbial communities in global biogeochemical processes, our knowledge of their community dynamics is rudimentary and their response to ocean acidification remains largely unknown (Allgaier et al. 2008, Liu et al. 2010, Newbold et al. 2012, Ray et al. 2012, Lindh et al. 2013, Sperling et al. 2013), although mesocosm work is broadening our understanding of effects on pelagic microbial communities (Roy et al. 2013). Studies on the effects of ocean acidification on marine pelagic microbes have mainly been *in vitro* experiments which have limitations when attempting to scale-up to effects on seawater (Krause et al. 2012). Recently, volcanic vents have started to be used as natural laboratories to evaluate the consequences of increase in seawater acidity on marine ecosystems *in situ*, revealing tipping-points beyond which seagrass, coral reef and rocky shore systems are radically altered due to adverse effects on calcified organisms (Fabricius et al. 2011, Rodolfo-Metalpa et al. 2011, Kroeker et al. 2013). Kitidis et al. (2011) performed the first field investigation of the impact of ocean acidification on sediment bacteria and found that although microbial ammonia oxidation was affected by ocean acidification in the water column, it was not affected in sediments, perhaps due to buffering within the sediments or adaptation of the ammonia oxidizing microbes to high CO_2 conditions.

In recent years, major advances in our understanding of marine microbial diversity and

community structure have been driven by advances in molecular techniques (Munn 2011). For marine sediments, 16S rRNA genes are now routinely amplified using PCR from nucleic acids extracted from the sediment; the PCR products are then cloned and sequenced (Hongxiang et al. 2008, Ghosh et al. 2010). A meta-analysis by Hendriks et al. (2010) concluded that it is likely that microbes will adapt to ocean acidification by genetic modification at the species level as well as by replacement of sensitive species by non or less sensitive ones at the community level. Studies of microbes along marine CO_2 gradients have so far confirmed that the community compositions of resident marine microbiota alter as a result of acidification (Johnson et al. 2011, Lidbury et al. 2012).

Here, we used 454-pyrosequencing of 16S rRNA gene to investigate the effect of a natural gradient in seawater pCO_2 on sediment bacterial communities off Vulcano in the Mediterranean. Our hypothesis was that a pCO_2 gradient would cause similar community shifts in sedimentary bacterial communities as those recently observed in biofilm grown on slides at the same field site (Lidbury et al. 2012). We predicted that significant changes in sedimentary bacterial communities would occur due to the direct effects of acidified seawater on sediment biota, as well as indirect effects due to documented changes in the biota of surrounding habitats (Johnson et al. 2011) which contribute to the supply of organic material to the sediment.

4.2 Importance of marine microbes

Marine microorganisms play a crucial role in aquatic life as primary producers. In contrast, in the terrestrial environment, the primary production is provided by higher plants, which are large and having longer life; in the oceans the microbes are the primary producers that have short life (for only a day or few). Nevertheless, these marine microbial communities are vital for the global ecosystems as they produce more than half of the total global oxygen produced by photosynthesis. Bacteria and archaea play also an important role by maintaining the productivity of the oceans via nutrients recycling that are used by the phytoplankton. Moreover, bacteria are involved in ocean biogeochemical processes and controlling the productivity in oceans. Understanding the fundamental role of microbes on biogeochemical cycles to maintain the oceans productivity is still poor investigated. In fact, it is more proficient to measure the chemical result of microbial activity (e.g. release of methane to the

atmosphere) than identifying organisms responsible of these biogeochemical processes, since that the diversity of ocean microbes using DNA sequencing is recent (Joint et al. 2009).

4.3 Key ecological services provided by marine microbes

Despite the poor knowledge on the functioning of marine microbes that are involved in the biogeochemical process, we know the importance of processes involved in almost all environmental services provided by marine microbes.

The responses of these biogeochemical processes to increases CO_2 emissions are still poor studied. Thus, the importance of experimental data to statistically predict the shift of microbial activity in the oceans (Joint et al. 2009).

Acclimation and/or adaptation

The next generation sequencing technologies led to a wide knowledge in marine microbial diversity. The diversity of microbial communities is huge but the role of these organisms in marine environment is still unknown. With the climate change, three possible scenarios are expected to happen. Firstly, because of the large diversity of microbial diversity, microorganisms always exist to carry out the fundamental marine biogeochemical cycles. In fact, community composition may change and tolerant species to environmental variation may dominate, but other species which are able to live in news environmental conditions, and to carry out certain ecosystem key processes such us nitrogen fixation will be present. Then, considering the plasticity of genes and the frequency of gene transfer, it is more than possible that most of marine microbial community will develop through acquisition of new genes. Consequently, less change on microbial community will be noticed and same dominant species will exist. In contrast, their ecological fitness will be adapted to the new environmental conditions. The last scenario that may happen at higher CO_2 in seawater is the loss of species diversity even the destruction of the whole community. This is unlikely, may occur to heterotrophic organisms except for some coccolithophore species (with calcareous plates), whose calcite coccoliths (external carbonate shell plates) will highly dissolve due to the less saturation of calcium carbonate in the oceans. Therefore, ocean acidification has more impact on coccolithophores with calcareous plates (Joint et al. 2009).

Acclimation

It is the adjustment of any organism in response to perturbations using existing cell mechanism. For this, changes in gene expression without changing the organism's genetics will occur. Acclimation may be achieved for few minutes to hours. It will be hard to precisely predict the species acclimation because of the lack of solid and sufficient information about either genotype or phenotype of present microbial diversity.

The identification of natural microbial communities is developing in many marine environments. However, species presenting key functional groups are not well described. This information on marine microbial communities is essential to monitor and predict the ability of species and functional groups to acclimation to environmental variations like increasing CO_2 or temperature of seawater (Joint et al. 2009).

Adaptation

It is the adjustment of any organism in response to perturbations using genetic change due to natural selection. It may last hundreds of generations. The adaptation rates are faster for microorganisms than multi-cellular organisms because of the lateral gene transfer of microbes. Climate change may also have an effect on microbial communities due to increasing temperature of surface seawater enhancing vertical stratification, declining of nutrient supplies, reducing of arctic sea-ice and increasing the occurrence and intensity of storms. Most of seawaters are experiencing the effects of these various perturbations such as increasing nutrients. As a consequence, the primary production will increase.

All these mechanisms will affect microbial communities but, it is still unclear how the responses of marine microorganisms to increasing CO_2 will interact with these alterations (Joint et al. 2009).

4.4 Why is ocean acidification an urgent problem?

Humans have depended on oceans for food over thousands of years. Even today, most of largest cities are located on the coastal area. In the few last decades, surface pH seawater is decreasing by about 0.1 pH unit, and it is expected to decline by 0.2 to 0.4 by next century. This change in pH will definitely cause an alteration of the services provided by oceans to humans. The effects of lower pH in seawater will depend on the speed of this variation; if

change occurs slowly, organisms can adapt so they are able to grow under these new environmental conditions. In addition, over time, the oceans will be buffered using geological reactions. However, if the changes happen in a short timescale, the risks of species extinctions will increase (Joint et al. 2009).

4.5 Benthic biogeochemistry

As a result of increasing CO_2 and declining seawater pH in the ocean, the carbonate saturation would lower significantly in the shallow sea sediment (Andersson et al. 2003). For example, in the North Sea, benthic ecosystems may be sensitive to increasing CO_2 in seawater shallow sea benthic ecosystems (Turley et al. 2006) that are characterized by strong geochemical gradients, comprising pH (Fenchel and Riedl 1970). Most of benthic communities could adapt to these gradients, but other existing organisms could not (Barnes and Hughes 1988). Shallow water is most densely populated, while they are the only species that can oxygenate their environment immediately, using ventilated holes for example. They have the ability to survive and settle under the redox discontinuity depth (Furukawa 2001). The biogeochemical cycles carried out by microbial communities such as nitrification and denitrification, even the communities are different between aerobic sediment and anaerobic environments. As a consequence, organisms inhabiting these environments are more tolerant to pH changes compared to non-holes produces. These bioturbating sediment communities change the physical and chemical characteristics of their environment (Rhoads and Young 1970) and as a result, change in the rate of nutrient fluxes at the sediment/water interface (Widdicombe and Austen 1998; Howe et al. 2004) and the maintenance of biodiversity (Widdicombe et al. 2000).

The calcareous organisms may be vulnerably affected by the decrease in carbonate ions. For example, sea urchin can survive even at highest CO_2 concentrations (550 ppm) which is expected to be reached by 2050. Even among these calcareous organisms, the tolerance to pH variations depend on the species, for example the echinoderms demonstrated less tolerance to pH change than molluscs (Shirayama et al. 2004). The difference in adaptation to pH variations lead to the selection of more tolerant species to pH change and thus substantial changes in the composition and sediment functioning.

Pelagic and benthic ecosystems are correlated, even in the deep seas. In shallow waters, up to

80% of the nitrogen required by photosynthesis comes from sediment organic matter regenerated by microbes. Thus, shifts in the nutrients benthic regeneration via shifts in sediment organisms' structure and their function. As a result, pelagic production could be affected. Conversely, changes in the structure and function of microbial communities and the primary production could strongly impact the food resources for benthic inhabitants and nutrient regeneration (OSPAR commission 2006).

4.6 Materials and methods

4.6.1 Study site

Sampling took place on Vulcano Island 25 km off the NE coast of Sicily (Figure 12). Samples were collected from Levante Bay, on the north-east side of Vulcano where CO_2 from volcanic activity is released through vents at 0-10 m depth creating a pH gradient from 5.6 to >8.0 along the bay (Boatta et al. 2013). The sediment along this pH gradient was deposited during a volcanic eruption in 1888-90 (Boatta et al. 2013). Samples were taken at 1 m depth from three sites, which had the same salinity, temperature and alkalinity (Figure 12). Site 1 (S1) was located outside an area influenced by the vents and had diurnal variations in pH that are typical for shallow vegetated habitats (Kerrison et al. 2011), with a minimum pH of 8.08 at dawn and a maximum pH of 8.29 at dusk. At Site 2 (S2) plumes of acidified water occasionally drove the pH down to 7.76, although this site was regularly flushed with ambient normal pH seawater. Site 3 (S3) was closest to the vents and had the widest pH variation; point measurements by Johnson et al. (2011) had minimum pH 7.07 although long-term monitoring revealed an unusual brief minimum of pH 6.6 at this station. Table 2 summarizes the seawater carbonate chemistry of these stations.

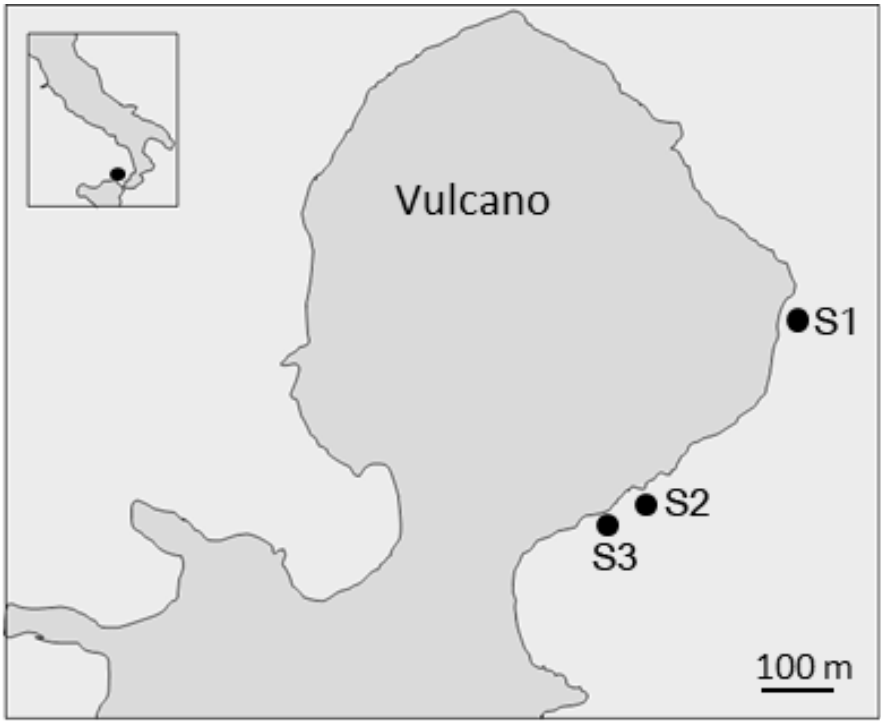

Figure 12: Location of sample sites off Vulcano Island, part of the Aeolian island chain, northeast Sicily (38°25′N, 14°57′E.

Table 2: Maximum, median and minimum seawater pH with corresponding carbonate chemistry measurements along a CO_2 gradient on Vulcano island (Johnson et al. 2011).

Station		**pH.range (NBS scale)**	***p*CO$_2$ (µatm)**	**TA (mmol kg^{-1})**	**DIC (mmol kg^{-1})**	**CO_3^{2-} (mmol kg^{-1})**	**HCO_3^- (mmol kg^{-1})**	**$\Omega_{calcite}$**	**$\Omega_{aragonite}$**
S1	Max	8.29	331	2.625	2.197	0.32	1.871	7.54	7.97
	Median	8.21	419		2.233	0.29	1.929	7	4.65
	Min	8.08	603		2.339	0.22	2.101	5.26	3.77
S2	Max	8.22	410	2.642	2.23	0.31	1.912	7.38	4.91
	Median	8.08	592		2.401	0.19	2.193	4.45	2.89
	Min	7.76	1429		2.512	0.12	2.349	2.96	1.96
S3	Max	8.1	599	2.736	2.409	0.25	2.14	6.05	4.02
	Median	7.71	1611		2.656	0.1	2.508	2.28	1.49
	Min	7.07	7454		2.95	0.02	2.682	0.54	0.35

Temperature (range 18.6-27.7^{o}C), pH (NBS scale) and salinity (=38) were measured on several occasions between September 2009 and October 2010 (n=18). Total alkalinity (TA) is point measurement taken on 02/10/10. The remaining parameters were calculated using CO_2 SYS program (using the constants of Roy et al. 1993 and Dickson for KSO_4)

4.6.2 Marine sediment sampling and DNA extraction

At each sampling site seven surface sediment samples were taken in May 2011. Each sample was taken using a 20 cm diameter cylindrical corer: the top 2 cm of sediment in each core was kept for analysis. At each site samples were taken at approximately 0.5 m apart along a transect running parallel to the shore at 1 m depth. One sample was retained for granulometry, the remainder were stored in sterile tubes at -20^{o}C until DNA extraction, carbon and nitrogen analyses.

The marine sediment genomic DNA was extracted from 0.3 g of sediment by using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the protocol described by the manufacturer. There was an error in labeling the small subsamples that were sent from UK to South Korea for DNA extraction, they were not labeled identically to the original sediment sample bags that they were taken from, and from which sediment parameters were taken. Consequently although pH zone is known for each DNA and sediment sample, the correspondence to the exact individual sample for sediment analysis is unknown. This precludes relating each sample individually to the sediment parameters in that exact sample. Instead, we analyzed 3 sediment samples at random from each pH zone.

4.6.3 PCR amplification and pyrosequencing

The sediment sample DNA was amplified using primers targeting the V1 to V3 hypervariable regions of the bacterial 16S rRNA gene (Unno et al. 2010). Polymerase chain reactions were performed in 50 μl reactions under the following conditions: initial denaturation at 94℃ for 5 min, followed by 10 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ to 55℃ with a touchdown program for 45 s, and elongation at 72℃ for 90 s. This was followed by additional 20 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 45 s, and elongation at 72℃ for 90 s. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and quantified using PicoGreen (Invitrogen) spectrofluorometrically (TBS 380, Turner Biosystems, Inc. Sunnyvale, CA, USA). 50 ng of purified PCR product for each sample were combined in a single tube and sent to Macrogen Incorporation (Seoul, Korea) for sequencing using 454/Roche GS FLX Titanium Instrument (Roche, NJ, USA).

4.6.4 Analysis of pyrosequencing data

The sequence data obtained after pyrosequencing were processed using mothur (Schloss et al. 2009). Sequences shorter than 200 nt with homo-polymers longer than 8 nt and all reads containing ambiguous base calls or incorrect primer sequences were removed. Next, the sequences were aligned using mothur (default settings: kmer searching with 8mers and the Needleman–Wunsch pairwise alignment method) and the SILVA database (available at http://www.mothur.org/wiki/Alignment_database) and further filtered to remove gaps. The sequences were then pre-clustered using the mothur implementation of pseudo-single linkage pre-clustering algorithm from Huse and colleagues (Huse et al. 2010). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mothur (Edgar et al. 2011) in *de novo* mode, which first splits sequences into groups and then checks each sequence within a group using the more abundant groups as reference. A distance matrix was constructed using the average neighbor algorithm at phylogenetic distances of 0.03 (equivalent to species) in mothur. Pairwise distances between aligned sequences were calculated at a 0.97% similarity cutoff and were then clustered into unique operational taxonomic units (OTUs).

All taxonomic classification was performed using mothur's version of the RDP Bayesian classifier, using a RDP training dataset number 9 (available at http://www.mothur.org/wiki/RDP_reference_files) normalized to contain six taxonomic levels for each sequence at 80% Naïve Bayesian bootstrap cutoff with 1000 iterations.

4.6.5 Statistical processing and analysis of results

All samples were standardized by random subsampling to 676 sequences per sample using the sub.sample command (http://www.mothur.org/wiki/Sub.sample) in mothur. Phylogenetic diversity was calculated as Faith's phylogenetic diversity in mothur by using a maximum-likelihood (ML) tree inferred from partial 16S rRNA gene sequences of representative OTUs using FastTree2 with default settings (Price et al. 2010). OTUs (at 97% similarity), Shannon diversity index and rarefaction values were also calculated using the mothur. OTU based abundance data was first square-root transformed to build the Bray-Curtis distance matrix using the vegdist function in the vegan package of R (Table S1). We performed an NMDS

plot using the metaMDS function in the vegan package of R (Oksanen et al. 2007). This used the Bray-Curtis distance matrix to assess whether bacterial community composition clustered according to different pH levels. PERMANOVA analyses and post-hoc t-tests were based on 9999 random permutations, using type III sums of squares and unrestricted permutation of raw data. Kruskall-Wallis tests were performed to assess the effect of pH on relative abundance of major phyla. ANOVA test was performed to check the relationship between pH and soil properties. PERMANOVA analyses were conducted in Primer 6.1.10. Kruskall-Wallis and rarefaction curve analysis were performed using R.

4.7 Results

Where gas bubbling was strongest, the benthic microbial community was clearly affected, as the seabed was coated with bacterial mats (Figure 13A). However, we chose sampling sites with less extreme levels of pCO_2 where bacterial mats were absent and the black volcanic sand habitat appeared to be homogenous (Figure 13B).

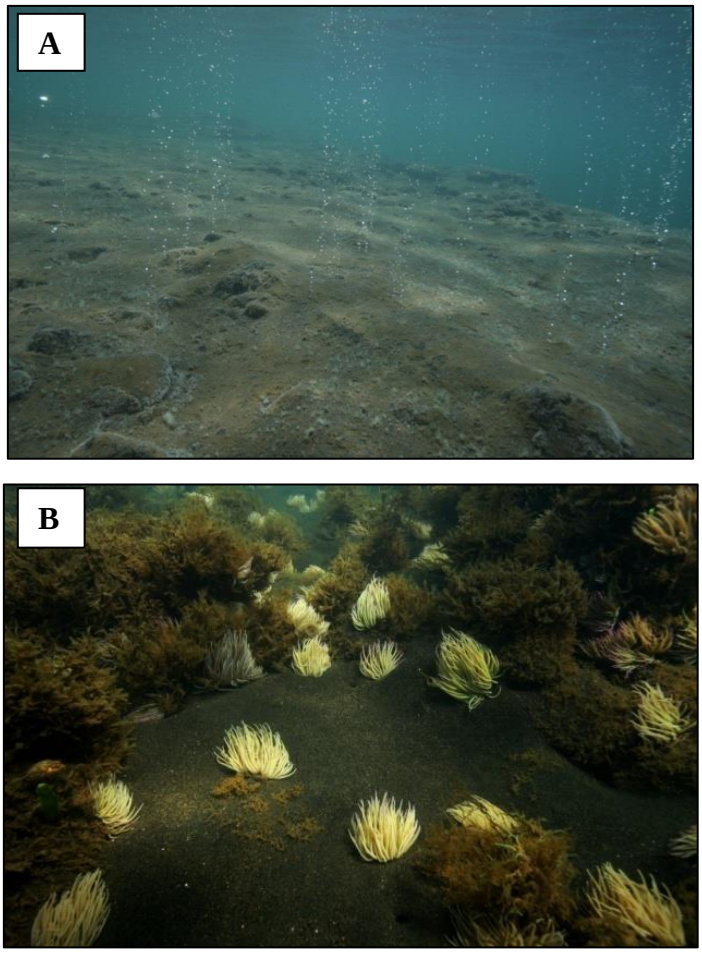

Figure 13: Sediment habitat at 1-m depth in Baia di Levante, Vulcano, May 2011. (A) CO_2 venting area (pH 5.6) showing bacterial mats on the sediment surface. (b) Patch of black volcanic sand typical of sites 1–3, surrounded by rock outcrops, anemones, and macroalgae.

We obtained 62238 quality sequences in total, which were classified into 1726 operational taxonomic units (OTUs) at 97% similarity level. On average, each individual sample was represented by 2973 classifiable sequences, with a range of 676 to 4255 sequences per sample. Most samples showed no sign of reaching an asymptote in OTU richness among the total number of reads available in the rarefaction analysis. This means that to cover the full taxonomic diversity, more sequences would be required (Figure 14).

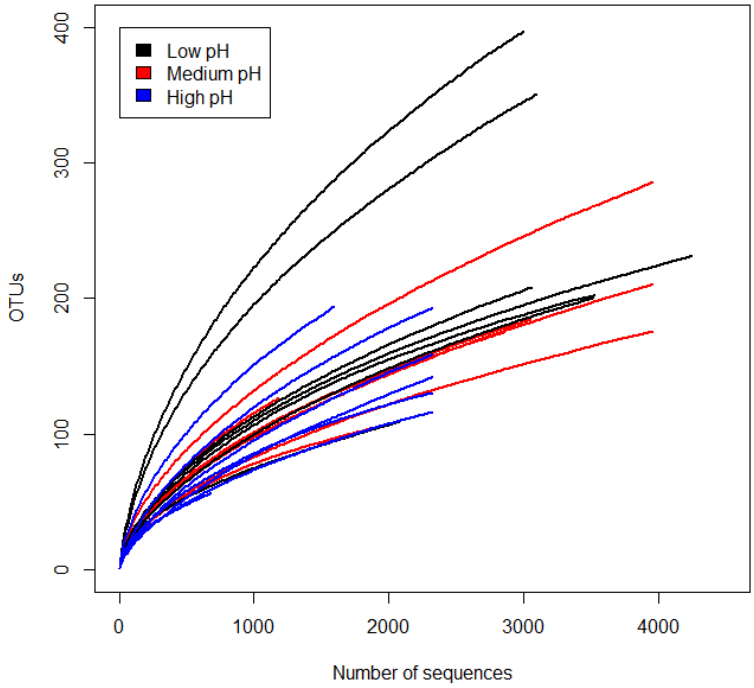

Figure 14: Rarefaction curves comparing surface sediment bacterial communities at 1-mdepthalonga seawater pH gradient caused by volcanic CO_2 vents off Vulcano, May 2011.

4.7.1 Effect of seawater *p*CO_2 on bacterial community composition

An NMDS plot reveals a shift in benthic sediment bacterial communities at the three different seawater CO_2 levels (Figure 15). Most of the samples from each pH zone cluster separately. The PERMANOVA test results show that the observed overall community differences among pH levels were highly significant (Pseudo-$F_{2,18}$=1.9635, P(perm)=0.0001) with significance between the pair-wise comparisons of the three different pH levels considered (High, Low: t=1.6374, P(perm)=0.001; High, Medium: t=1.1605, P(perm)=0.025; Low, Medium: t=1.3539, P(perm)=0.0045).

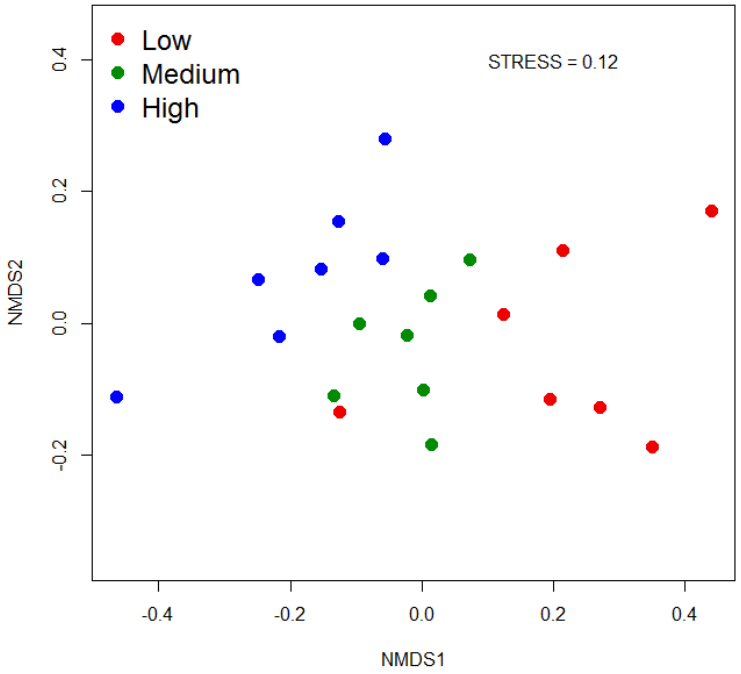

Figure 15: NMDS ordination, the low-pH (high CO_2) marine sediment bacterial communities cluster separately from the communities sampled at medium and high (i.e., normal) pH.

4.7.2 Effect on bacterial community diversity

Permutational univariate analysis of variance showed that pH change affected neither the OTU ('species') richness (Pseudo-$F_{2,18}$=1.9109, P(perm)=0.1672) nor the phylogenetic diversity (Pseudo-$F_{2,18}$=2.9526, P(perm)=0.0684). However, the Shannon Index (Pseudo-$F_{2,18}$=3.9597, P(perm)=0.031) revealed that pH significantly affected bacterial diversity, with the lower level of bacterial diversity observed at high pH (i.e., ambient pH/pCO_2) than the low pH (High, Low: t=2.3479, P(perm)=0.0433) sampling site (Figure 16).

