Microbial community analysis in soil (rhizosphere) and the marine (plastisphere) environment in function of plant health and biofilm formation

Caroline De Tender

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Promotors: Prof. Dr. Peter Dawyndt

Department of Applied mathematics, Computer Science and Statistics

Faculty of Science

Ghent University

Dr. Martine Maes

Crop Protection - Plant Sciences Unit

Institute for Agricultural, Fisheries and Food Research (ILVO)

Ir. Lisa Devriese

Fisheries – Animal Sciences Unit

Insitute for Agricultural, Fisheries and Food Research (ILVO)

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CONTENTS

LIST OF ACRONYMS AND ABBREVIATIONS

AL	Ammonium Lactate
ANOVA	Analysis of Variance
AUDPC	Area under the disease progress curve
bp	base pairs
BP	Beach pellets
BC	Biochar
BLAST	Basic local alignment search tool
CEN	Committee for standardization
CFU	Colony forming unit
CHOS	Chito-oligosaccharides
dai	days after inoculation
DGGE	Denaturing Gradient Gel Electrophoresis
DM	Dry matter
DNA	Deoxyribonucleic acid
DNA-SIP	DNA stable isotope probing
EC	Electrical conductivity
FAME	Fatty acid methyl ester
FDR	False discovery rate
FT-IR	Fourier-transformed infrared spectra
GlcNAc	N-acetylglucosamine
GO	Gene ontology
HAB	Harmful algal bloom
HDPE	High-density polyethylene
HTS	High-throughput sequencing
ICP-OES	Inductively coupled plasma optical emission spectrometry
ISO	International organization for standardization
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
LDPE	Low-density polyethylene
MAMP	Microbe-associated-molecular pattern
MANOVA	Multivariate Analysis of Variance
MPL	Marine plastic litter
mRNA	messenger ribonucleic acid
nMDS	non-Metric Multidimensional Scaling
OD	Optical density
OTU	Operational Taxonomic Unit
PAMP	Pathogen-associated-molecular pattern
PB	Phthalo blue
PBS	Phosphate buffered saline

PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PERMANOVA	Permutational Multivariate Analysis of Variance
PE	Polyethylene
PET	Polyethylene terephthalate
PGP	Plant growth promotion
PGPF	Plant growth promoting fungi
PGPR	Plant growth promoting rhizobacteria
PLFA	Phospholipid fatty acid
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
RLP	Receptor-like protein
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RT-qPCR	quantitative reverse transcription PCR
SD	Spinoladijk
SEM	Scanning electron microscopy
STAMP	Statistical analysis of metagenomic profiles
TOC	Total organic carbon
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TSA	Tryptone soya agar
VAMPS	Visual analysis of microbial population structure
WFPS	water-filled pore space

Summary

In this thesis, high-throughput sequencing methods were used to study microbial communities in two environments: the plant's rhizosphere and on plastic debris.

To prevent plant diseases and maintain a good crop productivity, crop cultivation often relies on high pesticide and fertilizer uses. There is a great interest in reducing this high use, and the effect of application of specific environment-friendly substrate amendments, such as biochar and chitin, to the soil or substrate is studied in this respect. These amendments can have a direct effect on the micro-organisms in **the rhizosphere**, the narrow zone of soil surrounding the plant root. This shift in rhizosphere microbiome should be directed towards beneficial micro-organisms such as plant-growth promotors and biocontrol agents.
Micro-organisms in the ocean are less-extensively studied compared to a soil environment. Nonetheless they can reach high numbers, especially when they can attach to substrates, and perform similar crucial roles as for soil environments. With an expected amount of 8 million tons entering our marine ecosystem yearly, the major contaminator of our oceans and seas is probably plastic. The microbial colonization of plastic debris in the marine environment, also referred to as **"the plastisphere"**, has been studied since the seventies, but the dynamics of this colonization and impact on plastic degradation, the marine ecosystem and animal and human health is currently poorly understood.

In the first part of this thesis, the effect of biochar and chitin on the rhizosphere microbiome in relation to crop growth, disease development or survival of human pathogens is studied. We showed that in nutrient-limiting conditions, biochar was able to change the physicochemical properties of soil and substrates and induced major changes in the bacterial composition of strawberry plants, redirecting the rhizosphere community towards a higher relative abundance of plant-growth promoters and biocontrol agents. No effect of biochar addition to peat was seen on the fungal composition of the strawberry rhizosphere. Both the increase in nutrient stock and the shift in bacterial community composition could be related to an increase in strawberry crop growth, a higher strawberry yield and an increase in resistance towards the fungal pathogen *Botrytis cinerea*. In addition, this aboveground infection also affected the rhizosphere bacterial community. This research indicates that upon biochar incorporation in peat, plants recruit rhizosphere bacteria that may help them in their defense and plant growth promotion.

In contrast, chitin addition to potting soil altered both the bacterial and fungal community composition of the lettuce rhizosphere, redirecting the microbiome towards higher abundances of chitin-degraders and plant growth promotors. These effects were correlated with an increase in lettuce growth and a reduction in the survival of *Salmonella enterica* on the leaves. Especially the consumption of contaminated leafy vegetables, such as lettuce, can be problematic for human health and reported to be the cause of *S. enterica* outbreaks. The use of chitin can thus be tested further as an interesting supplementary strategy for sustainable control of this zoonotic pathogen in the food chain.

In the second part of this thesis, the major contributors and dynamics of the bacterial and fungal colonization of marine plastic debris located at the seafloor in the Belgian part of the North Sea were studied. We showed that environmental properties, plastic-related properties and biofilm formation stages are probably the most important factors influencing the bacterial colonization on plastic. Dependent on the environment, this bacterial biofilm formation can go through progressive temporal stages, reaching a more or less stable community after a few months. In addition, we studied for the first time the fungal community on plastic debris, which was also quite diverse for different plastics, even when they were located at the same site. This microbial colonization could have major influences on the marine ecosystem. We showed that compared to seawater and sediment, other bacterial groups were attached on plastic, indicating that plastic could serve as a transport vector for micro-organisms to other environments. In contrast, the microbial colonization could also be beneficial in terms of biodegradation. Despite plastic-degradation has not been shown in this study, a *Mycobacterium* species was identified which is probably able to degrade the pigments of beach-located resin pellets, indicating that biodegradation of plastic-related chemicals is possible.

The use of high-throughput sequencing techniques made it possible to give insight in the microbial community composition. Here we focussed mainly on taxonomic identification using amplicon sequencing, but already a first step was taken towards functional annotations using shotgun metagenomics in function of chitinase detection. Future studies should elaborate more on this also including e.g. shotgun metagenomics, metatranscriptomics and stable-isotope probing in order to identify specific biochar-, chitin- and even plastic-metabolizers and related functions.

Samenvatting

Deze thesis beschrijft het gebruik van “high-throughput sequencing” methoden om microbiële gemeenschappen te bestuderen in twee omgevingen: de rhizosfeer van de plant en plastic afval.

Het telen van gewassen gaat vaak gepaard met een hoog gebruik van pesticiden en meststoffen om ziektes te voorkomen en een goede opbrengst te verkrijgen. Het gebruik van milieuvriendelijke substraat additieven zoals biochar en chitine kan daarbij een goed methode zijn om het gebruik van pesticiden en meststoffen in de landbouw te verminderen. Zowel biochar en chitine zijn in staat om de microbiële gemeenschap van de **rhizosfeer**, het kleine laagje grond die de wortel van de plant omvat, te veranderen in samenstelling. Om een goede gewasproductie en gezondheid van de plant te behouden, wordt de microbiële gemeenschap van de rhizosfeer daarbij gestuurd naar een hoger aantal plantengroei promotoren en biocontrole organismen.
In tegenstelling tot de bodem zijn de micro-organismen in de oceanen minder intensief bestudeerd. Desondanks bereiken ze hoge aantallen in de mariene omgeving, voornamelijk wanneer ze zich kunnen vasthechten aan substraten, en voeren ze net als in de bodem verscheidene cruciale processen uit. Recent onderzoek wijst uit dat jaarlijks ongeveer 8 miljoen ton plastic in het marien milieu terecht komt, wat plastic waarschijnlijk de belangrijkste vervuiler maakt van onze oceanen en zeeën. De micro-organismen die zich vastzetten op dit plastic, ook wel “**de plastisfeer**” genoemd, worden reeds bestudeerd sinds de jaren zeventig. Desondanks blijft de dynamiek van deze microbiële kolonisatie en de impact op plastic degradatie, het mariene milieu en de gezondheid van mens en dier grotendeels ongekend.

In het eerste deel van deze dissertatie wordt het effect van biochar en chitine toevoeging aan de bodem of substraat op het microbioom van de rhizosfeer in relatie tot plantengroei, ziekte ontwikkeling en de overleving van humane pathogenen op de plant bestudeerd. Onder nutriënt limiterende omstandigheden bleek biochar in staat om de fysicochemische eigenschappen van de bodem en substraten te veranderen. Daarnaast zorgde de toediening van biochar voor een wijziging in de bacteriële gemeenschap van de rhizosfeer van aardbei, met een verhoogd percentage aan plantengroei promotoren en biocontrole organismen. De schimmel gemeenschap van de aardbei rhizosfeer bleef daarentegen ongewijzigd. Biochar induceerde ook een groeipromotie van de aardbeiplant, een hogere vruchtopbrengst en een

hogere ziekteresistentie van de plant tegen *Botrytis cinerea*, allen gerelateerd aan het verhoogd aantal nutriënten en de wijziging in de bacteriële gemeenschap van de rhizosfeer. Ook een bovengrondse infectie met *B. cinerea* bleek de bacteriële gemeenschap van de rhizosfeer op eenzelfde manier te wijzigen in samenstelling. Dit onderzoek toont aan dat de toediening van biochar in substraat, de plant stimuleert om bacteriën aan te trekken die de plantengroei en ziekteweerbaarheid van de plant bevordert.

In tegenstelling tot biochar zorgde de toevoeging van chitine aan potgrond voor een wijziging in zowel de bacteriële als schimmel gemeenschap van de rhizosfeer van sla, waarbij zowel plantengroei promotoren en chitine afbrekers gestimuleerd werden. Deze verschuiving in samenstelling van het rhizosfeer microbioom was gecorreleerd met een promotie van de slagroei en een reductie in de overleving van *Salmonella enterica* op de bladeren van de plant. Verschillende *S. enterica* uitbraken zijn gelinkt met de consumptie van bladgroenten, en dus kan de consumptie van deze groenten zoals sla gevaarlijk zijn voor de menselijke gezondheid. Het gebruik van chitine in potgrond kan dus een interessante strategie zijn om deze uitbraken te voorkomen.

In het tweede deel van deze thesis werd de kolonisatie van plastic afval in het Belgisch deel van de Noordzee door bacteriën en schimmels bestudeerd. Omgevingsfactoren, plastic-gerelateerde factoren en verschillen in biofilm stadia zijn daarbij waarschijnlijk de belangrijkste factoren die de bacteriële kolonisatie van plastic afval in het marien milieu beïnvloeden. Afhankelijk van de omgeving waaraan de plastic wordt blootgesteld, kan de vorming van de bacteriële biofilm verschillende stadia doorlopen. Na enkele maanden van blootstelling van de plastic aan het marien milieu kan daarbij een min of meer stabiele bacteriële gemeenschap worden bereikt. Ook werd voor de eerste keer de kolonisatie van plastic afval door schimmels bestudeerd, die redelijk divers bleek tussen verschillende plastic fragmenten, zelfs als de plastic fragmenten op dezelfde plaats gelokaliseerd waren.

Deze microbiële kolonisatie van plastic kan het mariene ecosysteem op verschillende manieren beïnvloeden. Unieke bacteriële groepen, niet aanwezig in het zeewater of sediment, werden gevonden op plastic, wat erop wijst dat deze groepen afkomstig zijn uit een ander milieu en plastic dus als een transport vector kan dienen voor micro-organismen. De aanwezigheid van micro-organismen kan ook voordelig zijn, aangezien ze gekend zijn voor hun degradatie capaciteiten. In deze studie werd daarentegen geen biodegradatie van plastic door

micro-organismen aangetoond. Daarentegen werd een bacterieel taxon, *Mycobacterium* sp., geïdentificeerd op gekleurde industriële plastic pellets, een taxon die mogelijks pigmenten kan afbreken. Dit indiceert dus dat biodegradatie van plastic-gerelateerde chemicaliën mogelijk is.

Het gebruik van high-throughput sequencing technieken maakte het mogelijk om de taxonomische samenstelling van microbiële gemeenschappen en hun functioneel potentieel in het genoom in twee omgevingen te bestuderen. Daarbij werd voornamelijk gefocust op taxonomische identificatie met behulp van amplicon sequencing, maar de eerste stap naar functionele annotatie door gebruik te maken van shotgun metagenomics werd al gemaakt voor de studie van chitinases. Toekomstig onderzoek naar de microbiële gemeenschappen in een bodem of marien milieu zou daarbij gebruik moeten maken van een combinatie van technieken, bv. shotgun metagenomics, metatranscriptomics en stable-isotope probing om specifieke biochar-, chitine- en plastic-degradeerders en gerelateerde functies te identificeren.

Preface

This PhD is part of the Concerted Action 'Genomics' 2013-2017, an ILVO funded research project aimed at the establishment of the ILVO Genomics Platform and stimulating horizontal interaction across the wide spectrum of ILVO's researchers. In the frame of this platform, high-throughput DNA sequencing techniques are implemented for the genomic analyses of microbial communities and plant and animal (meta-)populations. The heart of the platform was formed by four PhD projects, each covering two topics, thus building bridges between different research units at ILVO.

In this PhD the microbial communities and their possible ecological impact are studied in two different environments: a terrestrial environment (the plant's rhizosphere) and a marine environment (plastic debris).

The first part of this thesis, focusses on the rhizosphere microbiome. This environment is already well-defined and has been examined intensively. The rhizosphere is the interface between the soil and plant and it contains several microbial species that could be beneficial for the plant in terms of plant growth and health. In this thesis, we describe how the addition of soil and substrate amendments (biochar, chitin), promotes a number of plant-beneficial organisms in the rhizosphere microbiome, which can result in the improvement of growth and disease resistance of the plant.

In the second part of the thesis, the microbial colonization of plastic debris located in the marine environment has been studied. So far, only a few studies related to the microbial colonization of marine-related plastic debris have appeared and this work is still in an exploratory phase. Within this thesis, the bacterial and fungal colonization of plastic debris, the factors influencing this colonization and the microbial source of this colonization are studied in the North Sea environment. Although the work presented here is still in an exploratory phase, it may provide a foundation for future research to establish if microbes may play a role in plastic degradation and mitigate effects of plastic pollution.

To study the microbial communities of these two environments, mainly high-throughput sequencing technologies have been used. Especially 16S rRNA gene and ITS2 gene amplicon sequencing were implemented, validated, and used to study bacterial and fungal communities in both environments. To get a deeper insight in the rhizosphere microbiome, changes in

function-related genes have also been studied using whole-genome shotgun sequencing. In addition, phospholipid fatty acids analysis (PLFA) has been applied to study changes in the microbial biomass induced by the soil and substrate amendments.

Chapter 1

General introduction

Parts of this introduction are published in:

De Tender C, Schlundt C, Devriese LI, Mincer TJ, Zettler ER, Amaral-Zettler LA. A review of microscopy and comparative molecular-based methods to characterize "Plastisphere" communities. Analytical methods 9,2132-2143.

1.1 ENVIRONMENTAL MICROBIOLOGY

The study of micro-organisms in natural or artificial environments is defined as environmental microbiology. More precisely, multi-species assemblages and species interactions of the microbial community are studied (Konopka, 2009). This microbial community includes all species not visible to the naked eye: bacteria, archaea, Protists, and even some members of the fungi and algae. Micro-organisms are extensively studied, because they carry out many important functions in the ecosystem of the earth, such as decomposition, oxygen production, plant symbiosis and contaminant degradation (Cong et al., 2015; Paul, 2015). Especially bacteria and archaea are extremely abundant in the biosphere, with an estimated total amount of 4 - 6 x 10^{30} cells. The highest numbers of bacteria and archaea can be found in the oceans (1.2 × 10^{29}) and in soils (2.6 × 10^{29}) (Whitman et al., 1998).

For any natural or artificial ecosystem, three central questions are asked regarding the discovery and characterization of microbial communities: (1) what type of micro-organisms are present in the environment?; (2) what do these micro-organisms do?; and (3) how do the activities of these micro-organisms relate to ecosystem functions (Rastogi and Sani, 2011)? Cultivating the micro-organisms in commercial growth media makes it possible to identify species by Sanger sequencing (Sanger et al., 1977). However, > 99% of the micro-organisms in any environment are not cultivable by standard cultivation techniques (Staley & Konopka, 1985), by which a major part of the microbial community remains underexplored. With the introduction of culture-independent methods, it became possible to explore the “uncultivable part” of micro-organisms in the environment.

The development of new molecular approaches during the 1980s, based on the extraction and characterization of DNA from the environment (e.g. water, soil, sediments), revolutionized the analysis of microbial communities in the environment (Fox et al., 1980). For the identification of bacteria, the 16S ribosomal RNA (rRNA) gene is generally used as molecular marker. The 16S rRNA gene is highly conserved between different species of bacteria and archaea and consists of two region types: the highly conserved sites that contain regions for primer design; and the hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria (Woese, 1987) (Figure 1.1). Similar to the 16S rRNA gene, the internal transcribed spacer (ITS) region is conserved across large groups of fungi, but contains enough variation to be phylogenetically informative (Lindahl et al., 2013). For most molecular methods, only a part of these phylogenetic marker genes can be used. In a soil environment,

the V3-V4 region of the 16S rRNA gene is generally used as it has a high coverage rate in most bacterial phyla (Vasileiadis et al., 2012; Klindworth et al., 2013). In case of the ITS region, the coverage of ITS1 and ITS2 region over the fungal phyla is comparable (Porras-Alfaro et al., 2014).

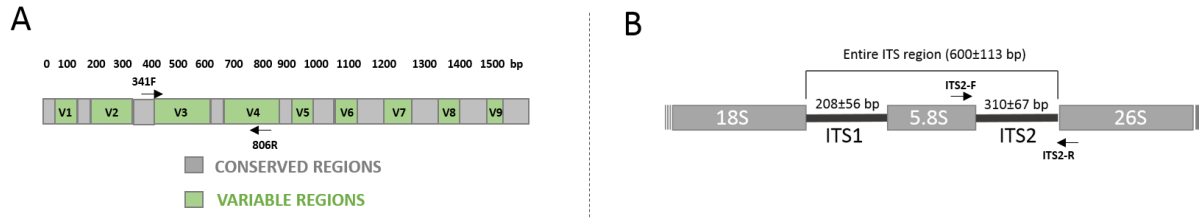


Figure 1.1 Structure of the 16S rRNA gene and internal transcribed spacer (ITS) region of bacteria and fungi respectively. A) Structure of the 16S rRNA gene sequence based on those of *Escherichia coli* (± 1500 bp). B) Structure of the ITS region (Modified from Poras-Alfarro et al., 2014). For both panels of the figure, the primer combinations used in this PhD dissertation are indicated by arrows.

The genetic fingerprint techniques Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) differentiate between microbial species in the environment by amplifying these specific molecular markers in environmental DNA and separating individual sequences based on melting properties (GC ratio) or length for DGGE and T-RFLP, respectively (Muyzer & Smalla, 1998; Thies, 2007). Fingerprinting techniques have the advantage that they are quite fast and that multiple environmental samples (10-16) can be analysed simultaneously, which makes comparison between samples possible. However, only 1-2%, of the total microbial population of an environment can be detected with these techniques, in which especially high abundant organisms will be identified (MacNaughton et al., 1999). In addition, T-RFLP fingerprint alone does not allow for conclusive taxonomic identification of individual phylotypes, because it is challenging to recover terminal fragments for direct sequencing (Dunbar, 2001).

These limitations were overcome with the introduction of the whole community analysis approaches or high-throughput sequencing (HTS) techniques (section 1.1.1). To study the microbial community in an environment, HTS approaches can be divided in three groups: (1) amplicon sequencing, (2) shotgun metagenomics, and (3) metatranscriptomics. Within this PhD project, both amplicon sequencing and shotgun metagenomics will be applied to study soil (rhizosphere) and marine (plastisphere) environment related questions, giving insight in the microbial community composition and their genomic potential. Metatranscriptomics, which is not applied within this thesis, can be used to study the microbial activity, by studying

gene expression in specific microbial environments. Especially in soil environments, metatranscriptomic approaches have not been widely used, probably due to the instability of messenger RNAs (mRNAs) and difficulties in their extraction from complex ecosystems (Lagos et al., 2015). In addition, soil microbial communities are quite diverse and there is still a lack of representative genomes which complicate the bioinformatics analysis of metatranscriptome data (Carvalhais et al., 2013). One of the drawbacks of these HTS techniques is that they are not able to provide information on the absolute microbial biomass. In soil environments, phospholipid fatty acids (PLFA) analysis can be used which will provide information on the absolute biomass of several microbial groups (section 1.1.2).

1.1.1 High throughput sequencing

Since 2005, several HTS techniques have been introduced. The first commercially successful HTS machine which was widely available for consumers, was the Roche 454, which uses pyrosequencing (Margulies et al., 2005). The 454 system can sequence relatively long reads (~700 bp) within a short period (10 hours). Since 2016, the system is however no longer supported by Roche, probably due to the higher sequencing cost compared to other systems and relatively high error rate in terms of poly-bases longer than 6 base pairs (bp) compared to other technologies (Liu et al., 2012). Substitutions deletions and insertions in Roche 454 are present for every 1,000 to 10,000 nucleotides, which is 10 times higher compared to Sanger Sequencing (Erwing & Green, 1998; Quinlan et al., 2008). Therefore the technology is expected to disappear from the metagenomics field. Currently, the most important HTS technique is the Solexa method, which was acquired by Illumina (www.illumina.com). Adaptor-ligated DNA fragments are passed over a flow-cell containing complementary oligonucleotides. Each individual flow-cell bound DNA strand will be amplified by “bridge amplification” to form clusters containing clonal DNA fragments (Figure 1.2). After amplification, fluorescent dNTPs and DNA polymerase are washed over the single-stranded flow-cell bound clusters in cycles, by which the incorporation of a nucleotide is monitored by exciting the fluorophores with appropriate lasers (Heather & Chain, 2016). In comparison to Roche 454, the number of frameshifts (introducted by homopolymer errors) and single-base sequencing errors are lower for Illumina sequencing (Luo et al., 2012). Illumina currently produces a suite of sequencers optimized for a variety of throughputs. The most commonly used Illumina platforms used in environmental microbiology are the MiSeq and HiSeq. The MiSeq is a benchtop sequencer

with a run time of 4 (1x 36 hours) to 56 hours (2x 300 bp) and outputs intended for targeted amplicon sequencing and the sequencing of small genomes such as those of bacteria. MiSeq sequencers can generate up to 25 million read clusters of up to 2x300 bp. The HiSeq is engineered for high-throughput applications, such as shotgun metagenomics (Reuter et al., 2015). The newest HiSeq sequencers can generate up to 5 billion read clusters of up to 2x125 bp per flow cell (which consists of 8 lanes). Older HiSeq sequencers such as the HiSeq2500 can produce longer reads in rapid run mode (2x250 bp), but have lower output (up to 600 million read clusters per flow cell).

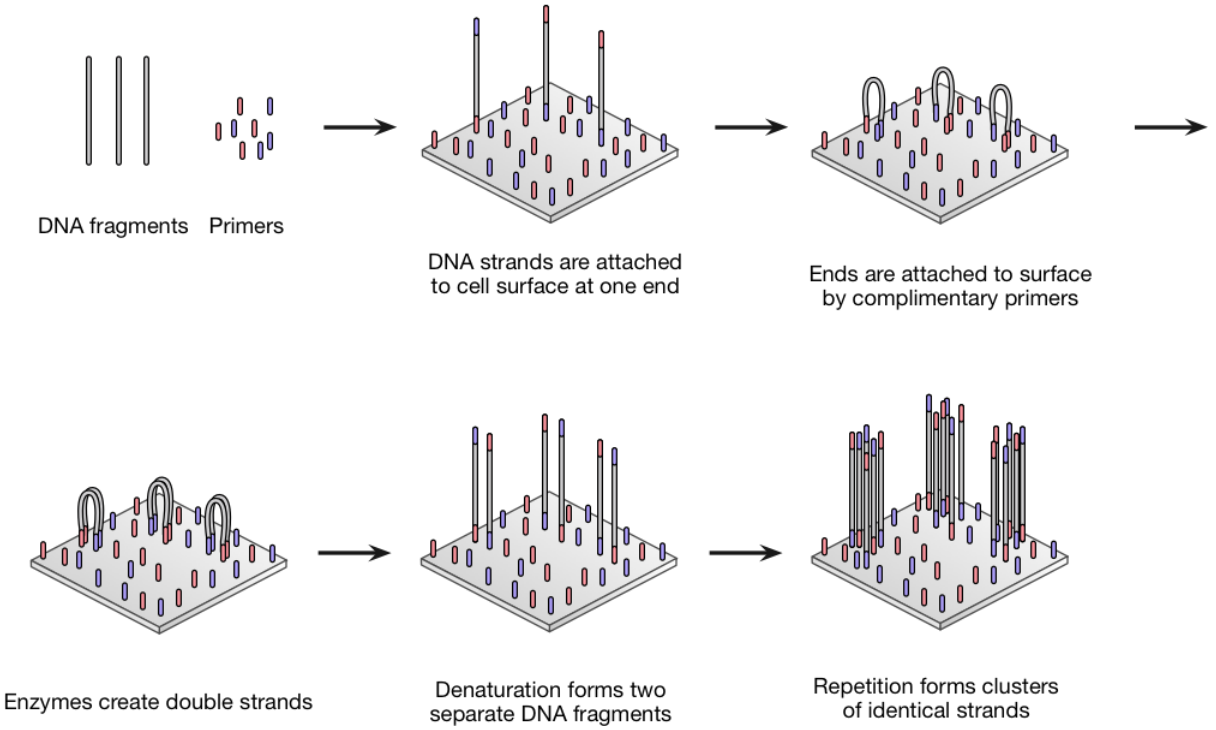


Figure 1.2 Illumina sequencing technology making use of bridge PCR. Adaptor-ligated DNA fragments are passed over a flow-cell containing complementary oligonucleotides. DNA fragments will bind to the flow cell and ends are attached to the surface by complementary primers. Enzymes will create double bind strands, which will be denaturated to form two separate DNA fragments. This process is repeated until clusters of identical strands are formed. (source: http://www.atdbio.com)

Amplicon sequencing targets a specific genomic locus for amplification, e.g. 16S rRNA gene (bacteria) or ITS (fungi). In general, amplicon sequencing consists of two steps. First, the specific genomic locus is amplified by polymerase chain reaction (PCR), making use of target-specific primers. Further on, nucleotide indices or barcodes are added to the target region by a second round of PCR amplification or by enzyme ligation (Figure 1.3A). This makes it possible to sequence multiple samples (> 100) simultaneously, and to compare many environmental samples. Amplicon sequencing can therefore currently be used as a diagnostic tool to study

the taxonomic composition and diversity of microbial communities (Vogl et al., 2012). Recently, the technique has also been applied to target “functional marker genes” encoding key enzymes of nitrogen cycling (e.g. nifH, amoA, urea), carbon cycling (e.g. mcrA, pmoA) and sulphur cycling (e.g. dsrAB, apsA). So far, only a few studies have applied this technique to identify protein encoding genes (Lüke et al., 2015).