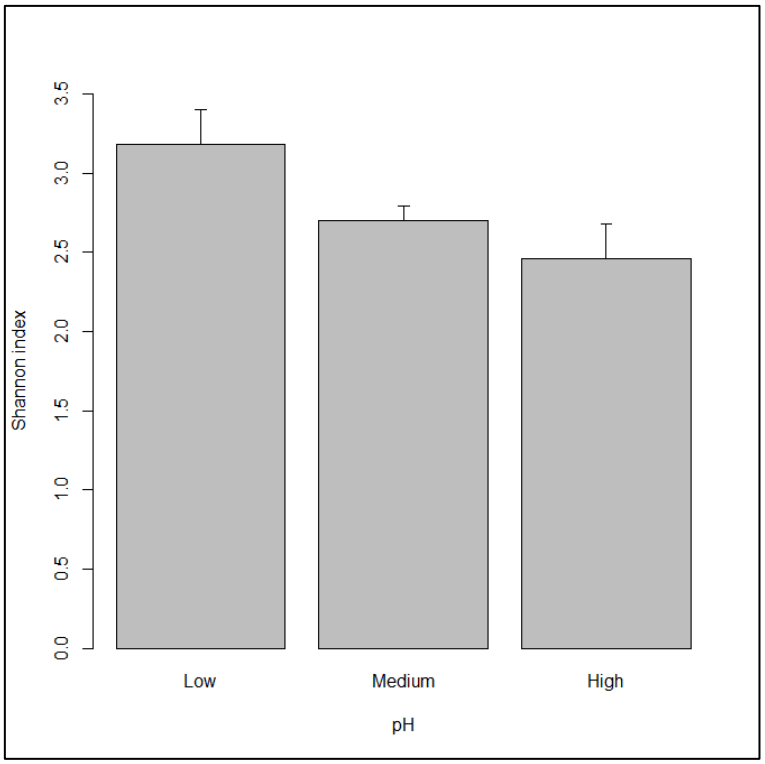

Figure 16: Marine sediment microbial Shannon Diversity (mean±SE) at different seawater pH levels, with high pH being the normal reference conditions (median pCO_2 419) and low pH corresponding to median pCO_2 1611µatm.

4.7.3 Effect of pCO_2 on relative abundance of dominant taxa

The dominant taxon across all sediment samples was *Gammaproteobacteria* (56%), followed by *Firmicutes* (14%), *Bacteroidetes* (12%), *Alphaproteobacteria* (11%), *Actinobacteria* (4%), and *Betaproteobacteria* (1.3%) respectively (Figure 17, Figure A5). They were, statistically, non-significant along the pCO_2 gradient (All P>0.1).

We found 21 genera of *Gammaproteobacteria*, of which *Pseudomonas* and *Vibrio* were predominant (Figure 18 and Figure A6) and we found no significant shift in the dominance of these bacteria along the pCO_2 gradient. However, five genera (Table 3) within the bacterial community were significantly affected by the pCO_2 gradient. These were *Georgenia* {$x^2(2)=9.63$, P=0.008}, *Lutibacter* {$x^2(2)=7.23$, P=0.02}, *Photobacterium* {$x^2(2)=14.26$, P=0.0008}, *Acinetobacter* {$x^2(2)=6.47$, P=0.03} and *Paenibacillus* {$x^2(2)=6.81$, P=0.03}. Together these genera accounted for less than 5% of the bacterial community sampled.

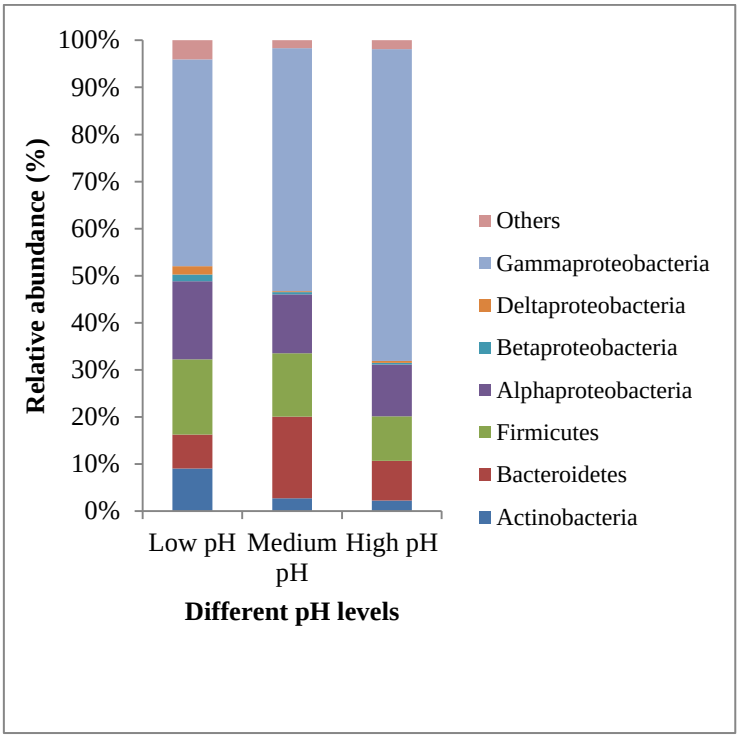

Figure 17: Relative abundance of sediment bacterial classes along a pH gradient in the overlying water column,Vulcano,May2011.

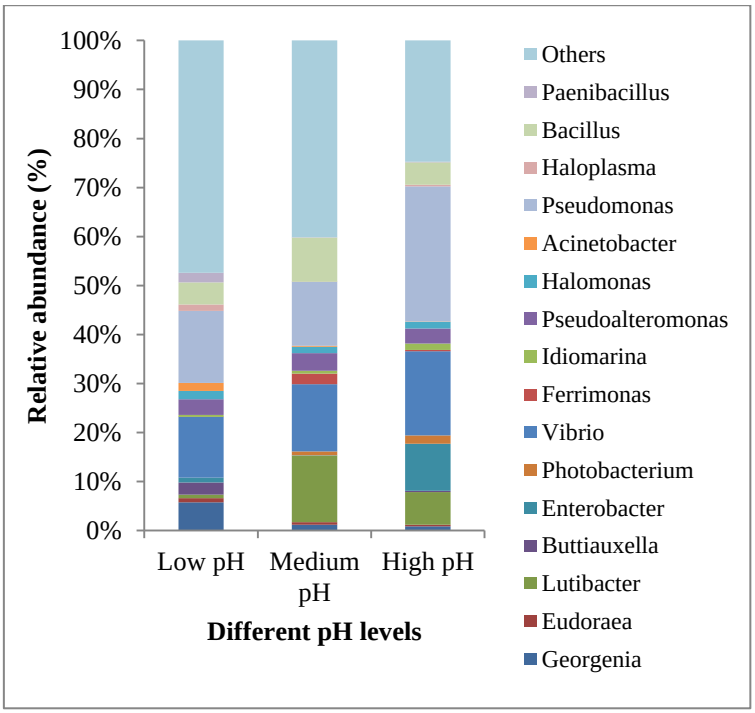

Figure 18: Relative abundance of dominant marine sediment bacterial taxa at different pH levels.

Table 3: Classification of the five taxa affected by pH change at different pH levels.

Taxon	Phylum	Class	Order	Family
Georgenia	Actinobacteria	Actinobacteria	Actinomycetales	Bogoriellaceae
Lutibacter	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobactereaceae
Photobacterium	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae
Acinetobacter	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae
Paenibacillus	Firmicutes	Bacilli	Bacillales	Peanibacillaceae

4.7.4 Relationship between seawater pCO_2 and sediment properties

There was no correlation between seawater pCO_2 and the TN (R^2= -0.16, P=0.67) and TOC (R^2= -0.2, P=0.73) content of the sediment (Table 4). There was also no correlation between pH zone and sediment granulometry; all samples were Medium Sand on the Wentworth scale (Site 1 mean Φ 1.497, Site 2 mean Φ 1.097, and Site 3 mean Φ 1.210) and were unusual, for coastal sediments, in that carbonates were absent.

Table 4: Marine sediment properties: Total organic carbon (TOC) and total nitrogen (TN) contents.

Sample	TOC%	TN%
Low pH1	0.29	0.018
Low pH2	0.16	0.016
Low pH3	0.28	0.025
Medium pH1	0.21	0.018
Medium pH2	0.17	0.012
Medium pH3	0.25	0.021
High pH1	0.16	0.014
High pH2	0.25	0.019
High pH3	0.37	0.018

4.8 Discussion

Our analyses of sediments along a natural pCO_2 gradient (from median 419 - 1611 µatm), revealed that the volcanic sand habitat of each of the three pH zones sampled was closely similar in composition with no differences in granulometry, carbonate, carbon or nitrogen content. In addition, the overlying water column in each of the three zones had no differences in salinity, temperature or alkalinity providing a consistent basis for comparison of the effects of seawater acidification.

Although significant changes in sediment bacterial community were observed along the chosen pCO_2 gradient, as revealed by the NMDS plot and Shannon Diversity, the majority of major taxa of sediment dwelling bacteria were not significantly affected by the steep gradients in seawater pH and carbonate saturation. Changes in sediment bacterial communities were obvious to the naked eye at the CO_2 vents themselves where *Beggiatoa*-like mats carpeted the sediment. In the present study we chose a gradient of pCO_2 that was representative of the changes that are predicted to occur due to ocean acidification, lacking confounding factors such as geothermal heating and the presence of H_2S that profoundly affect microbial communities (Simmons and Norris 2002, Rusch and Amend 2008, Boatta et al. 2013). *Gammaproteobacteria* were the dominant bacterial type at all the pCO_2 levels we studied and were also the most abundant bacterial group in a marine volcanic ash layer in the Sea of Okhotsk (Inagaki et al. 2003). *Gammaproteobacteria* are usually the most abundant type of bacteria found in marine sediments, often comprising >50% of the microbial community and the bulk of the marine sediment bacterial biomass (Ravenschlag 2001, Bowman and McCuaig 2003). Of the *Gammaproteobacteria* at the study site, *Pseudomonas* was the most common, and is generally abundant in marine sediments where they are able to use diverse organic compounds as a carbon source and electron donor (Hongxiang et al. 2008). The genera *Georgenia*, *Lutibacter*, *Photobacterium*, *Acinetobacter* and *Paenibacillus* were less abundant at our study sites and all of these genera exhibited significant shifts in their abundance along the pCO_2 gradient. *Georgenia* is a Gram-positive, non-sporulating and motile or non-motile aerobic bacterium, but the growth under anaerobic conditions can also occur. Temperature range for growth is 10–37^{o}C, with optimum growth at 28–30^{o}C. The pH range for growth is between 6.5 and 10, with optimum growth at pH 7 (Altenburger 2002, Li 2007). *Lutibacter* are Gram-negative and rod-shaped bacteria. They can grow in

heterotrophic and aerobic conditions. The growth occurs at 5–30°C (optimum 25–30°C) and at pH values of between 7 and 8 (Choi and Cho 2006, Park et al. 2010). *Photobacterium* are Gram-negative bacteria. They are widely distributed in the marine environment and are facultative anaerobes. The pH range for the optimal growth is between 5 and 9 (Nogi et al. 1998, Farmer and Hickman-Brenner 2006). *Acinetobacter* are nonmotile, Gram-negative and strictly aerobic bacteria. They are ubiquitous organisms (Wagner et al. 1994). *Paenibacillus* is a Gram-positive, facultative anaerobic bacterium. The optimum growth temperature is 28-30℃, but it grows also at 20℃ (Heyndrickx et al. 1996).

Other laboratory and field-based studies have shown detectable effects of CO_2 gradients on sediment bacterial community composition or activity. Krause et al. (2012) found, using seawater acidification studies on sediment in microcosms, that a reduction in pH caused major shifts in bacterial community composition that had direct effects on nutrient availability and use within the sediment although Kitidis et al. (2011) found that natural gradients of CO_2 had no significant effect on sediment microbial ammonia oxidation activity. Kitidis et al. (2011) suggested that this could be explained by adaptation of the microbial communities to high pCO_2 or due to interstitial pH buffering that may lessen the impact of ocean acidification on life within sediments (Widdicombe et al. 2011).

The relatively subtle shifts we observed in bacterial sediment communities contrast with the major shifts seen in benthic foraminifera and biofilms at similar pCO_2 levels. Dias et al. (2010) and Uthicke et al. (2013) noted remarkable reductions in foraminifera diversity and abundance. A significant increase in foraminifera dissolution along gradients of decreasing seawater carbonate saturation state at CO_2 vents in Italy, Mexico and Papua New Guinea was recorded by Dias et al. (2010), Pettit et al. (2013) and Uthicke et al. (2013). Also, Moy et al. (2009) document reduced calcification in Southern Ocean planktonic foraminifera and a decline in their abundance that correlates with declining carbonate saturation states. Successional processes in bacteria and Eukarya communities forming biofilms were also strongly affected by seawater CO_2 concentration (Lidbury et al. 2012). Johnson et al. (2011) observed a change of both the grazing sea urchin and the calcifying macroalgae *Padina* along increasing CO_2 gradients in Vulcano, Italy. Moreover, a significant alteration of periphyton communities was detected as CO_2 concentrations increased; the periphyton community

increased at the CO_2 enriched sites. This indicates an increase in primary productivity in the CO_2 enriched area. The reasons for the somewhat muted effects of pCO_2 gradients on sediment dwelling bacteria in our study, compared with the study of Krause et al. (2012), may lie in the differences of the composition of sediments tested. Furthermore, they used microcosms whereas we examined natural sediment affected by waves, currents, and macro benthic organisms; bioturbation for example will hinder the formation of a surface biofilm. The system used in the present study was open offering ample opportunity for the colonization by bacterial strains that are tolerant of acidified conditions. In addition, it has been present for decades providing more time for bacteria to evolve than has been available in manipulative experiments. A study of the microbial communities associated with anemones and corals along a volcanic pCO_2 gradient did not pick up any significant changes, which may be due to acclimation to pH variations, although the host organisms will modify the seawater chemistry of their surfaces that might mask any effects of the overlying seawater pCO_2 gradient (Meron et al. 2012, Meron et al. 2013).

Effects on diversity

Although ordination analyses showed less profound effects of pCO_2 levels on sediment bacterial community composition than those revealed in microcosm work by Krause et al. (2012), we found that enriched pCO_2 sites had a higher Shannon Index, which takes into account both the 'richness' and 'evenness' aspects of diversity (Shannon and Weaver 1948). This is in fact the opposite of the findings of Krause et al. (2012) and the predictions by Unno et al. 2010) that ocean acidification might cause changes in bacterial community of the world's ocean sediments with a shift towards communities more heavily dominated by a few species. It is also similar to that observed by Roy et al. (2013) on pelagic microbes, whereby most taxa were unaffected and a few rare taxa increased in abundance with higher CO_2. Higher bacterial diversity at lower pH sites has been also reported for coastal microbial biofilms exposed to lower-than-ambient natural CO_2, although not under present ambient CO_2 conditions (Lidbury et al. 2012). It is unclear why higher CO_2 might have increased diversity in our study, but have the opposite effect in others. No detailed studies have yet been done on the changing ecology and physiology of bacteria in such systems. There are certainly some chemical processes in sediment that may be expected to change as the pH shifts. For example, accelerated bacterial degradation of polysaccharides by extracellular

enzymes may occur in lower pH conditions, and the changes in resource availability may bring about community change (Piontek et al. 2010). Increased biofilm EPS (Extracellular Polymeric Substances) production with increased polysaccharide degradation in reduced pH could also be a cause of the change of community structure and the adjustment of available niches (Lidbury et al. 2012). How such changes should produce a shift in diversity, however, are presently unclear.

Studies on terrestrial soils generally show lower bacterial diversity as pH declines or increases away from neutral (Fierer et al. 2007, Tripathi et al. 2012). The explanation favoured in these studies, is that the internal pH of bacterial cells is always near neutral, and at higher and lower ambient pH values, high physiological demands are placed on bacterial cells which are maintaining their intracellular pH. This may limit niche viability, or the possibilities of evolutionary origin of new lineages, into these more physiologically demanding environments. It is challenging to measure the pH of pore water within the upper layer of marine sediment, as the process of measuring pH strongly perturbs the system. Hypothetically, some of these sediment systems could be near neutral pH – in which case as in soils, diversity could decline with pCO_2 increase. Other systems might be considerably above neutral pH, in which case lowering pH though higher pCO_2 may bring the sediment pH closer to neutral, and thereby increase diversity.

The effects of ocean acidification on sediment microbes are likely to differ between different sedimentary environments: we studied sediment that was somewhat unusual in that it lacked carbonates and was surrounded by dense stands of macroalgae. The microbes of carbonate sediments may be affected differently as corrosive waters begin to dissolve the carbonate. Sediment carbonate may act as a buffer, but also its physical loss may profoundly change the microscopic pore structure of the sediment. Such buffering effects of sedimentary carbonates seem most likely to affect time transgressive responses to a recent increase in pCO_2, slowing down changes. However, all of these perspectives remain purely hypothetical at present: detailed experimental and observational evidence on sediment changes under increased pCO_2 will be necessary for an improved understanding of bacterial community changes.

It is unclear what functional implications shifts in sediment microbial diversity might have

for the overall biogeochemical functioning of benthic communities. Some authors have claimed that increasing diversity makes ecosystems more resilient to changes (Chapin et al. 1997), although beyond a minimal low level of diversity the biogeochemical functions of communities may well be left unchanged by shifts in diversity, if multiple species can fulfill the same ecological role.

An important outcome of our meta-analysis is that almost all the parameters respond differently to ocean acidification amongst previous studies, suggesting the occurrence of confounding impacts. Thus, it is necessary to launch of major national and international projects on ocean acidification based on different natural CO_2 gradients to better constrain the large-scale changes expected in response to increases in CO_2 emissions and to provide available datasets over the coming decades which will lead to more conclusions on the effect of ocean acidification on microbial communities.

4.9 Conclusion

From a global change viewpoint, the response of benthic bacterial diversity to pCO_2 enrichment provides a warning that ocean acidification has the potential to significantly alter marine sediment bacterial communities worldwide. Bacterial community composition showed detectable shifts with increasing pCO_2, and bacterial diversity increased towards higher pCO_2. Given that these shifts occur, it is possible that the biogeochemical functions of marine sediments will also be significantly affected by ocean acidification.

Firmer conclusions will only be possible after the roles of pCO_2 sensitive marine sediment bacteria are known, following direct *in situ* analyses of sediment biogeochemical processes conducted, with a range of sediment types tested at a various locations.

CHAPTER 5: EFFECTS OF MULTI-WALLED CARBON NANOTUBES ON SOIL BACTERIAL COMMUNITIES

5.1 Introduction

Carbon nanotubes (CNTs) are widely used in novel industrial materials because of their particular chemical and physical characteristics (Salvetat et al. 1999, Biercuk et al. 2002, Chung et al. 2011). They are currently used for—or under development in—electron emission devices, energy storage devices, drug delivery mechanisms, and a range of other engineering applications (Derycke et al. 2001, Baughman et al. 2002, Yue et al. 2002, Han et al. 2004, Dong et al. 2009, Kim et al. 2010). Global carbon nanotube production is increasing by around 25% per year and by 2015 is expected to reach 9300 tons with a production value of $1.3 billion (Parish 2011). Nevertheless, there are no strict rules regulating CNTs production, usage and release. Consequently, substantial quantities of CNTs could be released into the environment with unknown potential to affect the environment and human health (Shrestha et al. 2013). The release of CNTs to the environment may occur during the production phase or the usage and disposal phases. Direct or indirect exposure pathways to CNTs have rarely been studied, and the risks to human health and the general environment are still poorly understood (Köhler et al. 2008). To assess the environmental risks presented by CNTs, it is important to understand their fate after release (their mobility, reactivity and persistence in environmental compartments), and their impact on living organisms (Simon-Deckers et al. 2009).

Despite their evident advantages in practical applications, the potential toxicity of CNTs is a major concern because of their potential impact in the environment (Poland et al. 2008). CNTs' toxicity has been studied in both *in vivo* and *in vitro*, and has been related to various factors such as CNTs' length, type of functionalization, concentration, duration of exposure, method of exposure, concentration of the solubilizing agent, and the surfactant used. So far, while many studies suggest that CNTs do not show toxicity, certain others suggest that CNTs are harmful to human health and the environment. These inconsistencies might be due to differences in experimental protocol (Firme and Bandaru 2010).

Presently, few studies have investigated the impact of CNTs on living organisms in the

environment (Nowack and Bucheli 2007, Klaine et al. 2008, Chung et al. 2011). Of those studied, only a few have focused on bacteria, despite their ubiquity and key role in ecosystem processes. Most ecotoxicological studies that have dealt with their effects on bacteria have been conducted only under culture conditions (Ghafari et al. 2008, Kang 2008). For example, Kang et al. (2007), demonstrating that the exposure of *Escherichia coli* to highly purified CNT aggregates can lead to cell death. In other studies, it has been shown that CNTs dispersed using a range of different surfactants can have antimicrobial properties when incubated with bacteria (Liu et al. 2009, Bai et al. 2011). It has been demonstrated that carbon nanotubes can also lower soil enzyme activities and microbial biomass when applied to soil (Kang et al. 2009, Chung et al. 2011, Jin et al. 2013).

Soil is likely to be one of the main ultimate recipients of nanomaterial contamination in the environment, more so than water and air (Nowack and Bucheli 2007, Klaine et al. 2008, Navarro et al. 2008). Soil microbial communities play a vital role in soil ecosystem activities such as nutrient cycling, and are known to be susceptible to alteration by heavy metals and a range of other chemical agents (Yang et al. 2006, Liu et al. 2009, Madsen, 2011). Therefore, it is important to study the impact of CNTs on these living soil systems (Chung et al. 2011, Rodrigues et al. 2012, Shrestha et al. 2013). If it were to turn out that CNTs strongly alter the composition or functioning of the soil ecosystem, and that the effects persist over the long term, precautionary measures for their manufacture, usage and disposal could be necessary.

Previous studies on CNTs in the soil system have been carried out by Chung et al. (2011) who demonstrate that short-term exposure (20 days) to multi-walled CNTs can lower most enzymatic activities and overall microbial biomass in soils, at exposures of around 5000 μg of MWCNTs per gram of soil. The same group (Jin et al. 2013) has observed similar effect of CNTs on soil enzyme activities and microbial biomass at the concentration 300-1000 μg/g but using single-walled CNTs (SWCNTs). Another short-term study indicates that SWCNTs may alter the structure of activated sludge microbial communities (Goyal et al. 2010). However, these studies were limited to describing overall soil processes and only very broad features of the bacterial community. They did not study the bacterial community down to a very detailed taxonomic level corresponding to the species or genus, and did not follow the effects on the soil system for more than 3 weeks. Furthermore, many of the previous studies used untreated CNTs, which are known to be hydrophobic, mixing poorly with soils. Only a subset of studies

has used the far more easily miscible acid–treated CNTs (functionalized or fCNTs), which are also commonly used in industry (Jin et al. 2013), and which seem more likely to interact with the soil ecosystem.

Here we set out to understand the effect of both treated (fMWCNTs) and untreated multi-walled CNTs on soil bacterial communities at a range of taxonomic levels. We consider shifts in both relative abundance and diversity. The relative abundance of certain groups might be important because particular taxa are consistently associated with ecological functions in the soil, especially at the finer taxonomic level. Diversity may be important because it is widely thought that ecosystem resilience is affected by taxonomic diversity (Tilman 1997, Cardinale et al. 2002, Elmqvist et al. 2003).

Furthermore, we continued our experiment for 8 weeks; most of the other studies ran for less than 4 weeks (Chung et al. 2011, Jin et al. 2013). Given that soil bacteria are generally thought to be slow growing with a high proportion of dormant cells much of the time, a longer duration of study was desirable as this would more likely show the relevant shifts in ecology. Shrestha et al. (2013) evaluated the impact of MWCNTs on soil microbial community structure and functioning in soil over 90 days of exposure and they found no effect on soil respiration, enzymatic activities and microbial community composition. However, at the highest (MWCNTs) concentration (10,000 mg/kg), shifts in microbial community composition and abundance of some bacterial genera were observed.

Essentially we hypothesize:

1) That fMWCNTs will have a greater effect on soil bacteria than untreated MWCNTs, due to their greater ability to mix with soil water and interact directly with bacterial cells. This higher concentration of MWCNTs will also have stronger effects.
2) That given the relatively slow and long-term nature of soil processes, including bacterial community shifts, the 8-week timeframe will show a different result from the much shorter 2-3 week timeframe used in most previous studies. The effect may intensify, as more cells die or are unable to reproduce under the effect of the MWCNTs.

In the present study, we use paired-end Illumina bar-coded sequencing of hypervariable V3

region of 16S rRNA gene to investigate the impact of multi-walled carbon nanotubes on soil bacteria.

5.2 Structure of carbon nanotubes

Carbon nanotubes are composed of honey comb lattices and they are built from sp^2 carbon units. Carbon nanotubes (CNTs) are single atom layers of hexagonal carbon rolled up into hollow cylinders. They are classified as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) (Figure 19). SWCNTs are single-layered graphitic cylinders with a diameter ranging from 0.4 to 2 nm and MWCNTs which are composed by 2 to 30 concentric cylinders with outer diameters ranging between 2 and 100 nm (Paradise and Goswami 2007).

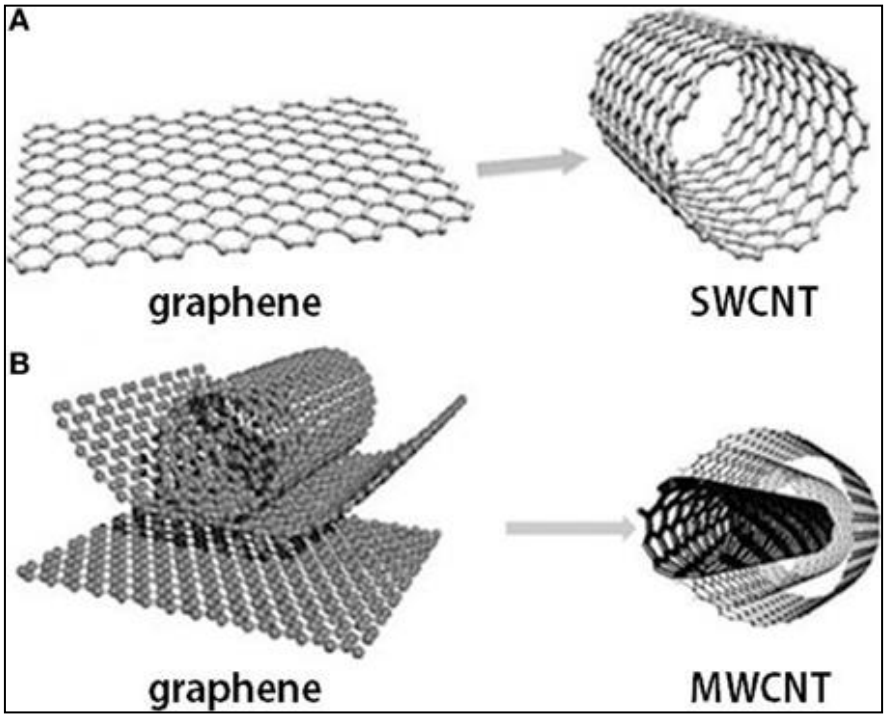

Figure 19: Illustration of single-walled carbon nanotube (SWCNT) and Multi-walled carbon nanotube (MWCNT) (Vidu et al. 2014).

Depending on how the graphitic walls of carbon nanotube are rolled together they can have various shapes resulting in an armchair, zigzag or chiral shapes (Figure 20). The unit cells of carbon nanotubes determine these different shapes by the chiral vector given by the following

equation: $C_h = n\hat{a}_1 + m\hat{a}_2$ where $n\hat{a}_1$ and $m\hat{a}_2$ are unit vectors in the two-dimensional hexagonal lattice, and n and m are integers. The chiral angle (angle between C_h and $\hat{a}_1$) is another important parameter. When n = m and the chiral angle is 30°, the shape is armchair. When m = 0 or n = 0 and the chiral angle is 0°, the nanotube has zigzag type. When the chiral angle is between 0° and 30°, the nanotube is known as chiral carbon nanotube (Paradise and Goswami 2007).

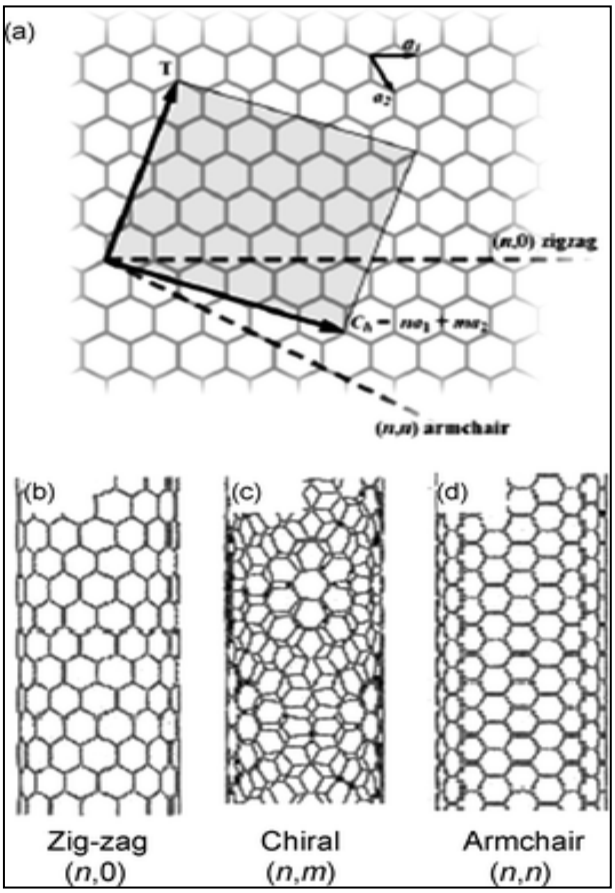

Figure 20: Illustrations of the atomic structure of a zigzag, a chiral, and an armchair carbon nanotube (Zhang and Zhao 2009).

Different types of carbon nanotubes have same properties. However, their electrical conductivity is different and these structural differences can have profound effects. For example, all armchair nanotubes (i.e., where m = n) have truly metallic electrical conductivity. In contrast, the other types of carbon nanotubes are essentially semiconducting.

Since nanotubes with different n and m are molecularly distinct, it is possible that different types of carbon nanotubes may exist according to their chemical proprieties, although at present, all produced nanotubes have random mixture (Paradise and Goswami 2007).

5.3 Properties of carbon nanotubes

Carbon nanotubes have been attracting wide interest because of their unique structures and particular electronic, mechanical, thermal, and optical properties. Because of the carbon–carbon sp^2 covalent bonds and the fact that each carbon nanotube is one supramolecule, carbon nanotubes have high stiffness and axial strength (Popov 2004). Tensile strength measures the force that an object can resist without tearing apart. The tensile strength of carbon nanotubes is approximately 100 times greater than that of steel of the same diameter.

Carbon nanotubes are not only strong but are also elastic which means it takes a lot of force to bend a nanotube. Atomic force microscopes were used to physically push nanotubes around and observe their elastic properties. The transmission electron microscopes (TEM) evaluations showed that the bonds in the atomic lattice did not break when a nanotube is bended or condensed (Dresselhaus et al. 1996). Carbon nanotubes are also lightweight, with a density about one quarter that of steel. Moreover, they are able to well conduct heat and cold because of their high thermal conductivity proprieties; their thermal conductivity is predicted to be more than 10 times that of silver. To conduct heat, metals depend upon the movement of electrons, but carbon nanotubes conduct heat by the vibration of the covalent bonds holding the carbon atoms together. The stiffness of the covalent bonds helps the transmission of this vibration throughout the nanotube, providing very good thermal conductivity (Paradise and Goswami 2007).

5.4 Applications of carbon nanotubes

5.4.1 Peptide delivery by carbon nanotubes

Carbon nanotubes were used as a template to present bioactive peptides to the immune system (Pantarotto et al. 2003a). To test the delivery of peptide using CNTs, a B-cell epitope of the foot-and mouth disease virus (FMDV) was covalently attached to the amine groups present on CNTs, using a bifunctional linker. The peptides attached to the CNTs adopt the appropriate secondary structure for recognition by specific monoclonal and polyclonal antibodies. The immunogenic features of peptide-CNTs conjugates were then evaluated in vivo (Pantarotto et al. 2003b). Immunization of mice with FMDV peptide-nanotube conjugates showed higher antibody responses compared to the free peptide. Using CNTs as potential novel vaccine delivery tools was confirmed by interaction with the complement. The complement is the part of the human immune system that is composed of a series of proteins responsible for recognizing, clearing and killing pathogens, apoptotic or necrotic cells and foreign materials (Bianco et al. 2005).

5.4.2 Cellular uptake of carbon nanotubes

Only small molecules are allowed to pass through cell membranes. Nutrient uptake and any communication between the cells and their environments are conducted through the plasma membrane via different biological mechanisms. Oxygen, carbon dioxide, water, and small hydrophobic or nonpolar molecules can diffuse over the plasma membrane due to their concentration gradients. Small molecules such as ions and amino acids cross the plasma membrane through the active transport system of integral membrane protein pumps or ion channels (Luo 2004); however nanoscale hydrophilic biomacromolecules enter the cell via endocytosis. Nanoparticles such as carbon nanotubes, fullerene, and quantum dots, could be transported into various types of cells such as endothelial cells, pulmonary epithelia, intestinal epithelia, alveolar macrophages, other macrophages, and neuron cells (Bianco et al. 2005). The main cellular uptake pathways of nanoparticles are summarized in the table 5 and figure 21. Endocytosis is an active transport in which a cell takes in objects by enclosing them in vesicles or vacuoles pinched off from its cytoplasmic membrane. The known endocytotic processes are mostly through phagocytosis, pinocytosis, and caveolae-dependent

or clathrin-mediated endocytosis (Murakami et al. 2004, Shi et al. 2004, Yinghuai et al. 2005). Phagocytosis is mainly conducted by specialized mammalian cells such as monocytes, macrophages, and neutrophils to engulf solid particles having diameters > 750 nm by the cell membrane to form an internal phagosome. Smaller particles with diameters ranging from a few to several hundred nanometers are transported into cell by pinocytosis or macropinocytosis.

Table 5: Description of the main cellular uptake processes of nanoparticles (Zhao et al. 2011).

Uptake pathway	**Description**
Clathrin-mediated endocytosis	Clathrin-coated vesicles containing plasma membrane proteins with receptor sites specific to the NPs being internalized The receptors are recycled to the plasma membrane An energy-dependent manner Up to 100–150 nm
Caveolae-dependent endocytosis	Caveolae, small flask-shape pits in the membrane, consisting of the cholesterol-binding protein caveolin Up to 200–500 nm
Pinocytosis/ Macropinocytosis	Cell-drinking, nonspecific 0.5–5 μm in diameter for Macropinocytosis
Phagocytosis	Internalization of whole large particles, neutrophils and macrophagocyte

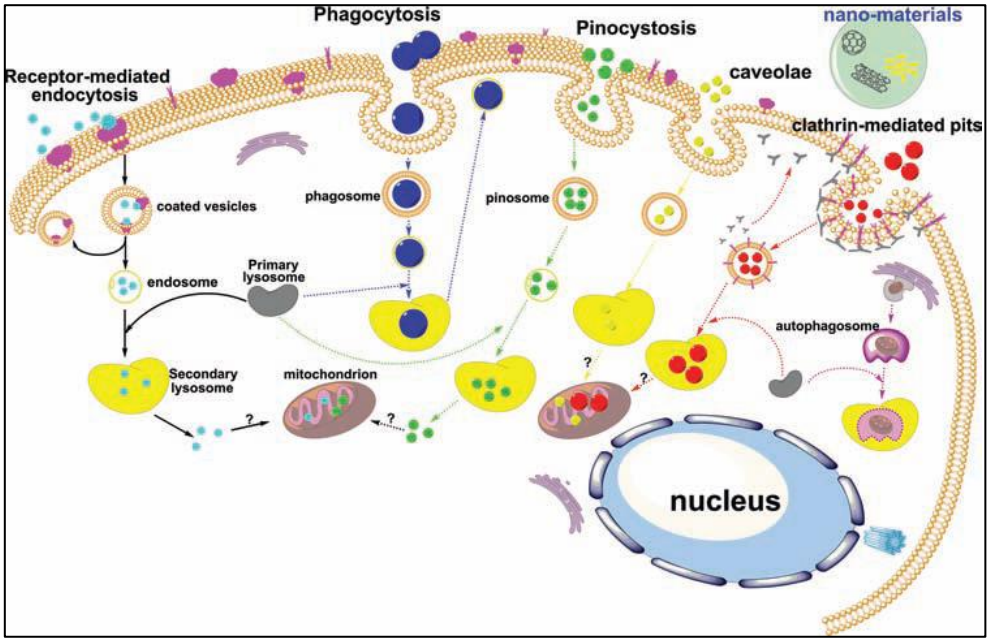

Figure 21: Schematic of the known pathways for intracellular uptake of nanoparticles (Zhao et al. 2011).

Cellular uptake of carbon nanotubes was confirmed by Dai and colleagues (Shi et al. 2004, Shi and Dai 2005) who used oxidized CNTs in further studies to covalently link fluorescein or biotin, allowing for a biotin–avidin complex formation with fluorescent streptavidin. Carbon nanotubes tagged with a fluorescent agent were easily tracked into the cell using epifluorescence and confocal microscopy (Pantarotto et al. 2004). They could also be observed inside the cells using transmission electron microscopy (TEM). Some carbon nanotubes were identified at the cell membrane during the process of translocation. The conformation of CNTs perpendicular inside the cell during uptake suggested a mechanism similar to nano needles, which could perforate and diffuse through the lipid bilayer of plasma membrane without killing the cell. Dynamic simulation studies have demonstrated that amphiphilic nanotubes can theoretically traverse artificial lipid bilayers via a similar mechanism. Recently, an efficient *in vitro* nano penetration called nanotube spearing was also proposed by Cai et al. (2005). Weismann et al. suggested another way to observe CNTs inside the cell using near-infrared fluorescence (Cherukuri et al. 2004). They indicated that

macrophage cells were able to ingest significant amounts of nanotubes; and no toxic effect was observed (Bianco et al. 2005).

5.4.3 Nucleic acid delivery by carbon nanotubes

Several cationic systems are being examined for the delivery of nucleic acids to cells (Luo 2004, Schmidt-Wolf and Schmidt-Wolf 2003). Ammonium-functionalized CNTs were also investigated to form supramolecular complexes with nucleic acids through electrostatic interactions. These tests were carried out to improve gene transfer and expression (Dean et al. 2005). The macromolecular cationic nature of functionalized carbon nanotubes has been used to condense plasmid DNA (Singh et al. 2005) like any non-viral vector (i.e. liposomes, cationic polymers, microparticles and nanoparticles). In order to investigate the possibility of CNTs as gene transfer vectors, plasmid DNA pCMV-Bgal expressing b-galactosidase was adsorbed on fCNTs carrying ammonium groups. Both single- and multi-walled cationic CNTs are able to form stable complexes, characterized by electron microscopy (TEM and SEM), surface plasmon resonance, electrophoresis and fluorescence dye exclusion. Gene transfer tests demonstrated that fCNTs has obvious effect on the expression of b-galactosidase following the formation of the complexes (Singh et al. 2005). It was also shown that the level of gene expression was five to ten times higher than that of DNA alone. Recently, the effectiveness of using fCNTs for DNA transfer was improved by covalent modification of the external walls of the tubes with polyethyleneimine (PEI). PEI-grafted MWCNTs complexed and delivered plasmid DNA to different cell types; but, the levels of luciferase expression were same as PEI alone (Bianco et al. 2005).

Other studies revealed that cationic carbon nanotubes are able to condense short oligodeoxynucleotide (ODN) sequences and improve their immune stimulating activity, using same approach. Carbon nanotubes were also used to deliver non-encoding RNA polymers into cells (Bianco et al. 2005).

The hybrids showed insignificant toxicity as found by monitoring cell growth. Since CNTs can form stable complexes with nucleic acids, this opens the way to diverse applications of CNTs including gene therapy, and genetic vaccination.

5.4.4 Drug delivery with carbon nanotubes

Nowadays, carbon nanotubes are highly researched in drug delivery and biosensing methods for disease treatment and health monitoring. Carbon nanotube technology has shown to have the potential of using fCNTs as vehicles for the delivery of small drug molecules; this is due to the ability of fCNTs to penetrate into the cells (Pantarotto et al. 2004). Carbon nanotubes are able to carry one or more therapeutic agents with recognition capacity, optical signals for imaging and/or specific targeting. This is an important and essential advantage, for example in the treatment of cancer and different types of infectious diseases (Ferrari 2005).

The antibiotic connected to the nanotubes did not show any toxicity effect while penetrating into mammalian cells compared to the antibiotic incubated alone. In addition, amphotericin B linked to CNTs conserved its high antifungal activity against diverse pathogens, including *Candida albicans*, *Cryptococcus neoformans* and *Candida parapsilosis*. Thus, functionalized carbon nanotubes represent a new and emerging technique of drug delivery into various mammalian cells. Despite no cytotoxicity of these CNTs conjugates was detected *in vitro*, it will be necessary to assess their metabolism, biodistribution and clearance from the body (Bianco et al. 2005).

5.5 Materials and methods

5.5.1 Soil sampling

Soil samples were collected in June 2013 from an overgrown flowerbed on Seoul National University campus, which is located in the Gwanak Mountain area, south of Seoul. This sampling site was selected because it represents a typical and widespread type of soil (slightly acidic sandy loam) in South Korea. The upper 10 cm of soil was sieved and thoroughly mixed in a sterile container. The soil was divided into pots containing 200 g soil each. Soil pH was measured using a soil pH meter (Hanna Instruments HI 99121N Direct Soil pH Meter). Soil pH was around 6.1 and it contained 10.4% clay, 18.4% silt and 71.2% sand. Soil texture and organic matter content were measured at National Instrumentation Center for Environmental Management (NICEM, South Korea) following the standard protocol of SSSA (Soil Science Society of America).

5.5.2 Preparation and characterization of MWCNTs

Pure commercial MWCNTs were purchased from Hanwha Nanotech, Republic of Korea. Two forms of CNTs were used in the present experiment: raw and functionalized forms of MWCNTs. The powder MWCNTs were not treated, but used in the form received from the manufacturer because this is a form that microorganisms are most likely to encounter if there is an accidental release from a manufacturing facility. The functionalized MWCNTs are more commonly used during fabrication processes of commercial products, so would more likely be released at those sites (Jin et al. 2013).

Two functionalisation approaches are used to modify carbon nanotubes (Figure 22). CNTs can be acid-treated using strong acids (this oxidized form was used in the present study), resulting in the reduction of their length while generating carboxylic groups, which increase their dispersibility in aqueous solutions. In addition, functionalized carbon nanotubes (fCNTs) can be linked to various active molecules, including peptides, proteins, nucleic acids and other therapeutic agents (Bianco et al. 2005).

Another type of functionalization is to functionalize the CNTs using an a-amino acid and an aldehyde. This type of fCNTs possesses high solubility in a large range of solvents. By carefully choosing the reactants, it is possible to modulate solubility in organic solvents or aqueous solutions, if the reactants are carefully chosen (Georgakilas et al. 2002). CNTs carrying ammonium groups (Figure 22b) are very soluble in water and have been exploited for their potential in the delivery of therapeutic molecules.

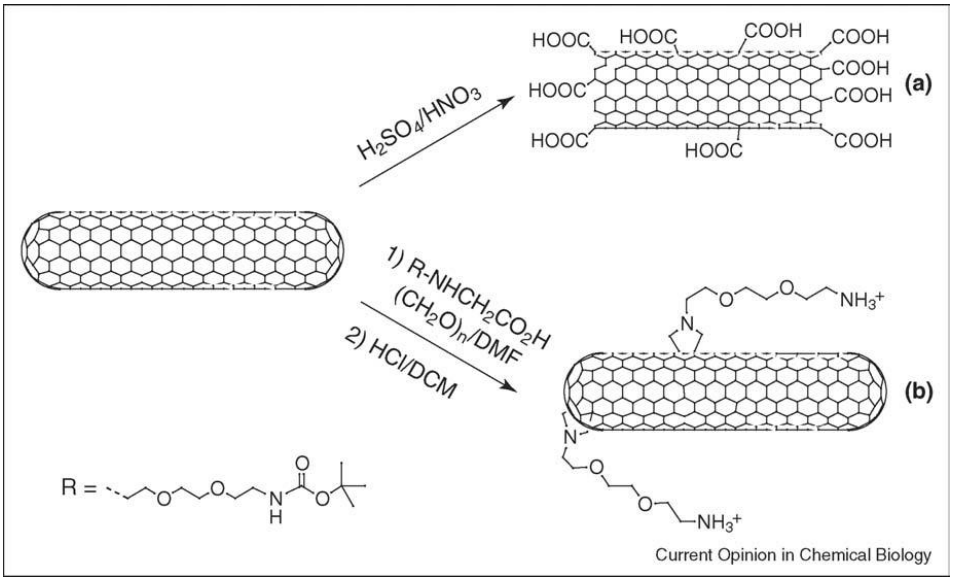

Figure 22: Functionalization of carbon nanotubes. Carbons nanotubes can be (a) acid-treated generate carboxylic groups at the terminal parts, or (b) reacted with amino acid derivatives and aldehydes (Bianco et al. 2005).

The MWCNTs were functionalized (fMWCNTs) by attaching carboxyl groups (-COOH) to their surfaces using acidic solutions (Park et al. 2010). A mixture of H_2SO_4:HNO_3=3:1 (v:v) were added to the raw MWCNTs at room temperature (Osorio et al. 2008). The mixture was bath sonicated for 24h, followed by vacuum filtration through 0.22 μm Millipore Teflon membrane (JGWP04700). Then, the membrane was thoroughly washed using deionized (DI) water, and was immersed in DI water according to established protocols (Kim et al. 2005). The MWCNTs were then dried overnight in the oven at 60℃.

5.5.3 Characterization of MWCNTs

MWCNTs were characterized using Energy-Filtering transmission electron microscopy (EF-TEM: LIBRA 120, Carl Zeiss, Germany) and field-emission scanning electron microscopy (FE-SEM: S-4800, Hitachi, Japan). These techniques were effective in characterizing the internal structure (diameter and wall number) of MWCNTs. Raman spectra were taken to determine the diameter distribution using LabRam Aramis (Horiba Jobin-Yvon, France).

The metal components in the MWCNTs are less than 5% in weight. They have been analyzed

by the manufacturer (Hanwha Nanotech, Republic of Korea) and are aluminum, iron, and molybdenum.

5.5.4 Soil incubation

The soil was divided into plastic self-draining pots containing 200 g soil each, to give 3 replicates to be exposed to each concentration of raw MWCNTs or fMWCNTs. The concentrations of MWCNTs applied to soil were 0 (DI water only), 50, 500, and 5000 µg/g soil. The soils were then well mixed to ensure homogenity before incubation in a BOD incubator at 25℃ for 8 weeks. The pots were not covered to allow free gas exchange to the soil microbial community. The positions of replicate pots of different treatments were randomized and randomly interchanged each week. Soil moisture was adjusted to 60% water holding capacity. Soil moisture content was maintained by weighing the pots twice a week and adjusting to initial weight by regular addition of DI water. Samples of 3 g of soil were collected from each pot, at different time points (0, 2, and 8 weeks), to be used for DNA extraction.

5.5.5 DNA extraction and sequencing

The soil DNA was extracted from 0.3 g of the mixed 3 g sample of soil, using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the protocol described by the manufacturer. DNA isolated from each sample was amplified using primers 338F (5=-XXXXXXXXGTACTCCTACGGGAGGCAGCAG-3=) and 533R (5=TTACCGCGGCTGCTGGCAC-3=), targeting the V3 hypervariable regions of the bacterial 16S rRNA gene (the X sequence denotes a barcode sequence) (Huse et al. 2008). The Polymerase chain reactions (PCR) were carried out under the following thermal profile: denaturation at 94℃ for 2 min, followed by 25 cycles of amplification at 94℃ for 30 s, 57℃ for 30 s and 72℃ for 30 s, followed by a final extension of 72℃ for 5 min. PCR products were analyzed by electrophoresis in 1% agarose gels and were purified using Wizard SV Gel and PCR Clean-up System (Promega, USA). The paired-end sequencing was performed at Kim lab incorporation (Yonsei University, Seoul), using a paired 150-bp HiSeq 2000

sequencing system (Illumina) according to the manufacturer's instructions. Library preparation, sequencing and initial quality filtering were performed as described previously (Zhou et al. 2011).

5.5.6 Quantitative PCR analysis

Relative abundance of bacterial subunit rRNA gene copies was quantified using quantitative PCR (qPCR). Standard curves were created using a 6-fold serial dilution (10^{-2} to 10^{-7}) of a plasmid containing a full-length copy of the *Escherichia coli* 16S rRNA gene, to estimate bacterial relative abundance. qPCR assays were conducted in 48-well plates. Each 10 µl reaction contained 5 µl of reaction mixture (2X Real-Time PCR Smart mix), 0.5 µl of forward and reverse primers (Eub 338 and Eub 518), and DNA-free water. PCR conditions were 2 min at 50℃, and 15 min at 95℃, followed by 40 cycles of 95℃ for 60 s, 53℃ for 30 s and 72℃ for 45 s. Melting curve analyses was performed to confirm that the amplified products were of the appropriate size. Each plate included triplicate reactions per DNA sample.

5.5.7 Data analysis

The sequenced data were processed using the mothur platform (Schloss et al. 2009). Illumina sequencing data was pair-assembled using pandaseq (Masella et al. 2012) with an assembly quality score of 0.9, which is the most stringent option to reduce errors. Next, the sequences were aligned against the EzTaxon-aligned reference (Chun et al. 2007). Sequences were denoised using the '*pre.cluster*' command in mothur, which applies a pseudo-single linkage algorithm with the goal of removing sequences that are likely due to pyrosequencing errors (Huse et al. 2010). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mother (Schloss et al. 2011). The taxonomic classification was performed using mothur's version of the RDP Bayesian classifier, using EzTaxon-e database for each sequence at 80% Naïve Bayesian bootstrap cutoff with 1000 iterations.

5.5.8 Statistical analysis

To perform the statistical analysis, all samples were standardized by random subsampling to 4,073 sequences per sample using the sub.sample command (http://www.mothur.org/wiki/Sub.sample) in mother. To assess the relationship between soil bacteria richness/diversity and CNTs concentration, as well as with time incubation, the number of OTUs and other diversity indices were calculated using mothur's platform (Schloss et al. 2009).

To test the effect of CNTs on bacterial community, we performed an analysis of similarity (ANOSIM) with pairwise Bray–Curtis distance as response variable and CNTs concentration, time incubation, and CNTs form as factors. We used a non-metric multidimensional scaling plot (NMDS) using the Bray-Curtis dissimilarities in Primer 6 to visualize the clustering of bacterial community composition. To test the effect of MWCNTs on bacterial community, we performed an analysis of similarity (ANOSIM) with pairwise Bray-Curtis distance as the response variable and MWCNTs concentration, time incubation and form as factors. We used a non-metric multidimensional scaling plot (NMDS) using the weighted UniFrac distance to visualize the clustering of bacterial community composition. We performed multiple regression analysis in R software package 2.15.2 using linear model (LM) for normal data, and a generalized linear model (GLM) for non-normal data to compare the relative abundance of detected phyla among different treatments, the richness and the diversity with MWCNTs concentrations and incubation time. To test whether bacterial abundance (qPCR) is correlated with fMWCNTs and raw MWCNTs across different sampling time, we performed regression analysis using linear functions in SigmaPlot.

5.6 Results

A total of 2,568,331 quality bacterial sequences were obtained from the 63 samples, with an average of 40,767 sequences per soil sample and with coverage ranging from 4,760 to 219,775 reads per sample.

5.6.1 MWCNTs characterization

The characterization of MWCNTs based on FE-SEM (Figure 23A) and EF-TEM (Figures 23B, 23C) images showed that the average diameter was around 13.4 nm and the number of walls was 11 in average. The size of CNTs is an important factor in toxicological studies. In fact, the interactions between carbon nanotubes and living cells decreased with the size increase (Kang et al. 2008).

Raman spectrum showed that the D-band/G-band ratio was approximately 1.305 and this result showed that defects have been generated from pristine MWCNT which D-band/G-band ratio is 1.087 (Figure 23D).