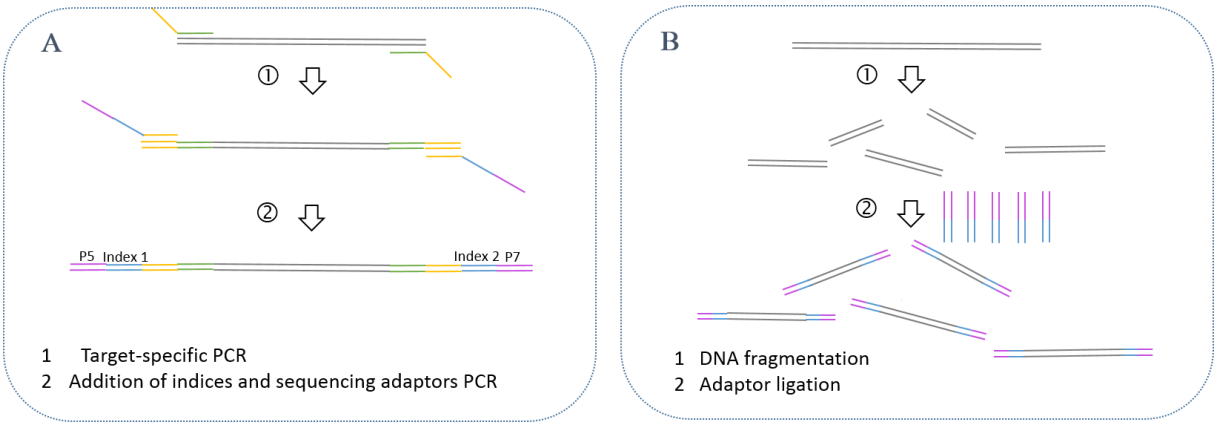


Figure 1.3 High throughput sequencing technologies. A) Amplicon sequencing library preparation consists of two successive PCRs to amplify the molecular marker sequence (amplicon PCR) and to attach the dual indices (index PCR). B) DNA fragmentation and adaptor ligation (shotgun metagenomics).

After sequencing the genomic loci of the environmental samples, the standard pipeline for amplicon analysis starts by clustering sequences with a percent sequence similarity threshold (typically 97% for bacteria) into “Operational Taxonomic Units” (OTUs). For each OTU, a representative sequence is selected and annotated using a 16S rRNA gene classification method and database (e.g. greengenes or SILVA) (DeSantis et al., 2006; Quast et al., 2013; Chaudhary et al., 2015). All sequences with a 97% similarity to this OTU will thus get the same annotation (Nguyen et al., 2016).

Before the arrival of sequencers such as Roche 454 or the Illumina MiSeq, 16S rRNA gene sequencing was carried out using labour intensive techniques including cloning in *E. coli*, colony picking and plasmid extraction, followed by Sanger sequencing. HTS therefore brought two major advances: massive parallelization of the sequencing reactions, which reduces sequencing cost extensively, and separation of templates without the need to insert gene fragments in a host (Sanschagrin & Yergeau, 2014).

Amplicon sequencing also faces some limitations. First, only a specific marker gene is targeted, which makes amplicon sequencing limited to taxonomic identification or restricted functional identification. Therefore it is impossible to directly resolve all biological functions associated

with these taxa (Sharpton, 2014). Second, primer mismatches or the chose of primer pair combination can result in reduced amplification for certain taxa, causing underrepresentation (or sometimes even absence) of those taxa in the data. In addition, bacterial species can contain multiple 16S rRNA gene copies. The number of 16S rRNA gene copies varies greatly from one in many species to up to 15 in some bacteria (Rainey et al., 1996). In microbial eukaryotes, the number of the SSU rRNA unit (18S) is positively correlated with genome size and can reach numbers of 100 or even thousands copies (Prokopowich et al., 2000). This can lead to an overestimation of certain microbial species in the environment and thus a distorted view on the community composition (Galtier & Steel, 2007; Vetrovský & Baldrian, 2013). Third, PCR errors can lead to a misinterpretation of the community composition. Additionally, high-throughput methods generate frequent errors during the sequencing procedure. Therefore, data sets derived by HTS must be subjected to extensive quality control measures (Kunin et al., 2010). Finally, extraction of the total DNA from environments may include material from dormant or even dead organisms. Therefore, it is possible that the most abundant organisms identified from the sequencing result are not the most abundant living organisms in the environment (Taberlet et al., 2012).

In **shotgun metagenomics**, all DNA extracted from a certain environment is analysed entirely, instead of targeting a specific genomic locus. This global sequencing approach is based on two steps. First the DNA molecules are split into tiny fragments, which are independently sequenced (Figure 1.3B). In a second step, these gene fragments can be reassembled (Bouchez et al., 2016). Some of these reads will give taxonomically relevant information (e.g. fragments from 16S rRNA gene), and others will provide insight in the biological functions encoded in the genome (fragments from coding sequences) (Sharpton, 2014).

In comparison with amplicon sequencing, shotgun metagenomics is advantageous for two reasons: (1) sequencing of the global metagenome offers the most comprehensive approach for a diversity and functionality study of micro-organisms in an environment; and (2) if already present in the database, new taxa or functions can be assigned to a certain environment where they were previously not identified (Bouchez et al., 2016). However, the application of shotgun metagenomics also has some limitations. First, the cost to obtain a good sequencing depth and the data complexity makes it limited for only a few samples, compared to amplicon sequencing. Second, the data is extremely large and highly complex which requires access to

servers or super computers with high storage and calculating capacities (Bouchez et al., 2016). Third, similar as for amplicon sequencing, the detection of DNA in a sample does not imply that this DNA originates from viable organisms or that the associated genes are functional (Nielsen et al., 2007).

The use of HTS approaches provides not only insight into the diversity of microbial species from a wide variety of environments, but can also provide insights or a solution for human health, agricultural and environmental concerns. Due to these enormous possibilities of HTS, the total number of microbial community studies is rising exponentially (Figure 1.4).

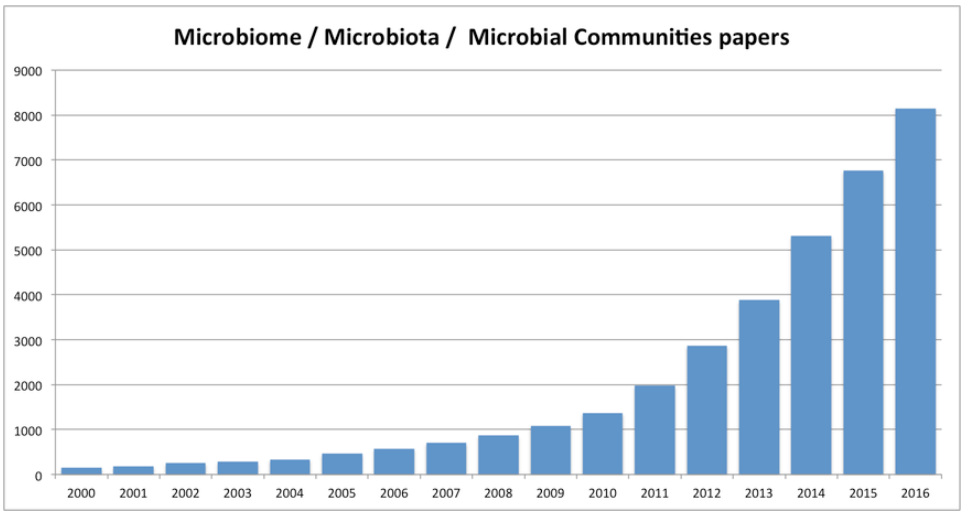


Figure 1.4 Evolution of the number of microbiome / microbial communities papers as registered in Pubmed (microbiomedigest.com, 9 January 2017).

Amplicon sequencing is often used in microbial ecology studies. In water quality assessments, HTS provides new insights into the ecology of microbial mediated processes that influence fresh water quality such as algal blooms, contaminant biodegradation, and pathogen dissemination, but can also be used to trace bio-indicators for sewage pollution (Tan et al., 2015).

In the human health sector, much attention is being paid to the characterization of diverse microbes resident to healthy human populations (Human Microbiome Project Consortium, 2012). They have shown that obesity and inflammatory bowel disease are associated with a decreased microbial diversity in the gut (Turnbaugh et al., 2009; Qin et al., 2010). In contrast, bacterial vaginosis is associated with an increase in microbial diversity (Fredricks et al., 2005). HTS can also be used to study the human genome and genome-related diseases. As an

example, Illumina sequencing is recently used to detect Down syndrome in human foetuses by tracing foetal DNA in the mother's blood (Palomaki et al., 2011).

1.1.2 PLFA analysis

The microbial cellular membranes of Bacteria and Eukarya contain PLFAs of different chain lengths and composition to maintain cell-membrane integrity and cellular function in response to their immediate environmental conditions. In soil, microbial PLFAs typically have a chain length of 14 to 20 carbon atoms and are considered to be either bacterial or fungal. In mixed cultures, such as in a soil environment, PLFA analysis cannot be used to identify individual species, but can provide an overall view of the microbial community by indicating microbial groups (Zelles, 1999).

PLFA analysis consists of four steps: (1) the extraction of lipids from soil samples using a single-phase chloroform mixture; (2) isolation of phospholipids from the other extracted lipids by fractionation using a solid phase extraction column; (3) methanolysis of phospholipids to produce fatty acid methyl esters (FAMEs); and (4) analysis of FAMEs by capillary gas chromatography (Quideau et al., 2016).

The use of PLFAs to characterize the microbial community has several benefits. First, PLFAs are only present in viable cells, which have an intact membrane (Zelles, 1999). Second, it gives an estimation of the absolute microbial biomass of the environment. Third, it has been proven that the technique is not only rapid and inexpensive, but usually also sensitive and reproducible (Frostegård et al., 2013). However, the technique also has some drawbacks. First, caution should be taken in the decision of the studied PLFAs. Some PLFAs can be present in several microbial groups, leading to misinterpretations. As an example, PLFA cy17:0 and cy19:0 are usually considered as indicators of Gram-negative bacteria, but are also found in large amounts in some Gram-positive bacteria (Frostegård et al., 2013). Second, fatty acid composition can be influenced by temperature and nutrition (Malik et al., 2008). Third, the technique cannot be used to assign taxonomy to the species. Individual fatty acids cannot be used to represent a specific species as a single micro-organism can have numerous fatty acids and the same fatty acids can occur in more than one species. In addition, diversity of the microbial community cannot be estimated using PLFA data (Frostegård et al., 2013).

1.1.3 Research objectives

In this PhD project, HTS was used to tackle two problems in the two prokaryotic richest biospheres: soil and oceans. First, amplicon sequencing, shotgun metagenomics and PLFA analysis have been used to study micro-organisms in close interaction with the plant root. Addition of organic amendments such as biochar or chitin to soils or substrates can enhance the disease-suppressiveness of the soil and disease resistance of the plant and increase plant growth (Sullivan, 2004; Meller-Harel et al., 2012; Cretoiu et al., 2013; Postma and Schilder, 2015), which is possibly associated with a change in the plant root microbiology, also referred to as **the rhizosphere**.

Second, microbial communities on plastic debris in the marine environment were characterized. Recently Eriksen et al. (2014) estimated that 5.25 trillion plastic particles are scattered over the oceans worldwide. This plastic pollution in our oceans will only grow in the following years. One of the possible approaches to tackle the problematic plastic pollution in the marine environment, is the characterization and application of plastic-biodegrading micro-organisms. Therefore, screening and a comprehensive knowledge of the microbial community on plastic, defined as "**the plastisphere"** (Zettler et al., 2013), is necessary.

In the following sections of the introduction, these two environments will be discussed in more detail.

1.2 ENVIRONMENT 1: THE RHIZOSPHERE

The rhizosphere comprises the narrow zone (1-2 mm) of soil surrounding or attached to and influenced by the plant roots (Hiltner, 1904). It consists of a mixture of soil particles and an overwhelming number of organisms, mainly bacteria and fungi, but also oomycetes, nematodes, protists, algae, viruses, archaea and arthropods (Figure 1.5) (Mendes et al., 2013). These organisms are attracted by and feed on rhizodeposits, including nutrients, exudates, border cells and mucilage released by the plant root (Philippot et al., 2013). Consequently, the rhizosphere microbiome is significantly different in composition from the microbial populations in the bulk soil (Lundberg et al., 2012).

The main drivers of the rhizosphere microbiome composition are the type of bulk soil (de Ridder-Duine et al., 2005; Berg & Smalla, 2009; Santos-Gonzalez et al., 2011; Lundberg et al., 2012; Schreiter et al., 2014), and the plant species and/or cultivar type (Ladygina & Hedlund, 2010; Inceoglu et al., 2012). Soil represents the major source of microbes for the rhizosphere

and is particularly important in the microbial composition through its physicochemical properties. Plant species not only influence the rhizosphere microbiome by differences or changes in rhizodeposit release, but also by differences in root morphology (Philippot et al., 2013). Furthermore, the rhizosphere microbiome is influenced by fertilizer addition, crop rotation and application of pesticides (Ikeda et al., 2011; Hilton et al., 2013; Jacobsen & HjelmsØ, 2014). A potential source for the assembly of the rhizosphere microbiota are the plant seeds. The composition of this prelaminar microbiome and the developmental stage of the plant, will lead to a different rhizosphere microbiome composition (Nelson, 2004; Schreiter et al., 2014).

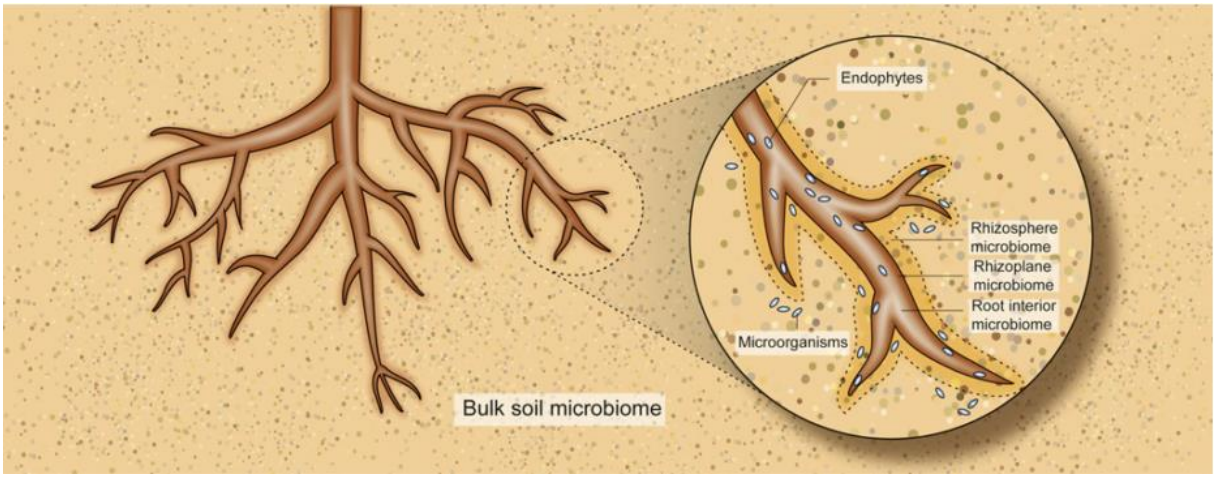


Figure 1.5 The narrow zone of soil surrounding the plant root is considered as the rhizosphere. It consists of soil particles, bacteria, fungi, oomycetes, viruses, archaea and small eukaryotes. (Figure adapted from Gaiero et al. (2013))

Rhizosphere microbes have profound effects on several plant processes such as seed germination, seedling vigour, plant growth and development, nutrition, diseases, and productivity (Mendes et al., 2013). In this introduction, it is not the intention to cover all available knowledge regarding the rhizosphere microbiome, given the enormous number of publications. Two important aspects however, the influence of the microbiome on plant growth and diseases, are discussed in more detail.

In general, the belowground microbial species richness has been proposed as a predictor of the aboveground plant productivity and can even help to maintain plant productivity under changing environmental conditions (Wagg et al., 2011). The predominantly studied group of rhizosphere organisms are those involved in plant growth and productivity. Well-known examples are mycorrhizal fungi, which acquire nutrients from the soil and deliver these to

plant roots, which in turn release photosynthates through the roots. Others are defined as nitrogen-fixing rhizobia, with *Rhizobium* as the most commonly known, which enter in close association with the plant and provide the plant of nitrogen (Richardson et al., 2009; Zamioudis & Pieterse, 2012). These micro-organisms live particularly in symbiosis with the plant and provide nutrients and minerals from soil to the plant through translocation (Mendes et al., 2013). Other beneficial associations with the plant include non-symbiotic plant-growth-promoting rhizobacteria (PGPR) and fungi (PGPF) which stimulate plant growth through the degradation of soil pollutants, production of phytostimulators, nutrient mobilization, improvement of soil structure, or by suppressing plant diseases or pests (Zamioudis & Pieterse, 2012; Ahemad & Kibret, 2014). The latter are also referred to as biocontrol micro-organisms, which are involved in plant health and disease suppression. A list of commonly known PGPR and their mechanism to improve plant growth are given in Table 1.1. An extensive overview of the mechanisms of PGP can be found in the review of Lugtenberg and Kamilova (2009). Caution should however be taken concerning these genera, because several not only contain PGP, but also pathogenic strains, e.g. Bacillus, Pseudomonas, Streptomyces. Therefore, a good taxonomic classification is necessary (Viaene et al., 2016).

Several mechanisms are known in which rhizosphere micro-organisms defend the plant towards pathogens: (1) production of antibiotics which inhibit the growth or activity of competing micro-organisms; (2) competition between micro-organisms for nutrients and trace elements; (3) parasitism; (4) interfering quorum sensing affecting virulence; and (5) induction of the systemic resistance response in plants (Mendes et al., 2013). Most of these mechanisms are suppressive for soil-borne pathogens, with the exception of the systemic resistance response induction which is also related to aboveground plant infections. The activation of the plant's induced systemic resistance (ISR) by micro-organisms can either be through the activation of the jasmonic acid and ethylene pathway or through the salicylic acid pathway in the plant (van de Mortel et al., 2012; Zamioudis & Pieterse, 2012).

Table 1.1 Commonly known plant growth promoting rhizobacteria (PGPR) and fungi (PGPF). The mechanism of plant growth promotion, if known, is given. However other mechanisms can be involved which are currently unknown are needs to be examined

PGPR genera	Specific strains	Mechanism	Reference
Acinetobacter	*A. calcoaceticus*	Gibberellin production, phosphate solubilization	Kang et al., 2009
Azetobacter sp.	*A. paspali*	Nitrogen fixation, auxin production	Okon et al., 1998
***Azospirillum* sp.**		Nitrogen fixation, increase in root development	Lugtenberg & Kamilova, 2009
	A. brasilense	Auxin production	Barbieri & Galli, 1993
Bacillus sp.	*B. amyloliquefaciens*	Release of volatiles (antifungal)	Ryu et al., 2003
	B. cereus	Antibiotic production	Lugtenberg & Kamilova, 2009
	B. subtilis	Release of volatiles, antibiotic production, activation ISR	Ryu et al., 2003; Rudrappa et al., 2008
	B. thuringiensis	Biocontrol against insects	
***Bradyrhizobium* sp.**		Nitrogen fixation / root nodulation	Lugtenberg & Kamilova, 2009
Enterobacter sp.	*E. cloacae*	Release of volatiles	Glick, 2012
Paenibacillus	*P. polymyxa*	Activation ISR	Timmusk & Wagner, 1999
Pseudomonas sp.	*P. fluorescens*	Auxin production, activation ISR, plant iron nutrition	Lugtenberg & Kamilova, 2009; Glick, 2012
	P. putida	Biocontrol	Pliego et al., 2008
***Rhizobium* sp.**		Nitrogen fixation / root nodulation, production of ACC deaminase	Lugtenberg & Kamilova, 2009
Sphingomonas sp.		Chitinase production	Wachowska et al., 2013; Zhu et al., 2007
Streptomyces sp.		Antibiotic production, siderophore production, auxin production (in planta tests still necessary)	Viaene et al. (2016)
	S. platensis	Volatile production (antifungal)	Wan et al. (2008)
PGPF genera		**Mechanism**	**Reference**
Gigaspora sp.		Promotion uptake phosphorus & minor elements	Allen, 1996
Glomus sp.		Promotion uptake phosphorus & minor elements	Allen, 1996
Lecanicillium sp.		Mycoparasitism, activation ISR	Goettel et al., 2008; Van Nam et al., 2014; Nguyen et al., 2015
***Trichoderma* sp.**		Predation & parasitism	Harman et al., 2004

To improve plant productivity and disease resistance of the plant, one of the strategies is to reshape the rhizosphere microbiome in favour of disease and pest suppressive and plant beneficial microbes. Soil amendments have the potential to induce these shifts in the rhizosphere microbiome, improve soil and substrate quality, plant growth and plant resistance. Within this research, two environment friendly soil amendments, biochar and chitin, are used to study the effect on strawberry and lettuce growth and their disease

susceptibility towards aboveground pathogens. Both amendments will be discussed further in this introduction.

1.2.1 Biochar

Biochar is the collective name of carbon-rich by-products which are produced during biomass pyrolysis for biofuel production (Goldberg, 1985). Differences in heating rates, pyrolysis temperature, residence time and especially feedstock material will affect the carbon and nutrient composition of biochar drastically. Several feedstocks can be used for biochar production, for which forestry wastes, animal manures and crop residues are the most used (Laghari et al., 2016). Despite the fact that nutrient composition and carbon content can differ significantly between several types of biochar, there are efforts to set-up a more strict definition (EBC, 2012).

Biochar is typically porous, has a high surface area and affinity for charged particles (Glaser et al., 2002; Keech et al., 2005; Steiner et al., 2008). Biochar plays a major role in sequestering atmospheric carbon dioxide, without releasing the carbon for centuries and even millennia, having the potential to reduce the CO_2 release back to the atmosphere (Barrow, 2012). Further on, biochar has been suggested to be used in soil management, feedstuff for livestock, water purification, health and sanitation, environmental remediation and as source of biomass-derived energy (Xie et al., 2015; Laghari et al., 2016). Some of these benefits are illustrated in Figure 1.6.

Biochar has the potential to increase soil water permeability, soil water retention and soil pH. In addition, biochar increases nutrient retention and the amount of organic carbon in soil or substrates (Chan et al., 2007; Asai et al., 2009; Laird et al., 2010). Biochar addition to soil can affect the soil microbial composition (Abujabhah et al., 2016; Dai et al., 2016), with even larger effects on the rhizosphere microbiome (Dai et al., 2016; Egamberdieva et al., 2016). It was proposed that biochar could act as an additional habitat for micro-organisms due to its highly porous structure, or could impact the microbiome due to an increase in water retention, nutrient release or pH (Quilliam et al., 2013). Further on, biochar addition does not only influence the microbial abundance, but also the microbial activity (Quilliam et al., 2013), however this is still questionable (Domene et al., 2014).

This change in microbial community in the soil or rhizosphere can affect the plant characteristics. Biochar has been extensively studied for its beneficial effects in agriculture.

Several studies have already shown a positive effect towards the growth of crops such as rice, wheat, maize, radish, soybean, tomato and sweet pepper (Chan et al., 2007; Asai et al., 2009; Graber et al., 2010; Major et al., 2010; Dong et al., 2015; Egamberdieva et al., 2016). Additionally, biochar can increase disease suppression and pathogen control, e.g. a higher resistance towards *Botrytis cinerea* (gray mold) and *Leveillula taurica* (powdery mildew) for pepper and tomato plants, suppression of *Fusarium* crown and root rot and a higher resistance towards a foliar mite on pepper plants (Matsubara et al., 2002; Elad et al., 2010; Elmer & Pignatello, 2011). These positive effects can be explained by the effect of biochar on the soil and rhizosphere microbiome, but can also be related to changes in physicochemical and biological properties of soils and substrates (Elad et al., 2011; Jeffery et al., 2011). The remark should be made however, that despite the high number of publications showing the benefits of biochar on plant growth and disease resistance, also neutral or negative effects of biochar on these plant parameters are reported (Revell et al., 2012; Gravel et al., 2013 Copley et al., 2015; Ebrahimi et al., 2016).

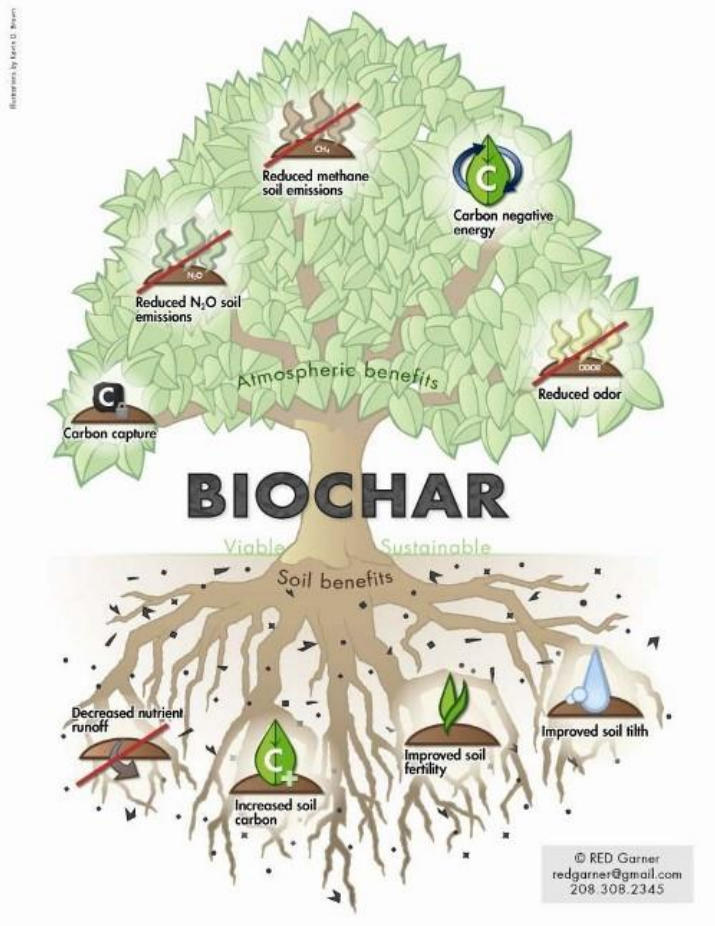


Figure 1.6 Atmospheric and soil benefits of biochar addition to soil or substrate (©REG garner).

1.2.2 Chitin

Chitin is composed of β-1,4-linked N-acetylglucosamine (GlcNAc) residues forming a linear homopolymer (Gooday et al., 1990). It has a biological role in both terrestrial and aquatic ecosystems as a major constituent of fungal cell walls, and insect, krill and shellfish exoskeletons (Gomes et al., 2001). As a result, chitin is the second most abundant biopolymer in nature after cellulose. Chitin has applications in several areas such as biofilms, drug release, wound dressing and agriculture (Younes & Rinaudo, 2015). Therefore, the commercial global production rate of chitin is enormous with an estimated rate of 10^{10} to 10^{11} tons $year^{-1}$ (Beier & Bertilsson, 2013). Pure chitin is mainly produced from crab and shrimp shells and can be extracted either chemically or biologically. In chemical processing, chitin is extracted by acid treatment to dissolve the calcium carbonate followed by alkaline solution to dissolve proteins. In addition, a decolourization step is often added in order to remove pigments and obtain a colourless pure chitin. In biological processing, various proteolytic micro-organisms deproteinize shrimp shells to extract chitin. The biological extraction of chitin offers high reproducibility in shorter time, simpler manipulation, smaller solvent consumption and lower energy input. Additionally, the structure of chitin is preserved better compared to the chemical method. However, the biological method is still limited to laboratory scale studies (Younes & Rinaudo, 2015).

Several organisms such as fungi, bacteria, archaea, rotifers, some algae and even plants can produce chitin-degrading enzymes (Hooday, 1990; Huber et al., 1995; Vrba et al., 1996; Strosjová & Vrba, 2005). Bacteria and fungi are however believed to be the major mediators of chitin degradation in nature (Beier & Bertilsson, 2013; Langner & Göhre, 2016). The degradation of chitin into GlcNAc residues consists of three steps: (1) cleaving the polymer into water-soluble oligomers, (2) splitting the oligomers into dimers and (3) cleavage of the dimers into monomers. The first two steps are catalysed by chitinases, while the last step involves N-acetylglucosaminidases (Hartl et al., 2012; Beier & Bertilsson, 2013). Growth on chitin is however not always accompanied by a direct degradation of the polymeric structure. Alternatively, chitin can be deacetylated to chitosan or even cellulose-like forms through deamination (Campbell & Williams, 1951). These in turn can be further degraded by the production of chitosanases and cellulases, in which the latter even can possess considerable chitosan-cleaving activity (Xia et al., 2008) (Figure 1.7).