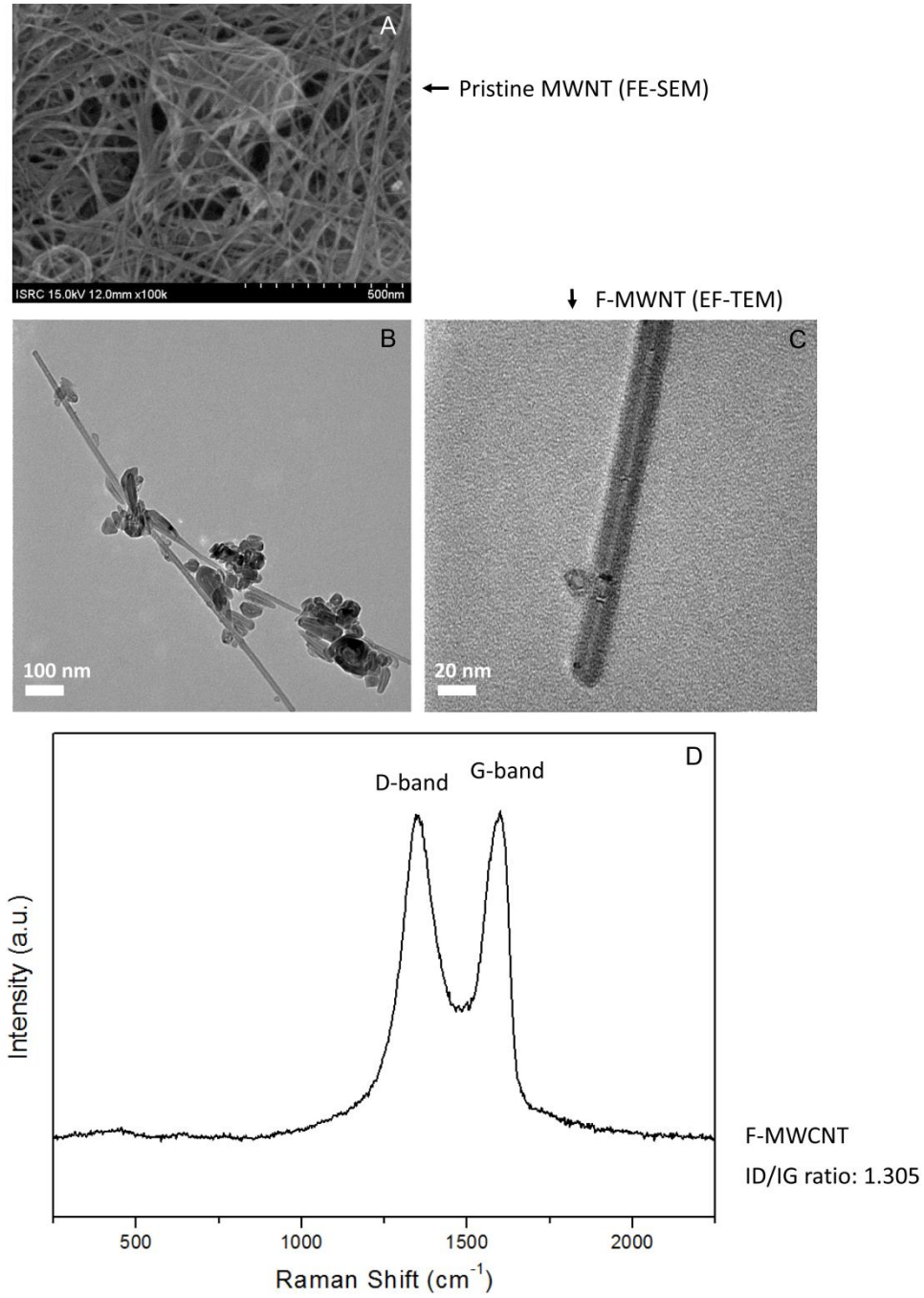

Figure 23: Characterization of multi-walled carbon nanotubes (MWCNTs) used in the study. (A) Field-emission scanning electron microscope (FE-SEM) image of the MWCNTs. (B,C) Energy-filtering transmission electron microscope (EF-TEM) images of the MWCNTs. (D) Raman spectrum of the MWCNTs.

5.6.2 Effect of MWCNTs on bacterial community diversity

The effect of multi-walled carbon nanotubes (MWCNTs) on bacterial community diversity and richness was evaluated using diversity indices. Bacterial richness (i.e. number of OTUs) showed a marginal difference among different treatments ($F_{1,63} = 4.03$, $P = 0.04$). Among the different α-diversity indices, only Chao and Ace indices were marginally affected by MWCNTs between the different treatments (Chao $F_{1,63} = 4.02$, $P = 0.04$; Ace, $F_{1,63} = 4.53$, $P = 0.03$; Shannon $X^2(3) = 2.62$, $P = 0.45$; and Simpson, $X^2(3) = 1.71$, $P = 0.63$).

The multiple regression analyses showed that the concentration of fMWCNTs showed a high correlation with OTUs richness and Chao index, whereas raw MWCNTs added to soil did not show any significant effect. In contrast, time was significantly correlated with OTUs richness and diversity for the two MWCNTs forms. Considering time and MWCNTs concentration together, there was an important correlation for OTUs richness and diversity indices, but only for acid-treated MWCNTs (Table 6).

5.6.3 Effect of MWCNTs on bacterial community composition

OTUs community composition did, however, differ between different concentrations of fMWCNTs but not of raw MWCNTs for Bray-Curtis dissimilarities (fMWCNTs, $R = 0.24$, $P = 0.001$). NMDS plots showed a clustering of soil samples according to MWCNTs concentrations; nevertheless samples from soil with highest concentration of fMWCNTs showed a greater dissimilarity in their bacterial community composition compared to soil treated with raw MWCNTs (Figure 24).

Considering the variation of the bacterial community over time, the NMDS plot showed a clustering of the soils sampled at different sampling time according to different MWCNTs concentrations used in this experiment for fMWCNTs, but not for raw MWCNTs (Figure 25); an ANOSIM test confirmed this result as indicated by the test results (Table 7).

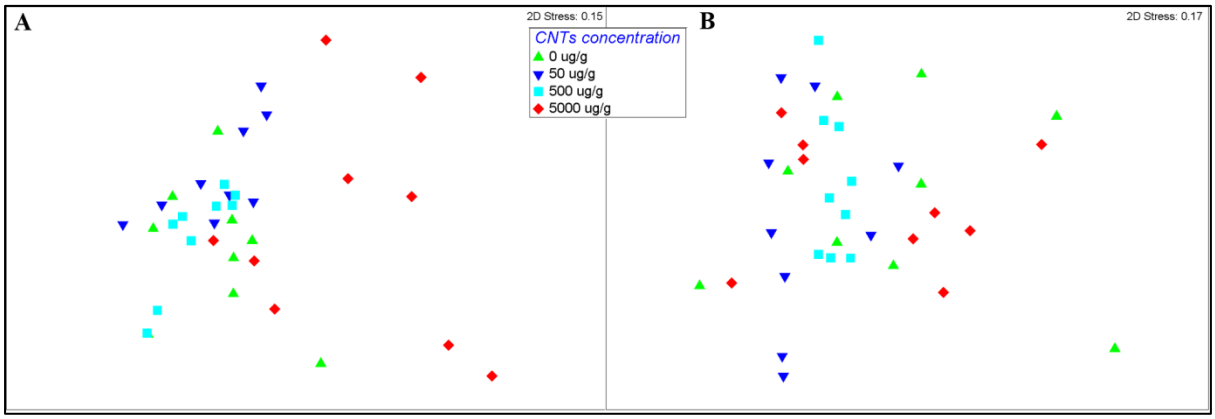

Figure 24: NMDS of Bray-Curtis Index of bacterial community composition in relation to (A) fMWCNTs and (B) raw MWCNTs concentrations applied to soil among the different treatments.

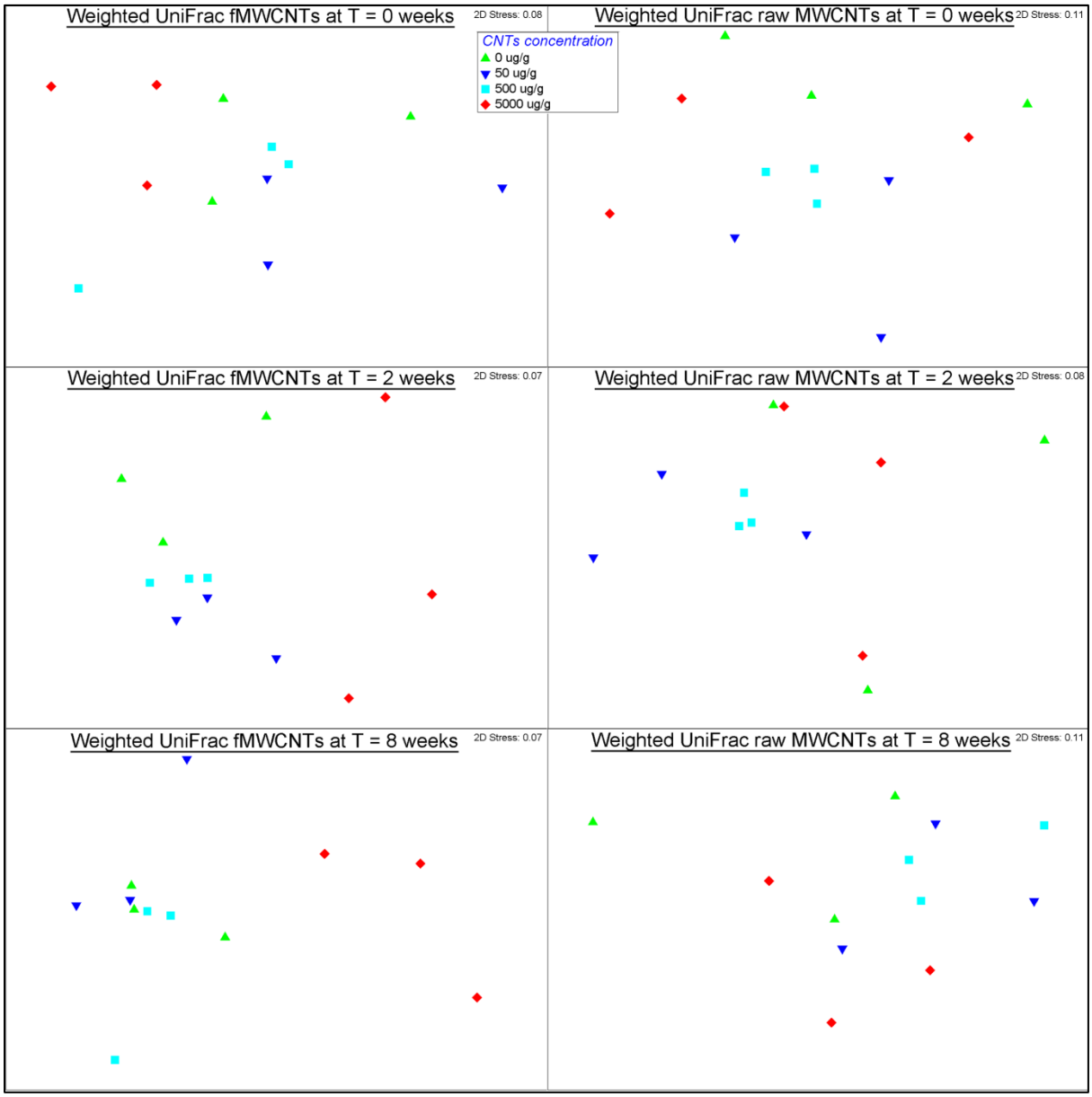

Figure 25: NMDS of weighted UniFrac indices of bacterial community composition in relation to MWCNTs concentrations applied to soil over time (at T = 0 weeks, 2 weeks, and 8 weeks).

Table 6: Multiple regression between richness (OTUs) and diversity indices with CNTs concentrations and incubation time for both acid treated (fMWCNTs) and raw MWCNTs.

fMWCNTs		OTUs (R^2=0.54***)	Shannon (R^2=0.53***)	Simpson (R^2=0.58***)	Chao (R^2=0.52***)
	Intercept	1.745e^{+03***}	6.5520***	7.585e^{-02***}	6086.9***
	Time	-4.144e^{+01***}	-5.808e^{-02**}	3.330e^{-03**}	-229.7***
	CNTs conc	-1.599e^{-02}	-2.563e^{-05}	7.721e^{-07}	-0.1159
	Time*CNTs conc	-8.115e^{-03}	-1.394e^{-05*}	1.460e^{-06**}	-0.0195
Raw MWCNTs		OUTs (R^2=0.83***)	Shannon (R^2=0.86***)	Simpson (R^2=0.76***)	Chao (R^2=0.75***)
	Intercept	1.815e^{+03***}	6.6820***	-5.4760***	6.390e^{+03***}
	Time	-6.114e^{+01***}	-8.951e^{-02***}	1.283e^{-01***}	-3.197e^{+02***}
	CNTs conc	-8.303e^{-04}	1.250e^{-05}	-4.735e^{-05}	-1.071e^{-01}
	Time*CNTs conc	-1.711e^{-03}	-3.182e^{-06}	1.027e^{-05}	-6.929e^{-03}

Significance level is shown at $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$.

Table 7: ANOSIM results for weighted UniFrac dissimilarity over time.

Form of MWCNTs	0 weeks		2 weeks		8 weeks	
	R value	P value	R value	P value	R value	P value
fMWCNTs	0.21	0.015	0.66	0.006	0.72	0.003
Raw MWCNTs	0.10	0.07	-0.015	0.63	0.27	0.3

5.6.4 Effect of MWCNTs on bacterial community abundance

Overall, the most abundant phyla were *Proteobacteria* with 29% of the sequences, followed by *Acidobacteria* with around 20%, *Actinobacteria* (15%), and to a lesser degree, *Chloroflexi* (9%), *Bacteroidetes* (7%), *Gemmatimonadetes* (3%), *TM7* (3%), and less than 2% for *Planctomycetes*, *Armatimonadetes*, *Cyanobacteria*, *Nitrospirae* and *Verrucomicrobia*; around 4% of the sequences were unclassified.

Of the most abundant phyla, we found significant differences in relative abundance between different concentrations of fMWCNTs except for *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, and *Bacteriodetes* (Figure 26). The multiple regression analyses showed that time and fMWCNTs together have an effect on soil bacteria (Table 8). However, the samples treated with raw MWCNTs showed less correlation with both time and concentration for some phyla (Table 8 and Figure 26). Consequently, the fMWCNTs showed a more highly significant change in the relative abundance of the dominant detected phyla.

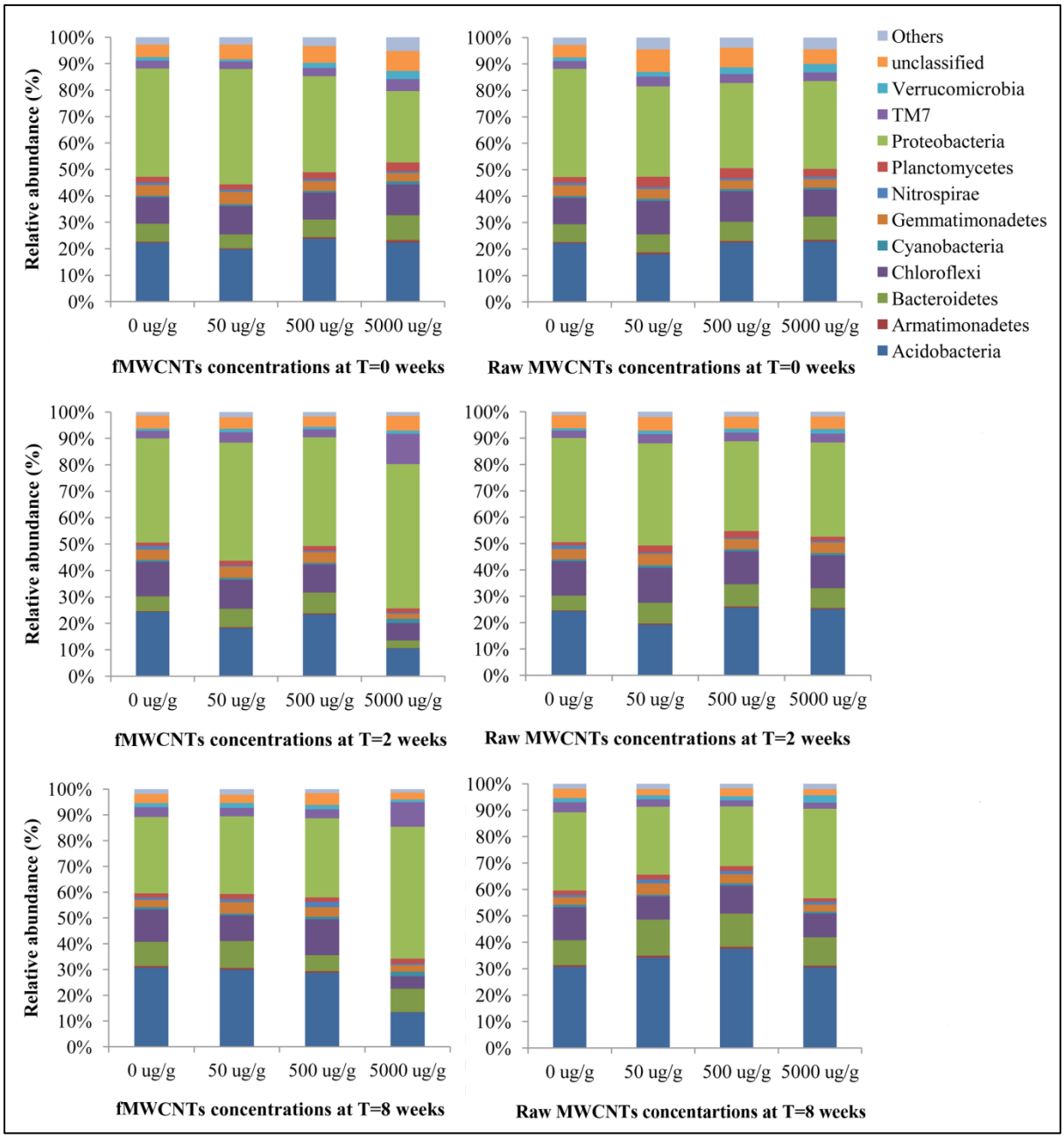

Figure 26: Bacterial composition of the most abundant phyla among different concentrations of fMWCNTs and raw MWCNTs over time (at T = 0 weeks, 2 weeks and 8 weeks).

Table 8: Multiple regression between the relative abundance of the dominant bacterial phyla and with the concentration and incubation time of fMWCNTs and raw MWCNTs.

fMWCNTs		*Acidobacteria* (R^2=0.51***)	*Proteobacteria* (R^2=0.46***)	*Actinobacteria* (R^2=0.20*)	*Bacteroidetes* (R^2=0.06)	*Chloroflexi* (R^2=0.55***)
	Intercept	17.2028***	34.8423***	17.2211***	5.0180***	8.7270***
	Time	1.1080**	-1.1033***	-0.6582	3.304e^{-01*}	2.502e^{-01*}
	CNTs conc	-0.0005	-0.0019***	0.0009	7.732e^{-05}	-1.746e^{-04}
	Time*CNTs conc	-0.0003**	0.0001	0.0001	-5.961e^{-05}	-1.706e-^{04***}
Raw MWCNTs		*Acidobacteria* (R^2=0.58***)	*Proteobacteria* (R^2=0.31**)	*Actinobacteria* (R^2=0.40***)	*Bacteroidetes* (R^2=0.50***)	*Chloroflexi* (R^2=0.01)
	Intercept	4.0410***	3.093e^{+01***}	1.733e^{+01***}	5.1920***	9.9320***
	Time	1.802e^{-01***}	-9.371e-01***	-9.190e^{-01***}	6.627e^{-01***}	-2.775e^{-02}
	CNTs conc	7.403e^{-05}	-5.010e^{-04}	-5.049e^{-04}	3.126e^{-04}	-7.906e^{-05}
	Time*CNTs conc	-1.699e^{-05}	2.102e^{-04*}	1.245e^{-04}	-7.143e^{-05}	-3.151e^{-05}

Significance level is shown at $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$.

Changes in the relative abundance of the predominant bacterial genera in response to exposure to fMWCNTs and raw MWCNTs are illustrated in Figure 27 using a heat map. The most abundant OTU across the various soil replicates/treatments was classified under the genus *Blastocatella* (Acidobacteria) represented by 5.6% of the total reads. This genus significantly decreased in abundance in the highest treatments with fMWCNTs at 2 weeks, but later in the experiment its abundance increased by the final time of sampling at 8 weeks. However, raw MWCNTs did not have any detectable effect on OTU relative abundances. Overall, even fMWCNTs did not have profound effects on the soil bacterial community, even though effects were detectable. Shifts in bacterial abundance were observed only for fMWCNTs with the greatest changes observed at highest concentrations (5000 µg/g).

For all the qPCR assays, there was a linear relationship between the log of the plasmid DNA copy number and the calculated threshold cycle value across the different concentration range ($R^2 > 94$ in all cases). The bacterial abundance, as determined using qPCR, did not show any correlation with fMWCNTs or raw MWCNTs across different concentrations.

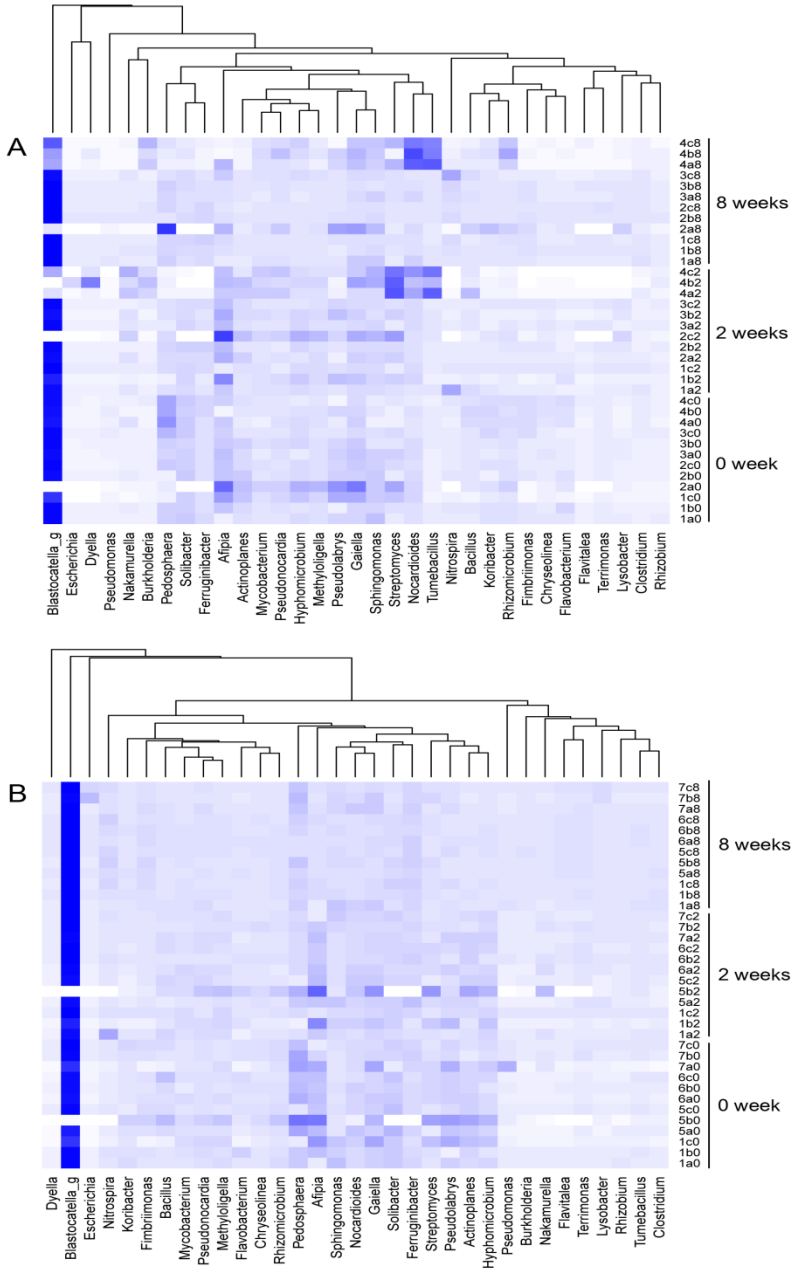

Figure 27: The heat map showing the relative abundances of the most abundant genera at different MWCNTs concentrations applied to soil over time (at T = 0 weeks, 2 weeks, and 8 weeks). The numbers written on Y-axis indicate the concentration of MWCNTs (1: 0 µg/g, 2: 50 µg/g, 3: 500 µg/g, 4: 5000 µg/g of fMWCNTs and 5: 50 µg/g, 6: 500 µg/g, 7: 5000 µg/g of raw MWCNTs), and the letters indicate the replicates.

5.7 Discussion

In our study, we had predicted that MWCNTs would significantly alter the soil bacterial community and its diversity. Yet during the two month-long experiment, we found no differences in diversity as a result of exposure to either raw or fMWCNTs. Nevertheless, fMWCNTs showed an effect on bacterial community composition detected using NMDS. Raw MWCNTs did not show any effect on bacterial composition.

However, a taxonomic breakdown of the reads obtained showed that significant effects on the relative abundance of some major bacterial taxa were present, even though these did not clearly show up on the NMDS. Most of these effects were seen only for the fMWCNTs, not the raw MWCNTs. The effect was also only evident for the highest fMWCNT concentration. Changes in the structure and abundance of the soil bacterial community in response to MWCNTs exposure have been observed in previous studies (Goyal et al. 2010, Shrestha et al. 2013). Our results showed that soils treated with fMWCNTs exhibited shifts in bacterial community abundance for different MWCNTs concentrations at both of the sampling times (2 weeks and 8 weeks). The relative abundance of the most common bacterial phyla was affected by the presence of fMWCNTs and showed different responses to exposure of fMWCNTs. The abundance of *Proteobacteria*, and *TM7* was comparatively higher in the highest fMWCNTs treatment, while there was decrease in *Chloroflexi* at the highest concentration of fMWCNTs. *Acidobacteria*, *Bacteriodetes* and *Gemmatimonadetes* showed a decrease at highest concentration of fMWCNTs then increased over time (8 weeks), whereas *Actinobacteria* increased at highest concentration of fMWCNTs, then later decreased.

These findings suggest that the exposure of soil to fMWCNTs could in fact impact carbon cycling by altering the microbial community (Rodrigues et al. 2010). *Actinobacteria* are important in the biogeochemical cycle of carbon in soils (Bradford et al. 2002, Zhou et al. 2004). In particular, they play a major role in the degradation of cellulolytic and hemicellulolytic compounds in soils (Freeman et al. 2009, Yousuf et al. 2012). *Acidobacteria* are ubiquitous and among the most abundant bacterial phyla in soil (Janssen 2006): their relative abundance is generally negatively correlated with soil carbon availability (Fierer et al. 2007). The *Chloroflexi* are commonly found in soils, also playing an important role in the biogeochemical cycle of carbon and the CO_2 dynamics in soils (Freeman et al. 2009, Yousuf et al. 2012, Yousuf et al. 2012). Such changes in bacterial abundance may be explained in

terms of a shift of microbial community towards bacterial species that are more tolerant of the effects of fMWCNTs and the decline of less tolerant species (Shi et al. 2002, Vivas et al. 2008, Yergeau et al. 2012).

Despite the evident effects of exposure to fMWCNTs, overall it appears from our experiment that the soil bacterial community is quite resilient to the environmental perturbation caused by high concentrations of fMWCNTs. The community largely recovers from exposure to fMWCNTs by 8 weeks. It also appears that the soil bacterial community is resistant to perturbation from raw MWCNTs, with almost no observed influence on bacterial community.

The observed lack of response to raw CNTs generally matches previous findings for untreated nanotubes and other carbon-based new materials (Nannipieri et al. 2003, Johansen et al. 2008). For instance Khodakovskaya et al. (2013), observed no impact on soil bacterial diversity of MWCNTs added by watering into soil at concentrations up to 200 g/ml. Other studies on the impact of a carbon-based nanomaterial, C60 fullerene by Chung et al. (2011) and Tong et al. (2007) demonstrated no effect of toxicity on soil bacterial diversity even at 1000 mg/kg concentration.

However, some studies have shown toxin-like effects of raw CNTs in cultures. Rodrigues et al. (2012) found that raw CNTs can negatively affect soil bacterial diversity. In fact, they observed a major effect of single-walled carbon nanotubes on the soil bacterial community after only 3 days of exposure, and then bacterial diversity recovered after 14 days' of exposure. If this is the case, the lack of an observed effect from raw CNTs after 2 weeks may be due to the system having already recovered from an initial perturbation. MWCNTs are chemically extremely inert, especially in relation to biological processes (Park et al. 2010), and this could be one of the possible reasons that we did not find any effect of MWCNTs on soil bacterial communities.

Why do fMWCNTs cause a shift in the soil bacterial community? fMWCNTs are acidic in nature: pure fMWCNTs after thorough washing have a pH of around 3, due to carboxyl groups that cover their surface. Our measurements of soil pH showed that pH was around 4 at 2 weeks for the highest fMWCNTs concentration, two units lower than the control without nanotubes, and around 5.5 at 8 weeks. There is abundant evidence that pH is crucial to bacterial community structure (Fierer and Jackson 2006, Lauber et al. 2008, Rousk et al.

2010); in fact, pH seems to be the strongest factor of all in structuring soil bacterial communities on a global scale (Hartman et al. 2008, Tripathi et al. 2012). Thus, it is no surprise that fMWCNTs caused significant changes in soil bacterial communities. One might hypothesize that fMWCNTs effects might decrease or even disappear on a time scale of months as their acidity becomes neutralized. Longer-term studies are warranted to confirm if this is indeed the case. Aside from the overall bacterial community, it will also be important to examine effects on biogeochemical processes, such as carbon and nitrogen cycling, in fMWCNTs contaminated soils.

Further detailed investigations and longer exposure period on the effects of multi-walled carbon nanotubes are needed to determine if these changes are permanent beyond the incubation time (8 weeks in the present study), and if there will be changes in soil metabolic capacity and soil organic matter dynamics.

5.8 Conclusion

The overall picture is rather weak but there are still detectable responses from fMWCNTs, combined with the lack of any clear observable effects from the raw MWCNTs. This gives a generally reassuring picture in terms of the effects of MWCNTs on the soil environment. Even though high concentrations were used here, MWCNTs apparently did not have profound effects on soil bacterial communities.

GENERAL CONCLUSIONS

Human actions on the environment are likely to cause significant shifts in microbial communities. As with larger organisms, the extent of this effect will depend on the dimension and magnitude of the changes occurring (Tilman and Lehman 2001).

In the present work, three different case studies were carried out to investigate the consequences of human disturbance in the environment. The results suggest that human activities drive changes in microbial community composition and diversity. The first study case here shows that logging the expansion of agricultural land (oil palm plantations in our study) in tropical forests resulted in a shift in microbial communities. This study allows us to conclude that the conversion of tropical forest to oil palm leads to a change in the fungal community composition and a decrease in fungal diversity. Moreover, logging produced significant changes in fungal community composition, lasting for decades after the logging event itself occurred. Thus, conservation strategies may be necessary to protect the fungal biodiversity of unlogged and logged forests. Our work was conducted in only one geographic region and on only one form of agriculture. Thus, long-term studies will be necessary to elucidate if the changes in soil fungal community composition observed here between forests and oil palm plantations are an intermediate disturbance effect or a more permanent one. Future studies should also focus on functional changes associated with changes in soil microbial communities.

A significant alteration of marine sediment bacterial communities was detected as CO_2 concentrations increased due to anthropogenic CO_2 emissions. Ocean acidification led to changes in bacterial community of the ocean sediments. In fact, marine species which may be especially vulnerable have little possibility to adapt, but some species which may exist in different forms e.g. with and without carbonate shells may shift towards communities more tolerant to this disturbance. Ecosystems are likely to change but in yet unpredictable ways. Marine sediment bacterial community composition showed significant changes with increasing CO_2, and bacterial diversity increased CO_2 enriched sites. These shifts could be explained by the possibility that the biogeochemical functions of marine sediments could also be significantly affected by

ocean acidification.

Ocean acidification is fundamentally irreversible phenomenon. It will take thousands of years for ocean chemistry to return to initial condition (i.e., the ocean chemistry at pre-industrial times which is about 200 years ago). Thus, the only truly effective way to minimize the risk of large-scale and long term changes to the oceans is to reduce atmospheric carbon dioxide emissions. If action is not taken now, ocean acidification may cause widespread disruption to marine ecosystems and a massive decline of biodiversity. Moreover, researchers need to collaborate quickly and make a standardized research protocol to survey the ocean acidification and how oceans around the world are changing. This will lead to make governmental policies to reduce the cause and effects of ocean acidification by promoting for alternative fossil-free energy sources such as wind power.

Finally, concerning the impact of carbon nanotubes on the environment, little is known about the fate and behavior of these synthetic nanomaterials in the ecosystem, and suitable methods to assess their risks are still in the development stage. Here, in the present study case, soils treated with functionalized carbon nanotubes exhibited changes in bacterial community. This change is explained by the shift of microbial community to species more tolerant to pollution by nanoparticles. In spite of the evident effects of exposure to functionalized carbon nanotubes, it appears that that microbial community could recover over time and that the soil is resistant to the environmental perturbation caused by carbon nanotubes and especially perturbations caused by non-treated carbon nanotubes where almost no impact on microbial community was observed. Functionalized carbon nanotubes are more toxic to the environment than raw carbon nanotubes because of their acidic nature. In fact, pH seems to be one of the most important factors in structuring soil microbial communities. This is why observed significant changes in soil microorganisms caused by exposure to functionalized carbon nanotubes are not surprising.

Further investigations and longer exposure period on the impact of carbon nanotubes in the environment are needed to assess their potential risk when these nonmaterials are in use. Full life cycle evaluation and analysis for all difference applications should be

conducted with constant attention. In addition, long-term studies are required to determine delayed impacts of environmental exposure to nanoparticles and to help find possible adaptive mechanisms. More studies on bioaccumulation, as well as on the interaction of these nanoparticles with other elements in the environment are also needed.

The human disturbance cases treated in this study suggest that the consequences of human activities on Earth are something we face now and something confined to the future. Anthropogenic changes in the environment are likely to cause significant loss of biodiversity, leaving many niches empty and creating communities dominated by tolerant species to disturbance. The study cases described above provide examples of themes and methodologies, which can be used for many future studies to explore human effects on the living world. All these ongoing perturbations are accelerating in many cases. Thus, these changes should be monitored and be taken as a warning of future intensification. By understanding how species are affected by human activities, it may be possible to provide effective and useful management decisions.

References

Abarenkov K, Nilsson RH, Larsson KH, Alexander IJ, Eberhardt U, et al. 2010. The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytologist 186: 281-285.

Acevedo-Whitehouse K and Duffus ALJ .2009. Effects of environmental change on wildlife health. Philos. Philosophical Transactions of the Royal Society B: Biological Sciences 364, 3429–3438

Achard F, Eva HD, Stibig HJ, Mayaux P, Gallego J, et al. 2002. Determination of Deforestation Rates of the World's Humid Tropical Forests. Science 297: 999-1002.

Acinas SG, Marcelino LA, Klepac-Ceraj V, Polz MF. 2004. Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. Journal of Bacteriology 186: 2629–2635.

Altenburger P, Kämpfer P, Schumann P, Vybiral D, Lubitz W, Busse HJ. 2002. Georgenia muralis gen. nov., sp. nov., a novel actinobacterium isolated from a medieval wall painting. International Journal of Systematic Evolutionary Microbiology 52 (Pt 3):875-881

Allgaier M, Riebesell U, Vogt M, Thyrhaug R, Grossart HP. 2008. Coupling of heterotrophic bacteria to phytoplankton bloom development at different pCO_2 levels: a mesocosm study. Biogeosciences 5 (4):1007-1022. doi:10.5194/bg-5-1007-2008

Amann R I, Ludwig W & Schleifer, KH. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology Review 59: 143–169

Andersson AJ, Mackenzie FT and Ver LM. 2003. Solution of shallow-water carbonates: An insignificant buffer against rising atmospheric CO_2. Geology 31(6): 513-516.

Anderson MJ, Crist TO, Chase JM, Vellend M, Inouye BD, et al. 2011. Navigating the multiple meanings of beta diversity: a roadmap for the practicing ecologist. Ecology Letters 14: 19-28.

Ansorge WJ. 2009. Next-generation DNA sequencing techniques. New Biotechnology 25: 195-203

Aird D, Ross MG, Chen WS, Danielsson M, Fennell T, Russ C, Jaffe DB, Nusbaum C, Gnirke A. 2011. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biology 12:R18

Ash et al. 1994, a later subjective synonym of Paenibacillus (formerly Bacillus) larvae (White 1906) Ash et al. 1994, as a subspecies of P. larvae, with emended descriptions of P. larvae as P. larvae subsp. larvae and P. larvae subsp. pulvifaciens. International Journal of Systematic Bacteriology 46 (1):270-279.

Asner GP, Rudel TK, Aide TM, Defries R, Emerson R. 2009. A contemporary assessment of change in humid tropical forests. Conservation Biology 23: 1386-1395.

Ba AM, Duponnois R, Moyersoen B, Diedhiou AG. 2012. Ectomycorrhizal symbiosis of tropical African trees. Mycorrhiza 22: 1-29.

Bai Y, Park IS, Lee SJ, Bae TS, Watari F, et al. 2011. Aqueous dispersion of surfactant-modified multiwalled carbon nanotubes and their application as an antibacterial agent. Carbon 49: 3663-3671.

Baraloto C, Hérault B, Paine C, Massot H, Blanc L, et al. 2012. Contrasting taxonomic and functional responses of a tropical tree community to selective logging. Journal of Applied Ecology 49: 861-870.

Barnes RSK and Hughes RN .1988. An Introduction to Marine Ecology. Blackwell Scientific Publications. 351pp.

Bartlett JMS, Stirling D. 2003. A short history of the polymerase chain reaction. Methods in Molecular Biology 226: 3–6.

Baughman RH, Zakhidov AA, de Heer WA. 2002. Carbon nanotubes - the route toward applications. Science 297: 787-792.

Berry NJ, Phillips OL, Lewis SL, Hill JK, Edwards DP, et al. 2010. The high value of logged tropical forests: lessons from northern Borneo. Biodiversity and Conservation 19: 985-997.

Bianco A, Kostarelos K, Prato M. 2005. Applications of carbon nanotubes in drug delivery. Current Opinion in Chemical Biology. 9:674–679

Biercuk MJ, Llaguno MC, Radosavljevic M, Hyun JK, Johnson AT, et al. 2002. Carbon nanotube composites for thermal management. Applied Physics Letters 80: 2767-2769.

Binnerup S, Ferrari BC, Winsley T, and Gillings, M. 2008. Cultivating previously uncultured soil bacteria using a soil substrate membrane system. Nature Protocols 3: 1261–1269.

Birzele F, Schaub J, Rust W, Clemens C, Baum P, Kaufmann H, Weith A, Schulz TW, Hildebrandt T. 2010. Into the unknown: expression profiling without genome sequence information in CHO by next generation sequencing. Nucleic Acids Research 38:3999-4010.

Blaser J. 2011. Status of tropical forest management .2011. International Tropical Timber Organization.

Boatta F, D'Alessandro W, Gagliano AL, Liotta M, Milazzo M, Rodolfo-Metalpa R, Hall-Spencer JM, Parello F. 2013. Geochemical survey of Levante Bay, Vulcano Island (Italy), a natural laboratory for the study of ocean acidification. Marine Pollution Bulltin:485–494.

Botkin DB, Caswell MF, Estes JE, Orio A.A. 1989. Changing the Global Environment: Perspectives on Human Involvement. Academic Press, New York, 459pp.

Bowman JP, McCuaig RD. 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. Applied Environmental Microbiology 69 (5):2463-2483.

Bradford MA, Jones TH, Bardgett RD, Black HIJ, Boag B, et al. 2002. Impacts of Soil Faunal Community Composition on Model Grassland Ecosystems. Science 298: 615-618.

Brearley FQ. 2012. Ectomycorrhizal Associations of the Dipterocarpaceae. Biotropica 44: 637-648.

Bridge PD, Newsham KK. 2009. Soil fungal community composition at Mars Oasis, a southern maritime Antarctic site, assessed by PCR amplification and cloning. Fungal Ecology 2: 66-74.

Brock T D. 1987. The study of microorganisms in situ: Progress and problems. Symposium of the Society for General Microbiology, 41, 1–17.

Brodie E, Edwards S, Clipson N. 2003. Soil fungal community structure in a temperate upland grassland soil. Fems Microbiology Ecology 45: 105-114.

Buchan A, Newel, SY, Moreta JIL, Moran MA. 2002. Analysis of internal transcribed spacer (ITS) regions of rRNA genes in fungal communities in a Southeastern U.S. Salt Marsh. Microbial Ecology 43: 329-340.

Cai D, Mataraza JM, Qin ZH, Huang Z, Huang J, Chiles TC, Carnahan D, Kempa K, Ren Z. 2005. Highly efficient molecular delivery into mammalian cells using carbon nanotube spearing. Nature

Methods 2:449-454.

Caldeira K, Wickett ME. 2003. Anthropogenic carbon and ocean pH. Nature 425 (6956):365-365.

Cardinale BJ, Palmer MA, Collins SL. 2002. Species diversity enhances ecosystem functioning through interspecific facilitation. Nature 415: 426-429.

Carney KM, Sydeman WJ. 1999. A review of human disturbance effects on nesting colonial waterbirds. Waterbirds 22: 68–79.

Chapin FS, Walker BH, Hobbs RJ, Hooper DU, Lawton JH, Sala OE, Tilman D. 1997. Biotic Control over the Functioning of Ecosystems. Science 277 (5325):500-504. doi:10.1126/science.277.5325.500.

Cherukuri P, Bachilo SM, Litovsky SH, Weisman RB. 2004. Near-infrared fluorescence microscopy of single-walled carbon nanotubes in phagocitic cells. Journal of American Chemical Society 126:15638-15639.

Chung H, Son Y, Yoon TK, Kim S, Kim W. 2011. The effect of multi-walled carbon nanotubes on soil microbial activity. Ecotoxicology and Environmental Safety 74: 569-575.

Chun J, Lee JH, Jung Y, Kim M, Kim S, et al. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 57: 2259-2261.

Cleary DF, Boyle TJ, Setyawati T, Anggraeni CD, Loon EEV, et al. 2007. Bird species and traits associated with logged and unlogged forest in Borneo. Ecological Applications 17: 1184-1197.

Cornejo FH, Varela A, Wright SJ. 1994. Tropical Forest Litter Decomposition under Seasonal Drought: Nutrient Release, Fungi and Bacteria. Oikos 70: 183-190.

Costerton B.2004. Microbial ecology comes of age and joins the general ecology community. Proceedings of the National Academy of Sciences 101, 16983–16984.

Courtois S. et al. 2003. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Applied Environmental Microbiology 69: 49–55.

Daniel R. 2005. The metagenomics of soil. Nature Reviews. Microbiology 3:470-478.

Dean DA, Strong DD, Zimmer WE. 2005. Nuclear entry of nonviral vectors. Gene Therapy 12:881-890.

Derycke V, Martel R, Appenzeller J, Avouris P. 2001. Carbon nanotube inter- and intramolecular logic gates. Nano Letters 1: 453-456.

Dias BB, Hart B, Smart CW, Hall-Spencer JM. 2010. Modern seawater acidification: the response of foraminifera to high-CO_2 conditions in the Mediterranean Sea. Journal of the Geological Society London 167 (5):843-846. doi:Doi 10.1144/0016-76492010-050.

Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, Brulc JM, Furlan M, Desnues C et al. 2008. Functional metagenomic profiling of nine biomes. Nature 452, 629–632.

Dresselhaus MS, Dresselhaus G, Eklund PC. 1996. Science of fullerenes and carbon nanotubes. New York: Academic Press.

Dong LF, Witkowski CM, Craig MM, Greenwade MM, Joseph KL. 2009. Cytotoxicity Effects of Different Surfactant Molecules Conjugated to Carbon Nanotubes on Human Astrocytoma Cells. Nanoscale Research Letters 4: 1517-1523.

Doolittle W. 1999. Phylogenic classification and the universal tree. Science 284, 2124–2128.

Dupré J and O′Malley MO'. 2007. Metagenomics and biological ontology. Studies in History and Philosophy of Biology and Biomedical Science 38:834–846.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460-2461.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194-2200.

Edwrds J and Rykiel JR. 1985. Towards a definition of ecological disturbance. Australian Journal of Ecology 10:361-365.

Edwards DP, Hodgson JA, Hamer KC, Mitchell SL, Ahmad AH, et al. 2010. Wildlife-friendly oil palm plantations fail to protect biodiversity effectively. Conservation Letters 3: 236-242.

Edwards DP, Larsen TH, Docherty TD, Ansell FA, Hsu WW, et al. 2011. Degraded lands worth protecting: the biological importance of Southeast Asia's repeatedly logged forests. Proceeding of the Royal Society B: Biological Sciences 278: 82-90.

Edwards F, Edwards D, Larsen T, Hsu W, Benedick S, et al. 2013. Does logging and forest conversion to oil palm agriculture alter functional diversity in a biodiversity hotspot? Animal Conservation 17(2): 163-173.

Elmqvist T, Folke C, Nystrom M, Peterson G, Bengtsson J, et al. 2003. Response diversity, ecosystem change, and resilience. Frontiers in Ecology and the Environment 1: 488-494.

Fabricius KE, Langdon C, Uthicke S, Humphrey C, Noonan S, De'ath G, Okazaki R, Muehllehner N, Glas MS, Lough JM. 2011. Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. Nature Climate Change 1 (3):165-169. doi:Doi 10.1038/Nclimate1122.

Farmer JJ, III, Hickman-Brenner FW. 2006. The Genera Vibrio and Photobacterium. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The Prokaryotes. Springer New York, pp 508-563. doi:10.1007/0-387-30746-x_18.

Fenchel T M & Riedl R J (1970). The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. Marine Biology 7, 255–268.

Ferrari M. 2005. Cancer nanotechnology: opportunities and challenges. Nature Reviews Cancer 5:161-171

Fierer N, Bradford MA, Jackson RB. 2007. Toward an ecological classification of soil bacteria. Ecology 88 (6):1354-1364.

Fierer N, Jackson RB. 2006. The diversity and biogeography of soil bacterial communities. Proceeding of the National Academy of Sciences of the United States of America 103: 626-631.

Firme III CP, Bandaru PR. 2010. Toxicity issues in the application of carbon nanotubes to biological systems. Nanomedicine: Nanotechnology, Biology and Medicine 6: 245-256.

Fisher B, Edwards DP, Giam XL, Wilcove DS. 2011. The high costs of conserving Southeast Asia's lowland rainforests. Frontiers in Ecology and the Environment 9: 329-334.

Food and Agriculture Organization of the United Nations. 2010. Global forest resources assessment

2010 : main report. Rome: FAO. 340 pp.

Fracetto GGM, Azevedo LCB, Fracetto FJC, Andreote FD, Lambais MR, et al. 2013. Impact of Amazon land use on the community of soil fungi. Scientia Agricola 70: 59-67.

Freeman KR, Pescador MY, Reed SC, Costello EK, Robeson MS, et al. 2009. Soil CO_2 flux and photoautotrophic community composition in high-elevation, 'barren' soil. Environmental Microbiology 11: 674-686.

French SS, DeNardo DF, Greives TJ, Strand CR and Demas GE. 2010. Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (Amblyrhynchus cristatus). Hormones and Behavior 58(5):792-799.

Furukawa, Y. (2001). Biogeochemical consequences of macrofauna burrow ventilation. Geochemical Transactions (supplement). ACS Division of Geochemistry Symposium, Biogeochemical consequences of dynamic interactions between benthic fauna, microbes and aquatic sediments, San Diego, CA.

Gabor E, Liebeton K, Niehaus F, Eck J and Lorenz P. 2007. Updating the metagenomics toolbox. Biotechnology J2, 201–206.

Garbuio FJ, Howard JL, Dos Santos LM. 2012. Impact of Human Activities on Soil Contamination. Applied and Environmental Soil Science. doi:10.1155/2012/619548.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Mol Ecol 2: 113-118.

Geml J, Laursen GA, Timling I, McFarland JM, Booth MG, et al. 2009. Molecular phylogenetic biodiversity assessment of arctic and boreal ectomycorrhizal Lactarius Pers. (Russulales; Basidiomycota) in Alaska, based on soil and sporocarp DNA. Molecular Ecology 18: 2213-2227.

Geml J, Laursen GA, Herriott IC, McFarland JM, Booth MG, et al. 2010. Phylogenetic and ecological analyses of soil and sporocarp DNA sequences reveal high diversity and strong habitat partitioning in the boreal ectomycorrhizal genus Russula (Russulales; Basidiomycota). New Phytologist 187: 494-507.

Georgakilas V, Tagmatarchis N, Pantarotto D, Bianco A, Briand JP, Prato M. 2002. Amino acid functionalisation of water soluble carbon nanotubes. Chemical Communications (Cambridge) : 3050-3051.

Ghafari P, St-Denis CH, Power ME, Jin X, Tsou V, et al. 2008. Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa. Nature Nanotechnology 3: 347-351.

Ghazoul J. 2002 Impact of logging on the richness and diversity of forest butterflies in a tropical dry forest in Thailand. Biodiversity Conservation 11: 521-541.

Ghosh A, Dey N, Bera A, Tiwari A, Sathyaniranjan K, Chakrabarti K, Chattopadhyay D. 2010. Culture independent molecular analysis of bacterial communities in the mangrove sediment of Sundarban, India. Saline systems 6 (1):1. doi:10.1186/1746-1448-6-1.

Gibbs HK, Ruesch AS, Achard F, Clayton MK, Holmgren P, et al. 2010. Tropical forests were the primary sources of new agricultural land in the 1980s and 1990s. Proceeding of National Academy of Sciences of the United States of America 107: 16732-16737.

Gibson L, Lee TM, Koh LP, Brook BW, Gardner TA, et al. 2011. Primary forests are irreplaceable for sustaining tropical biodiversity. Nature 478: 378-381.

Gomes NC, Fagbola O, Costa R, Rumjanek NG, Buchner A, et al. 2003. Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. Applied Environmental Microbiology 69: 3758-3766.

Goyal D, Zhang X, Rooney-Varga J. 2010. Impacts of single-walled carbon nanotubes on microbial community structure in activated sludge. Letters in applied microbiology 51: 428-435.

Gray MW, Burger G, Lang BF. 1999. Mitochondrial evolution. Science 283: 1476–1481.

Hamer K, Hill J, Benedick S, Mustaffa N, Sherratt T, et al. 2003. Ecology of butterflies in natural and selectively logged forests of northern Borneo: the importance of habitat heterogeneity. Journal of Applied Ecology 40: 150-162.

Hammond, S., Swanberg, J.C., Kaplarevic, M. and Lee, K.H. 2011. Genomic sequencing and analysis of a Chinese hamster ovary cell line using Illumina sequencing technology. BMC Genomics 12:67

Handelsman J, Rondon MR, Brady SF, Clardy J, and Goodman RM. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemistry and Biology 5: 245–249.

Han L, Wu W, Kirk FL, Luo J, Maye MM, et al. 2004. A direct route toward assembly of nanoparticle-carbon nanotube composite materials. Langmuir 20: 6019-6025.

Hansen MC, Stehman SV, Potapov PV, Loveland TR, Townshend JR, et al. 2008. Humid tropical forest clearing from 2000 to 2005 quantified by using multitemporal and multiresolution remotely sensed data. Proceeding of National Academy of Sciences of the United States of America 105: 9439-9444.

Hartman WH, Richardson CJ, Vilgalys R, Bruland GL. 2008. Environmental and anthropogenic controls over bacterial communities in wetland soils. Proceeding of National Academy of Sciences of the United States of America 105: 17842-17847.

Hassink J, Bouwman LA, Zwart KB, Brussaard L. 1993. Relationship between habitable pore space, soil biota, and mineralization rates in grassland soils. Soil Biology and Biochemistry 25: 47–55.

Haug I, Weib M, Homeier J, Oberwinkler F, Kottke I. 2005. Russulaceae and Thelephoraceae form ectomycorrhizas with members of the Nyctaginaceae (Caryophyllales) in the tropical mountain rain forest of southern Ecuador. New Phytologist 165: 923-936.

Head SR, Kiyomi Komori H, Traver Hart J, Shimashita J, Schaffer L, Salomon DR, Ordoukhanian PT. 2011. Method for improved Illumina sequencing library preparation using NuGEN Ovation RNA-Seq System. BioTechniques 50:177-181.

Hendriks IE, Duarte CM, Alvarez M. 2010. Vulnerability of marine biodiversity to ocean acidification: A meta-analysis. Estuar Coast Shelf S 86 (2):157-164.

Heyndrickx M, Vandemeulebroecke K, Hoste B, Janssen P, Kersters K, De Vos P, Logan NA, Ali N, Berkeley RC. 1996. Reclassification of Paenibacillus (formerly Bacillus) pulvifaciens (Nakamura 1984)

Hibbett DS, Ohman A, Glotzer D, Nuhn M, Kirk P, Nilsson RH. 2011. Progress in molecular and morphological taxon discovery in Fungi and options for formal classification of environmental sequences. Fungal Biology Reviews (25): 38–47.