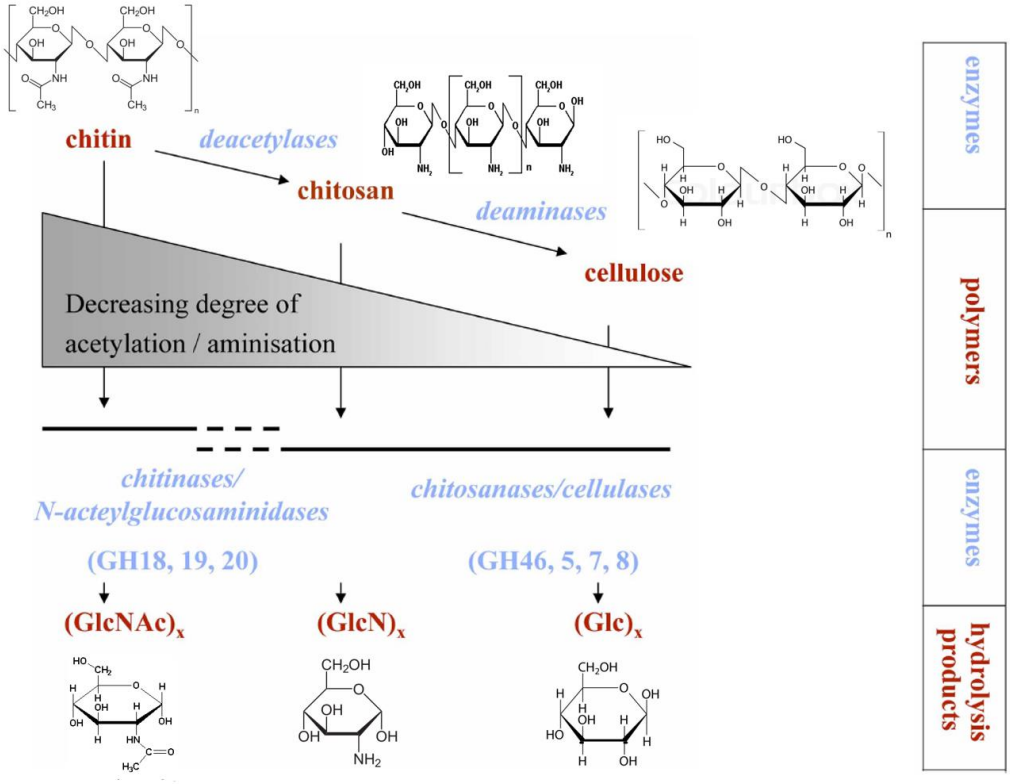


Figure 1.7 Processes involved in chitin degradation. Chitin can be degraded by the production of chitinases and N-acetylglucosaminidases to GlcNAc residues. However if deacetylation and deamination processes are very active, chitosan and even cellulose-like molecules may be produced. (GH: glycoside hydrolase family; GlcNAc: N-acetylglucosamine, GlcN: glucosamine; Glc: glucose. Adapted from Beier & Bertilsson, 2013).

Organisms capable of hydrolysing chitin will increase in number and/or activity once chitin is added to soil or substrates, leading to a different microbial community composition (Gryndler et al., 2002; Cretiou et al., 2013; Jacquiod et al., 2013; Vida et al., 2016). The degradation of chitin and microbial community change can affect plant growth, as carbon and nitrogen nutrients will be released in substantial amounts in the soil or substrate (Williamson et al;, 2000; Metcalfe et al., 2002). Further on, chitin and derivatives (e.g. chitosan) can enhance suppressiveness of plants towards pathogens. It has been shown that chitin or chitosan soil treatment reduces the density of nematodes in soil and results in a lower infection rate of plant roots by nematodes (Cretiou et al., 2013; Radwan et al., 2012). Furthermore a reduction in population density of *Verticillium dahliae* microsclerotia in soil and a reduction of disease severity caused by *V. dahliae* on antirrhinum can be noted (Dutta & Isaac, 1979; Cretiou et al., 2013). Chitinase-producing organisms, e.g. *Bacillus amyloliquefaciens*, *Burkholderia* sp. and *Streptomyces* sp., can even be used in agriculture as effective biocontrol agent against a

number of phytopathogenic fungi and nematodes, in which the addition of chitin can accelerate the response (Bhattacharya et al., 2007).

In addition, chitin does not only enhance suppressiveness of plants by the activation of chitinase production of micro-organisms which affect soilborne pathogens, but can also trigger plant immunity and act as a microbe/pathogen-associated-molecular pattern (MAMPs/PAMPs). In rice (*Oryza sativa*), Chito-oligosaccharides (CHOS) with a chain length n >= 8 formed from chitin due to chitinase activity, will be bound by the plant LysM containing receptor-like protein (RLP) CEBIP which will in a cascade reaction lead to the activation of plant defense genes (Figure 1.8) (Langner & Göhre, 2016). This chitin perception can however differ from the plant studied. In rice, both LysM proteins are required for chitin perception and signalling, whereis in the model plant Arabidospsi, the CEBiP-type molecules are not involved (Wan et al., 2008).

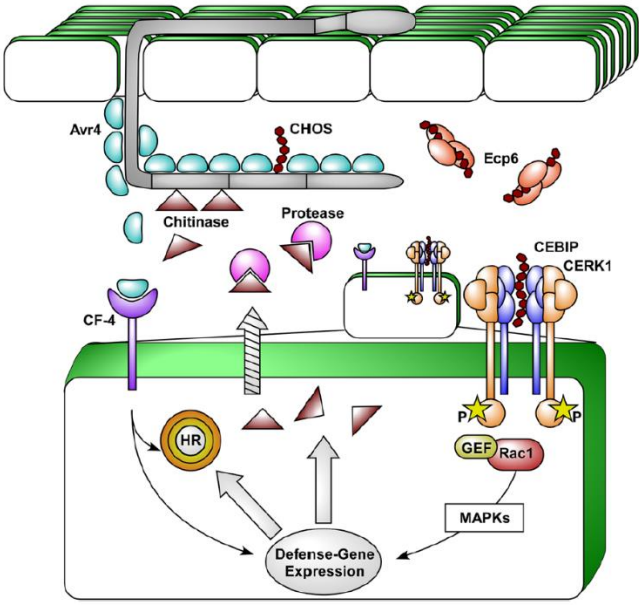


Figure 1.8 Chitin triggered immunity in plants, with rice as a model species. After entry into the host plant, the fungal cell wall is attacked by host secreted chitinases, which liberate soluble chito-oligosaccharides (CHOS) into the apoplastic space. CHOS with a chain length of n ≥ 8 are bound by the plant LysM containing receptor-like protein (RLP) CEBiP. Subsequently, the chitin elicited receptor kinase 1 (CERK1) gets recruited, which in turn leads to phosphorylation of its intracellular kinase domain. Activated CERK1 subsequently phosphorylates the Rac1 specific guanine exchange factor 1 (Rac1GEF1), which in turn activates a MAPK cascade that triggers expression of defense-genes, including hydrolytic enzymes (Langner & Göhre, 2016).

1.3 ENVIRONMENT 2: THE PLASTISPHERE

Since its first mass production in the 1950's, plastic has improved our quality of life in ways that make it impossible to imagine living without it. Plastics are cheap, lightweight, strong, and durable (Derraik, 2002), which makes them ideal for several applications. Nearly 300 million tons of plastic are produced each year, of which half are discarded after single use (PlasticOceans.com). This enormous production of plastic has however its consequences. By the early 1970s, plastic began appearing alongside plankton in oceanographic sampling nets (Carpenter and Smith, 1972; Carpenter et al., 1972). Now, plastic is the most abundant form of debris in the ocean, with an estimated number of around 5.25 trillion plastic particles scattered over our oceans and seas (Eriksen et al., 2014).

1.3.1 Plastic pollution in the marine environment

Nearly 80 percent of the plastic debris enters the marine environment through land sources. Coastal recreational activities, industrial manufacturing and handling of raw manufacturing materials are the major sources for littering and solid waste disposal. These plastics are eventually transported to the marine environment through river systems and wastewater treatments or through the occurrence of extreme weather conditions (e.g. flooding). The remaining 20 percent of plastic debris is derived from ocean-based sources, in which commercial fisheries is the major contributing human activity (Li et al., 2016) (Figure 1.9).

Once plastic enters the marine environment, it will be broken down to smaller particles due to mechanical degradation through waves, chemical changes through UV action, thermal degradation, photo-degradation and even biofouling and biological degradation (Pinto da Costa et al., 2016) (Figure 1.10). Plastic fragments which are eventually fragmented to pieces smaller than 5 mm in diameter, are defined as microplastics. These microplastics can also be directly produced for the use in e.g. cosmetics and cleaning products. In addition, washing of fleece materials will also release microplastics. Through domestic wastewaters, these microplastics will be deposited eventually in the marine ecosystem. A third source of microplastics is the manufacturing of resin powders or pellets, used as feedstock for the manufacturing of plastic products. Through spillage from freighters, these pellets also enter the marine environment (Pinto da Costa et al., 2016).

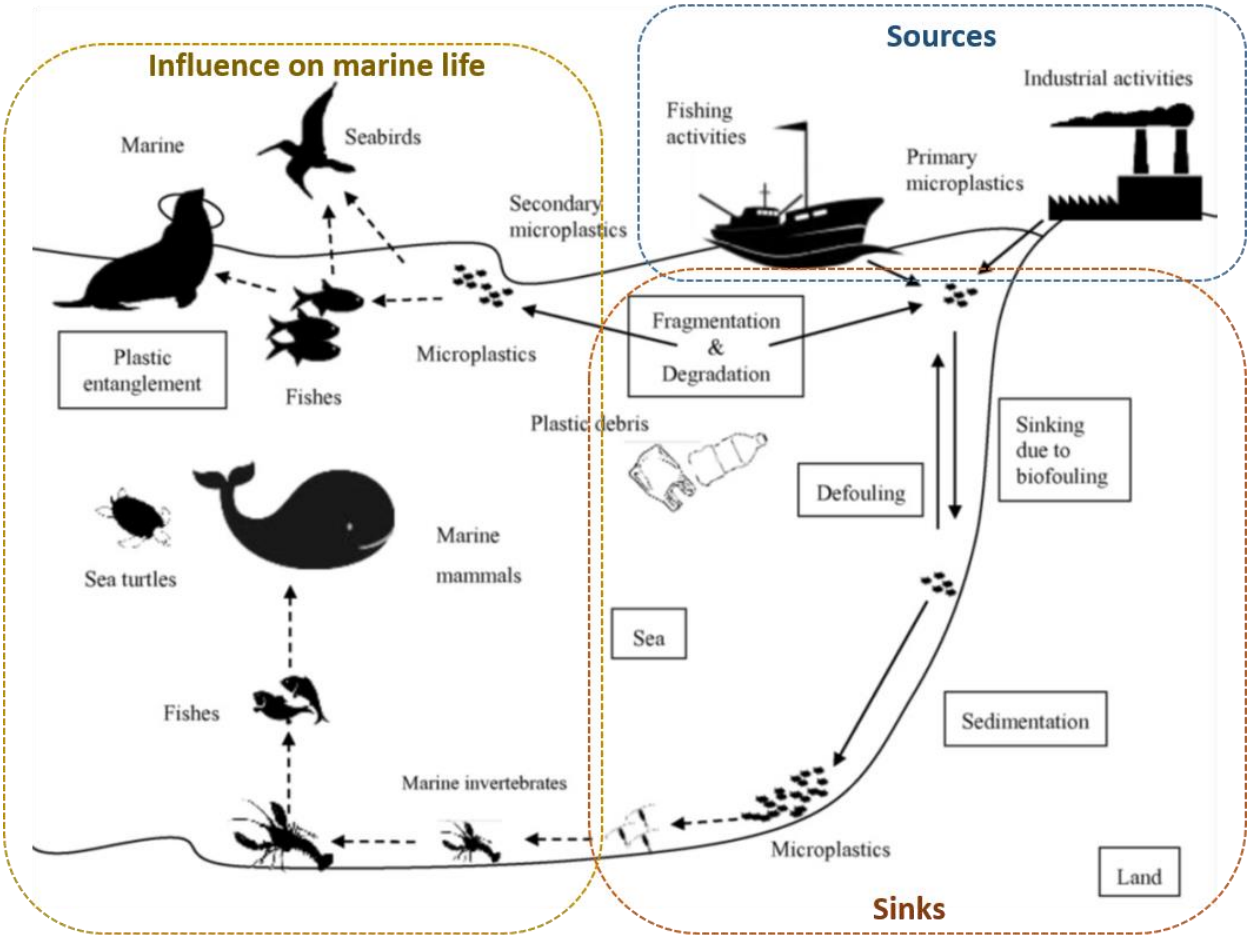


Figure 1.9 Sources, sinks and influence on marine life of plastic debris in the marine environment. Plastic is derived from land-based sources (industry, recreational activities) or ocean-based sources (fisheries). A small part of these plastics keeps floating, while the major part will sink to the seafloor eventually through biofouling and fragmentation to smaller plastic particles (microplastics). These plastics can eventually form a threat to marine life through ingestion and entanglement (adapted from Li et al., 2016).

Currently, the most widely used synthetic plastics are low- and high-density polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene tere-phthalate (PET), together representing around 90% of the total plastic production (Andrady and Neal, 2009). Most of these plastics are buoyant in water, with the exception of PVC. Consequently, most plastics float on seawater when entering the marine environment. Fragmentation and the colonization of micro-organisms on plastic debris however change their physicochemical properties, e.g. surface hydrophobicity and buoyancy (Ye & Andrady, 1991), which makes the plastic fragments sink to the seafloor (Figure 1.9; Figure 1.10). Therefore it is estimated that the major sink of plastic debris in the marine environment is the seafloor (70 %) and that a minor part is located in the seawater column (15 %) or is deposited on beaches (15 %) (Barnes, 2009; Sherrington, 2016).

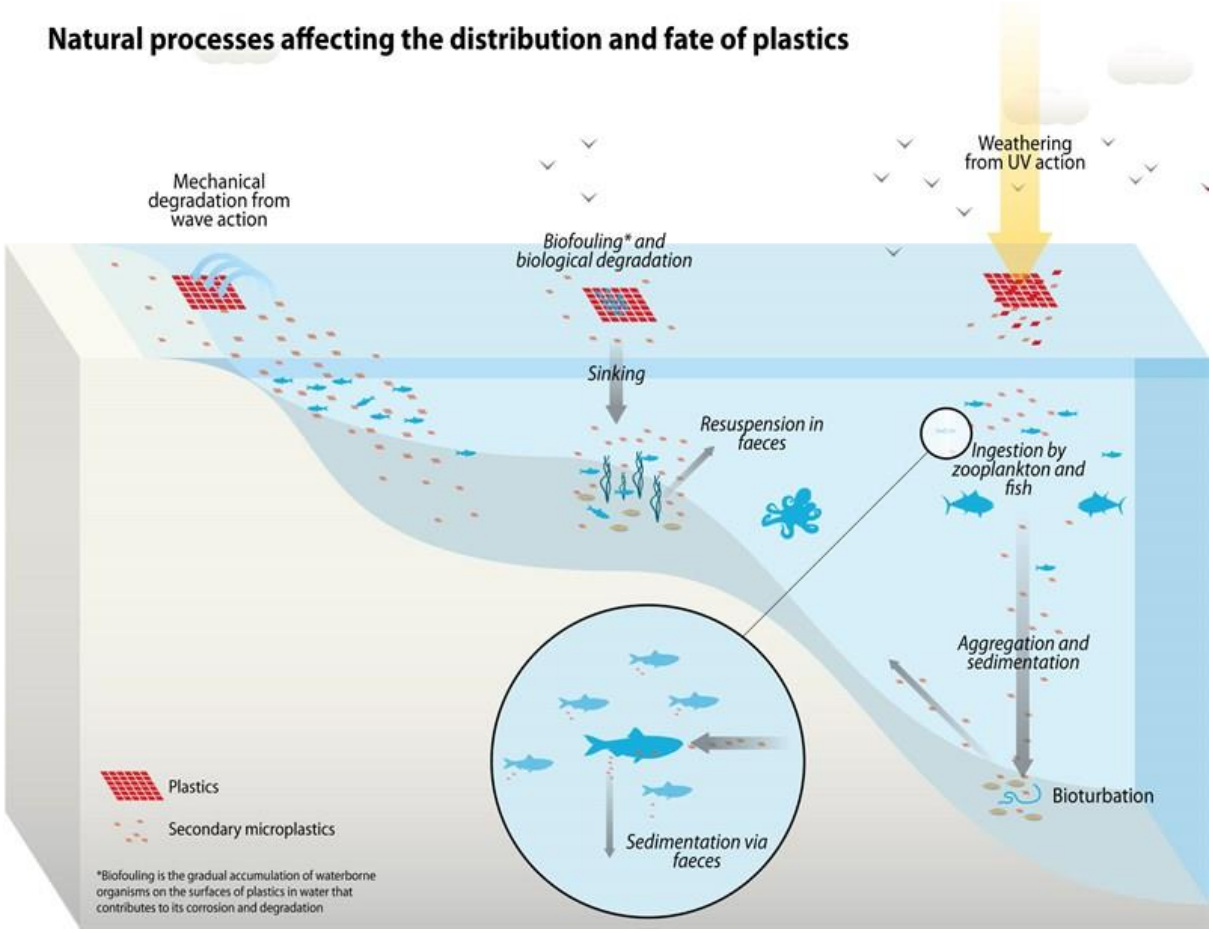


Figure 1.10 Natural processes affecting the distribution and fate of plastics. Once plastic enters the ocean, it will be fragmented through several processes such as mechanical, biological and chemical (UV) degradation. Plastics will eventually sink to the seafloor and/or be mistaken as food by marine organisms which ingest them. (source:http://www.grida.no/graphicslib/detail/natural-processes-aecting-the-distribution-and-fate-of-plastics_2cbc).

The presence of (micro)plastics in the marine environment has several consequences. Due to its hydrophobicity, plastic debris in the marine environment will adsorb toxic metals and persistent organic pollutants (Aston et al., 2010; Holmes et al., 2012; Pinto da Costa et al., 2016). In addition, several rafting species such as microalgae, Bryozoa, insects and even macrobenthos can attach to the plastic debris and use it as an aquatic vehicle to travel to new foreign habitats (Barnes, 2002; Gregory, 2009; Keswani et al., 2016).

Plastic can be a major threat for marine life in several ways. Entanglement, which is often caused by ghost fishing gear, is one of the most damaging effects of debris, and can represent up to 0.5% of captures, including animals like turtles; penguins; albatrosses, petrels and shearwaters; shorebirds, gulls and auks (Gregory, 2009; Galgani, 2015). (Micro)plastics are also ingested by animals which can lead to wounds of the digestive tract which can lead to

false satiation, starvation and often death of the organisms (GESAMP, 2016). The ingestion of microplastics by small marine organisms and zooplankton, can lead to the distribution of microplastics in the food web and can eventually result in microplastics in the human gastro-intestinal system (Keswani et al., 2016). Because plastic can act as a vector for both chemical and biological substances, pathogenic organisms and toxics can enter the food system through (micro)plastic ingestion (McCormick et al., 2014; Keswani et al., 2016).

In addition, it has been shown that even smaller plastics, the so-called nanoplastics, are able to penetrate cell membranes. In *Oryzias latipes*, nanoplastics were not only found in the intestines after exposure to the plastics, but also in the blood, liver and even in the brains, indicating that nanoplastics are able to cross the blood-brain barrier (GESAMP 2016; Almutairi et al., 2016).

To conclude, (micro)plastic pollution has severe consequences, most of them not quite yet understood and further research is necessary.

1.3.2 Microbial communities on plastic

In the early 1970s, Carpenter and Smith reported for the first time the presence of diatoms, bacteria and hydroids on the surfaces of plastic debris collected in the Sargasso Sea (Carpenter & Smith, 1972; Carpenter et al., 1972). A few years later, Sieburth (1975) noted the ubiquity of microbial colonization on man-made surfaces including high-density polyethylene plastic (HDPE) like bleach bottles in his pictorial essay book "Microbial Seascapes". His Scanning Electron Microscopy (SEM) images of pennate diatoms, filamentous cyanobacteria, coccoid bacteria and bryozoans were perhaps the first published glimpses of what has come to be referred to as the "plastisphere" (Zettler et al. 2013) - a name originally assigned to the diverse assemblage of taxa that inhabit the thin layer of life on the outer surface of plastic debris, but now more broadly referring to life on the surface of plastic litter in general (Figure 1.11).

The hard surface of plastics provides an ideal environment for microbial colonization, but several physicochemical factors influence microbial succession. In temperate coastal water, the *Roseobacter* clade (Rhodobacterales), appears as a primary surface colonizer (Dang and Lovell, 2000; Dang et al., 2008). Several studies that followed, employing a diversity of plastic substrates, revealed that diverse microbial biofilms colonise plastic debris after the initial colonization (Webb et al., 2009; Lobelle and Cunliffe, 2011; Harisson et al., 2014). The amount of microbial biomass increases with exposure time of the plastic to seawater or marine

sediment in both fossil-fuel-based and biodegradable/compostable plastic (Eich et al., 2015). Lab experiments have shown that the microbial cell densities are five to eight times higher on biodegradable plastic bags than on PE bags when both are buried in sediments, showing the influence of hydrophobic properties on microbial colonization (Nauendorf et al., 2016). In addition to hydrophobic surface properties (Oliveira et al., 2001), surface roughness (rugosity) of the plastic material also impacts colonization (Characklis et al., 1990; Bravo et al., 2011; Carson et al., 2013).

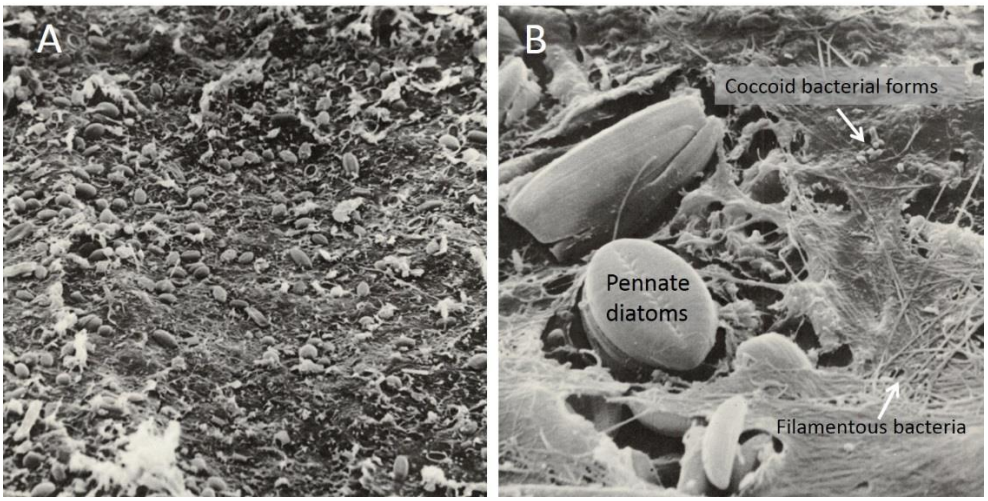


Figure 1.11 SEM images of microbial communities on plastic surfaces first recorded by Sieburth et al. (1975). Panel A shows diatoms colonizing the surface of a bleach bottle in Narragansett Bay, Rhode Island, USA. The magnification of the image is 950 X. Panel B shows a higher resolution view of the same microbial biofilm (5200 X magnification).

The colonization of plastic substrates and the formation of a microbial biofilm is however mainly influenced by three parameters: geographical location, season, and polymer type (Sudhakar et al., 2007; Artham et al., 2009; Hoellein et al., 2014; Oberbeckmann et al., 2014; Eich et al., 2015; Amaral-Zettler et al., 2015b). In freshwater ecosystems, location tends to be the major factor influencing biofilm composition, with polymer type being a secondary factor (Hoellein et al., 2014), a pattern also detected at global scales in the marine environment (Amaral-Zettler et al., 2015b). Given the impact of geography and season on the colonization of plastic substrates, it is challenging to compare studies across time and space.

All published studies covering “plastisphere” communities so far, making use of several visualisation techniques and molecular methods are described in Table 1.2. These studies primarily aim to understand the diversity and ecology of the microbial plastisphere. More specifically, three key questions are highlighted: (1) Does microbial degradation of plastic and

of adsorbed co-pollutants occur in marine environments?; (2) Are plastic surfaces a potential site for accumulation of pathogenic micro-organisms?; (3) How do the structure and function of plastisphere microbial communities change during transport? (Osborn & Stojkovic, 2014).

Table 1.2 Different methods used to study microbial composition of plastics in aquatic habitats ([a] Polyethylene, [b] Polypropylene, [c] Polystyrene , [d] Polyethylene terephtalate, [e] Polyvinyl alcohol, [f] Polyvinyl chloride).

Method	Plastic size	Geographic location	Habitat/laboratory	Plastic types	References
SEM	Microplastic	North Atlantic Ocean	Marine offshore	PE[a], PP[b]	Zettler *et al.* (2013)
		North Pacific Gyre	Marine offshore	PE, PP, PS[c]	Carson *et a*l. (2013)
		Lab test	Microcosm experiment	PE	Harrison *et al.* (2014)
		Australia-wide coastal and ocean region	Marine coastal waters and offshore	unidentified	Reisser *et al*. (2014)
		Urbanized river in Chicago, USA	River water	unidentified	McCormick *et al.* (2014)
		North Pacific and North Atlantic Ocean	Marine offshore	PE, PP, PS, PET[d], other	Amaral-Zettler *et al.* (2015b)
	Macroplastic	Mediterranean Sea	Marine offshore	PE, biodegradable plastic	Eich *et al*. (2015)
		North Sea, UK	Marine coastal waters and offshore	PET	Oberbeckman *et al.* (2014)
CARD-FISH	Microplastic	Lab test	Microcosm experiment	PE	Harrison *et al.* (2014)
Clone libraries	Microplastic	Lab test	Microcosm experiment	PE	Harrison *et al.* (2014)
	Macroplastic	West Pacific Ocean, Quingdoo	Marine coastal waters	PVA[e], PVC[f], unknown	Dang & Lovell (2000); Dang *et al.* (2008)
DGGE	Micro- and Macroplastic	North Sea, UK	Marine coastal waters and offshore	PET, PS, PE, PP	Oberbeckman *et al.* (2014)
T-RFLP	Microplastic	Lab test	Microcosm experiment	PE	Harrison *et al.* (2014)
Amplicon sequencing	Microplastic	North Atlantic Ocean	Marine offshore	PE, PP	Zettler *et al.* (2013)
		North Pacific Ocean, North Atlantic Ocean	Marine offshore and coastal	PE, PP	Amaral-Zettler *et al.* (2015b)
		Coast, BE	Beach	PE	De Tender *et al*. (2015)
		Urbanized river in Chicago, USA	River water	unidentified	McCormick *et al*. (2014)
	Macroplastic	North Sea, BE	Marine coastal waters and offshore	PE, PP	De Tender *et al*. (2015)
		North Sea, UK	Marine offshore	PET	Oberbeckmann *et al.* (2014)
Shotgun metagenomics	Micro- and macroplastic	North Pacific Subtropical Gyre	Marine offshore	unidentified	Bryant et al. (2016)

To date, little is known both about the temporal dynamics of colonization and biofilm formation on plastic debris and about the microbial interactions underlying the resulting **biodegradation process**. PE, one of the major pollutants of the marine environment, is reported as an inert polymer with strong resistance to microbial breakdown (Weiland et al., 1995). The degradation of polyethylene is probably a secondary process where an initial oxidation step first fragmentizes the polymer to smaller fragments (Scott, 1990). So far, the degradation of PE has been linked with lignin-degradation (fungi) under nitrogen- and carbon-limited conditions in soil, and with the production of manganese peroxidase (Wood, 1998). Until now, a limited number of micro-organisms in the marine environment have been detected that could potentially break down PE. Within the Arabian Sea, three isolates on low-density PE were identified that appear capable to degrade the polymer. They were classified

as *Kocuria palustris*, *Bacillus pumilis* and *Bacillus subtilis* (Harshvardhan and Bhavanath, 2013). In *in-vitro* conditions, two marine bacteria, *Arthhtrobacter* sp. and *Pseudomonas* sp., have been isolated that also appear capable to degrade HDPE (Balasubramanian et al., 2010). In addition, one fungal strain, *Zalerion maritimum*, has been identified as a potential PE biodegrader (Paço et al., 2017). Microbes have broad metabolic abilities, so finding bacteria that can degrade plastic is not unexpected, however, in all cases reported so far, the biological degradation of plastic is slow, and marine litter is expected to persist for years or even centuries in our marine environment (O'Brine and Thompson, 2010). Nevertheless, our knowledge of plastic-degrading bacteria in the marine environment remains limited. Using HTS techniques, screening for these organisms over wide areas is possible and can give us insight to which extend these organisms are present in the marine environment.