Hoff KJ, Tech M, Lingner T, Daniel R, Morgenstern B. and Meinicke P. 2008. Gene prediction in metagenomic fragments: a large scale machine learning approach. BMC Bioinformatics 9: 217.

Hongxiang X, Min W, Xiaogu W, Junyi Y, Chunsheng W. 2008. Bacterial diversity in deep-sea sediment from northeastern Pacific Ocean. Acta Ecologica Sinica 28 (2):479-485.

doi:http://dx.doi.org/10.1016/S1872-2032(08)60026-8.

Howe RL, Rees AP and Widdicombe S .2004. The impact of two species of bioturbating shrimp (Callianassa subterranea and Upogebia deltaura) on sediment denitrification. Journal of the Marine Biological Association of the United Kingdom 84, 629–632.

Hugenholtz P and Tyson GW. 2008. Metagenomics. Nature 455: 481-483.

Huse SM, Dethlefsen L, Huber JA, Welch DM, Relman DA, et al. 2008. Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. PLoS genetics 4: e1000255.

Huse SM, Welch DM, Morrison HG, Sogin ML. 2010. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environmental Microbiology 12: 1889-1898.

Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki K, Nealson KH, Horikoshi K. 2003. Microbial communities associated with geological horizons in coastal subseafloor sediments from the sea of okhotsk. Applied Environmental Microbiology 69 (12):7224-7235.

Jacob NM, Kantardjieff A, Yusufi FN, Retzel EF, Mulukutla BC, Chuah SH, Yap M, Hu WS. 2010. Reaching the depth of the Chinese hamster ovary cell transcriptome. Biotechnology and Bioengineering 105:1002-1009.

Janssen PH. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Applied Environmental Microbiology 72: 1719-1728.

Jin L, Son Y, Yoon TK, Kang YJ, Kim W, et al. 2012. High concentrations of single-walled carbon nanotubes lower soil enzyme activity and microbial biomass. Ecotoxicology and Environmental Safety 88: 9-15.

Johansen JR, Ashley J, Rayburn WR .1993. Effects of rangefire on soil algal crusts in semiarid shrub-steppe of the lower Columbia Basin and their subsequent recovery. Great Basin Nature 53:73–88

Johansen A, Pedersen AL, Jensen KA, Karlson U, Hansen BM, et al. 2008. Effects of C60 fullerene nanoparticles on soil bacteria and protozoans. Environmental toxicology and chemistry / SETAC 27: 1895-1903.

Johnson VR, Brownlee C, Rickaby REM, Graziano M, Milazzo M, Hall-Spencer JM. 2011. Responses of marine benthic microalgae to elevated CO_2. Marine Biology 1-12. doi:10.1007/s00227-

011-1840-2.

Joint I, Karl DM, Doney SC, Armbrust EV, Balch W, Beman M, Bowler C, Church M, Dickson A, Heidelberg J, Iglesias-Rodriguez D, Kirchman D, Kolber Z, Letelier R, Claudia Lupp C, Maberly S, Park S, Raven J, Repeta DJc), Riebesell U, Steward G, Tortell P, Zeebe RE, JonathanP. Zehr JP .2009. Consequences of high CO2 and ocean acidification for microbes in the global ocean. Report produced by Expert Meeting organized by the Plymouth Marine. Laboratory and the Center for Microbial Oceanography.

Jonasson J, Olofsson M, Monstein HJ. 2002. Classification, identification and subtyping of bacteria based on pyrosequencing and signature matching of 16s rDNA fragments. APMIS 115: 668–677.

Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Applied Environmental Microbiology 69: 7210–7215.

Kaeberlein T, Lewis K, Epstein S. 2002. Isolating ‘‘uncultivable’’ microorganisms in a simulated natural environment. Science 296: 1127–1129.

Kalis A, Merkt J, Wunderlich J. 2003. Environmental changes during the Holocene climatic optimum in central Europe - human impact and natural causes. Quaternary Science Reviews 22: 33–79.

Kang S, Pinault M, Pfefferle LD, Elimelech M. 2007. Single-walled carbon nanotubes exhibit strong antimicrobial activity. Langmuir 23: 8670-8673.

Kang S, Herzberg M, Rodrigues DF, Elimelech M. 2008. Antibacterial effects of carbon nanotubes: size does matter! Langmuir 24: 6409-6413.

Kang S, Mauter MS, Elimelech M. 2009. Microbial cytotoxicity of carbon-based nanomaterials: implications for river water and wastewater effluent. Environmental Sciences and Technology 43: 2648-2653.

Kerrison P, Hall-Spencer JM, Suggett DJ, Hepburn LJ, Steinke M. 2011. Assessment of pH variability at a coastal CO_2 vent for ocean acidification studies. Estuarine, Coastal and Shelf Science 94 (2):129-137. doi:http://dx.doi.org/10.1016/j.ecss.2011.05.025.

Khodakovskaya MV, Kim BS, Kim JN, Alimohammadi M, Dervishi E, et al. 2013. Carbon Nanotubes as Plant Growth Regulators: Effects on Tomato Growth, Reproductive System, and Soil Microbial Community. Small 9: 115-123.

Kim YJ, Shin TS, Choi HD, Kwon JH, Chung YC, et al. 2005. Electrical conductivity of chemically modified multiwalled carbon nanotube/epoxy composites. Carbon 43: 23-30.

Kim B, Chung H, Kim W. 2010. Supergrowth of Aligned Carbon Nanotubes Directly on Carbon Papers and Their Properties as Supercapacitors. Journal of Physics and Chemistry C 114: 15223-15227.

Kitidis V, Laverock B, McNeill LC, Beesley A, Cummings D, Tait K, Osborn MA, Widdicombe S. 2011. Impact of ocean acidification on benthic and water column ammonia oxidation. Geophysics Research Letters 38. doi:Artn L21603 Doi 10.1029/2011gl049095.

Klaine SJ, Alvarez PJ, Batley GE, Fernandes TF, Handy RD, et al. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry 27: 1825-1851.

Köhler AR, Som C, Helland A, Gottschalk F. 2008. Studying the potential release of carbon nanotubes throughout the application life cycle. Journal of Cleaner Production 16: 927-937.

Koleff P, Gaston KJ, Lennon JJ. 2003. Measuring beta diversity for presence-absence data. Journal of Animal Ecology 72: 367-382.

Kolasa J and Pickett STA .1991. Ecological heterogeneity. Springer-Verlag, New York, New York

Krause E, Wichels A, Gimenez L, Lunau M, Schilhabel MB, Gerdts G. 2012. Small Changes in pH Have Direct Effects on Marine Bacterial Community Composition: A Microcosm Approach. Plos One 7 (10): e47035. doi:10.1371/journal.pone.0047035.

Kroeker KJ, Micheli F, Gambi MC. 2013. Ocean acidification causes ecosystem shifts via altered competitive interactions. Nature Climate Change 3 (2):156-159. doi:Doi 10.1038/Nclimate1680.

Kuramae EE, Hillekens RHE, de Hollander M, van der Heijden MGA, van den Berg M, et al. 2013. Structural and functional variation in soil fungal communities associated with litter bags containing maize leaf. Fems Microbiology Ecology 84: 519-531.

Lauber CL, Strickland MS, Bradford MA, Fierer N. 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biology and Biochemistry 40: 2407-2415.

Lee-Cruz L, Edwards DP, Tripathi BM, Adams JM. 2013. Impact of Logging and Forest Conversion to Oil Palm Plantations on Soil Bacterial Communities in Borneo. Applied Environmental Microbiology 79: 7290-7297.

Lidbury I, Johnson V, Hall-Spencer JM, Munn CB, Cunliffe M. 2012. Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent ecosystem. Marine Pollution Bulltin 64 (5):1063-1066. doi:DOI 10.1016/j.marpolbul.2012.02.011.

Liedloff A. 1999. Mantel nonparametric test calculator. Version 2.0. School of Natural Resource Sciences, Queensland University of Technology, Australia.

Likens GE, Driscoll CT, Buso DC, Siccama TG, Johnson CE, Lovett GM, Fahey TJ, Reiners WA, Ryan DF, Martin C W, et al. 1998. Biogeochemistry 41: 89–173.

Lindh MV, Riemann L, Baltar F, Romero-Oliva C, Salomon PS, Graneli E, Pinhassi J. 2013. Consequences of increased temperature and acidification on bacterioplankton community composition during a mesocosm spring bloom in the Baltic Sea. Environmental Microbiology Reports 5 (2):252-262. doi:10.1111/1758-2229.12009.

Liu JW, Weinbauer MG, Maier C, Dai MH, Gattuso JP. 2010. Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. Aquatic Microbial Ecology 61 (3):291-305. doi:Doi 10.3354/Ame01446.

Liu SB, Wei L, Hao L, Fang N, Chang MW, et al. 2009. Sharper and Faster "Nano Darts" Kill More Bacteria: A Study of Antibacterial Activity of Individually Dispersed Pristine Single-Walled Carbon Nanotube. Acs Nano 3: 3891-3902.

Li WJ, Xu P, Schumann P, Zhang YQ, Pukall R, Xu LH, Stackebrandt E, Jiang CL. 2007. Georgenia ruanii sp nov., a novel actinobacterium isolated from forest soil in Yunnan (China), and emended description of the genus Georgenia. International Journal of Systematic and Evolutionary Microbiology 57:1424-1428. doi:DOI 10.1099/ijs.0.64749-0.

Luo D. 2004. A new solution for improving gene delivery. Trends in Biotechnology 22:101-103.

Lupatini M, Jacques RJS, Antoniolli ZI, Suleiman AKA, Fulthorpe RR, et al. 2013. Land-use change and soil type are drivers of fungal and archaeal communities in the Pampa biome. World Journal of Microbiology and Biotechnology 29: 223-233.

Madsen J, Fox AD. 1995. Impacts of hunting disturbance on water birds— a review. Wildlife Biology 1: 193–207.

Madsen EL. 2011. Microorganisms and their roles in fundamental biogeochemical cycles. Current Opinions of Biotechnology 22: 456-464.

Magurran AE. 2004. Measuring biological diversity. Maldan, MA: Blackwell Pub. viii, 256 pp.
Martin F, Cullen D, Hibbett D, Pisabarro A, Spatafora JW, et al. 2011. Sequencing the fungal tree of life. New Phytologist 190: 818-821.

McGrath DA, Smith CK, Gholz HL, Oliveira FdA. 2001. Effects of Land-Use Change on Soil Nutrient Dynamics in Amazônia. Ecosystems 4: 625-645.

McGuire KL, D'Angelo H, Brearley FQ, Gedallovich SM, Babar N, Yang N, Gillikin, CM, Gradoville R, Bateman C, Turner BL, Mansor P, Leff JW, Fierer N. 2014. Responses of soil fungi to logging and oil palm agriculture in Southeast Asian tropical forests. Microbial Ecology. DOI 10.1007/s00248-014-0468-4.

Meron D, Rodolfo-Metalpa R, Cunning R, Baker AC, Fine M, Banin E. 2012. Changes in coral microbial communities in response to a natural pH gradient. Isme Journal 6 (9):1775-1785.

Meron D, Buia MC, Fine M, Banin E. 2013. Changes in microbial communities associated with the sea anemone Anemonia viridis in a natural pH gradient. Microbial Ecology 65 (2):269-276. doi:10.1007/s00248-012-0127-6.

Montagna P, Holt S, Ritter C, Herzka S, Binney KF, Dunton K. 1998. Charaxterization of anthropogenic and natural disturbance on vegetated and unvegetated bay bottom habitats in the Chrpus Christi bay national estuary program study area. The University of Texas at Austin. Publication CCBNEP-25A. Vol 1. 127p.

Morris RJ. 2010. Anthropogenic impacts on tropical forest biodiversity: a network structure and ecosystem functioning perspective. Philosophical Transactions of the Royal Societyof London B: Biological Sciences 365: 3709-3718.

Moy AD, Howard WR, Bray SG, Trull TW. 2009. Reduced calcification in modern Southern Ocean planktonic foraminifera. Nature Geoscience 2 (4):276-280.

Moyersoen B, Becker P, Alexander I. 2001. Are ectomycorrhizas more abundant than arbuscular

mycorrhizas in tropical heath forests? New Phytologist 150: 591-599.

Munn CB. 2011. Marine microbiology : ecology and applications. 2nd edn. Garland Science, New York.

Murakami T, Ajima K, Miyawaki J, Yudasaka M, Iijima S, Shibe K. 2004. Drug-loaded carbon nanohorns: adsorption and release of dexamethasone in vitro. Molecular Pharmacology 399-405.

Murdoch WW and Oaten A .1975. Pre-dation and population stability. Ads'. Ecological Research. 9:2-131

Murty D, Kirschbaum MUF, McMurtrie RE, McGilvray H. 2002. Does conversion of forest to agricultural land change soil carbon and nitrogen? a review of the literature. Global Change Biology 8: 105-123.

Nannipieri P, Ascher J, Ceccherini M, Landi L, Pietramellara G, et al. 2003. Microbial diversity and soil functions. European Journal of Soil Science 54: 655-670.

Natarajan K, Senthilarasu G, Kumaresan V, Riviere T. 2005. Diversity in ectomycorrhizal fungi of a dipterocarp forest in Western Ghats. Current Science 88: 1893-1895.

Navarro E, Baun A, Behra R, Hartmann NB, Filser J, et al. 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17: 372-386.

Nelson K E. 2003. The future of microbial genomics. Environmental Microbiology 5, 1223–1225.

Newbold LK, Oliver AE, Booth T, Tiwari B, DeSantis T, Maguire M, Andersen G, van der Gast CJ, Whiteley AS. 2012. The response of marine picoplankton to ocean acidification. Environmental Microbiology 14 (9):2293-2307.

Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, et al. 2010. Use of ichip for high throughput in situ cultivation of ‘uncultivable’.

Nilsson RH, Veldre V, Hartmann M, Unterseher M, Amend A, et al. 2010. An open source software package for automated extraction of ITS1 and ITS2 from fungal ITS sequences for use in high-throughput community assays and molecular ecology. Fungal Ecology 3: 284-287.

Nilsson RH, Ryberg M, Abarenkov K, Sjokvist E, Kristiansson E. 2009. The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. FEMS

Microbiology Letters 296: 97–101.

Nogi Y, Masui N, Kato C. 1998. Photobacterium profundum sp. nov., a new, moderately barophilic bacterial species isolated from a deep-sea sediment. Extremophiles 2 (1):1-7. doi:DOI 10.1007/s007920050036.

Noller HF, Stolk BJV, Moazed D, Douthwaite S, Gutell RR. 1985. Studies on the structure and function of 16S ribosomal RNA using structure-specific chemical probes. Journal of Bioscience 8: 747–755.

O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R. 2005. Fungal community analysis by large-scale sequencing of environmental samples. Applied Environmental Microbiology 71: 5544-5550.

Odum EP. 1969. The strategy of ecosystem development. Science 164:262-270.

Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MHH, Oksanen MJ, Suggests M. 2007. The vegan package. Community ecology package Disponível em: http://www R-project org Acesso em 10 (01):2008.

Olafsson J, Olafsdottir SR, Benoit-Cattin A, Danielsen M, Arnarson TS, Takahashi T. 2009. Rate of Iceland Sea acidification from time series measurements. Biogeosciences 6 (11):2661-2668.

Oliver CD .1981. Forest development in North America following major dis-turbances. Forest Ecology and Management 3:153-68

Orgiazzi A, Lumini E, Nilsson RH, Girlanda M, Vizzini A, et al. 2012. Unravelling soil fungal communities from different Mediterranean land-use backgrounds. PloS One 7: e34847.

Oros-Sichler M, Gomes NC, Neuber G, Smalla K. 2006. A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. Journal of Microbiological Methods 65: 63-75.

Osorio AG, Silveira ICL, Bueno VL, Bergmann CP. 2008. H_2SO_4/HNO_3/HCl-Functionalization and its effect on dispersion of carbon nanotubes in aqueous media. Applied Surface Science 255: 2485-2489.

OSPAR Commission. 2006.Effects on the marine environment of ocean acidification resulting from elevated levels of CO_2 in the atmosphere. ISBN 978-1-905859-23-8

Pantarotto D, Briand JP, Prato M, Bianco A. 2004. Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chemical Communications (Cambridge):16-17.

Paradise M and goswami T. 2006. Carbon nanotubes-Production and industrial applications. Materials and Design 28 (2007) 1477–1489

Park S, Cha M, Lee J. 2010. Promotion of cell growth with magnetically enhanced single-walled carbon nanotubes. Diamond and Related Materials 19: 942-945.

Park S, Kang SJ, Oh TK, Yoon JH. 2010. Lutibacter maritimus sp nov., isolated from a tidal flat sediment. International Journal of Systematic Evolutionary Microbiology 60:610-614. doi:Doi 10.1099/Ijs.0.012401-0.

Parish A. 2011. Production and Application of Carbon Nanotubes, Carbon Nanofibers, Fullerenes, Graphene and Nanodiamonds: a Global Technology Survey and Market Analysis. Innovative Research and Products. Innovative Research and Products, Stanford, CT.

Pantarotto D, Partidos CD, Graff R, Hoebeke J, Briand JP, PratoM, Bianco A. 2003a. Synthesis, Structural characterization and immunological properties of carbon nanotubes functionalized with peptides. Journal of American Chemistry Society. 125:6160-6164.

Pantarotto D, Partidos CD, Hoebeke J, Brown F, Kramer E, Briand JP, Muller S, Prato M, Bianco A. 2003b. Immunization with peptide-functionalized carbon nanotubes enhances virusspecific neutralizing antibody responses. Chemistry and Biology 10:961-966

Paul EA and Clark FE. 1989. Soil microbiology and biochemistry. Academic Press, San Diego.

Peay KG, Kennedy PG, Davies SJ, Tan S, Bruns TD. 2010. Potential link between plant and fungal distributions in a dipterocarp rainforest: community and phylogenetic structure of tropical ectomycorrhizal fungi across a plant and soil ecotone. New Phytologist 185: 529-542.

Peay KG, Schubert MG, Nguyen NH, Bruns TD. 2012. Measuring ectomycorrhizal fungal dispersal: macroecological patterns driven by microscopic propagules. Molecular Ecology 21: 4122-4136.

Pearson TH and Rosenberg R. 1978. Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. Oceanography and Marine Biology Annual Review 16: 229-311.

Pettit LR, Hart MB, Medina-Sanchez AN, Smart CW, Rodolfo-Metalpa R, Hall-Spencer JM, Prol-Ledesma RM. 2013. Benthic foraminifera show some resilience to ocean acidification in the northern Gulf of California, Mexico. Marine Pollution Bulltin: 452–462. doi:10.1016/j.marpolbul.2013.02.011.

Pickett STA and White PS .1985. The ecology of natural disturbance and patch dynamics. Academic Press, San Diego, California.

Piesse J and Thirtle C. 2009. Three bubbles and a panic: An explanatory review of recent food commodity price events. Food Policy 34: 119-129.

Piontek J, Lunau M, Handel N, Borchard C, Wurst M, Engel A. 2010. Acidification increases microbial polysaccharide degradation in the ocean. Biogeosciences 7 (5):1615-1624. doi:DOI 10.5194/bg-7-1615-2010.

Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WAH, et al. 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nature Nanotechnology 3: 423-428.

Popov V.2004. Carbon nanotubes: properties and application. Materialas Science and Engineering R: Repeorts 43(3):61−102.

Price MN, Dehal PS, Arkin AP. 2010. FastTree 2-Approximately Maximum-Likelihood Trees for Large Alignments. Plos One 5 (3). doi:Artn E9490 Doi 10.1371/Journal.Pone.0009490.

Quince C, Lanzén A, Curtis TP, Davenport RJ, Hall N, Head IM, Fiona Read LF, Sloan WT. 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods 6 (9): 639-644.

Ramage BS, Sheil D, Salim HM, Fletcher C, Mustapha Nza , et al. 2013. Pseudoreplication in tropical forests and the resulting effects on biodiversity conservation. Conservation Biology 27: 364-372.

Ravenschlag K, Sahm K, Amann R. 2001. Quantitative molecular analysis of the microbial community in marine arctic sediments (Svalbard). Applied Environmental Microbiology 67 (1):387-395. doi:10.1128/AEM.67.1.387-395.2001.

Ray JL, Topper B, An S, Silyakova A, Spindelbock J, Thyrhaug R, DuBow MS, Thingstad TF,

Sandaa RA. 2012. Effect of increased pCO_2 on bacterial assemblage shifts in response to glucose addition in Fram Strait seawater mesocosms. Fems Microbiology Ecology 82 (3):713-723. doi:DOI 10.1111/j.1574-6941.2012.01443.x.

Rhoads DC and Young DK. 1970. The influence of deposit-feeding organisms on sedimen stability and community trophic structure. Journal Marine Research 28, 150–178.

Richter DD and Markewitz D. 1995. How deep is soil? Bioscience 45:600–609.

Riesenfeld CS, Schoss PD, Handelsman J. 2004. Metagenomics: genomic analysis of microbial communities. Annual Review of Genetics 38: 525–552.

Riviere T, Diedhiou AG, Diabate M, Senthilarasu G, Natarajan K, et al. 2007. Genetic diversity of ectomycorrhizal Basidiomycetes from African and Indian tropical rain forests. Mycorrhiza 17: 415-428.

Rodgers JC and Parker KC. 2003. Distribution of alien plant species in relation to human disturbance on the Georgia Sea Islands. Diversity and Distributions 9: 385–398.

Rodolfo-Metalpa R, Houlbreque F, Tambutte E, Boisson F, Baggini C, Patti FP, Jeffree R, Fine M, Foggo A, Gattuso JP, Hall-Spencer JM. 2011. Coral and mollusc resistance to ocean acidification adversely affected by warming. Nature Climate Change 1 (6):308-312.

Rodrigues DF, Jaisi DP, Elimelech M. 2012. Toxicity of Functionalized Single-Walled Carbon Nanotubes on Soil Microbial Communities: Implications for Nutrient Cycling in Soil. Environmental Science and Technology 47: 625-633.

Rodrigues JL, Pellizari VH, Mueller R, Baek K, Jesus Eda C, et al. 2013. Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. Proceeding of National Academy of Science of the United States of America 110: 988-993.

Rhoads DC, McCall PL, Yingst JY. 1978. Disturbance and production on the estuarine seafloor. American Scientist 66: 577-586.

Rousk J, Brookes PC, Bååth E. 2010. Investigating the mechanisms for the opposing pH relationships of fungal and bacterial growth in soil. Soil Biology and Biochemistry 42: 926-934.

Roy AS, Gibbons SM, Schunck H, Owens S, Caporaso JG, Sperling M, Nissimov JI, Romac S, Bittner L, Muhling M, Riebesell U, LaRoche J, Gilbert JA. 2013. Ocean acidification shows

negligible impacts on high-latitude bacterial community structure in coastal pelagic mesocosms. Biogeosciences 10 (1):555-566. doi:DOI 10.5194/bg-10-555-2013.

Rusch A, Amend JP. 2008. Functional characterization of the microbial community in geothermally heated marine sediments. Microbial Ecology 55 (4):723-736. doi:DOI 10.1007/s00248-007-9315-1

Sakio H. 1997. Effects of natural disturbance on the regeneration of riparian forests in a Chichibu Mountains, central Japan. Plant Ecology 132: 181–195, 1997.

Salvetat JP, Briggs GAD, Bonard JM, Bacsa RR, Kulik AJ, et al. 1999. Elastic and shear moduli of single-walled carbon nanotube ropes. Physical Review Letters 82: 944-947.

Sait M, Hugenholtz P, Janssen PH. 2002. Cultivation of globally-distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environmental Microbiology 4: 654–666.

Schlesinger WH. 1997. Biogeochemistry: An Analysis of Global Change (Academic, San Diego). 588 pp.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Applied Environmental Microbiology 75 (23):7537-7541. doi:Doi 10.1128/Aem.01541-09.

Schloss PD, Gevers D, Westcott SL. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One 6: e27310.

Schoch CL, Seifert KA, Huhndorf A et al. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America, 109: 6241–6246.

Shannon CE and Weaver W. 1948. A mathematical theory of communication. vol 27. American Telephone and Telegraph Company.

Shendure J and Hanlee J. 2008. Next-generation DNA sequencing. Nature Biotechnology 26 (10): 1135-1145.

Schmidt-Wolf GD, Schmidt-Wolf IG. 2003. Non-viral and hybrid vectors in human gene therapy: an

update. Trends in Molecular Medicine 9:67-72.

Shi KamNW, Jessop TC, Wender PA, Dai H. 2004. Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into mammalian cells. Journal of American Chemistry Society 126:6850-6851

Shi W, Becker J, Bischoff M, Turco RF, Konopka AE. 2002. Association of microbial community composition and activity with lead, chromium, and hydrocarbon contamination. Applied Environmental Microbiology 68: 3859-3866.

Shi Kam NW, Dai H. 2005. Carbon Nanotubes as Intracellular Protein Transporters: Generality and Biological Functionality. Journal of American Chemistry Society 127:6021-6026.

Shirayama Y et al .2004. Special Section on Advances in Biological Research for CO_2 Ocean Sequestration, Journal of Oceanography 60 (4), 691-816.

Shrestha B, Acosta-Martinez V, Cox SB, Green MJ, Li S, et al. 2013. An evaluation of the impact of multiwalled carbon nanotubes on soil microbial community structure and functioning. Journal of Hazardous Materials 261: 188-197.

Simmons S, Norris PR. 2002. Acidophiles of saline water at thermal vents of Vulcano, Italy. Extremophiles 6 (3):201-207. doi:DOI 10.1007/s007920100242.

Simon-Deckers A, Loo S, Mayne-L'hermite M, Herlin-Boime N, Menguy N, et al. 2009. Size-, composition-and shape-dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria. Environmental Science and Technology 43: 8423-8429.

Singh R, Pantarotto D, McCarthy D, Chaloin O, Hoebeke J, Partidos CD, Briand JP, Prato M, Bianco A, Kostarelos K. 2005. Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: towards the construction of nanotube-based gene delivery vectors. Journal of American Chemistry Society 127:4388-4396

Sleator RD, Shortall C, Hill C. 2008. Metagenomics. The Society for Applied Microbiology Letters in Applied Microbiology 47: 361–366.