Plastic has the ability to serve as a vector for harmful micro-organisms. Due to the long residence time of plastic in the marine environment, plastic can travel over long distances, favouring the survival of colonists (Barnes, 2002). Zettler et al. (2013) documented the presence of *Vibrio*, a genus that includes pathogenic species on plastic fragments in the Atlantic, and a potentially pathogenic *Vibrio parahaemolyticus* was detected using MALDI-TOF MS on a number of microplastic particles from the North Sea and Baltic Sea (Kirstein et al., 2016). Additionally, Masó et al. (2003) has found temporary cysts of a harmful algae bloom (HAB) species, the dinoflagellate *Alexandrium taylori*, attached to plastic debris found in coastal and open ocean waters in the Mediterranean Sea; even in marine regions that have not been affected by HAB events before. The authors suggested a high potential of increasing HAB dispersion due to increasing plastic debris accumulation in the oceans, when they are able to survive attached on plastic in foreign environments. Thus, plastic can be a potential vector for the wide-scale dissemination of harmful micro-organisms, but the impact of this plastic pollution and potential pathogen/HAB distribution on human and environmental health is still unknown (Amaral-Zettler et al. 2015b; Vethaak & Leslie, 2016).

Problem statement and thesis outline

Micro-organisms constitute a major fraction of the total biomass and are one of the main sources of biodiversity on Earth (Vieites et al., 2010). The succession of microbial community structure and functions remains a central topic in environmental microbiology studies, as micro-organisms drive the Earth's biogeochemical cycles (Cong et al., 2015). With the development of high-throughput sequencing tools, the ability to characterize their temporal and spatial variation has become achievable (Gonzalez et al., 2012). These high-throughput "omics" technologies, such as amplicon and shotgun metagenome sequencing, allow for the analysis of microbial communities without tedious cultivation efforts (Vieites et al., 2010).

In this thesis, "omics" technologies are applied to study microbial communities in two of the richest biospheres on earth: the soil and marine environment. In soils, the distribution of micro-organisms and the processes they are involved in, such as nutrient cycling, improvement of the soil structure and degradation of pollutants, are well studied (Paul, 2015). The distribution and activities of marine organisms are however less explored. Nonetheless they are highly abundant and perform several important processes such as photosynthesis, degradation of organic compounds and recycling of nutrients (Munn, 2011). This thesis is divided in two central parts, reflecting these two microbial-rich environments studied in terms of agricultural and marine environmental problems.

Part I (**Chapters 2, 3 and 4**) is dedicated to the microbial characterization of the rhizosphere, the narrow zone of soil which is influenced by the growth and nutrient exchange of plants (Hiltner, 1904). The rhizosphere microbiome has profound effects on several plant processes, such as plant productivity and plant health (Mendes et al., 2013). To reshape the rhizosphere microbiome towards a higher abundance of plant-beneficial micro-organisms, one of the strategies is to use soil or substrate amendments. In Part I, biochar (Chapter 2 and 3) and chitin (Chapter 4) were used as soil/substrate amendments to modify the rhizosphere microbiome. The title and rational behind each of these research chapters are listed below:

- **Changes in the rhizosphere microbiome in response to biochar and in relation to plant health (Chapter 2)**: Can biochar alter the bacterial and fungal community of the rhizosphere microbiome towards plant-beneficial organisms and can this be related to changes in the physicochemical properties of soil and peat substrate, crop growth and plant disease susceptibility? To take into account the effect of different plant species

and growing medium on the rhizosphere microbiome, two target crop-soil biosystems were chosen: lettuce grown in field soil and biochar grown in peat. At the end of the growing medium, biological, physicochemical and plant health parameters were measured and correlated with changes in the rhizosphere microbiome, which was studied either by phospholipid fatty acid (PLFA) analysis and 16S rRNA and ITS2 gene amplicon sequencing.

- **Dynamics of the rhizosphere microbiome in response to biochar and aboveground plant inoculation (Chapter 3):** Based on the results of Chapter 2, new questions raised: how dynamic is the effect of biochar during the plant growth cycle and is the rhizosphere microbiome not only altered by the addition of biochar, but also by the aboveground leaf infection with *B. cinerea* and can this interfere with the effect of biochar?

- **Taxonomic and functional changes in the lettuce rhizosphere microbiome in response to chitin and in relation to human health (Chapter 4):** Chitin is believed to be one of the most economical and practical options to improve soil and substrate quality, plant growth and plant resilience (El Hadrami et al., 2010; Sharp, 2013). In this chapter, we studied the role of chitin as a mediator of plant growth (model plant lettuce) and its ability to reduce the survival of zoonotic pathogens through a change in the rhizosphere microbiome. A combination of PLFA, amplicon sequencing and shotgun metagenomics, was used to cover the composition of the rhizosphere microbiome.

Part II (Chapters 5 and 6) focusses on the microbial community structure and dynamics of plastic debris in the North Sea, typically referred to as “the plastisphere”. Plastic is the most abundant form of debris in the marine environment (Laist, 1987). Since the nineteenseventies it has been shown that micro-organisms are able to colonise marine plastic debris (Carpenter et al., 1972), however the number of studies describing the composition of this microbial community remains limited. In Part II, the “plastisphere” microbiome is studied for plastic debris sampled in the Belgian part of the North Sea. The title and rational behind each of these research chapters are listed below:

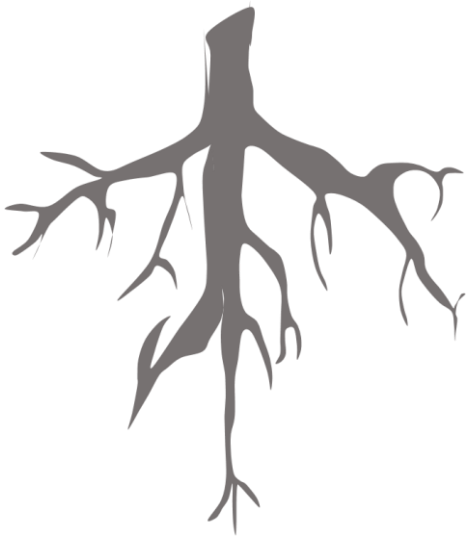
- **Bacterial community profiling of marine plastic debris in the North Sea (Chapter 5):** Are bacteria able to colonise plastic debris, how variable is this colonization and where do these bacteria originate from? Despite most "plastisphere" studies focus on floating plastic, most of this debris is located at the seafloor (Eunomia, 2016). In this study, we examine the main drivers influencing the bacterial colonization of seafloor plastic debris. Plastics were sampled at five locations in the Belgian part of the North Sea and at the beach of Ostend (resin pellets). The bacterial community was studied using 16S rRNA gene amplicon sequencing and compared with sediment and seawater samples, to track the origin of the bacterial members of plastic.

- **Microbial biofilm dynamics of the plastisphere (Chapter 6):** How dynamic is the bacterial colonization of plastic? Are other microbial organisms, more specifically fungi, also able to colonise plastic debris in the marine environment? Based on the experimental set-up, it was not possible to reconstruct the bacterial biofilm formation of plastics studied in Chapter 5 because we do not know the history of this plastic. Therefore a controlled exposure experiment was done to reconstruct the bacterial colonization process. Polyethylene samples, the polymer that was most abundant in the study of Chapter 5, were used and collected from the North Sea at two locations: the harbour of Ostend and offshore at the Thornton windmill farm. In addition, the research was expanded to the study of fungal communities using ITS2 gene amplicon sequencing.

In the last part of this thesis (**Chapter 7**) the major findings of each Chapter and future perspectives are discussed. For the rhizosphere microbiome, economical, ecological and human health impacts of biochar and chitin are evaluated. In terms of the "plastisphere" microbiome, the contribution of this thesis on the knowledge of "plastisphere" influences and impacts is discussed.

Part I

The Rhizosphere



Chapter 2

Changes in the rhizosphere microbiome in response to biochar and in relation to plant health

Modified from:

De Tender CA, Debode J, Vandecasteele B, D'Hose T, Cremelie P, Haegeman A, Ruttink T, Dawyndt P & Maes M. (2016a). Biological physicochemical and plant health responses in lettuce and strawberry in soil or peat amended with biochar. Applied Soil Ecology 107, 1-12.

Several pot and field trials have shown that biochar addition to soil or peat substrate can change the physicochemical properties of soil or substrate (Rondon et al., 2007; Asai et al., 2009; Laird et al., 2010), enhance productivity and performance of crops (Chan et al., 2007; Asai et al., 2009; Graber et al., 2010), and act in disease suppression against fungal foliar pathogens (Elad et al., 2010; Meller Harel et al., 2012). Biochar addition has also been proven to affect the soil and substrate microbial community structure (Kolb et al., 2009; Anderson et al., 2011; Lehmann et al., 2011; Abujabhah et al., 2016; Grunert et al., 2016), whereas its effect on the microbial community structure and diversity of the rhizosphere is less well understood. The rhizosphere microbiome can however play an important role in the development, nutrition and health of the plant, as it is in close interaction with the plant root. Addition of biochar to peat or soil can thus enhance plant growth and resistance through an indirect effect via the root-associated micro-organisms, which may shift in composition due to the addition of biochar (Egamberdieva et al., 2016). Especially plant growth promoting rhizobacteria (PGPR) and fungi (PGPF) in the rhizosphere play an important role in plant growth and health. These organisms can stimulate plant growth through the degradation of soil pollutants, nutrient mobilization, production of phytostimulators or suppression of plant pathogens (Zamioudis & Pieterse, 2012). In addition, PGPR and PGPF can enhance the resistance of the plant towards plant pathogens through the induction of the induced systemic resistance (ISR) of the plant (Mehari et al., 2015).

Previous studies examined generally only one or two of the above mentioned effects of biochar on agricultural properties (e.g. soil physicochemical and biological properties, crop productivity and performance, plant health or rhizosphere microbiology). However, in order to define biochar as a positive or negative operator on the crop-soil/substrate system, all these individual factors need to be integrated in order to estimate the overall impact and to understand the underlying mechanism. Therefore, in this chapter, two target crop systems were selected for bio-assays: lettuce and strawberry. Lettuce is typically well adapted for growth in field-soil, representing a complex environment for the plant roots. Strawberry grows well in a soilless system such as white peat, a well standardised environment with a low nutritional and microbial background. Lettuce is known to be responsive to nutrients (Upadhyay et al., 2014) and *Rhizoctonia solani* was chosen as pathogen system, as it is the predominant pathogen causing basal rot on lettuce (Van Beneden et al., 2009). Strawberry was infected with *Botrytis cinerea*, known as a serious pathogen reported to cause fruit losses

up to 50% (Jarvis, 1962). However, the leaves are also very important in the infection cycle, as infection of leaves by *B. cinerea* may lead to increased inoculum production when leaves are senescing in a perennial growing system (Braun and Sutton 1988; Sutton and Peng, 1993).

The effect of 1% or 3% biochar incorporation on the lettuce-soil and strawberry-substrate systems will be monitored at the end of the bioassays for the composition of the plant growth media, the plant and root growth, plant and fruit disease susceptibility, fruit yield, and the rhizosphere microbiology (bacteria and fungi), in which we expect a different outcome for the two systems. To study the rhizosphere microbiology, both amplicon sequencing and phospholipid fatty acid (PLFA) analysis were used. For the strawberry system, the effect of additional fertilization and liming of the peat on these properties will also be tested. This resulted in two bioassays referred to as the 'unfertilized peat experiment' and the 'fertilized peat experiment' for strawberry plants grown in unfertilized/unlimed peat and fertilized/limed peat respectively.

Biochar is expected to affect the composition of the plant growth media, such as pH, carbon content, nutrient availability, microbiology and water management and availability. It has previously been suggested that the effect of biochar on crop productivity would be dose and crop dependent (Gravel et al., 2013), but we realize that it should also be soil or substrate dependent. Therefore, in the present chapter the plant growth media were well characterized before and after the plant growth tests. The used field soil had an optimal pH and stored relevant nutrient concentrations for growth of lettuce. The white peat of the strawberry was confirmed to be low in plant nutrients and microbial diversity, and had a low pH. Based on previous reports, we expected that biochar has a neutralising effect on the peat pH and a fertilising effect for the plant (Carter et al., 2013). Effects beyond these two factors were tested by also combining biochar mixed in white peat with liming and extra addition of plant nutrient compounds. This Chapter aims to increase our understanding of the effect of biochar on the relation between the physicochemical properties of the plant growth media, crop growth, disease susceptibility and the rhizosphere microbial community. This kind of information is needed to fully appreciate the role of biochar as a soil or substrate amendment for agriculture.

2.1 MATERIALS AND METHODS

In this section, an overview of the materials and methods for the physicochemical characterisation of the plant growth media (2.1.1-2.1.3), the lettuce and strawberry bioassays (2.1.4-2.1.6), and the rhizosphere microbiology (2.1.7-2.1.8) is provided. In the end, an overview of statistical methods is given (2.1.9).

2.1.1 Chemical characterization of biochar and plant growth medium

Biochar (BC) was prepared from holm oak at 650 °C for 12-18 h and was kindly provided by Proininso S.A. (Malaga, Spain). This biochar consists of 72.4% dry matter (DM) (%/fresh), 77.8% organic matter (%/DM) and 74.2% C (%/DM) and was previously used and fully characterized by Vandecasteele *et al.* (2014, 2016).

Field soil used in the lettuce assay was sampled from the arable layer 0-20 cm of an ongoing field experiment at ILVO (D'Hose et al., 2016) and its chemical properties at the beginning and end of the experiment were measured as described below (2.1.2) and are listed in Additional Information (AI) Table AI2.1 and Table AI2.2, respectively. This sandy loam soil (pH-KCl = 5.79; clay = 5.3%; silt = 37.7%; sand = 57.0%) was sieved (1 cm), air-dried (99% dry matter/fresh), and stored at room temperature until use.

Peat used in the strawberry assays was NOVOBALT white peat 100% (AVEVE Lammens, Wetteren, Belgium). The chemical properties of the 'NOVOBALT peat' at the beginning (week 1) and end (week 13) of the experiments are listed in Table AI2.3 and Table AI2.4, respectively.

2.1.2 Chemical characterization of soil and amended soil

Methods for the chemical characterisation of soil and peat are based on European Standards developed by the European Committee for standardization (CEN) or by the International Organization for Standardization (ISO). European Standard EN numbers or ISO numbers refer to the specific standards.

Soil was sampled at the start and the end of the lettuce experiment for chemical analysis. At the start of the experiment, 1 L of thoroughly mixed soil was sampled after one week of pre-incubation. At the end of the experiment the soil that remained after sampling for rhizosphere microbiology (section 2.1.7 and 2.1.8) was used (± 1 L).

Prior to chemical analysis, the soil samples were thoroughly mixed and divided into three sub-samples. The first sub-sample was used immediately for pH-KCl, Electrical Conductivity (EC) and soil mineral N (NO_3^--N + NH_4^+-N) determination. Soil dry matter content was determined

by oven drying at 105 °C. The pH was measured potentiometrically in a 1:5 soil:KCl (1 M) extract according to ISO 10390. The EC was measured by means of a temperature compensating conductivity meter (E SK 10B electrode, 25°C) in a 1:5 soil:H_2O extract according to EN 13038. Soil mineral N was determined in a 1 M KCl extract according to ISO TS14256-1:2003 with a Skalar San++ mineral N analyser. The second and third sub-sample were oven dried at 45 °C and 70 °C, respectively. The samples were ground in a mortar and passed through a 2 mm and 250 µm sieve, respectively, prior to analysis of chemical soil properties. Ammonium lactate (AL) extractable elements were assessed on the second sub-sample by extracting plant-available concentrations of P, K, Ca, Mg, Fe, Mn and Na with ammonium lactate (extraction ratio 1:20) in dark polyethylene bottles, shaken for 4 hours (Egnér et al., 1960). The suspension was filtered in dark polyethylene bottles that were stored at 4 °C until analysis. Elements were analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian Vista-Pro) with an axial torch. Total organic carbon (TOC) was measured on the third sub-sample by dry combustion at 1050 °C using a Skalar Primacs SLC TOC analyser (ISO 10694).

2.1.3 Chemical characterization of peat and amended peat

The substrate was sampled at the start and the end of the strawberry. At the start of the experiment, 1 L of thoroughly mixed peat was sampled after one week of pre-incubation. At the end of the experiment, all the remaining peat after sampling for studying the rhizosphere microbiology (section 2.1.7 and 2.1.8) was used (± 1 L).

Dry matter content was determined according to EN 13040. EC (EN 13038) and pH_{H2O} (EN 13037) were measured in a 1:5 soil to water (v/v) suspension. Determination of organic matter content and ash was done according to EN 13039. Extraction (1:5 v/v) of water soluble nutrients and elements (NO_3-N, NH_4-N, Cl, Na, SO_4 and PO_4-P) was done according to EN 13652, and measured with a Dionex DX-600 IC ion chromatography (Dionex, Sunnyvale, CA), and for NH_4-N with a Skalar San++ mineral N analyzer. Plant-available concentrations of P, K, Ca, Mg, Fe and Mn were extracted (1:5 v/v) in ammonium acetate buffered at pH 4.65, and measured by CCD simultaneous ICP-OES (VISTA-PRO, Varian, Palo Alto, CA).

2.1.4 Lettuce-soil bioassay

Air-dried field soil was either used as pure growing medium (1323 g field soil), or mixed with 1% biochar (18 g BC + 1310 g field soil) or 3% biochar (54 g BC + 1273 g field soil) and pre-incubated for one week at 15 °C. Initial moisture content was set to 40% water-filled pore space (WFPS), and bulk density was adjusted to 1400 g L^{-1}. Subsequently, the mixed field soil was put in 1.5 L pots. Per pot, one 1 month old butterhead lettuce (*Lactuca sativa*, cultivar Alexandria) seedling was planted and placed in a growth chamber at 20 °C, 16 h/8 h day-night light regime and 80% relative humidity. Per treatment, seven plants were grown and this was done for two independent experiments. So, in total, fourteen biological replicates were grown for each treatment in which pots were placed in the growing chamber according to a completely randomised design. For each replicate separately, the soil moisture was adjusted weekly to 40% WFPS on the basis of measured mass loss and the supplied amount of water was recorded. No fertilizers were added during the experiment.

After 8 weeks, the lettuce heads were harvested and weighed (fresh weight and dry weight (2 days at 70 °C)).

To determine the susceptibility of the lettuce plants towards *Rhizoctonia solani* infection, the *R. solani* bio assay was done after 7 weeks on the lettuce leaves as described below (section 2.1.6). In each experiment, five plants were inoculated, thus in total 80 lesions per treatment were scored (5 plants x 4 leaves x 2 plugs x 2 experiments).

To differentiate between the evaporation from the soil surface and transpiration by the lettuce plant, a separate experiment was run in the growth chamber with an identical set-up as described above, but without lettuce grown in the pots. For each replicate separately, the soil moisture was adjusted weekly to 40% WFPS on the basis of measured mass loss and the supplied amount of water was recorded.

2.1.5 Strawberry-peat bioassays

In the strawberry bioassays, peat was used as growing medium and used as either pure growing medium (298 g peat) or mixed with 1% biochar (3.2 g BC + 295 g peat) or 3% biochar (9.4 g BC + 289 g peat). In the 'fertilized peat' experiment, 1.33 g L^{-1} fertilizer (PGMix, Peltracom, Ghent, Belgium) and 3 g L^{-1} lime (Dolokal extra, Ankerpoort NT, Maastricht, The Netherlands) were added to both the peat and peat/biochar mixtures (Table 2.1). No additional fertilizer was applied during plant growth for all experiments. The 'unfertilized peat'

and 'fertlized peat' experiments were both repeated. For each experiment, all substrates were wetted to obtain 40% WFPS, and bulk density was adjusted to 200 g L^{-1}. Each mixture (peat, biochar/peat) was put in a closed bag and pre-incubated at 15 °C for one week. Subsequently, 1.5 L pots were filled with the mixed substrates and a cold-stored bare-root strawberry (*Fragaria x ananassa*, cultivar Elsanta) transplant was planted in each pot. The plants were then arranged in the greenhouse in a completely randomized design and grown at 20 °C for up to 13 weeks. Every week, the moisture content of the substrate was adjusted to 40% WFPS based on mass loss.

Table 2.1 Experimental set-up of the strawberry experiments

	Lime (3 g L^{-1} substrate)	Fertilizer (1.33 g L^{-1} substrate)	Biochar dose (% on DM basis)	Rhizosphere sampling time (weeks)	Repeated
Unfertilized peat experiment	-	-	0%, 1%, 3%	13	✓
Fertilized peat experiment	+	+	0%, 3%	13	✓

In the 'unfertilized peat' and 'fertilized peat' experiments, 24 strawberry plants were grown per treatment. After 12 weeks of plant growth, the *B. cinerea* leaf inoculation was done as described in section 2.1.6. In each experiment, 12 plants per treatment were inoculated, thus in total 216 lesions per treatment were scored (12 plants x 3 leaves x 3 plugs per leaf x 2 repetitions). Fruits were harvested, weighted and infected with *B. cinerea* isolate 895 as described below (section 2.1.6). Thirteen weeks after planting, the strawberry plants were collected and weighed (fresh weight and dry weight (2 days at 70°C)) and the rhizosphere was sampled from non-inoculated plants for PLFA analysis and amplicon sequencing as described in sections 2.1.7 and 2.1.8. Five replicates were sampled for each growing medium, which were used for both PLFA and amplicon sequencing. This was not repeated.

2.1.6 *Botrytis cinerea* and *Rhizoctonia solani* bioassay

To determine the susceptibility of lettuce plants towards *R. solani*, the detached leaf test of Van Beneden *et al*. (2009) was conducted. Four detached leaves of five lettuce plants were inoculated with two mycelial plugs (4 mm) of the *R. solani* AG1-1B isolate S014-22, one on each side of the leaf. Control leaves were inoculated with sterile potato dextrose agar (PDA) plugs. The leaves were placed in sealable plastic containers with moistened tissue paper and

incubated at 20 °C in the dark. The resulting lesions on the leaflets were recorded using a 0-4 disease scale with 0 = 0% of the leaf area infected (no symptoms); 1 = <25% of the leaf area is affected; 2 = 25-50% of the leaf area is affected; 3 = 51-75% of the leaf are is affected; 4 = >75% of the leaf area is infected. An example of this disease scale on strawberry leaves is given in Figure 2.1.

Strawberry plants and fruits were inoculated with *B. cinerea* at pre-set time points for all experiments. Plant leaves were inoculated using the method of Meller-Harel *et al.* (2012). Briefly, the *B. cinerea* isolate 895 (Debode et al., 2013) was cultured on PDA at 20 °C for four days. Agar discs (4 mm) containing pathogen mycelium and conidiophores were cut out from the colony edge and placed, mycelium side down, on the surface of three young fully expanded strawberry leaves per plant, with one disc per leaflet. Control leaves were inoculated with sterile PDA plugs. All plants were sprayed with water and each pot (volume: 1.5 L) was covered with a plastic box for one week to create conditions of high humidity. The resulting lesions on the leaflets were recorded one week after inoculation using the same 0-4 disease scale described in the *R. solani* bioassay (Figure 2.1). After scoring the infection, inoculated leaves were removed from the plant.

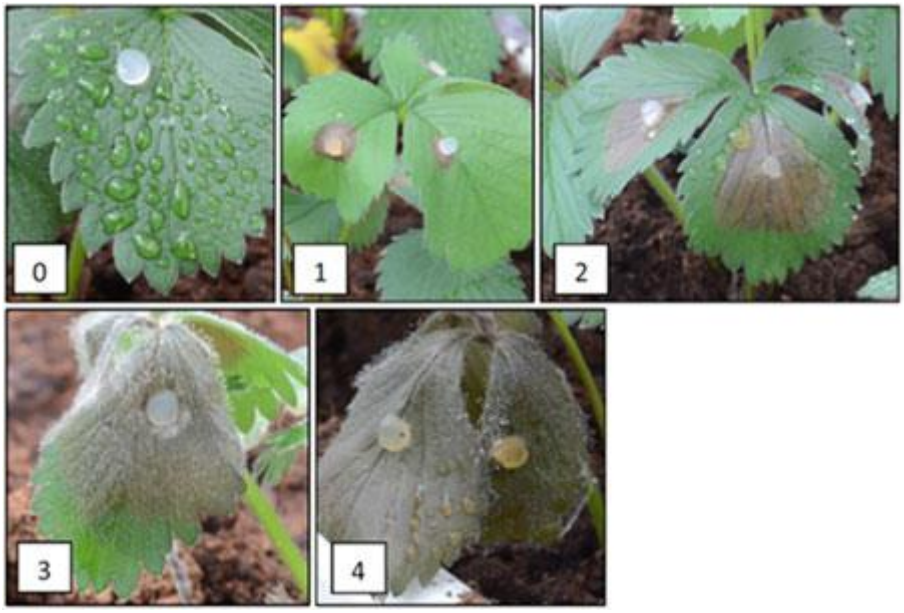


Figure 2.1 Disease scale used for both the lettuce and strawberry leaf infection with *Rhizoctonia solani* and *Botrytis cinerea* respectively. 0 = 0% of the leaf area infected (no symptoms); 1 = <25% of the leaf area is affected; 2 = 25-50% of the leaf area is affected; 3 = 51-75% of the leaf are is affected; 4 = >75% of the leaf area is infected.

Inoculation of the strawberry fruits was based on the method of Bhaskara Reddy *et al.* (2000). Briefly, individual ripe strawberry fruits were inoculated with 20 µL conidial suspension (2 ×

105 conidia mL^{-1}) of *B. cinerea* and incubated at 11 °C under humid conditions. When the first symptoms appeared, the strawberries were evaluated daily and spoiled fruits were discarded to avoid secondary infection. The area under the disease progress curve (AUDPC) was calculated for the infected fruits (Campbell & Madden, 1990).

2.1.7 Rhizosphere microbiology: PLFA analysis

Soil samples including plant roots (+/- 50 g) were taken from each pot and stored at -20 °C and then freeze-dried. In total 4 biological repetitions within the lettuce bioassay and 5 repetitions per treatment within the 'unfertilized peat' and 'fertilized peat' experiments of the strawberry bioassay were used for PLFA analysis. Total lipids were isolated from 6 g of freeze-dried soil or 1 g of freeze-dried substrate in a phosphate buffer:chloroform:methanol mixture (0.9:1:2). Neutral, glycol- and phospholipids were separated by solid phase extraction, eluting, respectively, chloroform, acetone and methanol. Phospholipids were saponified to obtain free fatty acids, which were subsequently methylated using 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were analysed with a capillary gas chromatograph-flame ionisation detector (Perkin Elmer Clarus 600, Perkin Elmer, Waltham, USA) with a col-elite-2560 column (100 m length x 0.25 mm ID, 0.25 µm film thickness, Perkin Elmer). The temperature program started at 75 °C, followed by a heating rate of 10 °C $minute^{-1}$ up to 180 °C and followed by a final heating rate of 2 °C $minute^{-1}$ up to 240 °C. PLFA's were identified and quantified from the retention time and response factor of each FAME in the external FAME and BAME mix (Sigma Aldrich, St Louis, MO, USA). The C values were corrected using a working standard C19:0.

The abundance of individual PLFAs was calculated in absolute C amounts (PLFA-C, C_x [nmol g^{-1}]) based on the concentrations in the liquid extracts using the following formula:

$$C_x[\text{nmol g}^{-1}] = \frac{\text{A}_\text{x} \,.\, \text{c}_\text{i}[\mu\text{g}] \,.\, 1000}{\text{A}_\text{i} \,.\, \text{W [g]} \,.\, \text{M [g µmol}^{-1}]}$$

Where C_x is the concentration of the fatty acid studied, A_x is the peak area of the fatty acid studied, A_i is the peak area of the internal standard, c_i is the absolute amount of internal standard in the vial [µg], W is the amount of soil [g], M is the molecular weight of the fatty acid [g $µmol^{-1}$].

Twenty PLFAs were selected because of their use as biomarker fatty acids for six distinct microbial groups: Gram-positive bacteria (i-C15:0, a-C15:0, i-C16:0, i-C17:0), Gram-negative

bacteria (C16:1c9, C16:1t9, C17:0cy, C18:1c11, C19:0cy), bacteria (non-specific) (C14:0, C15:0, C16:0, C17:0, C18:0), actinomycetes (10Me-C16:0, 10Me-C17:0, 10Me-C18:0), fungi (C18:1c9, C18:2n9,12) and mycorrhiza (C16:1c11). PLFAs were used as markers for specific bacterial or fungal groups according to Kroppenstedt *et al.* (1984), Brennan (1988), O'Leary & Wilkinson (1988), Frostegård & Bååth (1996), Stahl & Klug (1996), Zelles (1997) and Olsson (1999).

2.1.8 Rhizosphere microbiology: HTS

The rhizosphere was sampled from all strawberry and lettuce roots at the end of the plant bioassay experiments. Rhizosphere sampling was done according to Lundberg *et al.* (2012). Briefly, roots were placed in a sterile 50 mL tube containing 25 mL phosphate buffer. Tubes were vortexed at maximum speed for 15 s, which released most of the rhizosphere soil from the roots and turned the water turbid. The turbid solution was then filtered through a 100 µm nylon mesh cell strainer into a new 50 mL tube to remove plant parts and large sediment particles. The turbid filtrate was further centrifuged for 15 min at 3,200 g to form a loose pellet containing fine sediment and micro-organisms. These pellets (250 mg) were immediately used for DNA extraction with the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, USA), according to the manufacturer's instructions, or treated further for storage and later use. For storage, 1 mL subsamples were transferred to 1.5 mL microfuge tubes, spun at 10,000 g for 5 min to form tight pellets. These rhizosphere pellets, averaging 25 mg, were flash-frozen in liquid nitrogen and stored at -80 °C until DNA extraction.

Extracted DNA was used for either identifying bacterial rhizosphere populations (V3-V4 16S rRNA gene) or fungal rhizosphere populations (ITS2). For all experiments ('unfertilized peat', 'fertilized peat' experiments of the strawberry bioassay and for the lettuce bioassay), 5 replicates were taken of non-inoculated plants.

<u>16S rRNA gene and ITS2 amplicon sequencing</u>

Illumina amplicon sequencing of the bacterial and fungal rhizosphere populations was done on the V3-V4 fragment of the 16S rRNA gene and the ITS2 gene fragment, respectively, using Illumina technology (Illumina, San Diego, CA, USA). The V3-V4 fragments were amplified using the primers S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC), as described by Klindworth *et al.* (2013), extended with Illumina specific adaptors. Following PCR conditions were used: initial denaturation at 95 °C

for 3 min, followed by 25 cycles consisting of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30 min) and a final extension step at 72 °C for 5 min. To amplify the fungal rDNA-ITS2 region an adapted forward primer of fITS7bis from Ihrmark *et al.* (2012) (GTGAATCATCRAATYTTTG) and the ITS4NGSr reverse primer (CAWCGATGAAGAACGYAG) (Tedersoo et al. 2014) were used, both extended with Illumina specific adaptors. The ITS2-PCR conditions were as above, except for 30 cycles with an annealing time of 1 min. A second PCR was done to attach dual indices and sequencing adaptors to all fragments, using the Nextera XT index kit (Illumina, San Diego, CA, USA). Same PCR conditions were used as in the first PCR, but 8 cycles were used instead of 25 or 30 PCR cycles. Mastermixes for all PCRs were prepared using the Kapa HiFi Hotstart ReadyMix (Kapabiosystems, Wilmington, MA, USA) according to the manufacturer's instructions and total reaction volumes were 25 and 50 µL for the first and second PCR, respectively. Each PCR was followed by a PCR product clean-up using the HighPrep PCR reagent kit (MAGBIO, Gaithersburg, MD). The final libraries were quality controlled using the Qiaxcel Advanced, with the Qiaxcel DNA High Resolution kit (QIAGEN, Germantwon, MD, USA), and concentrations were measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The final barcoded libraries of each sample were diluted to 10 nM and pooled in equal amounts. Resulting libraries were sequenced using Illumina MiSeq v3 technology (2 x 300 bp, paired-end) by Macrogen, South-Korea, using 30% PhiX DNA as spike-in.

Sequence reads processing

Demultiplexing of the amplicon dataset and removal of the barcodes was performed by the sequencing provider. The raw demultiplexed sequence data is available in NCBI's Sequence Read Archive under the submission PRJNA294259 for the bacterial sequences and PRJNA317548 for the fungal sequences of the lettuce bioassay and the 'unfertilized peat' and 'fertilized peat' strawberry experiments. Trimmomatic v0.32 was used for removing the primers (Bolger et al., 2014). Raw Illumina forward and reverse reads were merged using the program PEAR v.0.9.8 (Zhang et al., 2014). Length cut-off values for the merged sequences were set between 400 and 450 bp for the V3-V4 16S rRNA gene region and between 200 and 480 bp for the ITS2 region. A minimum overlap size of 120 bp and quality score threshold of 30 were used for all sequences. To extract the ITS2 gene sequences from the complete amplicon sequence, which includes parts of the neighbouring, highly conserved, ribosomal

genes, the ITSx program v.1.0.11 was used (Bengtsson-Palme et al. 2013). In the following steps, different programs of the Usearch software v7.0.1090 were used (Edgar, 2014). Merged sequences were quality filtered with a maximum expected error of 3 with the "fastq_filter" option. Next, sequences of all samples that needed to be compared to each other were merged, dereplicated and sorted by size. Clustering the reads into Operational Taxonomic Units (OTUs) was done using Uparse, with an identity level of 97% for bacterial sequences and 98.5% for fungal sequences (Edgar, 2014; Ihrmark et al., 2012). Chimeras were removed from the V3-V4 gene fragments using "uchime_ref" with the RDP Gold database as a reference (Edgar et al., 2011). Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm at 97% identity, and converted into an OTU table (McDonald et al., 2012).

For each individual library, two negative controls were included, one for the fungal and one for the bacterial primer amplifications. After quality filtering, all negative controls gave a very low number of reads, indicating that probably no contamination of the samples occurred during the sample preparation.

2.1.9 Downstream data analysis and statistics

In the lettuce bioassay and the 'unfertilized peat' and 'fertilized peat' strawberry experiments, chemical soil and substrate properties and water use were analysed as a one-way ANOVA with treatment (i.e. % biochar) as a factor using SPSS 16.0 software. Significant differences ($p < 0.05$) between means were determined by Scheffe's test. To use the ANOVA analysis, homogeneity of the variances was tested using the Levene's test.

The plant properties data was analysed for three dependent variables: plant fresh weight, dry weight and leaf lesions. Homogeneity was tested using Levene's test. If variances were equal, a t-test was used, otherwise the Wilcoxon-rank sum test was used. For the strawberry fruit inoculation experiment with *B. cinerea*, the effect of biochar addition (factor) was studied using a repeated measures ANOVA, as fruit rot was evaluated over 3 time points.

Statistical differences in the PLFA profiles between the different treatments were determined using a MANOVA analysis for the absolute abundances. Statistical differences of the relative abundances of these PLFA data were determined using ANOVA analyses by the Statistical

Analysis of Metagenomic Profiles (STAMP) program (Parks and Beiko, 2010). Correction of multiple testing was done using the Benjamini-Hochberg False Discovery Rate (FDR) method.

OTU tables of the 16S V3-V4 and ITS2 amplicon sequencing were analysed using the QIIME software package (v1.9.0) (Caporaso et al., 2010a). Taxonomy was assigned with the script "assign_taxonomy.py" using the uclust method considering maximum 3 database hits, with the Silva v119 97% rep set (as provided by QIIME) as reference for the bacterial sequences and UNITE v7 (dynamic) for fungal sequences (Caporaso et al., 2010b; Quast et al., 2012; Kõljalg et al. 2013).

For the microbial analysis, both differences in community composition and in community richness were studied. Within the lettuce bioassay and the strawberry 'unfertilized peat' and 'fertilized peat' experiments, we first focused on the total community composition differences between groups, in which treatment of biochar is the main factor in the experiment. The multivariate analysis was done using the specific R package vegan (version 2.0-10) (Oksanen et al., 2010). The dissimilarity matrix, based on the Bray-Curtis dissimilarity index, was calculated from the OTU table as generated by Usearch, for both the bacterial and fungal sequences. Using the betadisper function, the homogeneity of the variances was checked on this dissimilarity matrix. Further, the significance of biochar treatment was analyzed using PERMANOVA analysis, in which the Bray-Curtis dissimilarity index matrix was used as input. Second, the STAMP analysis software was used to study individual differences in the bacterial groups (Parks and Beiko, 2010). For each experiment, ANOVA analyses were done on a species table to determine the effect of biochar addition on the individual groups (phyla, species). To correct for multiple testing, we used the Benjamini-Hochberg FDR method.

To study community diversity and richness in the lettuce bioassay and the strawberry 'unfertilized peat' and 'fertilized peat' experiments, Shannon-Wiener diversity indices and the total number of observed OTUs were calculated. The Shannon-Wiener diversity indices were calculated using the "alpha_diversity.py" script in the QIIME software package, while the number of observed OTUs were counted per sample on a rarefaction depth of 10,000 sequences. Rarefaction analysis was done using the "alpha_rarefaction.py" script. In total, two samples (1 sample of the lettuce 1% BC treatment, 1 sample of the lettuce 3% BC treatment) contained a lower number of bacterial reads than the established rarefaction depth and were therefore deleted for further analysis. To study differences among mean richness and diversity

indices, ANOVA analysis was done. Tukey HSD test was used to find the mean richness and diversity indices that are significantly different from each other. Both analyses were done using R (version 3.1.0) (R core team, 2015). To correlate chemical data with the bacterial OTU tables, a distance-based redundancy analysis was done, using Bray-Curtis as dissimilarity index. Analysis was done using the function “capscale” in the R package vegan (Oksanen et al., 2010).

2.2 RESULTS

In this section, the effect of biochar on the physicochemical properties of the plant growth media (2.2.1), the plant growth and disease susceptibility (2.2.2) and the rhizosphere microbiome (2.2.3) are studied. In addition, the relation of the microbiome community and the plant-soil/substrate properties are investigated (2.2.4).

2.2.1 Effect of biochar on physicochemical properties of plant growth media

Changes in chemical properties and water use of the plant growth media that were caused by the addition of biochar were measured at the beginning and end of each bio-assay. Results are listed in Table AI2.1 and Table AI2.2 for the field soil used for lettuce growth and Table AI2.3 and Table AI2.4 the peat used for strawberry growth. Only those properties in which we expected changes to occur during the short period of the bio-assay were measured at the end of the experiment (Nelissen et al., 2015).

Addition of 3% biochar to the field soil significantly increased the plant-available concentrations of the macronutrients P, K, Ca and Mg. Potassium concentrations also increased in the 1% biochar/field soil variant. Moreover, addition of biochar in a 1% or 3% concentration increased pH and TOC content, which was still observed at the end of the experiment. A lower mineral nitrogen concentration was observed for both biochar dosages at the start of the experiment. This mineral N concentration was reduced at the end of the experiment with no significant differences in concentration between soils treated with different biochar dosages (Table AI2.1). At the end of the experiment, the EC value increased for soils treated with 3% biochar (Table AI2.2). Water use was poorly correlated with the fresh lettuce biomass, but was significantly lower for the 1 and 3% biochar treatment when compared to the 0% biochar control. Biochar addition reduced the evaporation from the soil rather than affecting the water use by the plants, as shown by the similar decline (12%) in water use by the pot mixes without lettuce plants (0% biochar: 611 ml 3% biochar: 536 ml).

Similarly, chemical properties of the biochar amended and unamended peat were determined for both the ‘unfertilized’ and ‘fertilized’ peat experiments. In the ‘fertilized peat’ experiments, statistical variations between the non-biochar and biochar treated peat at the beginning of the experiment could not be determined due to a too low number of replications (n=2). This was also the case for the 1% biochar application on of the non-fertilized peat experiment (Table AI2.3).

For both the 'unfertilized' and 'fertilized' peat experiment, the total amount of mineral N was depleted after 13 weeks (mineral N concentrations < 5 mg/L peat). Application of biochar in unfertilized peat (experiment 1; Table 1) significantly raised the water-extractable K, Ca and Mn concentrations and the water-soluble P concentrations. However values remained extremely low compared to the reference values. Additionally the substrate with biochar had a significantly lower organic matter (OM) content and Cl concentration at the beginning of the experiments (Table AI2.3). Peat that was additionally fertilized and limed still showed an increase in Ca and Mn concentrations due to biochar application, but this could not statistically be proven. The increase of water dissolved P due to biochar addition was still detected at the end of the unfertilized peat experiments (Table AI2.4), but compared to the 'fertilized peat' experiment, these concentrations are extremely low. In contradiction with the lettuce growth in the field soil/biochar mixes, water use in the strawberry experiment was strongly positively correlated with the plant biomass.

2.2.2 Effect of biochar on plant growth and disease susceptibility

The effects of biochar on lettuce and strawberry growth and disease susceptibility are reported in Table 2.2. Overall, biochar addition to the field soil had no effect on the growth of lettuce, expressed as fresh and dry shoot weight (Table 2.2). In contrast, 3% biochar application raised the strawberry plant weight significantly, with 166.5% in fresh weight and 114.3% in dry weight. Supplemental addition of lime and fertilizer to the soil reduced the growth stimulating effect of the biochar itself, although a distinct but not significant increase in fresh weight due to biochar in the mix was still noted (Table 2.2).

Biochar addition did not affect lettuce resistance to *R. solani*. No remarkable differences in lesion sizes could be observed on the biochar/field soil grown lettuce leaves (Table 2.2), while biochar addition to peat did reduce the susceptibility of the strawberry plants to *B. cinerea*. A significant reduction of lesion sizes was observed for the leaves of plants grown in peat treated with 3% biochar compared to the control group (0% biochar). However, this effect of biochar was absent when the peat was supplemented with lime and fertilizer. A trend towards a lower infection rate was still seen, but was not significant. It should be noted that during these experiments an overall low infection rate was observed (Table 2.2), which may explain the non-significant effect.

Table 2.2 Properties of lettuce (soil – 8 weeks of plant growth) and strawberry (peat – 13 weeks of plant growth) plants with and without biochar addition (lettuce bioassay, n=14; strawberry bioassays, n=40)

	Fresh weight (g plant material)	Dry weight (g plant material)	Disease resistance (leaf inoculation)[b]
Lettuce in soil			
0% biochar	38.56±0.32	3.88±0.08	2.16±0.30
1% biochar	36.88±0.47	3.62±0.09	2.02±0.27
3% biochar	34.26±0.34	3.20±0.06	2.70±0.23
Strawberry in peat			
0% biochar	2.21±0.06	0.63±0.01	1.96±0.07
1% biochar	2.24±0.17	0.66±0.03	2.07±0.08
3% biochar	**5.89±0.15**	**1.35±0.04**	**1.19±0.08**
Strawberry – peat + lime + fertilizer			
0% biochar + lime + fertilizer	39.22±1.54	14.90±0.60	0.50±0.08
3% biochar + lime + fertilizer	42.04±1.37	15.77±0.72	0.44±0.07

[a]Values marked in bold are statistically different from the control treatment (= without biochar).
[b]Lesions of *Rhizoctonia solani* on lettuce and *Botrytis cinerea* on strawberry using a disease scale of 0-4.

In addition to the infection of the strawberry leaves, the strawberry fruits were infected with *B. cinerea* and the number of symptomatic fruits was recorded over time for the 'fertilized peat' experiment (Figure 2.2). Due to a low number of fruits developed in the strawberry grown in unfertilized peat, fruit inoculation was excluded in this experiment. Strawberry fruits produced on peat/biochar (3%) were less susceptible to the grey mold pathogen *B. cinerea.* The infection process was slowed down, as indicated by a reduced number of symptomatic fruit at time point 1 and 2 ($p < 0.05$) (Figure 2.2).

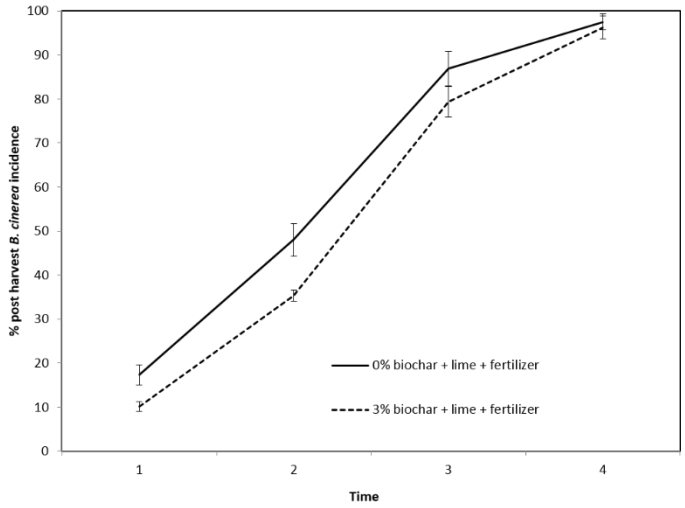


Figure 2.2 Effect of 3% biochar treatment on the postharvest decay of strawberry fruit caused by *Botrytis cinerea*. Both lime and PGMix were added to the peat. Data were pooled across 2 plant experiment repetitions and 4 picks per experiment. In total, 109 fruits were inoculated per treatment. (Time: number of days measured since the first symptoms of infection.

2.2.3 Effect of biochar on the rhizosphere microbiology

The effect of biochar on the bacterial and fungal community was first of all analysed through **PLFA**. Application of biochar at 1% or 3% in the field soil had no significant effect on the microbial groups, except for the stimulation of arbuscular mycorrhizae, represented by one biomarker (C16:1c11) (data not shown).

In the peat substrate, the biochar addition did not change the total microbial biomass (Table AI2.5) and the absolute number of fatty acids per microbial group, but induced some specific changes in the microbial content. The relative abundances of six biomarkers were significantly different in the peat/biochar mix compared to the control (0% biochar), representing a decrease in Gram negative bacteria (ANOVA, p=0.047) and non-specific bacteria (ANOVA, $p=5.57*10^{-3}$) and a significant increase in fungi (ANOVA, p=0.042) (Table AI2.6). Once biochar was enriched with fertilizer and lime however, these shifts in microbial relative abundances due to biochar application were absent in peat (Table AI2.6).

Second, we used **amplicon sequencing to study the effect of biochar on the bacterial community**. Differences in bacterial community composition of the lettuce rhizosphere grown in field soil with variable dosage of biochar (0%, 1% and 3%) were studied using PERMANOVA analysis. No significant differences were observed between the treatments (p=0.127), indicating that biochar application did not influence the bacterial community composition in the lettuce rhizophere.

For the bacterial community composition of the rhizosphere of strawberry plants of the 'unfertilized peat' and 'fertilized peat' experiments, an interaction effect between the addition of lime/fertilizer and biochar was present (PERMANOVA, p=0.035). Therefore the effect of biochar on the bacterial community composition was analysed individually for these experiments. There were significant differences due to the addition of biochar in the rhizosphere bacterial communities developed in unfertilized and unlimed peat (PERMANOVA, p=0.01), and also in fertilized and limed peat (PERMANOVA, p=0.019).

To determine the bacterial groups influenced by the addition of biochar, the taxonomic profiles of the strawberry rhizosphere bacteria were studied at the end of plant growth. Both a significant reduction in the relative abundance of *Proteobacteria* (ANOVA, corrected p-value=0.020) and a significant increase in the relative abundance of *Planctomycetes* (ANOVA, corrected p-value = 0.024) resulted from addition of biochar to the peat substrate (Table 2.3).

Furthermore a relatively large, but not significant reduction in *Acidobacteria* and increase in *Actinobacteria* occurred upon addition of 3% biochar to peat. In the 'fertilized experiment' biochar addition only induced changes in two, relatively small bacterial groups: the *Armatimonadetes* (reduction) and the *Cyanobacteria* (increase) (Table 2.3).

Table 2.3 Relative abundances (%) (average±standard error) of the eight most dominating phyla (relative abundance >1%) in the strawberry rhizosphere. These comprise almost 95% of the total bacterial community in the rhizosphere (n>=5). Phyla that are statistically significant ($p<0.05$) in abundance for the biochar treatments compared to the control group (0% biochar) are indicated with an asterix.

Phylum	**Experiment 1**				**Experiment 2**		
	Control	1% biochar	3% biochar		control	3% biochar	
Proteobacteria (%)	53.0±1.4	50.8±2.0	44.1±4.4	*	49.3±2.5	44.9±1.0	
Acidobacteria (%)	17.6±1.0	16.4±0.4	15.6±0.5		15.2±0.5	16.4±0.5	
Actinobacteria (%)	7.6±1.0	7.2±0.6	10.6±0.4		8.7±0.8	9.5±0.5	
Verrucomicrobia (%)	9.0±0.5	9.5±0.7	10.2±0.9		5.8±0.6	7.1±0.3	
Bacteroidetes (%)	5.2±0.7	6.9±0.7	4.2±0.4		7.0±1.0	7.5±0.6	
Planctomycetes (%)	2.0±0.3	2.5±0.3	1.1±0.3	*	3.1±0.5	3.7±0.2	
Candidate division TM7 (%)	0.7±0.1	0.5±0.1	1.1±0.1		2.1±0.2	2.5±0.2	
Armatimonadetes (%)	0.7±0.1	0.6±0.1	1.1±0.1		2.7±0.3	1.8±0.3	*
Cyanobacteria (%)	0.2±0.1	0.2±0.1	0.3±0.1		0.8±0.2	1.4±0.2	*

Genera that shift in relative abundance due to biochar addition at the end of strawberry growth ('unfertilized peat' and 'fertilized peat' experiments) were also determined for either peat and fertilized and limed peat. Genera were first subsampled by only retaining those with a relative abundance of at least 0.1%. In total, eleven genera of the 190 of the strawberry rhizosphere in the unfertilized and unlimed peat shifted in relative abundance due to the addition of biochar, among which three genera known to harbour plant-beneficial agents, two with microbial N cycling and one playing a major role in plant growth promotion (Table 2.4). For the strawberry rhizosphere of fertilized and limed peat, an additional treatment of biochar gave significant changes in the relative abundances of thirteen genera of the 148, of which two are described in literature as potential biocontrol agents (increase) and one is involved in N cycling (increase) (Table 2.5).

Additionally, the bacterial composition of pure biochar was studied to see if biochar could serve as an additional habitat for micro-organisms. Eighty-two different genera (with relative abundance >0.01%) were detected, and three of these genera (*Bauldia*, *Devosia*, *Opitutus*) were also among the bacterial types enriched by adding biochar to peat.

Table 2.4 Relative abundances of genera (mean ± standard error) of the strawberry rhizosphere ('Unfertilized peat experiment', no lime or fertilizer added), which are significantly changed by the addition of biochar. Functions of the genera related to promotion of plant growth and biocontrol, are listed as previously described in literature. The total number of OTUs classified as the genus respectively are indicated in the column N°.

Species	N°	Peat			Function	References
		0% biochar	1% biochar	3% biochar		
Acidocella (%)	5	9.85±3.15	4.78±2.62	2.55±0.94	/	
Bdellovibrio (%)	11	0.05±0.02	0.04±0.01	0.11±0.05	Biocontrol agent gram - bacteria	Dori-Bachash *et al.* (2008); Jurkevitch *et al.* (2000)
Devosia (%)	3	0.15±0.12	0.2±0.12	0.42±0.07	N fixation	Rivas *et al.* (2002); Hoque *et al.* (2011)
Haliangium (%)	30	0.05±0.01	0.13±0.09	0.25±0.04	Possible biocontrol agent (production haliangicin-antifungal)	Fudou *et al.* (2001); Kundim *et al.* (2003)
Marmoricola (%)	3	0.01±0.01	0.04±0.02	0.07±0.03	/	
Phenylobacterium (%)	3	0.25±0.13	0.44±0.10	0.62±0.17	/	
Rhizobium (%)	4	0.04±0.04	0.06±0.02	0.15±0.05	N fixation	Courty *et al.* (2015); Meng *et al.* (2015)
Schlesneria (%)	5	0.30±0.10	0.38±0.07	0.65±0.11	/	
Singulisphaera (%)	10	0.16±0.04	0.15±0.03	0.23±0.04	/	
Sorangium (%)	10	0.04±0.03	0.07±0.02	0.11±0.03	Potential biocontrol agent (antifungal activity)	Ligon & Hill (2001); Kim & Yun (2011)
Variovorax (%)	2	0.02±0.01	0.05±0.04	0.13±0.03	Plant growth promotion	Chen *et al.* (2013); Zhang *et al.* (2013)

Table 2.5 Relative abundances of genera (mean ± standard error) of the strawberry rhizosphere ('Fertilized peat experiment', lime and fertilizer added to peat), which are significantly changed by the addition of biochar. Functions of the genera related to promotion of plant growth and biocontrol, are listed as previously described in literature. The total number of OTUs classified as the genus respectively are indicated in the column N°.

Genera	N°	Peat+lime+fertilizer		Function	References
		0% biochar	3% biochar		
Aquicella (%)	34	0.33±0.08	0.25±0.05	/	
Bauldia (%)	3	0.41±0.03	0.26±0.04	/	
Devosia (%)	3	0.60±0.04	0.87±0.03	N fixation	Rivas *et al.* (2002); Hoque *et al.* (2011)
Haliangium (%)	30	0.30±0.11	0.98±0.09	Possible biocontrol agent (production haliangicin-antifungal)	Fudou *et al.* (2001); Kundim *et al.* (2003)
Inquilinus (%)	2	0.07±0.01	0.15±0.02	/	
Nocardia (%)	3	0.34±0.01	0.08±0.01	/	
Opitutus (%)	16	0.34±0.12	1.10±0.07	/	
Planctomyces (%)	38	0.05±0.01	0.12±0.01	/	
Prosthecobacter (%)	6	0.30±0.02	0.16±0.04	/	
Pseudolabrys(%)	2	0.31±0.07	0.94±0.07	/	
Reyranella (%)	5	1.22±0.01	0.13±0.02	/	
Rhodanobacter (%)	2	1.20±0.25	3.01±0.12	Possible biocontrol agent	De Clercq *et al.* (2006)
Taibaiella (%)	6	0.02±0.00	0.10±0.03	/	

To have insight in the complexity of the rhizosphere bacterial communities of both the lettuce and strawberry rhizospheres, community richness (number of observed OTUs) and diversity (Shannon-Wiener diversity indices) were estimated in the different treatments (Table 2.6).

Table 2.6 Effect of biochar addition on the number of observed OTUs (calculated at a rarefaction depth of 10.000 sequences) and Shannon-wiener diversity indices (mean±standard error) for the strawberry and the lettuce rhizosphere.