Smith ME, Henkel TW, Aime MC, Fremier AK, Vilgalys R. 2011. Ectomycorrhizal fungal diversity and community structure on three co-occurring leguminous canopy tree species in a Neotropical rainforest. New Phytologist 192: 699-712.

Smith SE, Read DJ. 2010. Mycorrhizal symbiosis: Academic press.

Soussa WP .1985. The role of disturbance in natural communities. Annual Review of Ecology and Systematics 15: 353-391

Sperling M, Piontek J, Gerdts G, Wichels A, Schunck H, Roy AS, La Roche J, Gilbert J, Nissimov JI, Bittner L, Romac S, Riebesell U, Engel A. 2013. Effect of elevated CO_2 on the dynamics of particle-attached and free-living bacterioplankton communities in an Arctic fjord. Biogeosciences 10 (1):181-191. doi:DOI 10.5194/bg-10-181-2013.

Stankowich T. 2008. Ungulate flight responses to human disturbance: A review and meta-analysis. Biological Conservation 141 (2008) 2159–2173.

Steele HL and Streit WR. 2005. Metagenomics: Advances in ecology and biotechnology. FEMS Microbiology Letters 247: 105–111.

Stevenson BS, Eichors, SA, Wertz JT, Schmidt TM, Breznak JA. 2004. New strategies for cultivation and detection of previously uncultured microbes. Applied Environmental Microbiology 70: 4748–4755.

Streit WR, and Schmitz RA. 2004. Metagenomics – the key to the uncultured microbes. Current Opinion in Microbiology 7: 492–498.

Suenaga H. 2012. Targeted metagenomics: a high-resolution metagenomics approach for specific gene clusters in complex microbial communities. Environmental Microbiology 14(1): 13–22.

Tedersoo L, Jairus T, Horton BM, Abarenkov K, Suvi T, et al. 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. New Phytologist 180: 479-490.

Tedersoo L, May T, Smith M. 2010. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20: 217-263.

Tedersoo L, Sadam A, Zambrano M, Valencia R, Bahram M. 2010. Low diversity and high host preference of ectomycorrhizal fungi in Western Amazonia, a neotropical biodiversity hotspot. Isme Journal 4: 465-471.

Tilman D, Lehman CL, Thomson KT. 1997. Plant diversity and ecosystem productivity: Theoretical

considerations. Proceeding of National Academy of Science of the United States of America 94: 1857-1861.

Tilman D and Lehman C. 2001. Human-caused environmental change: Impacts on plant diversity and evolution. PNAS 98(10): 5433–5440.

Tong Z, Bischoff M, Nies L, Applegate B, Turco RF. 2007. Impact of fullerene (C60) on a soil microbial community. Environmental Science and Technology 41: 2985-2991.

Torsvik V, Daae FL, Sandaa RA, Ø vreås L. 1998. Novel techniques for analyzing microbial diversity in natural and perturbed environments. Journal of Biotechnology 64: 53–62.

Torsvik V, Daae FL, Sandaa RA, Ø vreås L. 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology 5: 240–245.

Tripathi B, Kim M, Singh D, Lee-Cruz L, Lai-Hoe A, et al. 2012. Tropical Soil Bacterial Communities in Malaysia: pH Dominates in the Equatorial Tropics Too. Microbial Ecology 64: 474-484.

Turley C, Blackford J, Widdicombe S, Lowe D, Nightingale PD and Rees A P .2006. Reviewing the impact of increased atmospheric CO_2 on oceanic pH and the marine ecosystem. In: Avoiding Dangerous Climate Change, Schellnhuber, H J., Cramer,W., Nakicenovic, N., Wigley, T. and Yohe, G (Eds). Cambridge University Press, 8, 65-70

Tyson GW, Chapman J, Hugenholt P, Allen EE, Ram RJ, Richardson PM et al. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428: 37–43.

Unno T, Jang J, Han D, Kim JH, Sadowsky MJ, Kim OS, Chun J, Hur HG. 2010. Use of Barcoded Pyrosequencing and Shared OTUs To Determine Sources of Fecal Bacteria in Watersheds. Environmental Science and Technology 44 (20):7777-7782. doi:Doi 10.1021/Es101500z.

Uthicke S, Momigliano P, Fabricius KE. 2013. High risk of extinction of benthic foraminifera in this century due to ocean acidification. Scientific Reports 3:1769. doi:10.1038/srep01769.

Verhulst S, Oosterbeek K, Ens BJ. 2001. Experimental evidence for effects of human disturbance on foraging and parental care in oystercatchers. Biological Conservation 101: 375–380.

Viaud M, Pasquier A, Brygoo Y. 2000. Diversity of soil fungi studied by PCR-RFLP of ITS. Mycological Research 104 (9): 1027-1032.

Vidu R, Rahman M, Mahmoudi M, Enachescu M, Poteca TD, Opris I. 2014. Nanostructures: a platform for brain repair and augmentation. Frontiers Systems Neurosciences. doi: 10.3389/fnsys.2014.00091

Vivas A, Moreno B, del Val C, Macci C, Masciandaro G, et al. 2008. Metabolic and bacterial diversity in soils historically contaminated by heavy metals and hydrocarbons. J Environ Monit 10: 1287-1296.

Voelkerding KV, Dames SA, Durtschi JD. 2009. Next-Generation Sequencing: From Basic Research to Diagnostics. Clinical Chemistry 55(4): 641–658.

Wagner M, Erhart R, Manz W, Amann R, Lemmer H, Wedi D, Schleifer KH. 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus Acinetobacter and its application for in situ monitoring in activated sludge. Applied Environmental Microbiology 60 (3):792-800.

Waldrop MP, Balser TC, Firestone MK. 2000. Linking microbial community composition to function in a tropical soil. Soil Biology & Biochemistry 32:1837–1846.

Walker KR and Alberstadt LP. 1975. Ecological succession as an aspect of structure in fossil communities. Paleobiology 1: 238-257.

Walker LR. 1999. Ecosystems of disturbed ground. Elsevier Science B.V. 868 pp.

Wang Y and Qian PY. 2009. ConservativeFragmentsinBacterial16SrRNAGenesand Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. PLoS One 4(10): e7401.

Ward N and Fraser CM. 2005. How genomics has affected the concept of microbiology. Current Opinion in Microbiology, 8, 564–571.

Widdicombe S, Spicer JI, Kitidis V. 2011. Effects of ocean acidifi cation on sediment fauna. In: Gattuso JP, Hansson L (eds) Ocean acidification. Oxford University Press, Oxford, pp 176-191.

Wikelski M and Cooke SJ .2006. Conservation physiology. Trends in Ecology and Evolution 21, 38–46.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18: 315-322.

Whitmore TC. 1990. An introduction to tropical rain forests. Oxford: Clarendon Press. 226 pp.

Whittaker RH. 1960. Vegetation of the Siskiyou mountains, Oregon and California. Ecological Monographs 30: 279-338.

Widdicombe S and Austen MC .1998. Experimental evidence for the role of Brissopsis lyrifera (Forbes, 1841) as a critical species in the maintenance of benthic diversity and the modification of sediment chemistry. Journal of Experimental Marine Biology and Ecology 228(2), 241–255.

Widdicombe S, Austen MC, Kendall MA, Warwick RM and Jones MB .2000. Bioturbation as a mechanism for setting and maintaining levels of diversity in subtidal macrobenthic communities. Hydrobiologia 440, 369–377.

Wilcove DS, Giam X, Edwards DP, Fisher B, Koh LP. 2013. Navjot's nightmare revisited: logging, agriculture, and biodiversity in Southeast Asia. Trends in Ecology and Evolution 28: 531-540.

Woese CR. 1987. Bacterial evolution. Microbiology Review 51: 221–271.

Woodcock P, Edwards DP, Newton RJ, Vun Khen C, Bottrell SH, et al. 2013. Impacts of intensive logging on the trophic organisation of ant communities in a biodiversity hotspot. PLoS One 8: e60756.

White P S .1979. Pattern, process, and natural disturbance in vegetation. Botanical Review 45:229-99

Wright SJ. 2005. Tropical forests in a changing environment. Trends in Ecology and Evolution 20: 553-560.

Wubet T, Christ S, Schöning I, Boch S, Gawlich M, et al. 2012. Differences in soil fungal communities between European beech (Fagus sylvatica L.) dominated forests are related to soil and understory vegetation. PloS One 7: e47500.

Wu TH, Chellemi DO, Martin KJ, Graham JH, Rosskop EN. 2007. Discriminating the effects of agricultural land management practices on soil fungal communities. Soil Biology and Biochemistry 39: 1139-1155.

Yang Y, Campbell CD, Clark L, Cameron CM, Paterson E. 2006. Microbial indicators of heavy metal

contamination in urban and rural soils. Chemosphere 63: 1942-1952.

Yergeau E, Sanschagrin S, Beaumier D, Greer CW. 2012. Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. PLoS One 7: e30058.

Yousuf B, Keshri J, Mishra A, Jha B. 2012. Application of targeted metagenomics to explore abundance and diversity of CO_2-fixing bacterial community using cbbL gene from the rhizosphere of Arachis hypogaea. Gene 506: 18-24.

Yousuf B, Sanadhya P, Keshri J, Jha B. 2012. Comparative molecular analysis of chemolithoautotrophic bacterial diversity and community structure from coastal saline soils, Gujarat, India. BMC Microbiology 12: 150.

Yinghuai Z, Peng AT, Carpenter K, Maguire JA, Hosmane NS, Takagaki M. 2005. Substituted carborane-appended water-soluble single-wall carbon nanotubes: new approach to boron neutron capture therapy drug delivery. Journal of American Chemistry Society 127:9875-9880.

Yue GZ, Qiu Q, Gao B, Cheng Y, Zhang J, et al. 2002. Generation of continuous and pulsed diagnostic imaging x-ray radiation using a carbon-nanotube-based field-emission cathode. Applied Physics Letters 81: 355-357.

Zak JC. 1992. Response of soil fungal communities to disturbance. In: The Fungal Community: Its Organization and Role in the Ecosystem. 2nd edn. (eds. G. Carroll and D.T. Wicklow). Marcel Dekker, New York: 403-425.

Zengler K, Toledo G, Rappé M, Elkins J, Mathur EJ, Keller M. 2002. Cultivating the uncultured. Proceeding of National Academy of Science of the United States of America 99: 15681–15686.

Zhang LL and Zhao XS. 2009. Carbon-based materials as supercapacitor electrodes. Chemical Society Review 38, 2520-2531

Zhao F, Zhao Y, Liu Y, Chang X, Chen C, Zhao Y. 2011. Cellular Uptake, Intracellular Traffi cking, and Cytotoxicity of Nanomaterials. Small. 7, No. 10, 1322–1337

Zhou HW, Li DF, Tam NF, Jiang XT, Zhang H, et al. 2011. BIPES, a cost-effective high-throughput method for assessing microbial diversity. ISME Journal 5: 741-749.

Zhou J, Xia B, Huang H, Palumbo AV, Tiedje JM. 2004. Microbial diversity and heterogeneity in

sandy subsurface soils. Applied Environmental Microbioliogy 70: 1723-1734.

Zhou J et al. 2002. Spatial and resource factors influencing high microbial diversity in soil. Applied Environmental Microbiology 68: 326–334.

APPENDIX

Figure A1: Species accumulation curve of fungal overall community (dotted lines, 95% CI for unlogged forest) in Malaysian, Borneo.

Figure A2: β-diversity (average distance to group centroid) of fungal overall community among different land uses in Malaysian, Borneo.

Figure A3: Rarefaction curves comparing soil fungal communities across different land use types in Malaysian, Borneo.

Figure A4: The heat map showing the relative abundances of the most abundant genera at different pH levels off Vulcano, Italy.

Figure A5: Relative abundance of the main phyla of sediment bacterial classes at different pH levels off Vulcano, Italy.

Figure A6: Relative abundance of dominant benthic marine sediment genera at different pH levels off Vulcano, Italy.

Figure A7: Relative abundance (means ± SD) of the most abundant phyla among different concentrations of fMWCNTs applied to soil samples.

Figure A8: Relative abundance (means ± SD) of the most abundant phyla among different concentrations of raw MWCNTs applied to soil samples.

Table A1: Soil characterizations and properties in Malaysian, Borneo.

Table A2: Bray-Curtis distance matrix of marine samples off Vulcano, Italy.

Table A3: Phylotype richness (OTUs) and diversity indices using mothur platform (Schloss et al. 2009) calculated for fungal samples of Malaysian, Borneo.

Table A4: Phylotype richness (OTUs) and diversity indices using mothur platform (Schloss et al. 2009) calculated for bacterial marine samples of Italy.

Table A5: Phylotype richness (OTUs) and diversity indices using mothur platform (Schloss et al. 2009) calculated for bacteria for carbon nanotube samples.

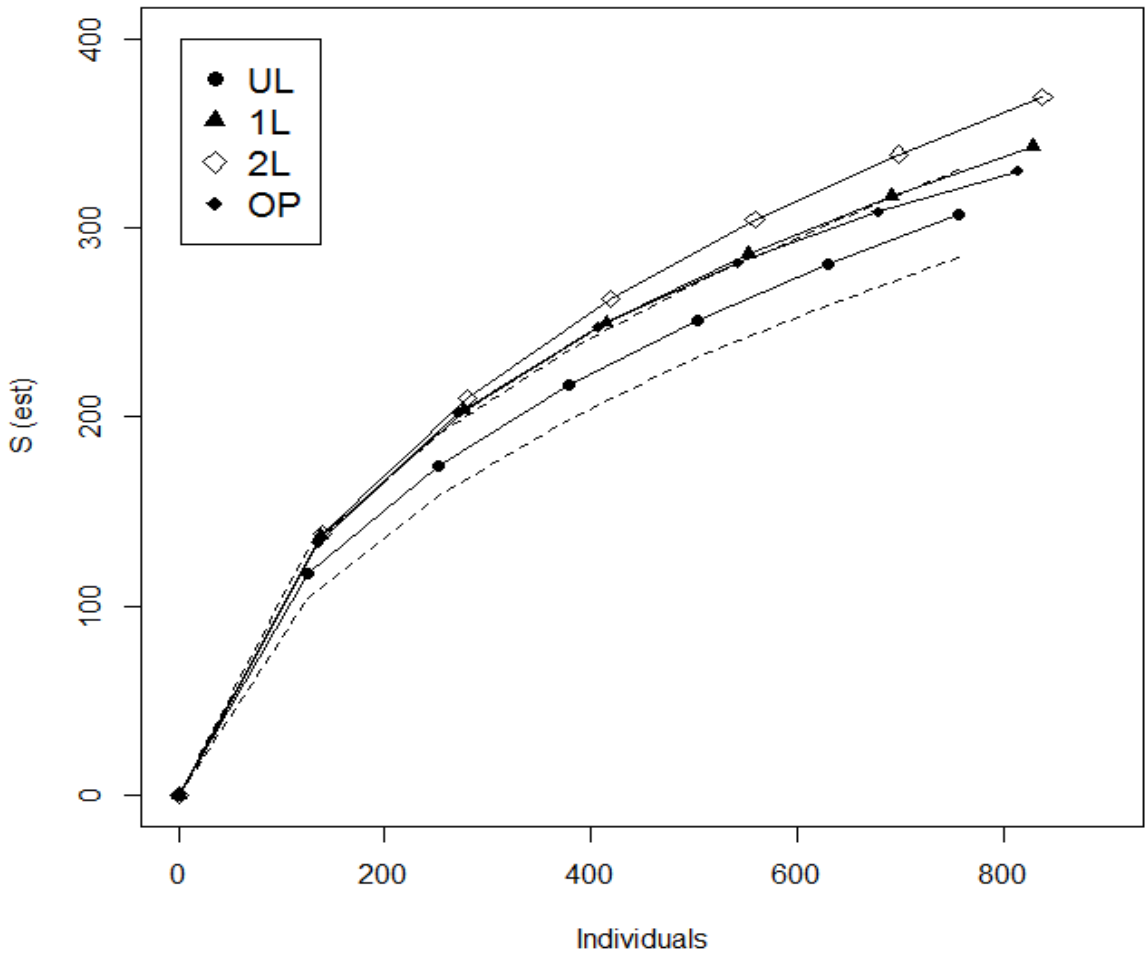

Figure A1: Species accumulation curve of fungal overall community (dotted lines, 95% CI for unlogged forest) in Malaysian, Borneo.

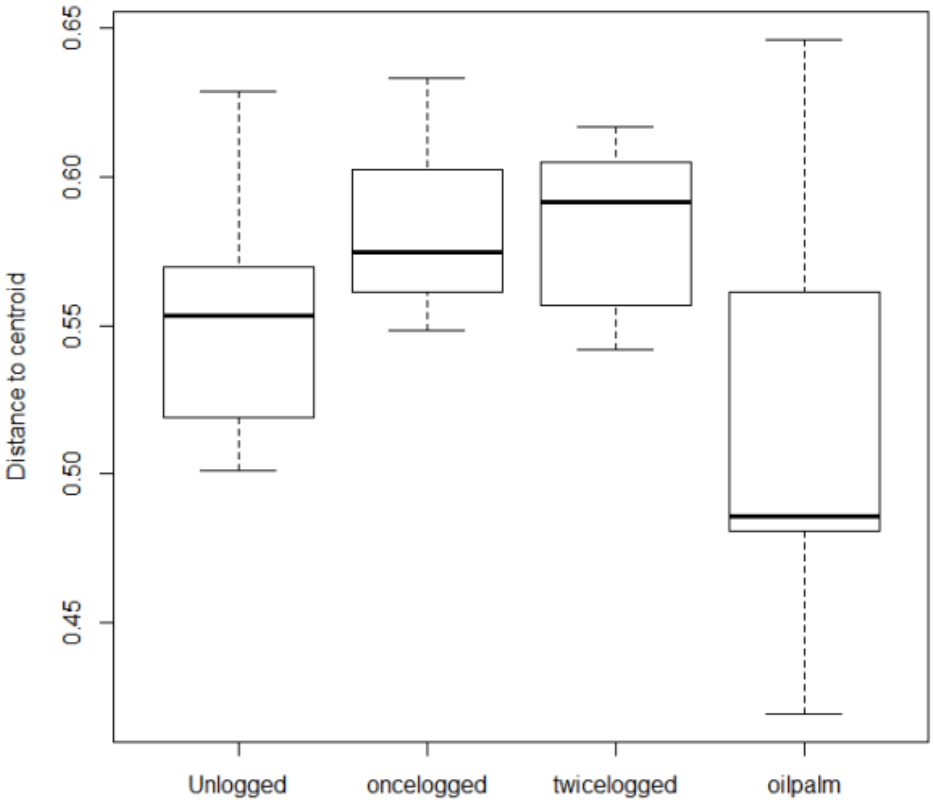

Figure A2: β-diversity (average distance to group centroid) of fungal overall community among different land uses in Malaysian, Borneo.

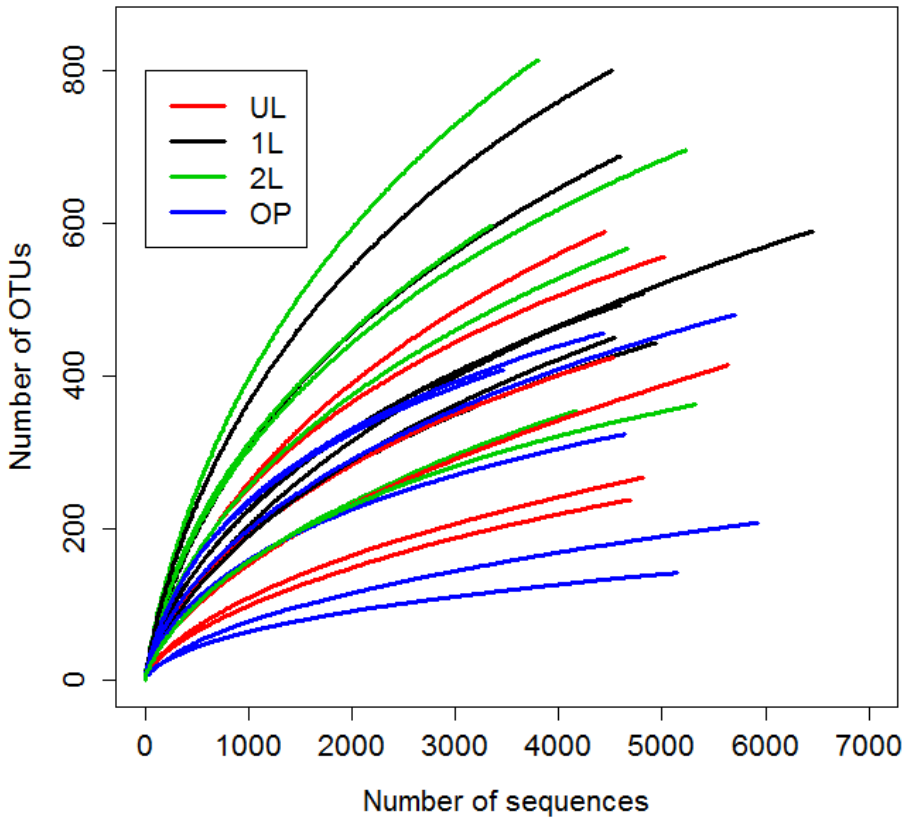

Figure A3: Rarefaction curves comparing soil fungal communities across different land use types in Malaysian, Borneo.

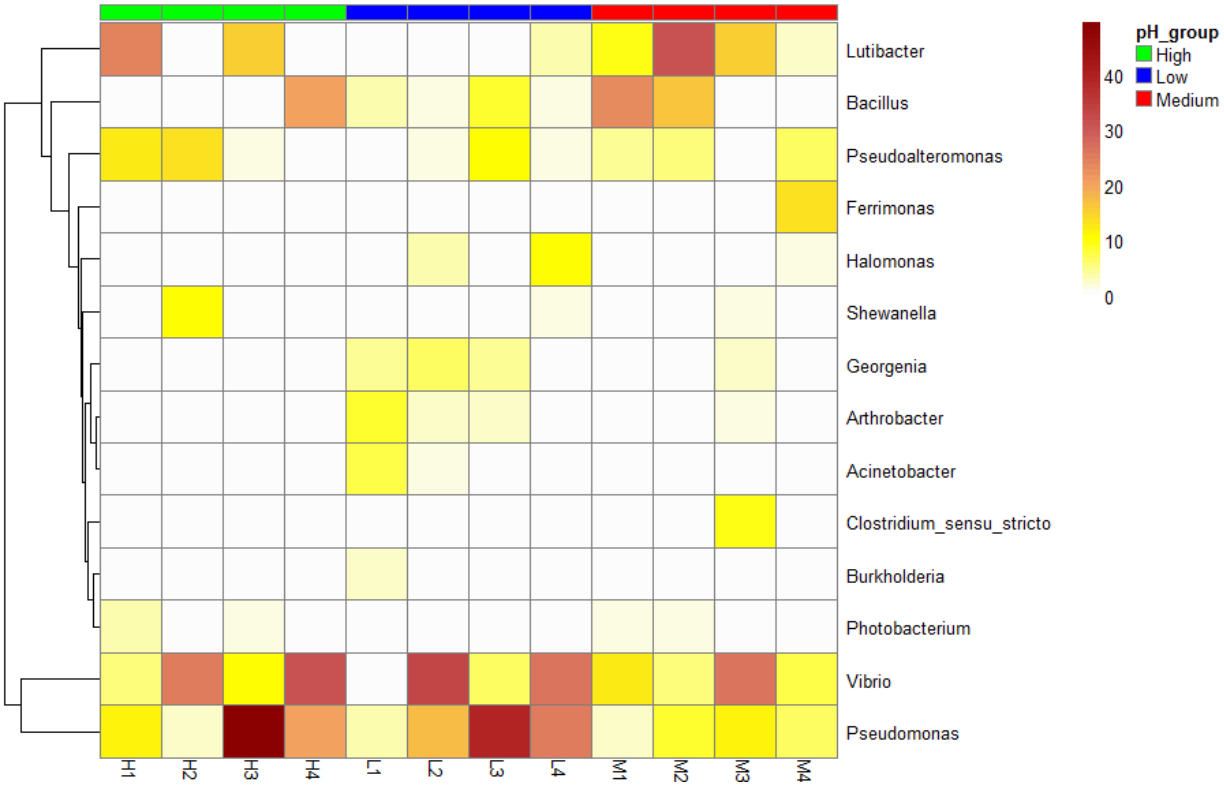

Figure A4: The heat map showing the relative abundances of the most abundant genera at different pH levels off Vulcano, Italy.

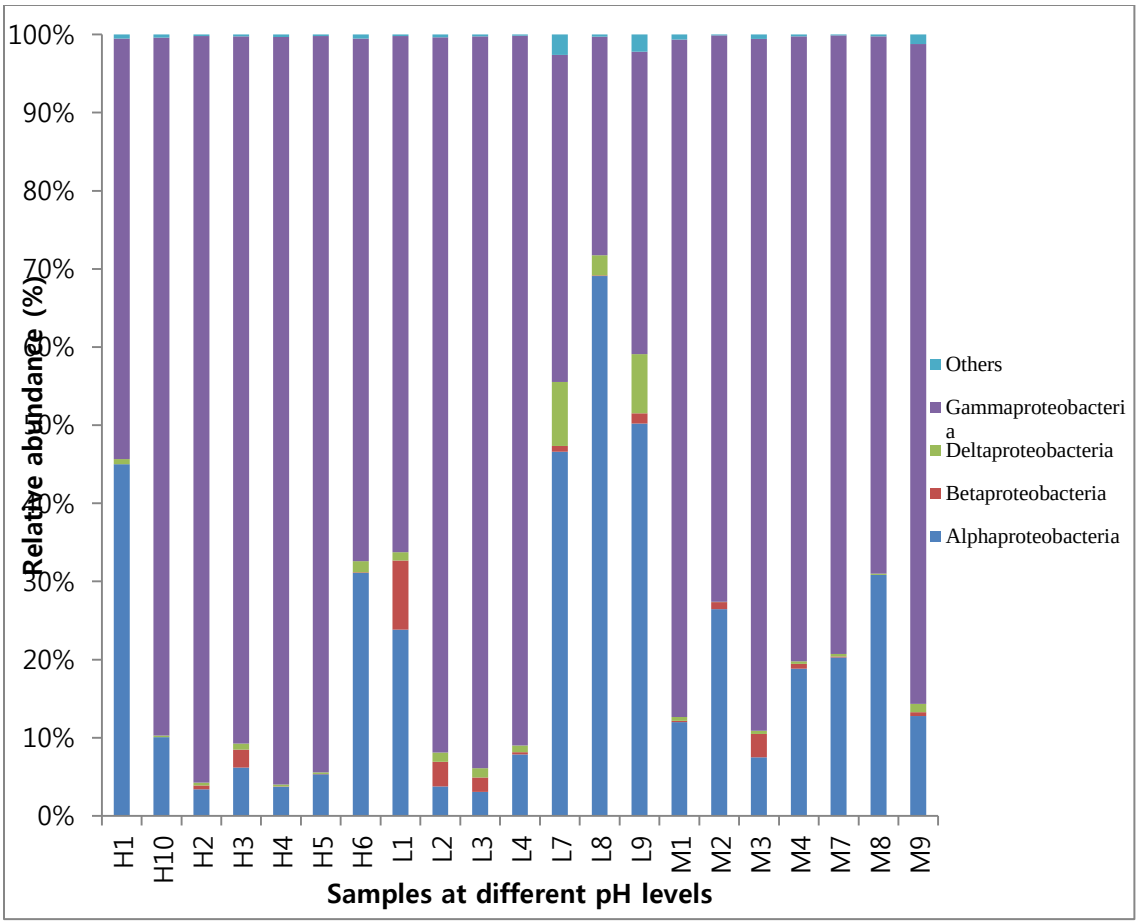

Figure A5: Relative abundance of the main phyla of sediment bacterial classes at different pH levels off Vulcano, Italy.