BACTERIA		**Number of observed OTUs**	**Shannon-Wiener diversity index**
Strawberry - peat	Control	901±60 (a)	7.63±0.21 (a)
	1% biochar	993±36 (ab)	8.07±0.12 (ab)
	3% biochar	1198±74 (b)	8.61±0.14 (bc)
Strawberry – peat + lime + fertilizer	Control	857±29 (a)	7.69±0.21 (a)
	3% biochar	946±34 (a)	8.13±0.08 (ab)
Lettuce – soil	Control	1642±33 (c)	9.29±0.09 (cd)
	1% biochar	1726±83 (c)	9.31±0.18 (cd)
	3%biochar	1706±54 (c)	9.41±0.10 (d)
FUNGI		**Number of observed OTUs**	**Shannon-Wiener diversity index**
Strawberry – peat + lime + fertilizer	Control	462±23 (a)	5.59±0.23 (a)
	3% biochar	446±38 (a)	5.66±0.08 (a)

Significant differences in the number of observed species (ANOVA, $p=5.8*10^{-11}$) and in the Shannon-Wiener diversity indices (ANOVA, $p=2*10^{-16}$) were observed (Table 2.7). Highest richness and diversity were measured in the lettuce rhizosphere grown in field soil. Richness and diversity of the strawberry rhizosphere microbiome grown in peat substrate, were significantly lower. Addition of biochar however raised both the number of observed OTUs as the diversity of the rhizosphere microbiome. This effect was significant for the strawberry microbiome developed in peat, but not in the fertilized and limed peat. Nonetheless, a trend towards a higher diversity and richness in the rhizosphere microbiome due to biochar was observed.

The **effect of biochar on the fungal community composition** was solely studied for the strawberry 'fertilized peat' bioassay using ITS2 gene amplicon sequencing. However, no significant effect of biochar addition to fertilized and limed peat medium was seen on the fungal community (PERMANOVA) for both experiments.

Also for the fungal richness and diversity, no significant effects of the addition of biochar were observed at the end of plant growth in the 'fertilized peat' experiment (Table 2.6).

2.2.4 Relation of microbiome community and plant-soil/substrate properties

As shown above, biochar had an effect on the physicochemical composition of the substrate of the strawberry bioassay. These changes could be correlated with the shifts in the rhizosphere microbiome observed when peat was treated with biochar. Therefore we did a distance-based redundancy analysis to correlate the physicochemical parameters with the bacterial communities on the data of the strawberry rhizosphere obtained from plants grown in unfertilized peat (Figure 2.3). This figure illustrates that the microbial communities of unfertilized peat without lime addition are correlated with changes in the concentration of Mn, Ca, P and Cl when biochar was added to the peat. A similar analysis could be done for the limed and fertilized peat, but would be less meaningful because of the less clear clustering (data not shown). An analysis for the lettuce experiment was not done, because no significant effects of biochar amendment were observed on the bacterial composition, as described above.

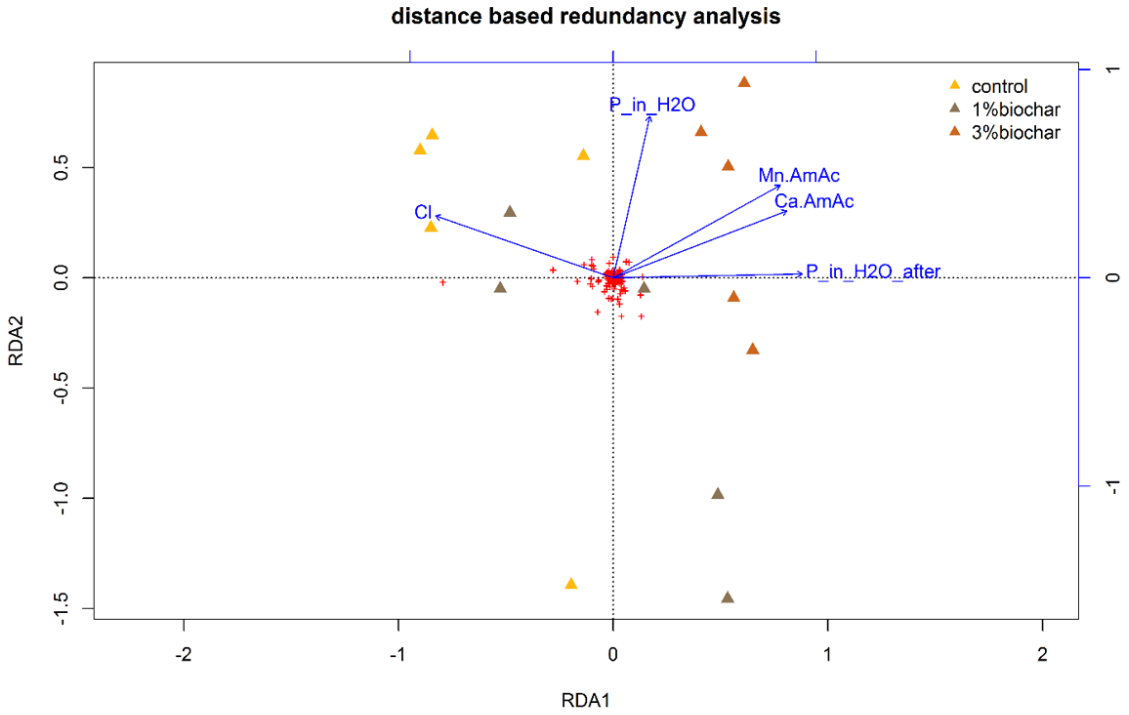


Figure 2.3 Distance based redundancy analysis of the 16S amplicon sequencing data shows differential taxonomic composition in the strawberry rhizosphere upon biochar addition to substrate. Substrate physicochemical parameters that are significantly different (Table AI2.3; Table AI2.4) are fitted to the plot. Red crosses represent the OTUs. The labels control, 1% biochar and 3% biochar indicate bacterial communities of the strawberry rhizosphere grown in non-fertilized peat, 1% biochar amended non-fertilized peat and 3% biochar amended non-fertilized peat respectively.

2.3 DISCUSSION AND CONCLUSIONS

In the present Chapter, we studied the effect of biochar on two different crop-soil/substrate systems: lettuce grown in soil and strawberry grown in peat. Changes in the physicochemical properties of the soil and substrate were observed for both the lettuce and strawberry assays. Adding biochar to the field soil affected the carbon content, the pH, the soil water evaporation and the nutrient availability. In contradiction with the lettuce growth in the field soil/biochar mixes, water use in the strawberry experiment was strongly positively correlated with the plant biomass. The water use in the substrate was thus driven by plant growth with a minor or undetectable effect of the biochar amendments on the evaporation from the substrate. Adding biochar to the peat thus affected the nutrient availability, but had in this case only small effect on pH or soil water evaporation.
Moreover, biochar addition induced major changes in (i) rhizosphere microbiology, (ii) plant growth and (iii) plant health exclusively in the strawberry bio-assays, especially when no additional fertilization or liming of the peat was done.

We observed **shifts in the composition and diversity of the microbial community of the strawberry rhizosphere** due to the addition of biochar. Previously, it has been described that biochar can alter the microbial composition of bulk soil, but the factors that drive these changes in microbial composition are still unknown (Graber et al., 2014a). The following hypotheses have been suggested: (1) Biochar could provide an additional habitat for bacteria and fungi (Ezawa et al., 2002; Thies and Rillig, 2009) and may provide places of refuge for fungal grazers for microbes (Warnock et al., 2007), (2) Biochar may interfere with microbial intercellular signalling (Masiello et al., 2013), (3) Due to its chemical composition, biochar could have an effect on microbial composition. Biochar borne organic chemicals may suppress some members of the microbial community and promote others (Kolton et al. 2011), (4) Biochar may change the physicochemical properties (e.g. pH, EC), which could have an effect the microbial communities (Graber et al., 2014b). Based on our observations, we suggest two additional hypotheses: (5) An effect of biochar on the microbial community due to its effect on the nutrient composition of soil and substrate. The biggest changes in rhizosphere microbial composition and diversity were observed when peat was not limed and fertilized. Additional application of fertilizer and lime reduced the effects on the strawberry rhizosphere microbiology. Complementary, no effects of biochar application on the lettuce rhizosphere

microbiology were observed in nutrient rich soil. This strengthens our hypothesis that in nutrient limiting conditions, biochar provides an additional nutrient source for the present microbial community, which can alter the proportion and composition of microbial communities. (6) Biochar could serve as a source of micro-organisms. We showed that from the 82 bacterial species present on pure biochar, at least three species were found in the strawberry rhizosphere after 12 weeks of growth in peat-biochar mixtures. Adding biochar to soil or substrate can therefore enhance species to the habitat.

Biochar addition **promoted plant growth** solely in the strawberry assay with unfertilized peat that was not limed. This made us suspect that biochar only promotes plant growth in nutrient limiting conditions. Following explanations for this assumption are proposed: (1) Biochar could serve as a fertilizer (Altland and Locke, 2013). In both lettuce and strawberry bio-assays, an increase of nutrients, e.g. P, K, Ca and Mg, was observed when biochar was added to soil or peat, respectively. In nutrient-rich conditions, the soil or substrate already stored a relevant concentration of nutrients, and nutrients are not expected to be limiting for plant growth. Adding more nutrients will increase the nutrient stock but will not directly enhance plant growth. In nutrient-limiting conditions, these changes in nutrients will supply necessary nutrients for the plant and the microbial community, e.g. P, resulting in plant growth promotion. (2) Biochar could have an effect on the plant growth by changing the rhizosphere microbiome. First a higher number of bacteria involved in the microbial N cycling were observed in the rhizosphere of strawberry plants grown in unfertilized peat. This can lead to a higher amount of plant-available N, which subsequently can promote plant growth (Brewin, 2010). Second a higher number of PGPR were observed, which can ease the uptake of nutrients for the plants and enhance the plant availability of phosphorus (Egamberdiyeva, 2007; Krey et al., 2013). Additionally, it has been shown that these stimulatory effects are higher in nutrient deficient soil than in nutrient rich soil (Egamberdiyeva, 2007). Finally, the increased diversity of bacteria in the strawberry rhizosphere might enhance the N mineralization and therefore improve plant nutrition and plant growth (Weidner et al., 2015). These effects of biochar on the rhizosphere microbiome were only observed in the strawberry assay where peat was not fertilized and limed. The hypothesis stated above might explain partly why this is the only assay where we observed improvement of plant growth by biochar addition.

Within the lettuce bio-assay no effect of leaf infection with *Rhizoctonia* was observed. However, here we used a detached leaf assay, instead of an attached leaf assay as in the strawberry bio-assays. Nonetheless these detached leaf assays are common in phytopathological research, this could have affected our results (Liu et al., 2007). In the strawberry bio-assay, where we used an attached leaf assay, **addition of biochar had an effect on plant disease resistance against *B. cinerea*** for both the leaves and fruits. We suggest following explanations: (1) Biochar could have an effect on the plant's resistance towards pathogens as fertilizer. Plants that are deficient in potassium are less resistant to pests, diseases and nematode attacks. Addition of K to K-deficient soils can therefore reduce the incidence of plant diseases (Römheld and Kerkby, 2010). In the strawberry assay we observed a significant increase of the K concentration in the substrate when no fertilizer or lime was added. The higher amount of nutrients, especially K, could thus partly explain the higher disease resistance measured of the strawberry plants to *B. cinerea* in nutrient limiting conditions, as indicated by the concentrations being lower than the reference values. In the lettuce bio-assay, K concentrations also increased significantly. However, concentrations of K were already higher than the reference values before biochar was added to the soil, which could explain why the higher amount of K did not have an effect on the disease resistance. (2) The effect of biochar on the rhizobiome could have an effect on the plant's resistance to pathogens. First, we identified species previously described as potential biocontrol agents that increased significantly in relative abundance after biochar addition to the peat. Second, we also detected a higher number of PGPR, which can promote the ISR of the plant. Our observations thus confirm previously published suggestions that biochar can promote the ISR of the plant, resulting in increased disease resistance (Mehari et al., 2015).

The extensive amount of literature on biochar application as a soil improver or fertilizer in agriculture shows the high expectations for this product in our society. However, next to the frequently described benefits of the use of biochar mainly in poor tropical soils (Alling et al., 2014), also neutral or in a few cases negative effects are observed, most often in more rich soils (Nelissen et al., 2015). This raises the question if biochar can really improve soil quality and crop production in general. These dissimilarities across studies could be due to the absence of a standardized protocol for biochar production, differences in feedstock and pyrolysis process in the biochar production process, differences in biochar application rate, or

as we showed, could even be crop or soil/substrate dependent. From our study we can conclude that the effect of biochar on the plant-soil system is not singular and multiple factors are involved. We show that biochar can alter physicochemical properties of the soil/substrate, plant growth, disease resistance and microbial communities in the plant-soil/substrate system and that these changes are related to each other. Therefore we suggest that future studies should focus on the effect of biochar on the plant, the soil and the microbial system simultaneously. Depending on the crop-soil/substrate system used, biochar can thus be valuable in agriculture. Analysing two different plant-soil systems revealed that biochar is useful under nutrient-limiting conditions, and we hypothesize that it could serve as a fertilizer, affecting plant growth, disease resistance and the microbial composition of the rhizosphere.

ADDITIONAL INFORMATION CHAPTER 2

Table AI2.1: Chemical and physical properties of field soil one week after starting the experiment. Values are averages ± standard deviations for 3 replicates. (AL: ammonium lactate extractable concentration)

	pH-KCl	EC (µS/cm)	NO_3-N (mg/kg DM)	NH_4-N (mg/kg DM)	Min N (mg/kg DM)	P-AL (mg/100g DM)	K-AL (mg/100g DM)
Optimal Range	**6.2-6.6**	**Not applicable**	**Not applicable**	**Not applicable**	**Not applicable**	**12-18**	**14-20**
Lettuce-soil							
0% biochar	5.77±0.03 (a)	84.67±4.73	13.71±0.07 (c)	9.09±0.23 (ab)	22.80±0.29 (c)	22.10±0.40 (a)	23.87±0.25 (a)
1% biochar	6.80±0.09 (b)		9.75±0.37 (b)	9.89±0.43 (b)	19.63±0.60 (b)	22.33±0.72 (a)	28.80±0.96 (b)
3% biochar	7.31±0.06 (c)		6.68±0.38 (a)	8.29±0.27 (a)	14.97±0.34 (a)	24.63±0.57 (b)	39.87±0.87 (c)

	Ca-AL (mg/100g DM)	Mg-AL (mg/100g DM)	Fe-AL (mg/100g DM)	Mn-AL (mg/100g DM)	Na-AL (mg/100g DM)	TOC (%/DM)
Optimal Range	**100-240**	**9-14**	**Not applicable**	**Not applicable**	**3.1-6.0**	**1.2-1.6**
Lettuce-soil						
0% biochar	70.20±2.16 (a)	13.13±1.29 (a)	48.27±0.80 (a)	15.28±0.46 (a)	0.96±0.00 (a)	0.86±0.01 (a)
1% biochar	105.70±16.20 (a)	13.37±0.59 (a)	46.00±2.79 (a)	15.14±0.97 (a)	0.96±0.00 (a)	1.53±0.13 (b)
3% biochar	186.13±18.08 (b)	16.77±0.75 (b)	47.40±0.60 (a)	16.22±0.35 (a)	0.96±0.00 (a)	2.67±0.15 (c)

Values within the same columns and following the same letter are not significantly different ($p<0.05$)

Table AI2.2: **Chemical and physical properties of field soil at the end of the experiment** (week 8). Values are averages ± standard deviations for 10 replicates.

	pH-KCl	EC (µS/cm)	NO_3-N (mg/kg DM)	NH_4-N (mg/kg DM)	Min N (mg/kg DM)	TOC* (%/DM)	Water availability (ml)	Water in plant (ml)
Lettuce-soil								
0% biochar	5.73±0.14 (a)	135.00±37.03 (a)	3.15±3.53 (a)	1.30±0.45 (a)	4.45±3.72 (a)	0.88±0.03 (a)	725±240 (b)	34.7±3.9 (a)
1% biochar	6.86±0.12 (b)	136.10±35.64 (a)	2.67±2.65 (a)	1.91±0.50 (ab)	4.57±3.00 (a)	1.48±0.16 (b)	667±227 (b)	33.3±5.9 (a)
3% biochar	7.67±0.05 (c)	173.10±50.39 (b)	2.07±1.78 (a)	2.07±0.95 (b)	4.13±2.60 (a)	2.60±0.53 (c)	443±223 (a)	31.1±4.2 (a)

* Interaction effect between treatment and repetitions of experiments

Values within the same columns and following the same letter are not significantly different ($p<0.05$)

Table AI2.3: **Chemical and physical properties of peat one week after starting the experiment.** Values are averages ± standard deviations for 4 replicates. AmAc: ammonium acetate extractable concentration(OM: organic matter, DM: dry matter, EC: electrical conductivity)

	pH-H_2O	EC (µS/cm)	NO_3-N in H_2O (mg/L peat)	NH_4-N in H_2O (mg/L peat)	NO_3-N+NH_4-N (mg/L peat)	P-AmAc (mg/L peat)	K-AmAc (mg/L peat)	Ca-AmAc (mg/L peat)
Optimal Range	**3.8-6.0**	**200-400**	**Not applicable**	**Not applicable**	**60-140**	**30-70**	**150-360**	**325-2100**
Strawberry-peat								
0% biochar	4.50±0.08 (a)	47.50±4.20 (a)	2.50±0.00 (a)	6.60±3.28 (a)	9.10±3.28 (a)	7.00±0.00 (a)	20.65±1.48 (a)	
1% biochar	4.48±0.08	43.00±4.24	2.50±0.00	3.95±2.05	6.45±2.05	7.00±0.00	27.55±3.46	
3% biochar	4.63±0.14 (a)	42.50±1.29 (a)	2.50±0.00 (a)	5.20±3.13 (a)	7.70±3.13 (a)	7.00±0.00 (a)	36.15±1.58 (b)	
Strawberry-peat+lime+fertilizer								
0% biochar	4.80±0.01	150.50±3.54	25.05±0.78	27.00±0.57	52.05±1.34	32.75±0.92	93.40±2.12	
3%biochar	5.05±0.01	128.50±9.19	19.70±3.68	20.95±3.60	40.65±7.28	28.65±3.32	108.30±4.10	

	Fe-AmAc (mg/L peat)	Mn-AmAc (mg/L peat)	SO_4 in H_2O (mg/L peat)	Na in H_2O (mg/L peat)	Cl in H_2O (mg/L peat)	OM (%OM/DM)	P in H_2O (mg/L peat)	Mg-AmAc (mg/L peat)
Optimal Range	**1-5**	**0.5-10**	**0-100**	**0-50**	**0-100**	**Not applicable**	**Not applicable**	**150-300**
Strawberry-peat								
0% biochar	1.50±0.00 (a)	0.75±0.00 (a)	26.85±3.77 (a)	16.45±4.59 (a)	16.03±4.41 (b)	98.15±0.17 (b)	0.38±0.52 (a)	63.63±17.58 (a)
1% biochar	1.50±0.00	0.75±0.00	20.95±2.76	12.50±0.00	13.45±0.35	97.9±0.00	0.45±0.07	38.00±0.00
3% biochar	1.50±0.00 (a)	1.825±0.09 (b)	23.325±2.00 (a)	15.65±3.64 (a)	11.05±1.16 (a)	97.38±0.13 (a)	1.00±0.12 (b)	64.10±18.83 (a)
Strawberry-peat+lime+fertilizer								
0% biochar	1.50±0.00	2.55±0.07	114.50±3.68	12.50±0.00	5.00±0.00	93.25±0.21	61.20±2.82	141.70±2.40
3%biochar	1.50±0.00	3.05±0.21	98.80±16.54	12.50±0.00	10.00±7.07	93.00±0.00	39.15±4.74	132.90±3.68

Values within the same columns following the same letter are not significantly different ($p < 0.05$)

Table AI2.4: **Chemical and physical properties of peat at the end of the experiment (week 13)** (DM: dry matter, EC: electrical conductivity). Values are averages ± standard deviations for 10 (Strawberry-Peat) or 38 (Strawberry – peat+lime+fertilizer) replicates.

	pH-H_2O	EC (µS/cm)	SO_4 in H_2O (mg/L peat)	Cl in H_2O (mg/L peat)	P in H_2O (mg/L peat)	Water availability (ml)	Water in plant (ml)	NO_3-N (mg/L peat)	NH_4-N (mg/L peat)
Strawberry-peat									
0% biochar	4.49±0.19 (a)	35.10±10.24 (a)	11.93±5.25 (a)	8.54±3.68 (a)	0.33±0.25 (a)	1038±427 (a)	5±4 (a)*		
1% biochar	4.51±0.12 (a)	30.40±10.90 (a)	11.77±9.44 (a)	8.78±5.18 (a)	0.54±0.05 (a)				
3% biochar	4.53±0.22 (a)	36.40±8.95 (a)	17.47±9.55 (a)	6.71±2.63 (a)	1.30±0.51 (b)	2788±776 (b)	17±16 (b)*		
Strawberry-peat+lime+fertilizer									
0% biochar	4.62±0.07 (a)*	65.47±6.59 (a)	49.94±5.40 (a)	9.81±1.62 (a)	5.05±1.02 (a)*	1255±285 (a)	23±5 (a)		
3%biochar	4.86±0.03 (b)*	65.89±3.26 (b)	59.50±3.91 (b)	9.35±0.57 (a)	4.68±0.44 (a)*	1246±211 (a)	24±7 (a)		

* Interaction effects between experiment replications and properties were measured with higher values in the second replicate. However if significant values were measured, this was for both experimental replicates.

Values within the same columns, following the same letter are not significantly different ($p < 0.05$)

Table AI2.5: **Absolute abundances of the PLFA of strawberry 'unfertilized peat' and 'fertilized peat' experiments.** Asterix indicate the level of significance between control groups and biochar treated peat for each experiment (*p<0.05; **p<0.01 by analysis of variance n=5). Values are expressed in nmol g^{-1} (average±standard error).

Community	PLFA	Peat			Peat+lime+fertilizer		
		Control	3% biochar		control	3% biochar	
Gram positive bacteria	i-C15:0	7.8±3.3	8.1±1.3		39.7±12.8	34.5±3.3	
	a-C15:0	4.0±2.0	3.4±1.1		14.1±4.1	11.6±1.0	
	i-C16:0	3.2±2.0	2.6±1.0		10.5±4.3	9.4±1.5	
	i-C17:0	2.3±1.5	1.9±0.8		6.6±2.1	5.6±0.6	
Non-specific bacteria	C14:0	1.5±0.5	1.0±0.3		3.8±0.7	2.9±0.2	*
	C15:0	0.9±0.2	0.7±0.1	*	2.9±1.1	2.9±0.6	
	C16:0	16.4±6.1	13.0±4.8		44.7±10.8	42.1±2.6	
	C17:0	0.5±0.3	0.5±0.3		1.7±0.6	1.5±0.2	
	C18:0	3.0±1.5	2.8±1.4		9.8±2.4	9.5±0.4	
Gram negative bacteria	C16:1c9	5.3±2.1	5.6±1.5		17.7±4.5	16.3±1.5	
	C16:1t9	2.2±1.7	1.6±1.1		3.9±1.8	3.0±0.6	
	C17:0cy	1.0±0.5	1.4±0.6		6.7±1.4	6.7±0.7	
	C18:1c11	10.3±3.1	7.6±2.6		22.1±4.4	22.0±1.8	
	C19:0cy	7.9±3.0	6.5±2.1		25.3±4.9	24.7±2.6	
Actinomycetes	10Me-C16:0	4.1±2.7	3.6±1.7		9.2±6.6	8.9±2.4	
	10Me-C17:0	2.5±1.3	2.7±0.8		8.6±3.0	7.6±0.8	
	10Me-C18:0	0.7±0.5	0.5±0.3		1.7±1.4	1.6±1.3	
AM Fungi	C16:1c11	2.4±1.9	1.9±0.8		7.7±3.9	6.0±0.9	
Fungi	C18:1c9	14.0±5.3	15.4±6.0		46.9±10.1	41.9±2.8	
	C18:2n9,12	9.0±4.7	12.1±7.0		29.2±7.0	28.2±2.5	
Total PLFA biomass		**98.9±14.9**	**90.76±12.3**		**310.9±31.6**	**285.2±8.4**	

Table AI2.6: **Relative abundances of the PLFA of strawberry 'unfertilized peat' and 'fertilized peat' experiments.** Asterix indicate the level of significance between control groups and biochar treated peat for each experiment (*p<0.05; **p<0.01 by analysis of variance n=5). Values represent percentages (average±standard error).

Community	PLFA	Peat			Peat+lime+fertilizer	
		Control	3% biochar		control	3% biochar
Gram positive bacteria	i-C15:0	8.02±0.34	9.5±0.86		12.59±0.51	12.02±0.14
	a-C15:0	3.97±0.26	3.86±0.30		4.51±0.20	4.06±0.11
	i-C16:0	3.13±0.15	2.91±0.08		3.28±0.34	3.26±0.13
	i-C17:0	2.13±0.18	2.02±0.06		2.08±0.09	1.94±0.04
Non-specific bacteria	C14:0	1.53±0.08	1.30±0.26		1.24±0.05	1.02±0.04
	C15:0	0.96±0.07	0.80±0.08	**	0.91±0.12	1.01±0.07
	C16:0	16.95±0.72	14.26±0.27	*	14.33±0.11	14.70±0.09
	C17:0	0.50±0.03	0.57±0.03		0.52±0.03	0.53±0.02
	C18:0	2.89±0.13	2.95±0.19		3.16±0.08	3.31±0.06
Gram negative bacteria	C16:1c9	5.49±0.18	6.33±0.26	*	5.70±0.28	5.67±0.09
	C16:1t9	2.07±0.45	1.60±0.19		1.19±0.11	1.04±0.07
	C17:0cy	1.02±0.03	1.51±0.04	**	2.15±0.06	2.32±0.04
	C18:1c11	10.87±0.52	8.41±0.16	**	7.15±0.27	7.67±0.15
	C19:0cy	8.02±0.19	7.19±0.22		8.20±0.37	8.60±0.12
Actinomycetes	10Me-C16:0	4.00±0.25	3.60±0.21		2.86±0.71	3.07±0.27
	10Me-C17:0	2.40±0.08	2.90±0.10	**	2.71±0.11	2.65±0.04
	10Me-C18:0	0.79±0.18	0.70±0.15		0.55±0.15	0.55±0.17
AM Fungi	C16:1c11	2.16±0.29	2.04±0.09		2.39±0.23	2.08±0.10
Fungi	C18:1c9	14.61±0.37	15.89±0.35		15.13±0.51	14.61±0.14
	C18:2n9,12	8.49±0.73	11.64±1.23		9.35±0.35	9.89±0.57

Chapter 3

Dynamics in the strawberry rhizosphere microbiome in response to biochar and aboveground plant inoculation

Modified from:

De Tender C, Haegeman A, Vandecasteele B, Clement L, Cremelie P, Dawyndt P, Maes M & Debode J. (2016b). Dynamics in the strawberry rhizosphere microbiome in response to biochar and Botrytis cinerea *leaf infection. Frontiers in Microbiology 7, 2062.*

In Belgium and The Netherlands, strawberry cultivation relies mainly on cold-stored strawberry plants used as planting material. They are taken from a nursery field (field soil) in December/January and cold-stored at -1.5 °C. Starting in January until the end of August, strawberry plants can be planted and cultivated, which greatly extends the traditional production season and provides an important economic benefit (Lieten et al., 1995; Lieten, 2013). The cold-stored strawberry plantlets are generally planted into peat substrate. The main cultivar used in this cultivation system is "Elsanta" (Lieten, 2013). This cultivar is susceptible to fungal diseases, including *Botrytis cinerea* (gray mold), which is one of the most destructive diseases on strawberry worldwide. Controlling the disease with fungicides is difficult, mainly because of the long latency period between inoculation and the appearance of symptoms, the prolonged and overlapping flowering and fruiting periods, the explosive fungal development that occurs at or near harvest time and the onset of fungal strains resistant to fungicides (Sutton, 1990).

In Chapter 2, strawberry plantlets of the cultivar Elsanta were grown for thirteen weeks in peat with and without biochar. Adding biochar to the peat resulted in a lower susceptibility of the plants towards *B. cinerea*. After these thirteen weeks an increased bacterial biodiversity was noted in the strawberry rhizosphere and a shift towards bacterial genera including species previously reported to be involved in biological control and induced resistances. In addition, there were also small differences in the chemical composition of the peat versus the biochar-amended peat.

However, these biological and physicochemical changes were only measured at the end of the experiment (thirteen weeks after planting). The moment that the shift in bacterial community composition took place within the rhizosphere remained unknown. Furthermore, the effect of the *B. cinerea* inoculation on the rhizosphere community has not been monitored, as only rhizosphere samples of non-inoculated plants have been studied. Therefore, two major questions still remain: (1) what are the dynamics of the biochar-mediated shift in the strawberry rhizosphere microbiome, and (2) is there an effect of an aboveground infection with *B. cinerea* on the rhizosphere microbiome of the strawberry plants? An additional aim was to gain more insight in the role of fungal communities in the strawberry rhizosphere.

To study the rhizosphere microbiome, both bacterial and fungal communities were studied using 16S rRNA V3-V4 gene region and ITS2 metabarcoding, respectively. Two experiments were set-up: (1) a time course experiment in which the effect of biochar and the dynamics

over time on the rhizosphere microbiome were studied, and (2) an inoculation experiment in which the effect of *B. cinerea* leaf inoculation on the bacterial community in the rhizosphere was studied. For both experiments, plants were grown in peat or biochar-amended peat for thirteen weeks, in which the rhizosphere was sampled at the end in the inoculation experiment, and at eight pre-set time points during the plant growth cycle in the time course experiment. In addition to the rhizosphere microbiome analysis, chemical properties of peat and biochar-amended peat were analyzed and the effect of biochar on plant and root growth, fruit yield, and disease susceptibility against *B. cinerea* inoculation on both plant leaves and fruits were analyzed.

3.1 MATERIALS AND METHODS

In this section, an overview of the materials and methods for the physicochemical characterisation of the plant growth media (3.1.1), the strawberry bioassays (3.1.2) and the rhizosphere microbiology (3.1.3) is provided. In the end, an overview of statistical methods is given (3.1.4).

3.1.1 Chemical characterization of biochar, peat and amended peat

The biochar used in this chapter is the same as described in Chapter 2 section 2.1.1.

The substrate was sampled at different time points (described below) during the strawberry experiments. Dry matter content was determined according to EN 13040. Electrical Conductivity (EC) (EN 13038) and pH-H_2O (EN 13037) were measured in a 1:5 soil to water (v/v) suspension. Determination of organic matter content and ash was done according to EN 13039. Extraction (1:5 v/v) of water soluble nutrients and elements (NO_3-N, NH_4-N, Cl, SO_4 and PO_4-P) was done according to EN 13652, and measured with a Dionex DX-600 IC ion chromatography (Dionex, Sunnyvale, CA), and for NH_4-N with a Skalar San++ mineral N analyzer.

3.1.2 Strawberry bioassays

Peat was used as either pure growing medium (298 g peat) or mixed with 3% biochar (9.4 g biochar + 289 g peat). Additionally, 1.33 g L^{-1} fertilizer (PGMix, Peltracom, Ghent, Belgium) and 3 g L^{-1} lime (Dolokal extra, Ankerpoort NV, Maastricht, The Netherlands) were added to both the peat and the peat/3% biochar mixture. No additional fertilizer was applied during plant growth. Both substrates were wetted to obtain 40% water-filled pore space (WFPS), and bulk density was adjusted to 200 g L^{-1}. Each mixture was put in a closed bag and pre-incubated at 15 °C for one week. Subsequently, 1.5 L pots were filled with the mixed substrates and a cold-stored bare-root strawberry (*Fragaria x ananassa*, cultivar Elsanta) transplant was planted in each pot. The plants were then arranged in the greenhouse in a completely randomized design and grown at 20 °C for up to 13 weeks. Every week, the moisture content of the substrate was adjusted to 40% WFPS based on mass loss.

Two experiments were done. In a first experiment, referred to as the **'time course experiment'**, in total 24 plants were grown in peat and 24 in peat amended with 3% biochar. Plants were sampled in a completely randomized way for rhizosphere microbiome analysis at nine time points: before planting and 1, 2, 3, 6, 9, 10, 12 and 13 weeks after planting. Three

replicates were sampled for each time point x growing medium combination. After 9 and 12 weeks of plant growth, the *B. cinerea* bio-assay was done on the plant leaves as described in Chapter 2 section 2.1.6.

In a second experiment, further referred to as the **'the inoculation experiment'**, in total 24 plants were cultivated in peat and 24 in peat amended with 3% biochar (n=48). Half of the plants in each treatment were inoculated with *B. cinerea* on a leaf at 12 weeks after planting using the method described in Chapter 2 section 2.1.6. This resulted in four treatments: peat non-inoculated (peat NI), peat inoculated (peat I), peat amended with biochar non-inoculated (Peat+BC NI) and peat amended with biochar inoculated (Peat+BC I). Additionally, fruits were harvested, weighed and inoculated with *B. cinerea* as in Chapter 2 section 2.1.6. At one week after inoculation (i.e., after 13 weeks of plant growth) the rhizosphere of six biological replicates per treatment was sampled (section 3.1.3).

At 13 weeks after planting, the strawberry plants were collected and weighed (fresh weight and dry weight (48 hours at 70°C)) and the root development was measured. This was done by observing the root systems that show up at the substrate surface when removing the pot. Depending on the number of visible lateral roots (lateral roots and root hairs), a 0-3 developmental score was given, with 0 = no lateral roots , 1 = a few lateral roots, 2 = lateral roots all over the substrate surface , and 3 = substrate surface fully covered with lateral roots.

3.1.3 Rhizosphere microbiology: high-throughput sequencing

In the time course experiment, the rhizosphere was sampled from strawberry roots before planting, followed by sampling at 1, 2, 3, 6, 9, 10, 12 and 13 weeks after planting. Three replicates were taken at each time point for the plants grown in peat and biochar-amended peat. For the inoculation experiment, the rhizosphere of strawberry plants was sampled after 13 weeks of plant growth. For each condition (peat NI, peat I, peat+BC NI and peat+BC I; in which NI= non-inoculated, I=inoculated, BC= biochar addition) six biological replicates were taken. Rhizosphere sampling was done according to Lundberg *et al.* (2012), in which 25 mL of root material was used. The resulting pellets (250 mg), which are considered as the rhizosphere sample, were immediately used for DNA extraction with the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, USA), according to the manufacturer's instructions. DNA was stored at -20 °C until further use.

16S rRNA gene amplicon sequencing

Amplicon sequencing of the bacterial and fungal rhizosphere populations was done on the V3-V4 fragment of the 16S rRNA gene and the ITS2 gene fragment respectively, using Illumina technology (Illumina, San Diego, CA, USA). Using an amplification and dual-index PCR successively, fragments were amplified and extended with Illumina specific adaptors, which is described in detail in Chapter 2 section 2.1.8. Each PCR step was followed by a PCR product clean-up using the CleanPCR reagent kit (MAGBIO, Gaithersburg, MD, USA).

Final libraries were quality controlled using the Qiaxcel Advanced, with the Qiaxcel DNA High Resolution kit (QIAGEN, Germantown, MD, USA), and concentrations were measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The final barcoded libraries of each sample were diluted to 10 nM and pooled in a 2:1 ratio for bacterial and fungal libraries respectively. Resulting libraries were sequenced using Illumina MiSeq v3 technology (2 x 300 bp) by Macrogen, South-Korea, using 30% PhiX DNA as spike-in.

Sequence reads processing

Demultiplexing of the amplicon dataset and removal of the barcodes was performed by the sequencing provider. The raw sequence data is available in the NCBI Sequence Read Archive under the accession number SRA399532 for the time course experiment and SRA416875 for the inoculation experiment. A detailed description of the sequence read processing can be found in Chapter 2 section 2.1.8.

3.1.4 Downstream data analysis and statistics

All statistical analyses were done using the R statistical software, version 3.2.2 (R core team). Chemical substrate properties were analyzed as a two-way ANOVA with biochar treatment and time as the two factors within the time course analysis, and biochar treatment and the presence of infection as the factors in the inoculation experiment. To use the ANOVA analysis, first Levene's test was used to study homogeneity of the variances.

The plant properties data was analyzed for seven dependent variables: plant fresh weight, dry weight, root development, number of fruits picked, fruit weight per plant, leaf lesions and AUDPC for the fruit rot. Homogeneity was tested using Levene's test. If variances were equal, a t-test was used, otherwise the Wilcoxon-rank sum test was used.

OTU tables of the 16S V3-V4 gene region and ITS2 amplicon sequencing were analyzed using the QIIME software package (v1.9.0) (Caporaso et al., 2010a). Taxonomy was assigned with

the script “assign_taxonomy.py” using the uclust method considering maximum 3 database hits, with the Silva v119 97% rep set (as provided by QIIME) as reference for the bacterial sequences and UNITE v7 (dynamic) for fungal sequences (Caporaso et al., 2010b; Quest et al., 2012; Kõljalg et al., 2013).

For the microbial analysis, both differences in community composition and in community richness were studied.

Within the **time course analysis**, we first focused on the total community composition differences between groups, in which treatment of biochar and time were indicated as the main factors in the experiment. The multivariate analysis was done using the specific R package vegan (version 2.0-10) (Oksanen et al., 2010). The dissimilarity matrix, based on the Bray-Curtis dissimilarity index, was calculated from the OTU table as generated by Usearch, for both the bacterial and fungal sequences. Using the betadisper function, the homogeneity of the variances was checked on this dissimilarity matrix. Further, the significance of biochar treatment, time and the interaction effect between treatment of biochar and time were analyzed using PERMANOVA analysis, in which the Bray-Curtis dissimilarity index matrix was used as input.

Secondly, we assessed differential abundance using likelihood-ratio tests. We tested for (1) the effect of time within non-biochar treated samples and the biochar treated samples, separately and (2) the effect of treatment within each time point. The analyses were done upon clustering the bacterial and fungal OTU tables generated by QIIME at family level for research question 1 above and on genus level for research question 2 above. In a filtering step OTUs with low count number in most samples were removed. For both fungal and bacterial OTU tables on family or genus level, only those families/genera with a count of four in at least three samples were kept for analysis. Normalization is based on the trimmed mean of M-values (TMM) in which we correct for effective library size of the count tables (Robinson and Oshlack, 2010). This normalization takes the sequencing depth into account and corrects for the presence of highly abundant families. The counts are modeled OTU by OTU using a negative binomial (NB) model with main effects for time and biochar, as well as a biochar x time interaction. The effective library size was used as an offset in the model for normalization purposes, hence, all model parameters have an interpretation in terms of changes in relative abundance. Empirical Bayes estimation of the overdispersion parameters of the NB model

was adopted using the quantile-adjusted conditional maximum likelihood (qCLM) method by shrinking the OTU-level overdispersion towards the common dispersion across all OTUs. Statistical tests were adopted on the appropriate contrasts of the model parameters to assess the research questions of interest. We adopted the Benjamini-Hochberg False Discovery Rate procedure to correct for multiple testing. All of these analyses were done using edgeR package, version 3.12.0 (Robinson et al., 2010).

Third, statistical differences in richness between groups was studied for both the bacterial and fungal sequences. Rarefaction analysis was done using the "alpha_rarefaction.py" script of QIIME. A plateau was reached at 50,000 sequences for the bacterial OTUs and 20,000 sequences for the fungal OTUs. Richness of the bacteria and fungi was determined on rarefied data, for which the number of sequences was set on the reached plateau. The temporal evolution of richness is expected to be nonlinear. Therefore an additive model is used with two thin plate regression spline components:

$$y_i = f_a(t_i) + x_i\, f_b(t_i) + \varepsilon_i,$$

with y_i the richness of observation i, t_i the time in weeks at which observation i is taken, $f_a(t_i)$ a smoother to model the evolution in average richness, x_i an indicator variable, which is x_i=0.5 when observation i is treated with biochar and x_i=-0.5 when observation i originates from the control treatment and $f_b(t_i)$ a smoother modeling the average difference in richness between biochar amended medium and the control medium. The knots of the splines are placed at the 9 observed time-points (t=0, 1, 2, 3, 6, 9, 10, 12, 13 weeks) and the smoothness penalty is tuned by exploiting the link between smoothing and mixed models (Ruppert et al., 2003; Wood, 2006). Upon fitting, the additive model can used to study the average evolution of the richness in peat ($f_a(t_i)$ - 0.5 $f_b(t_i)$) and biochar amended medium ($f_a(t_i)$ - 0.5 $f_b(t_i)$), separately. However, it is more appealing to assess the first derivative of these average richness profiles since it indicates if the richness is increasing or decreasing over time. Next, inference on smoother $f_b(t_i)$ is adopted to study the effect of biochar addition over time. Note, that the additive model can provide inference on average richness, difference in richness and their first derivatives at any timepoint t, however, if we want to assess these effects at multiple time-points we have to adjust for multiple testing. We address the multiple testing issue by using a grid based approach for constructing approximate simultaneous confidence intervals (Ruppert

et al., 2003) and adjusted p-values (Yang et al., 2013). To control the multiple testing burden, we consider a grid that is spanned by the 9 observed time-points in the experiment.

Within the **inoculation experiment**, the total community composition differences between groups were analyzed similar to the time course experiment, using PERMANOVA analyses. Within these experiments, both the main effect of biochar treatment and infection with *B. cinerea* were studied, as the interaction effect between the factors. Second, we wanted to test which genera show differences in relative abundances between: (1) peat NI versus peat I, (2) peat NI versus peat + BC NI, (3) peat I versus peat + BC I and (4) peat + BC NI versus peat + BC I. To do so we used the bacterial OTU tables clustered on genus level, as generated by QIIME. Differential abundance at OTU-level was assessed using the EdgeR procedure described above. Third, the significant differences in richness were estimated on the rarefied data, obtained from the OTU table as generated by QIIME. Data was rarefied at 50,000 sequences. Equality of variances between groups was tested using Levene's test. Statistical differences in richness were analyzed using a linear model with main effects for infection and biochar addition and the infection x biochar interaction.

3.2 RESULTS

In this section first the effect of biochar on the chemical properties of the substrates (3.2.1) and the plant properties (3.2.2) is described. Further on, results concerning the time course experiment (3.2.3) are described. In the end, the effects of aboveground *B. cinerea* infection of the leafs on the rhizosphere microbiome are noted (3.2.4).

3.2.1 Effect of biochar on chemical properties of the substrates

Within the time course experiment, changes in chemical parameters of the strawberry substrates (peat and peat amended with 3% biochar) were measured over time. Additionally, the effect of biochar amendment in peat was studied for each time point (Additional Information (AI) Table AI3.1). Over time, no significant changes were observed in the chemical parameters within 13 weeks of plant growth for both the peat and biochar amended peat treatment. The addition of biochar to the growing medium, however, significantly raised the pH and reduced the amount of NH_4-N, independent of the time point at which the parameters were measured.

In the inoculation experiment, the effects of biochar incorporation and *B. cinerea* leaf infection on peat chemical parameters were measured at the end of the experiment, after 13 weeks of plant growth (Table AI3.2). The aboveground infection did not affect the plant available nutrients and pH of the peat. Similar to the time series experiment, adding biochar to the growing medium of strawberry raised the pH. Other chemical parameters were not altered by the addition of biochar, however.

3.2.2 Effect of biochar on plant growth, root growth and strawberry production

Seven dependent plant properties were measured at the end of the strawberry growth cycle: plant fresh weight, dry weight, root development, number of fruits picked, fruit weight per plant, leaf lesions and AUDPC for evaluating fruit rot caused by *B. cinerea*. For these results, measurements of strawberry plants of both the time course and inoculation experiment were pooled, as no interaction effect of biochar treatment and experiment was noticed.

Addition of 3% biochar to peat significantly increased the development of lateral roots, the number and weight of the strawberry fruits and the resistance of the fruits to *B. cinerea*. Biochar had no effect on leaf and petiole fresh and dry weight nor on the leaf lesions caused by *B. cinerea* (Table 3.1). For the leaf lesions, it should be noted that the infection rate was

very low (mean score was <1, with about 15% of the leaf area was affected). The effect on disease severity after adding 3% biochar to fresh and dry weight of peat corresponds to the results of Chapter 2.

Table 3.1 Properties of strawberry plants (n=6) grown for 13 weeks in peat with (3%) and without biochar.

	Leaf and petiole weight (g per plant)		**Root development (0-3)**	**Fruits per plant**		**Disease severity (*B. cinerea*)**	
	Fresh	**Dry**		**Amount**	**Weight (g)**	**Leaf lesions (0-4)**	**Fruits decay (AUDPC)**
Peat	42.19±2.15	16.92±0.73	**1.54±0.18**	**2.23±0.22**	**12.43±1.15**	0.68±0.07	**65.31±6.09**
Peat + 3% biochar	44.78±1.91	18.09±0.80	**2.76±0.15**	**4.22±0.35**	**21.95±1.96**	0.69±0.07	**45.46±2.27**
P-value	0.18	0.36	**<0.001**	**0.02**	**0.02**	0.90	**0.04**

3.2.3 Time course experiment

Within this experiment we questioned how rhizosphere microbiome compositions evolve during growth of the plants by analyzing successive time points. Subsequently, the effect of biochar mixed in the peat substrate was analyzed for each time point, comparing the rhizospheres in peat and in the peat/biochar mix. To study these effects, changes in community composition as well as differences in richness are analyzed on both the bacterial and fungal community.

First, **shifts in the bacterial and fungal community structure** were studied. The bacterial and fungal community composition of the strawberry rhizosphere were studied during a 13 week growth period. The main effects of the biochar treatment and time, and their interaction effect were estimated using the complete OTU dataset. The OTUs were homoscedastic between groups, and therefore general effects could be studied using PERMANOVA analysis. For the bacteria, the interaction effect between biochar addition and time was extremely significant ($p<0.001$). In other words, both the time and biochar treatment affect the bacterial community composition; further, the temporal changes in community structure are different for the biochar-amended and unamended peat. For the fungi however, only a significant effect of time ($p<0.001$) was measured. Biochar treatment did not alter the fungal community composition of the rhizosphere, which is in accordance to the results of Chapter 2.

Second**, the temporal variation in bacterial and fungal community structure** was studied**.** Both the bacterial and fungal communities of the rhizosphere changed significantly over time, both for the plants grown in peat and biochar-amended peat separately.

These temporal changes in bacterial and fungal community are represented in Figure 3.1, in which the number of significantly altered families within a three-week timeframe are represented. Especially within the first six weeks of plant growth, a high number of bacterial families changed in relative abundances over time, both for plants grown in biochar-amended or unamended peat (Figure 3.1A and Figure 3.1B).

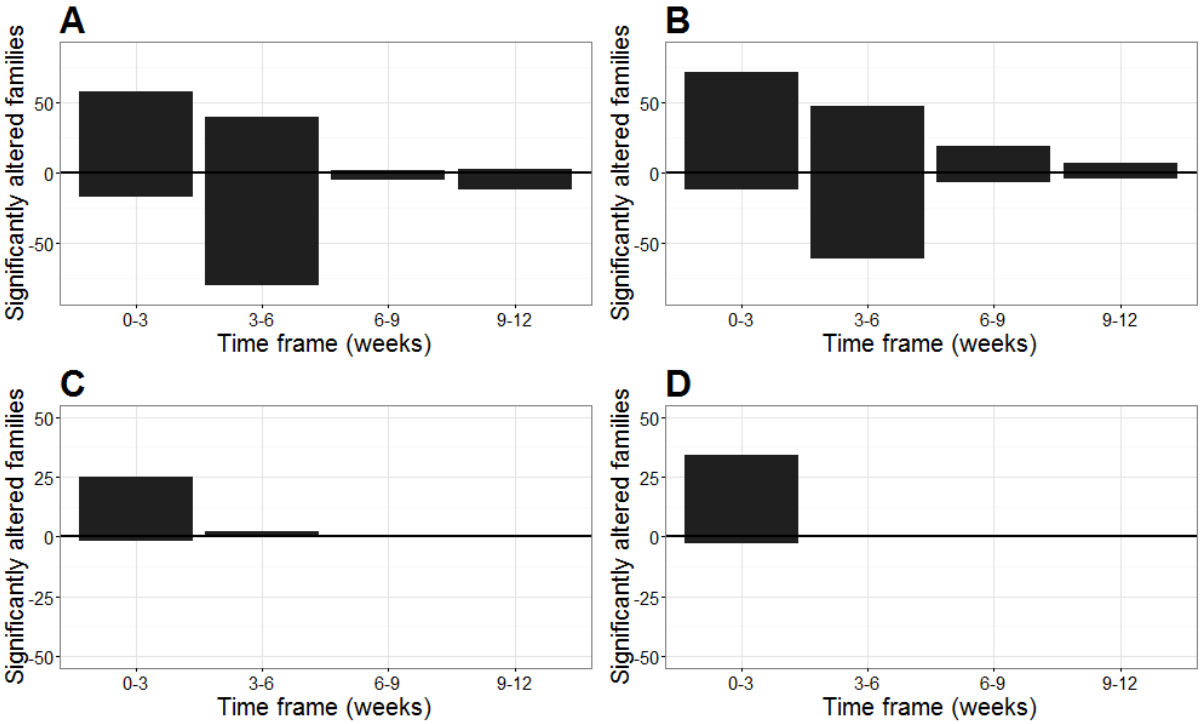


Figure 3.1 Number of bacterial and fungal families of the strawberry rhizosphere that altered significantly over time (weeks). The number of families that increased and decreased in relative abundance within a three week timeframe are shown above and below the horizontal line, respectively. Number of significantly altered bacterial families in the rhizosphere of strawberry grown in **(A)** peat, and **(B)** biochar amended peat. Number of significantly altered fungal families in the rhizosphere of strawberry grown in **(C)** peat and **(D)** biochar amended peat. Plants were leaf-inoculated with *B. cinerea* at the beginning of weeks 9 and 12.

In total 77 bacterial families changed significantly in relative abundance between at least two successive time points within the strawberry rhizosphere of plants grown in peat or biochar amended peat. In total, 45 of these families represented at least 0.1% of the total community for at least one time point (Figure 3.2).

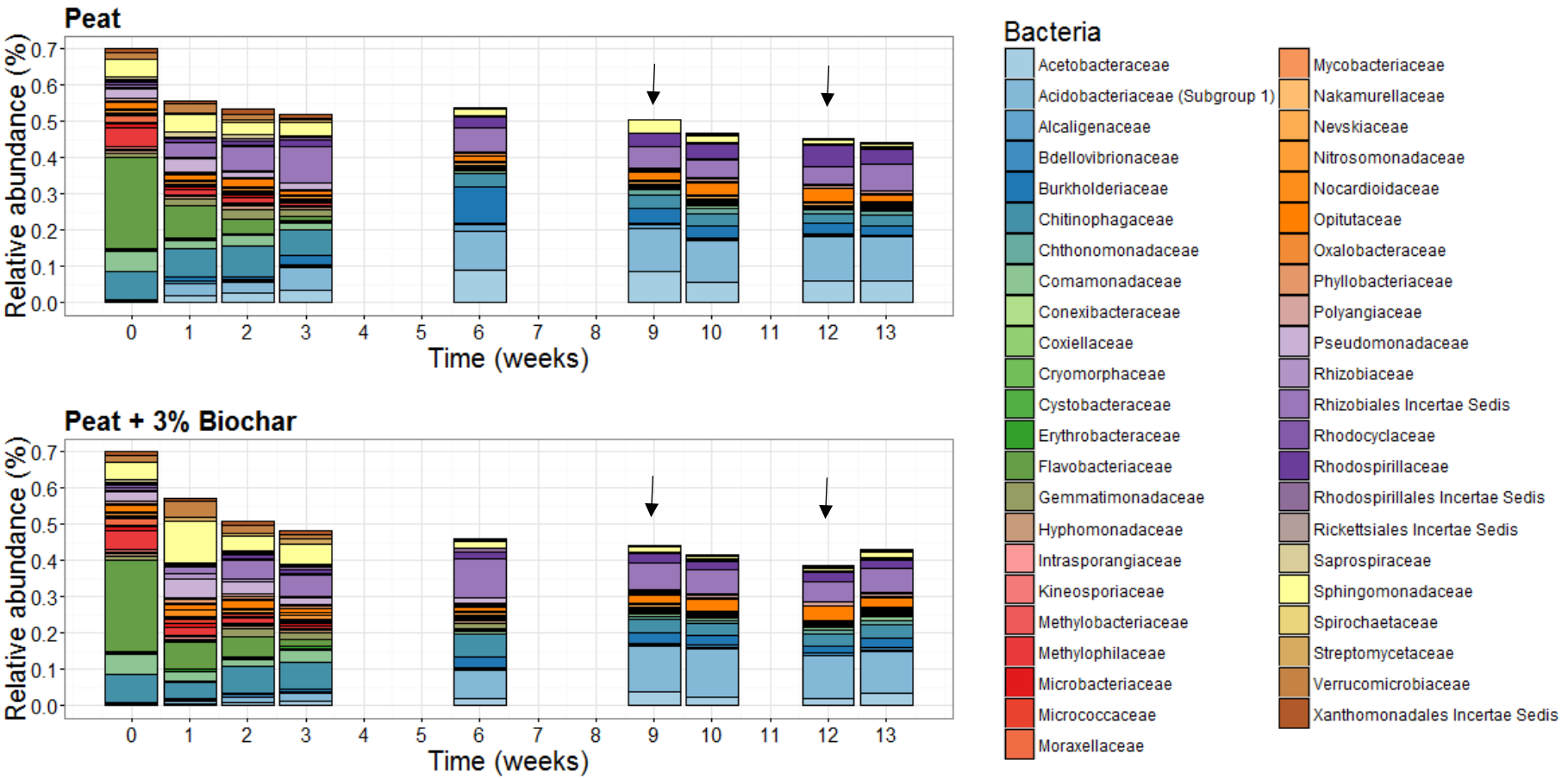


Figure 3.2 Representation of the bacterial families that change significantly in relative abundance (%) over time. The mean relative abundance (n=3) of the bacterial families that change significantly over time between at least two successive time points are represented. Only those families that contribute for at least 0.1% of the total community for at least one time point are represented in the graph. Time points where no sampling was done are visualized as an empty space. Time points of infection with *B. cinerea* are indicated using arrows Upper figure: The rhizosphere of strawberry grown in peat. Bottom figure: The rhizosphere of strawberry grown in peat amended with 3% biochar.

Within the first three weeks of plant growth, a reduction in the relative abundance of especially the *Flavobacteriaceae*, *Sphingomonadaceae* and *Microbacteriaceae* and an increase in relative abundance of the *Rhizobiales incertae sedis* were noticed. Between weeks 3 and 6, a reduction in the relative abundance of *Chitinophagaceae* was mainly observed, especially in the non-amended peat. The period from week 6 onwards, was characterized by a significant increase in relative abundance of the *Acidobacteriaceae* for plants grown in peat and the peat/biochar mixture and of the *Acetobacteriaceae* and *Rhodospirillaceae* solely in the biochar-amended peat (Figure 3.2).

In contrast with the bacteria, the main changes in fungal community composition occurred during the first week of plant growth, both for the plants grown in peat and in biochar-amended peat (Figure 3.1C and Figure 3.1D). This was characterized by a prominent decrease of the *Amphisphaeriaceae* and an increase of the *Morteriellaceae* and *Lasiosphaeriaceae* (Figure 3.3). From week 3 onwards, no important shifts occurred in the fungal community, with the exception of *Auriculariales Incertae sedis*, which increased significantly between weeks 3 and 9, and then decreases again at the end of the strawberry growth period in week 13 (Figure 3.3). In total, 38 fungal families of the strawberry rhizosphere altered in relative abundance between at least two successive time points for plants grown in peat or biochar amended peat. In total, 10 of these families represented at least 0.1 % of the total fungal community and are represented in Figure 3.3.