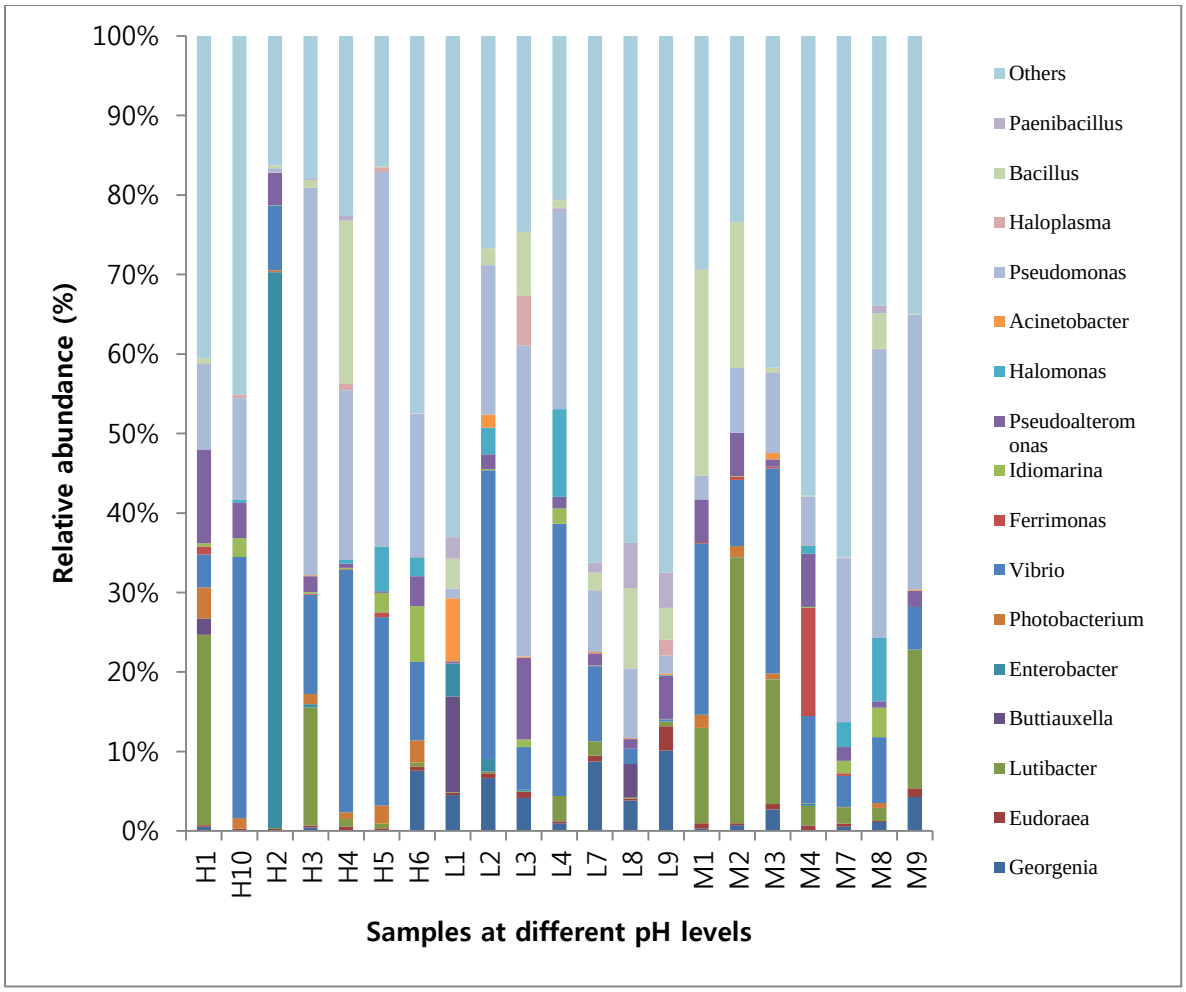

Figure A6: Relative abundance of dominant benthic marine sediment genera at different pH levels off Vulcano, Italy.

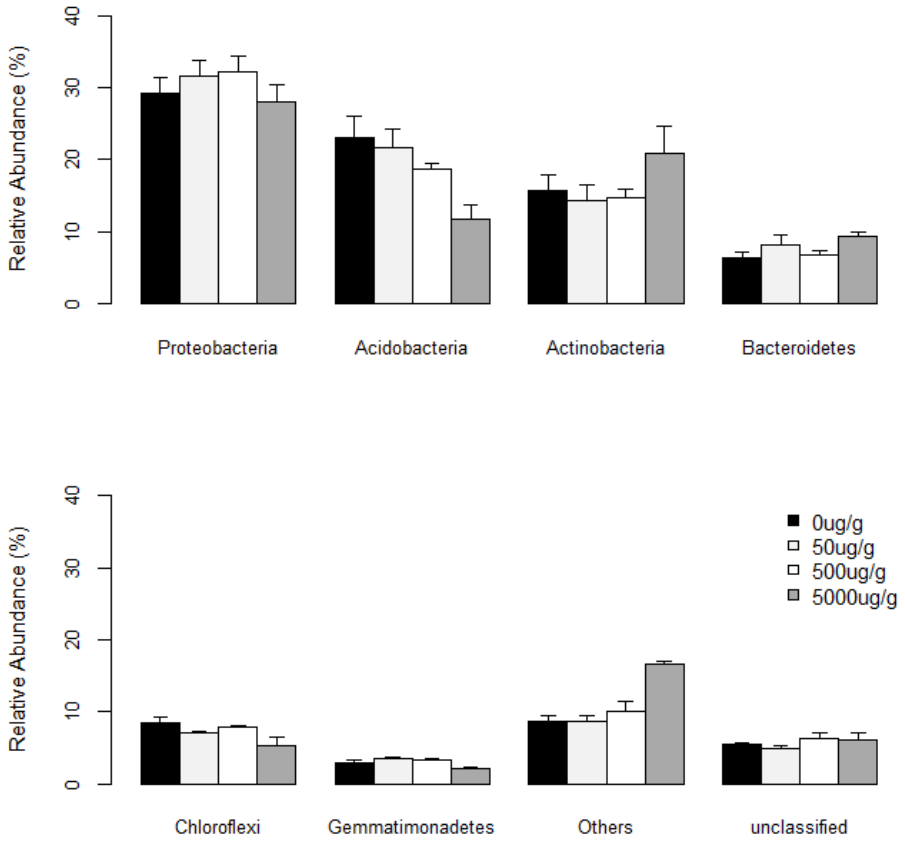

Figure A7: Relative abundance (means ± SD) of the most abundant phyla among different concentrations of fMWCNTs applied to soil samples.

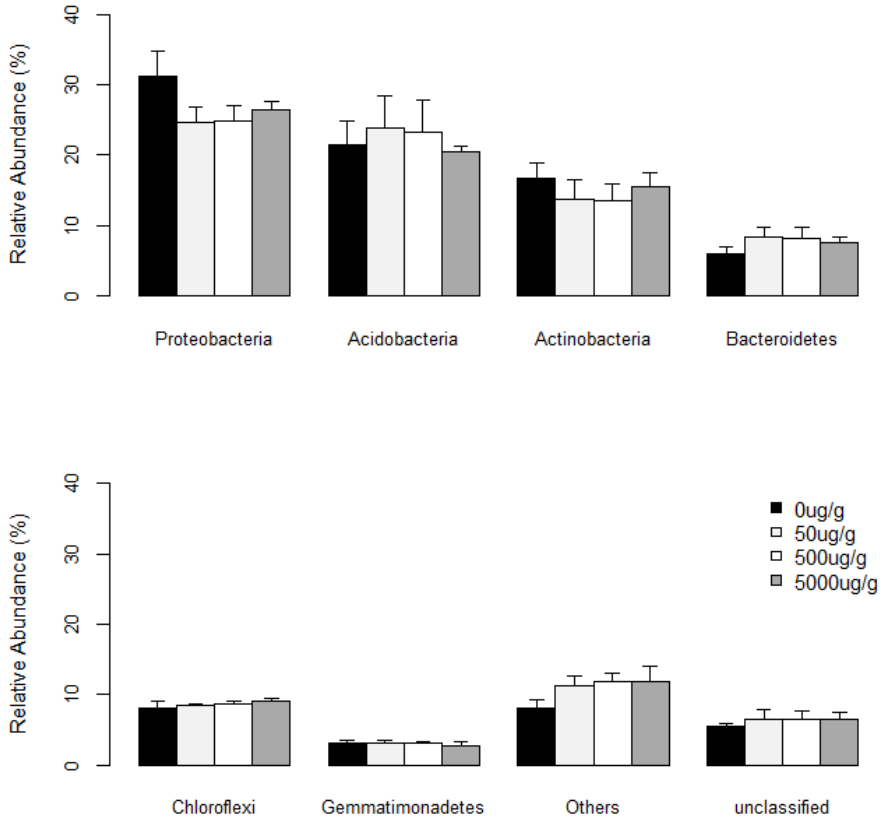

Figure A8: Relative abundance (means ± SD) of the most abundant phyla among different concentrations of raw MWCNTs applied to soil samples.

Table A1: Soil characterizations and properties in Malaysian, Borneo.

sample	Site	land use	Lattitude	Longitude	C	N	C/N	P
T41	BRL	UL	5.02092	117.752	3.421	0.344	9.94477	28.66
T13	Tembaling	UL	4.9498	117.807	2.877	0.246	11.6951	18.19
T14	Tembaling	UL	4.95909	117.804	3.203	0.322	9.9472	32.89
T17	Elephant Ridge	UL	4.97136	117.786	5.359	0.323	16.5913	17.82
T54	Takala	UL	5.02156	117.79	3.629	0.443	8.19187	25.58
T55	Takala	UL	5.02812	117.787	3.767	0.287	13.1254	24.44
T20	Danum 1L	1L	4.97052	117.816	2.623	0.283	9.26855	24.07
T12	Danum 1L	1L	4.96495	117.821	3.137	0.303	10.3531	21.3
T38	MBP	1L	5.09517	117.646	4.487	0.265	16.9321	28.39
T63	West Ulu-Segama	1L	5.07986	117.706	4.714	0.345	13.6638	22.57
T64	West Ulu-Segama	1L	5.08484	117.708	2.101	0.255	8.23922	21.24
T45	INFAPRO	1L	4.98051	117.865	5.083	0.309	16.4498	37.28
T46	INFAPRO	1L	4.98554	117.86	4.533	0.35	12.9514	38.77
T28	N Benta	2L	4.92604	117.949	6.143	0.505	12.1644	16.24
T34	West Malua	2L	5.09316	117.636	4.904	0.359	13.6602	30.71
T35	West Malua	2L	5.09575	117.63	5.046	0.308	16.3831	38.02
T52	Mid Benta	2L	4.9334	117.981	3.651	0.2888	12.642	6.66
T60	East Malua	2L	5.08979	117.681	3.717	0.273	13.6154	25.03
TDB	N Benta	2L	4.93717	117.947	2.484	0.135	18.4	14.33
T71	Danum OP	OP	5.08372	117.776	2.298	0.199	11.5477	118.03
T72	Danum OP	OP	5.07999	117.783	3.442	0.232	14.8362	103.52
T73	Dumpas East	OP	4.49215	117.773	0.936	0.098	9.55102	16.64
T74	Dumpas East	OP	4.49784	117.781	1.386	0.147	9.42857	69.88
T75	Dumpas West	OP	4.49018	117.724	1.467	0.161	9.1118	14.46
T76	Dumpas West	OP	4.49794	117.727	2.271	0.274	8.28832	16.64

Table A2: Bray-Curtis distance matrix of marine samples off Vulcano, Italy.

	H1	H10	H2	H3	H4	H5	H6	L1	L2	L3	L4	L7	L8	L9	M1	M2	M3	M4	M7	M8	M9
H1	0	0.7515	0.6574	0.6309	0.6835	0.6754	0.6507	0.8254	0.7444	0.7226	0.7014	0.7421	0.7502	0.8145	0.696	0.6069	0.6738	0.6754	0.613	0.6797	0.6732
H10	0.7515	0	0.764	0.7842	0.7293	0.7046	0.74593	0.9488	0.7972	0.83309	0.7114	0.8487	0.8565	0.9109	0.8085	0.7501	0.8067	0.763	0.7622	0.6956	0.7783
H2	0.6574	0.764	0	0.6781	0.7293	0.7117	0.70342	0.7792	0.7196	0.73689	0.7563	0.7797	0.7872	0.8031	0.753	0.7343	0.6914	0.6923	0.6982	0.7335	0.7298
H3	0.6309	0.7842	0.6781	0	0.6029	0.632	0.63713	0.7699	0.6252	0.71987	0.6852	0.7178	0.7918	0.7581	0.6264	0.6094	0.5845	0.6351	0.664	0.6934	0.6872
H4	0.6835	0.7293	0.7293	0.6029	0	0.6212	0.66778	0.8692	0.6907	0.76735	0.6779	0.7646	0.8159	0.8204	0.695	0.6394	0.6954	0.6992	0.7051	0.6946	0.7317
H5	0.6754	0.7046	0.7117	0.632	0.6212	0	0.65958	0.8762	0.6692	0.78416	0.5666	0.7664	0.7873	0.8301	0.7107	0.6793	0.6721	0.6681	0.6161	0.6257	0.7021
H6	0.6507	0.7459	0.7034	0.6371	0.6678	0.6596	0	0.8558	0.7054	0.70232	0.7066	0.7175	0.757	0.7544	0.7036	0.6939	0.6802	0.7137	0.6611	0.6566	0.7054
L1	0.8254	0.9488	0.7792	0.7699	0.8692	0.8762	0.85585	0	0.648	0.70528	0.8204	0.7283	0.6946	0.7747	0.8442	0.8543	0.7265	0.7512	0.7602	0.8127	0.7847
L2	0.7444	0.7972	0.7196	0.6252	0.6907	0.6692	0.70542	0.648	0	0.61571	0.5959	0.624	0.6723	0.7071	0.6974	0.6963	0.6041	0.6659	0.6609	0.6742	0.7102
L3	0.7226	0.8331	0.7369	0.7199	0.7674	0.7842	0.70232	0.7053	0.6157	0	0.7416	0.7118	0.7066	0.6972	0.7413	0.7336	0.6577	0.6921	0.7184	0.7451	0.6907
L4	0.7014	0.7114	0.7563	0.6852	0.6779	0.5666	0.70659	0.8204	0.5959	0.74162	0	0.6937	0.7174	0.7951	0.7164	0.6761	0.7039	0.6882	0.6388	0.4953	0.6523
L7	0.7421	0.8487	0.7797	0.7178	0.7646	0.7664	0.71754	0.7283	0.624	0.71178	0.6937	0	0.5848	0.5991	0.7053	0.7106	0.6883	0.7168	0.7108	0.6962	0.6903
L8	0.7502	0.8565	0.7872	0.7918	0.8159	0.7873	0.75698	0.6946	0.6723	0.70664	0.7174	0.5848	0	0.652	0.7264	0.7534	0.7198	0.7375	0.6845	0.6693	0.7167
L9	0.8145	0.9109	0.8031	0.7581	0.8204	0.8301	0.75437	0.7747	0.7071	0.69721	0.7951	0.5991	0.652	0	0.7351	0.7896	0.7229	0.7794	0.7373	0.814	0.7492
M1	0.696	0.8085	0.753	0.6264	0.695	0.7107	0.70356	0.8442	0.6974	0.74128	0.7164	0.7053	0.7264	0.7351	0	0.627	0.6172	0.678	0.7047	0.7399	0.6799
M2	0.6069	0.7501	0.7343	0.6094	0.6394	0.6793	0.69392	0.8543	0.6963	0.73364	0.6761	0.7106	0.7534	0.7896	0.627	0	0.6526	0.6359	0.6625	0.6971	0.6648
M3	0.6738	0.8067	0.6914	0.5845	0.6954	0.6721	0.68025	0.7265	0.6041	0.65767	0.7039	0.6883	0.7198	0.7229	0.6172	0.6526	0	0.6511	0.6742	0.7324	0.7161
M4	0.6754	0.763	0.6923	0.6351	0.6992	0.6681	0.71367	0.7512	0.6659	0.6921	0.6882	0.7168	0.7375	0.7794	0.678	0.6359	0.6511	0	0.5715	0.6827	0.6555
M7	0.613	0.7622	0.6982	0.664	0.7051	0.6161	0.66106	0.7602	0.6609	0.71838	0.6388	0.7108	0.6845	0.7373	0.7047	0.6625	0.6742	0.5715	0	0.5734	0.6342
M8	0.6797	0.6956	0.7335	0.6934	0.6946	0.6257	0.65665	0.8127	0.6742	0.74507	0.4953	0.6962	0.6693	0.814	0.7399	0.6971	0.7324	0.6827	0.5734	0	0.6612
M9	0.6732	0.7783	0.7298	0.6872	0.7317	0.7021	0.70544	0.7847	0.7102	0.69073	0.6523	0.6903	0.7167	0.7492	0.6799	0.6648	0.7161	0.6555	0.6342	0.6612	0

Table A3: Phylotype richness (OTUs) and diversity indices using mothur platform (Schloss et al. 2009) calculated for fungal samples of Malaysian, Borneo.

Label	Sample	OTUs	Coverage	Shannon	Simpson	Chao	Ace
0.03	T12	420	0.932	3.525	0.138	757.513	1077.994
0.03	T13	465	0.936	4.465	0.052	733.786	928.406
0.03	T14	517	0.922	4.455	0.042	884.645	1207.638
0.03	T17	217	0.964	3.057	0.099	440.125	618.048
0.03	T20	580	0.915	4.640	0.063	980.693	1275.542
0.03	T28	597	0.911	5.044	0.024	1080.033	1418.047
0.03	T34	768	0.889	5.575	0.012	1247.965	1596.610
0.03	T35	576	0.920	4.998	0.026	960.710	1159.724
0.03	T38	372	0.946	3.752	0.106	655.983	639.398
0.03	T41	195	0.969	2.231	0.311	319.558	574.929
0.03	T45	425	0.940	4.275	0.039	685.269	908.010
0.03	T46	421	0.937	4.215	0.048	781.742	971.079
0.03	T52	322	0.946	3.203	0.145	692.227	883.452
0.03	T54	368	0.948	3.517	0.112	576.562	753.311
0.03	T55	302	0.951	3.406	0.092	583.875	860.861
0.03	T60	483	0.930	4.622	0.030	846.480	1038.127
0.03	T63	686	0.906	5.363	0.015	1060.659	1090.374
0.03	T64	382	0.939	3.620	0.092	729.508	962.140
0.03	T71	281	0.966	3.425	0.129	433.442	420.022
0.03	T72	404	0.952	4.490	0.035	593.851	586.582
0.03	T73	151	0.977	1.633	0.467	249.276	387.239
0.03	T74	411	0.950	4.502	0.035	622.881	616.366
0.03	T75	114	0.986	2.082	0.243	153.808	173.298
0.03	T76	383	0.945	4.124	0.045	627.000	836.010
0.03	TDB	314	0.950	3.427	0.086	599.313	817.055

Table A4: Phylotype richness (OTUs) and diversity indices using mothur platform (Schloss et al. 2009) calculated for bacterial marine samples of Italy.

Label	Sample	OTUs	Coverage	Shannon	Simpson	Chao	Ace
0.03	H1	133	0.968	2.547	0.161	202.194	300.819
0.03	H3	212	0.940	2.487	0.279	503.667	628.970
0.03	H4	163	0.951	2.652	0.157	413.143	698.382
0.03	L1	185	0.952	3.436	0.062	341.484	511.820
0.03	L2	178	0.950	2.856	0.152	426.182	591.379
0.03	L3	160	0.958	2.759	0.168	296.714	416.498
0.03	L4	120	0.966	2.539	0.168	285.667	283.691
0.03	M1	136	0.964	2.468	0.188	294.333	360.894
0.03	M2	134	0.961	2.625	0.145	336.500	439.516
0.03	M3	212	0.942	3.209	0.116	446.194	592.436
0.03	M4	167	0.951	2.859	0.131	412.286	619.812

Table A5: Phylotype richness (OTUs) and diversity indices using mothur platform (Schloss et al. 2009) calculated for bacteria for carbon nanotube samples.

Label	Sample	OTUs	Coverage	Shannon	Simpson	Chao	Ace
0.03	1a0	1835	0.683	6.623	0.005	6212.764	10913.902
0.03	1a2	1560	0.739	6.293	0.007	4863.720	9332.252
0.03	1a8	1424	0.774	5.843	0.022	4111.582	7454.387
0.03	1b0	1841	0.674	6.680	0.004	6834.437	13323.640
0.03	1b2	1643	0.700	6.343	0.009	5791.757	11529.212
0.03	1b8	1409	0.768	5.987	0.012	4499.081	8142.639
0.03	1c0	1708	0.701	6.490	0.007	5708.938	10557.558
0.03	1c2	1654	0.699	6.383	0.006	5952.436	11380.930
0.03	1c8	1386	0.774	5.975	0.011	4435.242	8687.427
0.03	2a0	1669	0.711	6.459	0.007	5156.548	10507.573
0.03	2a2	1572	0.726	6.241	0.009	5275.777	10791.858
0.03	2a8	1491	0.764	6.084	0.011	4555.961	8050.250
0.03	2b0	1861	0.664	6.650	0.005	6929.087	13590.504
0.03	2b2	1591	0.735	6.276	0.008	4632.282	7978.023
0.03	2b8	1535	0.753	6.111	0.010	4778.823	9231.853
0.03	2c0	2014	0.637	6.780	0.004	7432.000	15308.325
0.03	2c2	1557	0.734	6.271	0.009	4657.781	8624.268
0.03	2c8	1438	0.788	6.126	0.009	3549.627	5463.768
0.03	3a0	1823	0.673	6.618	0.005	7106.742	13257.584
0.03	3a2	1610	0.723	6.299	0.009	4945.103	10161.432
0.03	3a8	1535	0.750	6.198	0.010	4659.492	9051.868
0.03	3b0	1890	0.666	6.701	0.004	6877.890	11988.274
0.03	3b2	1620	0.727	6.409	0.007	4745.377	8909.136
0.03	3b8	1668	0.721	6.339	0.007	5613.789	11263.814
0.03	3c0	1992	0.624	6.820	0.004	7278.457	15562.456
0.03	3c2	1627	0.716	6.309	0.009	5140.332	10289.763
0.03	3c8	1629	0.727	6.384	0.006	5166.144	9011.779
0.03	4a0	2066	0.627	6.971	0.002	7155.689	14045.242

0.03	4a2	1134	0.813	5.510	0.020	3625.417	6533.844
0.03	4a8	1142	0.823	5.466	0.026	3238.938	6343.324
0.03	4b0	1961	0.652	6.865	0.003	6374.971	11974.658
0.03	4b2	1120	0.845	5.580	0.017	2788.520	4713.197
0.03	4b8	1139	0.827	5.584	0.022	3173.773	5618.617
0.03	4c0	1923	0.655	6.839	0.003	6234.581	12169.005
0.03	4c2	1210	0.801	5.541	0.021	4047.462	7684.659
0.03	4c8	1166	0.820	5.592	0.027	3533.077	6052.980
0.03	5a0	1966	0.635	6.844	0.003	6976.297	13015.972
0.03	5a2	1793	0.695	6.484	0.007	6136.634	12446.102
0.03	5a8	1434	0.770	6.001	0.010	4288.343	8381.565
0.03	5b0	1935	0.654	6.848	0.003	6403.763	12062.008
0.03	5b2	1600	0.732	6.379	0.006	4906.051	8157.400
0.03	5b8	1408	0.781	6.024	0.010	4024.966	6943.876
0.03	5c0	1985	0.628	6.874	0.003	7728.483	14712.379
0.03	5c2	1665	0.716	6.475	0.006	4879.114	9051.762
0.03	5c8	1339	0.829	6.070	0.009	2917.918	4464.832
0.03	6a0	1905	0.653	6.825	0.003	6748.702	13113.650
0.03	6a2	1704	0.705	6.533	0.005	5495.990	10116.559
0.03	6a8	1417	0.785	5.970	0.011	3584.991	6621.277
0.03	6b0	2010	0.633	6.894	0.003	7400.712	13786.964
0.03	6b2	1666	0.713	6.456	0.006	6053.156	9609.314
0.03	6b8	1424	0.774	6.004	0.010	4271.759	7736.150
0.03	6c0	1911	0.646	6.826	0.003	6906.005	13677.514
0.03	6c2	1617	0.718	6.384	0.007	5678.000	10209.564
0.03	6c8	1556	0.751	6.228	0.008	4709.157	8108.672
0.03	7a0	1748	0.729	6.644	0.004	4922.449	8309.901
0.03	7a2	1682	0.698	6.483	0.006	5429.591	10495.742
0.03	7a8	1327	0.808	5.799	0.018	3258.689	5506.428
0.03	7b0	1937	0.659	6.840	0.003	6254.447	12594.229
0.03	7b2	1691	0.731	6.516	0.005	4505.259	8477.458
0.03	7b8	1426	0.804	6.046	0.013	3449.572	5792.030

0.03	7c0	1932	0.656	6.821	0.003	7125.446	13602.570
0.03	7c2	1798	0.687	6.564	0.005	6043.455	12424.282
0.03	7c8	1308	0.829	5.959	0.011	2894.222	4468.460