Third**, the effect of biochar was studied per sampling time point.** An effect of biochar was only observed for the bacterial rhizosphere. This effect was further studied for each time point, using the OTU table clustered on genus level. The bacterial genera that changed significantly in relative abundance ($p<0.05$) within each time point as a response to the addition of biochar are listed in Table 3.2. Three groups could be discriminated, representing different effects of biochar on three time frames. The first group (group A), consisted of genera that changed in relative abundance within the first 6 to 9 weeks of plant growth as a response to the biochar supplemented to the peat. Group B contained genera that changed in relative abundance from week 6 of plant growth until the end of the experiment. Finally, group C contains bacterial genera that change in relative abundance from week 9 to 13 of plant growth.

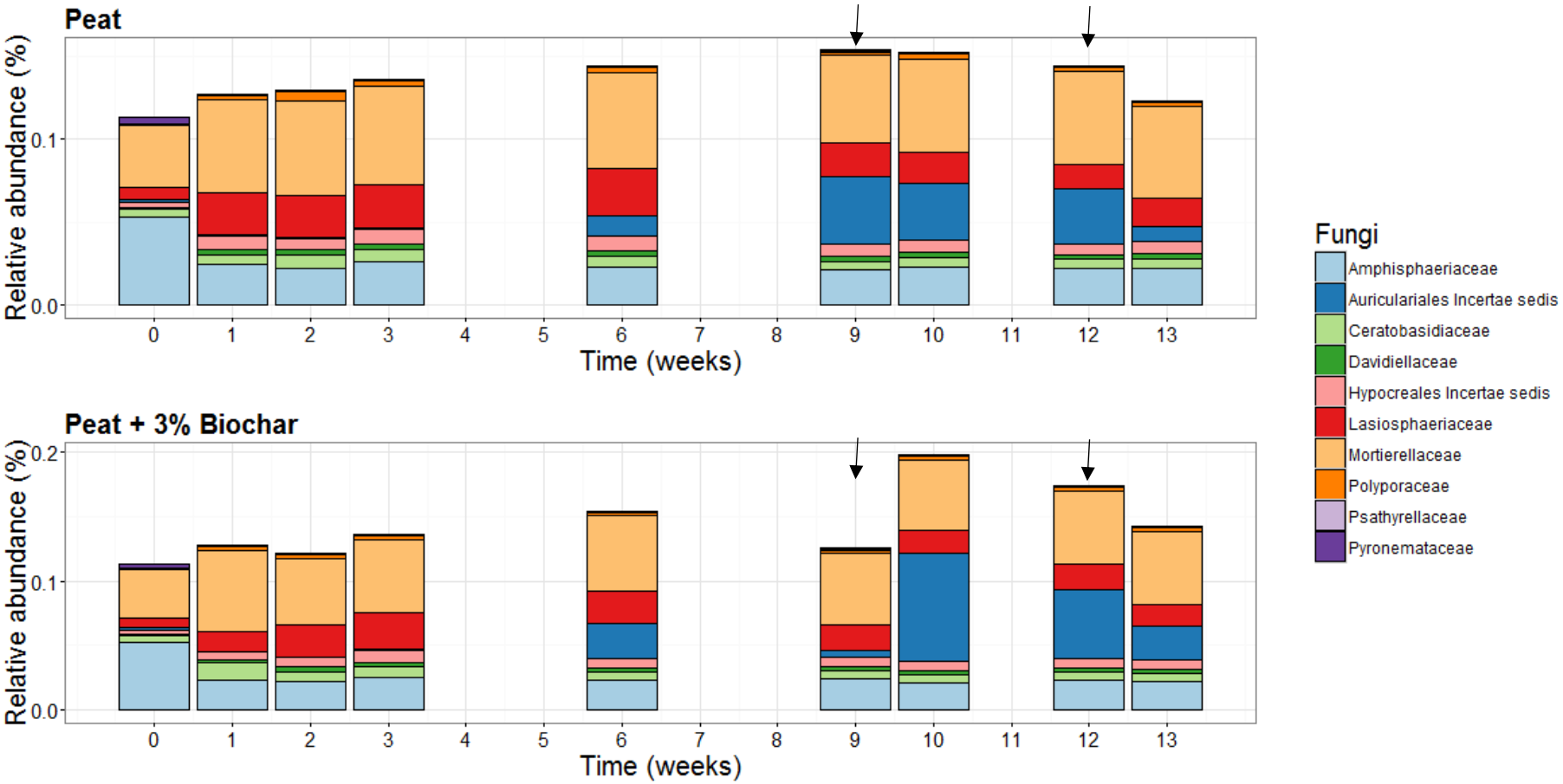


Figure 3.3 Representation of the fungal families that change significantly in relative abundance (%) over time. The mean relative abundance (n=3) of the fungal families that change significantly over time between at least two successive time points are represented. Only those families that contribute for at least 0.1% of the total community for at least one time point are represented in the graph. Time points where no sampling was done are visualized as an empty space. Time points of infection with *B. cinerea* are indicated using arrows Upper figure: The rhizosphere of strawberry grown in peat. Bottom figure: The rhizosphere of strawberry grown in peat amended with 3% biochar.

Table 3.2 Bacterial genera showing significant differences in relative abundance (%) according to presence or absence of biochar. Bacterial genera showing significant differences according to the presence of biochar are listed. Only those genera significantly different in relative abundance between the biochar and non-biochar treated peat for at least two time points are listed. Genera that decrease in relative abundance in the rhizosphere of biochar-treated peat compared to the control (non-biochar) are indicated with a "-", those that increase in relative abundance are indicated with a "+". The total number of bacterial genera that increased or decreased in relative abundance are indicated at the bottom of the table. Genera that were also identified in strawberry 'unfertilized peat' (underlined) and 'fertilized peat' (bold) experiments are indicated.

	GENUS	t1	t2	t3	t6	t9	t10	t12	t13
	Achromobacter	-	-	-	-				
	Dyella	-	-	-					
	Granulicella	-		-					
	Bordetella		-	-					
	Asticcacaulis		+		-				
A	*Aquincola*		+		+				
	Byssovorax		+		+				
	Gemmata		+			+			
	Acidobacterium	-	-						
	Acidicapsa	-		-	-	-			
	Telmatobacter	-		-	-				
	Burkholderia	-		-	-				
	Sporocytophaga			-	-				
	Chthonomonas			+		-			
	Telmatospirillum			-		-	-		
	Acidocella	-			-	-	-	-	-
	Aeromicrobium	+			+	+		+	+
	Mesorhizobium	+			+	+		+	+
	Nitratireductor	+			+	+		+	+
	Bradyrhizobium				+	+	+	+	+
	Rhodoplanes				+	+	+	+	+
	Alkanibacter				+	+	+	+	+
B	*Streptomyces*				+	+		+	+
	Gemmatimonas				+	+		+	+
	Planctomyces				+	+		+	+
	<u>*Marmoricola*</u>	+	+		+	+		+	
	Rickettsia				+	+	+	+	
	Parvibaculum				+	+		+	
	<u>Devosia</u>	+			+	+			
	Verrucomicrobium	+			-	-			
	Holophaga				+	+			
	Woodsholea				+	+			
	Arthrobacter				+			+	
	Steroidobacter				+			+	
	Massilia				-				+
	Pseudomonas				+				+
	Pseudoxanthomonas				+		-		
	Nocardioides	+				+		+	+
	Jatrophihabitans					+	+	+	+
	Crossiella					+	+	+	+
	Pseudolabrys					+	+	+	+
	Novosphingobium					-		-	
C	*Nitrosospira*					+		+	
	Spirochaeta					+		+	
	Bryocella					+	+		
	Ferruginibacter							+	+
	Taibaiella						+	+	+
	Dongia							+	+
	Sediminibacterium						+	+	
	Total +	**7**	**5**	**1**	**21**	**23**	**10**	**24**	**18**
	Total -	**8**	**4**	**9**	**9**	**6**	**3**	**2**	**1**

The major changes in the bacterial community of the rhizosphere due to the addition of biochar were observed from week 6 onwards, however (groups B and C). In general, these were mainly increases in relative abundances noticed for those genera that differed significantly between the biochar and non-biochar treatment at the specified time point. In contrast, in group A we observed decreases in relative abundance due to the addition of biochar (Table 3.2).

Fourth**, the bacterial and fungal richness was studied** based on the number of observed OTUs**.** The bacterial and fungal community richness of the strawberry rhizosphere was estimated over 13 weeks of plant growth (Figure 3.4). After planting the cold-stored bare-root strawberry plants, a significant increase in bacterial richness is seen at time points 0, 1 and 2 ($p<0.05$), for plants grown in peat and in peat amended with biochar. Subsequently, the bacterial richness continued increasing till the end of the strawberry growing period (week 13), although not significantly (Figure 3.4A). The number of fungal OTUs in the strawberry rhizosphere increased significantly at week 0 and 1 of plant growth ($p<<0.01$), but stabilized thereafter, independent whether biochar had been added to the peat (Figure 3.4B).

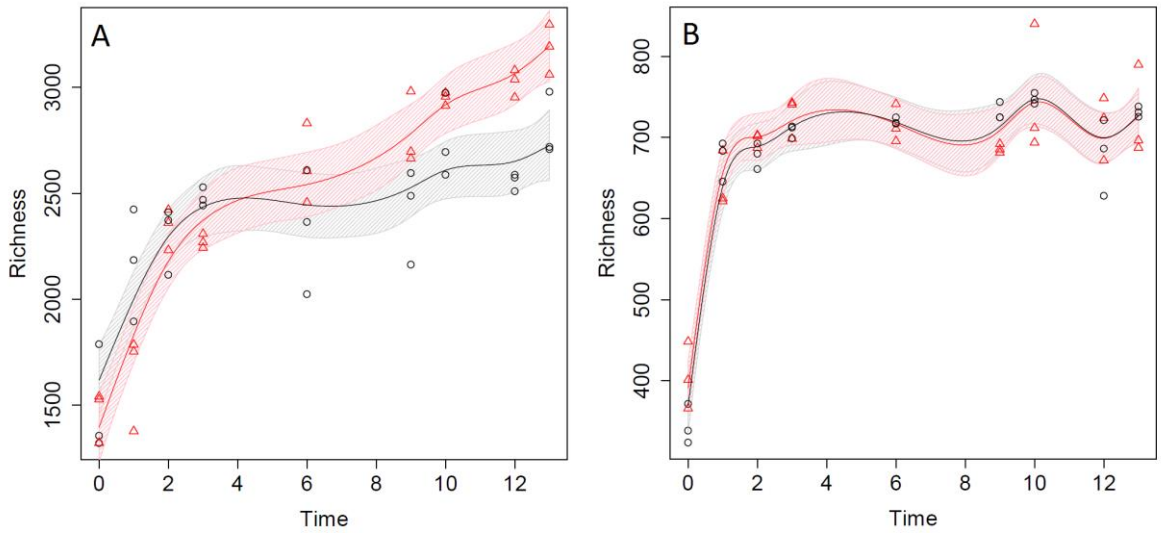


Figure 3.4 Richness of the microbial community in the strawberry rhizosphere measured over 13 weeks of plant growth. The richness derived from peat and biochar-amended samples are indicated in black and red, respectively. The observed rarefied richness's of the biological replicates are depicted with dots. The lines represent the fitted average richness using an additive model with thin plate regression smoothers. The shaded areas are simultaneous 95% confidence bands that are estimated on a grid spanned by the observed time points (t=0,1,2,3,6,9,10,12,13 weeks). **(A)** Bacterial community richness of the strawberry rhizosphere. **(B)** Fungal community richness of the strawberry rhizosphere.

The average bacterial richness of the strawberry rhizosphere was significantly larger in the biochar amended peat than in the non-amended peat from week 9 of plant growth onwards ($p<0.01$). Before week 9 the effect of biochar addition was not significant. In contrast, no significant effects of the addition of biochar were observed for the fungal rhizosphere richness ($p>0.65$).

3.2.4 *B. cinerea* leaf inoculation experiment

In this experiment, the effect of an aboveground infection (plant leaf) on the bacterial rhizosphere was studied. In addition, the effect of biochar addition to the peat and the interaction with the aboveground infection was studied. Rhizosphere samples were taken only at the end of plant growth (13 weeks of growth), one week after the inoculation with *B. cinerea*. Only the effects on the bacterial community were observed, as the time course experiment showed that the number and composition of fungal OTUs did not change from week three onwards and were not affected by the addition of biochar.

First**, the composition of the bacterial community** was studied**.** The main effects of biochar addition and inoculation of the strawberry leaves with *B. cinerea* on the bacterial community composition of the strawberry rhizosphere and the interaction between both were studied using PERMANOVA analysis. This could be done because the condition of homogeneity of variances was fulfilled. The addition of biochar had a significant effect on the bacterial community ($p<0.001$), but there was no general effect of infection and no interaction effect was revealed. This is illustrated with a PCoA plot, in which the first axis seems to corresponds to the variability in the community composition due to the addition of biochar and the second axis to the percentage of variability due to infection (Figure 3.5).

Subsequently, four individual comparisons were made, studying: (1) the effect of biochar on the rhizosphere bacterial community of non-inoculated plants, (2) the effect of biochar on the rhizosphere bacterial community of inoculated plants, (3) the effect of *B. cinerea* inoculation on the rhizosphere bacterial community of strawberry plants grown in peat and (4) the effect of *B. cinerea* inoculation on the rhizosphere bacterial community of strawberry plants grown in biochar-amended peat. For these analyses, the OTU table was clustered on genus level. For non-infected plants, the 13 weeks of growth in substrate with biochar had a significant influence on the relative bacterial abundances. In total, 38 bacterial genera increased and 25 bacterial genera decreased in relative abundance when compared to the rhizosphere

microbiome of the plants grown in the unamended peat. The effect of biochar was less prominent when plants were infected: 12 bacterial genera increased and 17 decreased in relative abundance when compared to the rhizosphere microbiomes of the infected plants grown in the unamended peat.

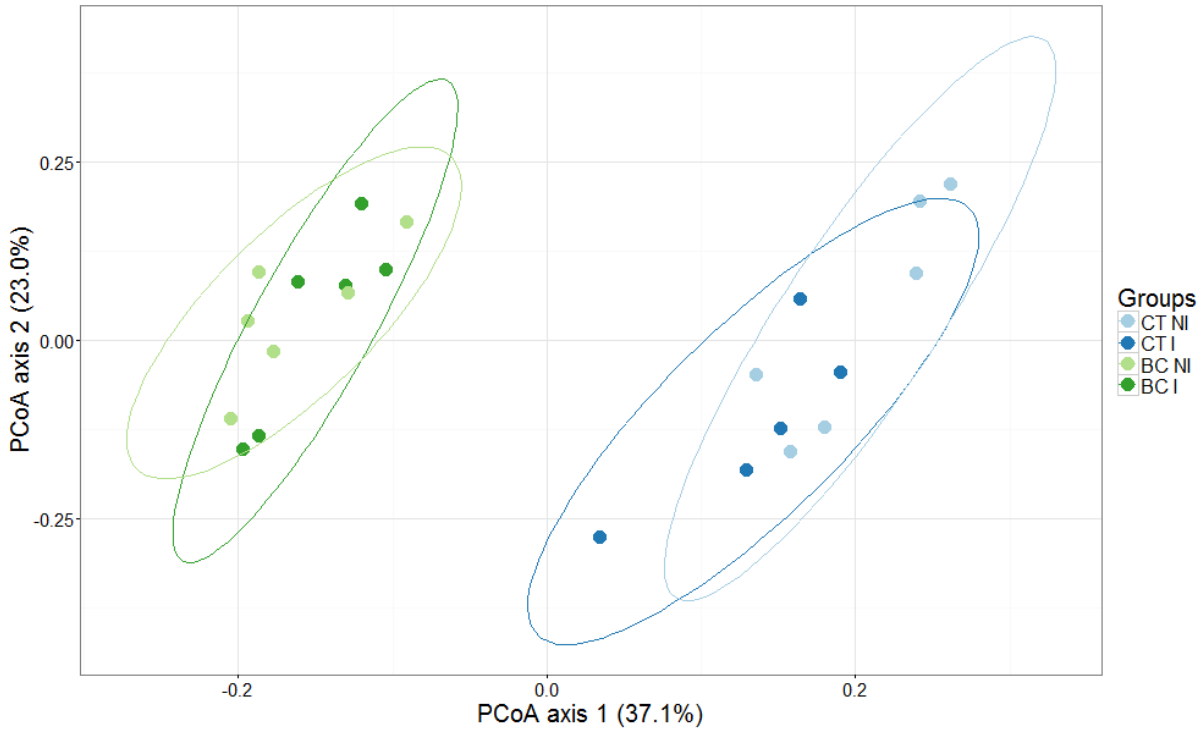


Figure 3.5 Principal Coordinate Analysis (PCoA) profile of pairwise community dissimilarity (Bray-Curtis) indices of 16S V3-V4 sequencing data of the strawberry rhizosphere grown in biochar-amended (green) and unamended (blue) peat. Ellipses represent the 95% confidence intervals. Half of the plants were infected (I) with *B. cinerea* (dark coloured), the other half were not (NI) (light coloured). The first and second axes represent 37.1% and 23.0% of the variance in the dataset, respectively. A clear separation is seen in the first axis, representing the major amount of variance in the dataset due to the biochar (BC) addition. Microbiome sequences of plants grown in non-biochar treated peat are indicated as control (CT).

Second, the effect of infection on the bacterial community composition was studied. In total, 31 bacterial genera increased and three genera decreased significantly in relative abundance in the strawberry-peat bio-assay due to inoculation of the leaves with *B. cinerea.* The aboveground infection did not alter the bacterial community of the rhizosphere of strawberry grown in biochar amended peat (Table 3.3).

Table 3.3 Significant differences in the relative abundance of bacterial genera (%) ± standard error between strawberry rhizospheres in peat with and without infection of *B. cinerea* on the strawberry leaves (n = 6). Genera followed by an asterisk indicate a significant increase or decrease in the relative abundance in the infected samples as compared to the non-infection samples for the non-biochar treatment. As a comparison, the values of the biochar treated samples are included in the table in grey, in which no significant differences were observed. The total number of OTUs classified as the genus respectively are indicated in the column N°.

Phylum	Family	Genus	N°	Peat - NI	Peat - I		Peat + BC - NI	Peat + BC - I
Acidobacteria	*Unknown Family*	*Bryobacter*	20	2.13±0.23	1.09±0.09	*	0.64±0.04	0.71±0.04
	Acidobacteriaceae	*Edaphobacter*	2	0.50±0.04	0.29±0.04	*	0.28±0.02	0.31±0.02
Actinobacteria	*Acidothermaceae*	*Acidothermus*	4	0.33±0.04	0.84±0.14	*	0.77±0.09	0.92±0.09
	Cellulomonadaceae	*Cellulomonas*	1	<0.01	0.06±0.06	*	<0.01	0.01±0.00
	Conexibacteraceae	*Conexibacter*	3	0.13±0.02	0.38±0.05	*	0.23±0.02	0.30±0.03
	Frankiaceae	*Jatrophihabitans*	3	0.06±0.01	0.14±0.06	*	0.34±0.03	0.39±0.04
	Iamiaceae	*Iamia*	4	<0.01	0.02±0.01	*	0.01±0.01	0.01±0.00
	Intrasporangiaceae	*Phycicoccus*	1	<0.01	0.04±0.01	*	0.02±0.01	0.08±0.05
	Micromonosporaceae	*Actinoplanes*	1	0.00±0.00	0.01±0.01	*	<0.01	0.03±0.03
	Mycobacteriaceae	*Mycobacterium*	2	0.06±0.01	0.26±0.02	*	0.30±0.03	0.30±0.05
	Nocardiaceae	*Nocardia*	2	0.03±0.01	0.08±0.02	*	0.06±0.01	0.08±0.01
		Williamsia	1	<0.01	0.08±0.07	*	<0.01	<0.01
	Nocardioidaceae	*Aeromicrobium*	1	<0.01±<0.0	0.02±0.01	*	0.01±0.00	0.03±0.01
		Marmoricola	2	<0.01	0.03±0.01	*	0.02±0.01	0.04±0.01
		Nocardioides	10	0.05±0.01	0.19±0.05	*	0.36±0.03	0.45±0.06
	Patulibacteraceae	*Patulibacter*	7	<0.01	0.02±0.01	*	<0.01	0.01±0.00
	Pseudonocardiaceae	*Pseudonocardia*	1	0.01±0.00	0.03±0.01	*	0.03±0.00	0.03±0.00
	Solirubrobacteraceae	*Solirubrobacter*	1	<0.01	0.02±0.01	*	0.02±0.00	0.02±0.00
	Streptomycetaceae	*Streptomyces*	3	0.01±0.00	0.05±0.05	*	0.08±0.02	0.11±0.04
Armatimonadetes	*Chthonomonadaceae*	*Chthonomonas*	3	1.31±0.34	0.38±0.17	*	0.45±0.06	0.46±0.07
Bacteroidetes		*Flavobacterium*	12	0.02±0.01	0.16±0.10	*	0.01±0.00	0.01±0.00
	Sphingobacteriaceae	*Pedobacter*	6	0.01±0.01	0.10±0.05	*	<0.01	0.01±0.00
Planctomycetes	*Planctomycetaceae*	*Gemmata*	14	0.25±0.02	0.47±0.10	*	0.50±0.04	0.50±0.02
		Pirellula	8	0.00±0.00	0.01±0.01	*	0.01±0.00	0.01±0.01
		Planctomyces	16	0.02±0.00	0.04±0.00	*	0.11±0.01	0.10±0.01
		Singulisphaera	11	0.03±0.00	0.06±0.01	*	0.07±0.00	0.07±0.00
Proteobacteria	*Bradyrhizobiaceae*	*Rhodopseudomonas*	1	0.01±0.00	0.02±0.02	*	0.05±0.01	0.05±0.00
	Hyphomicrobiaceae	*Devosia*	2	0.44±0.07	0.78±0.18	*	0.96±0.05	1.12±0.08
	Hyphomonadaceae	*Hirschia*	1	0.01±0.01	0.09±0.04	*	0.02±0.01	0.02±0.00
		Woodsholea	4	<0.01±0.00	0.02±0.02	*	0.04±0.01	0.03±0.01
	Phyllobacteriaceae	*Mesorhizobium*	1	0.01±0.01	0.04±0.01	*	0.04±0.01	0.05±0.02
		Nitratireductor	1	0.01±0.001	0.04±0.01	*	0.05±0.01	0.06±0.00
	Rhizobiaceae	*Shinella*	1	0.02±0.01	0.07±0.05	*	0.06±0.01	0.07±0.02
	Rhodospirillaceae	*Defluviicoccus*	3	<0.01	0.01±0.01	*	<0.01	<0.01

Second**, the effect of biochar addition and aboveground infection on the bacterial richness** was studied**.** The number of OTUs was estimated for each of the four groups (peat NI, peat I, peat+BC NI and peat+BC I). For strawberry plants grown in peat, the average bacterial richness of the rhizosphere was 1327.5±9 OTUs and 1509.3±73.8 OTUs for the non-infected and the *B. cinerea* infected plants, respectively. For strawberry plants grown in biochar-amended peat, the average bacterial richness was 1698.1±32.1 OTUs and 1654.2±38.9 OTUs for the non-infected and the *B. cinerea* infected plants respectively. In general, two significant effects were measured. First, infection of the plants induced a significant increase in bacterial richness

($p=0.039$) in the rhizosphere when the plants were grown in unamended peat. Second, biochar amended to the peat substrate also induced a significant increase in bacterial richness ($p<0.01$) in the rhizosphere, and this condition was not further influenced by the infection of the plants.

3.3 DISCUSSION AND CONCLUSIONS

In previous studies, the effect of biochar on the microbial community of the rhizosphere was analyzed on a single sampling date in pot trials (Chapter 2; Egamberdieva et al., 2016, Kolton et al., 2011). In the present study we have evaluated the temporal dynamics of bacterial and fungal communities of the strawberry rhizosphere by sampling at nine time points during the 13-week strawberry growing period. Two factors were evaluated: (1) the change in the rhizosphere microbiome community over time, and (2) the effect of biochar addition to peat on the bacterial and fungal community of the strawberry rhizosphere. First, we showed that the bacterial and fungal community changed in composition and richness over time. The fungal community composition changed mainly during the first week of plant growth and stabilized thereafter, with the exception of the *Auriculariales*. In contrast, the composition of the bacterial community changed especially during the first six weeks of plant growth. We believe that the major shift in bacterial and fungal community composition in the rhizosphere, that occurs in the first week of plant growth, is mediated by a change in growing medium. At the beginning of the experiment, the growing medium of the strawberry plants changed from field soil (nursery fields) to peat, which is common practice in Belgian strawberry cultivation (Lieten et al., 1995; Lieten, 2013). One of the main drivers of the rhizosphere microbiome is soil type (Berg and Smalla, 2009). Therefore, changes brought in this bulk soil will eventually affect the rhizosphere microbial community (Mendes et al., 2014). During the experiment, no significant changes in chemical parameters of the peat were observed over time. This indicates that the shift in bacterial community profile of the rhizosphere measured from week 1-6 was not mediated by a shift in chemical composition of the growing medium. Within the experiment, plants were either in a vegetative phase (week 1 to 3) or in a flowering and fruiting stage (from week 4 onwards). It has already been shown that the rhizosphere composition could be different between plant stages and that this could be related to the excretion of chemical compounds through the roots (Chaparro et al., 2014). We therefore hypothesize that the observed changes in the first weeks of plant growth were plant-driven, as plants can influence the rhizosphere by the release of rhizodeposits from the roots (Philippot et al., 2013). An alternative hypothesis could be that the rhizosphere microbiome is influenced by an interplay between the microorganisms of the plant root itself (Dennis et al., 2010).

Second, the effect of biochar on the bacterial and fungal community of the rhizosphere was evaluated over time. During the strawberry growth period, no effect on the fungal rhizosphere

community was observed due to the addition of biochar which is in accordance to the results of Chapter 2. In contrast, starting at week nine of plant growth onwards, increased richness was observed in the biochar-treated bacterial rhizosphere community as compared to the non-biochar treated rhizosphere. Additionally, a higher number of genera were induced from week six onwards. Some of these genera contain species previously reported to be involved in the N-cycle, e.g. *Nitratireductor*, *Devosia*, *Nitrospira* and *Taibaiella* (Hoque et al., 2011; Kox and Jetten, 2015; Penton et al., 2013; Rivas et al., 2002; Zhang et al., 2013) and some contain species that can act as biocontrol agent, e.g. *Streptomyces* and *Nocardioides* (Carrer et al., 2008; Saharan and Nehra, 2011; Viaene et al., 2016). These so-called plant-growth promoting rhizobacteria (PGPR) have been extensively studied because of their beneficial effects on plant growth and health (Berendsen et al., 2012). This is in accordance with the results of Chapter 2 in which we studied the rhizosphere community at the end of the strawberry growth period in peat and also found PGPR linked genera which increased by biochar addition to peat. Based on the results of the current Chapter, we believe that the effect of biochar on the rhizosphere bacterial community is postponed, as it is measurable from week six of strawberry growth. This is in contrast to the study of Kolton *et al.* (2016), who measured the biggest effect of biochar on the bacterial community the first six weeks of tomato growth. This could however be due to the difference in growing medium (dune sand versus peat).

Within the time course experiment and the inoculation experiment, the effect of biochar on some plant properties after thirteen weeks of plant growth was evaluated. The addition of biochar to fertilized and limed peat resulted in an increase in plant root development and strawberry fruit production. We suggest the following explanations: (1) biochar could have a nutrient-addition effect, i.e. could act as a fertilizer in the growing medium. In previous experiments biochar addition to unfertilized peat resulted in an increase in plant growth (De Tender et al., 2016). These effects however were less obvious once the peat was fertilized, indicating a role of fertilization on the effect of biochar on strawberry growth (Chapter 2). Those experiments also revealed that incorporating biochar to peat resulted in an increase in potassium (K) concentration of 20.65 mg L^{-1} peat to 36.15 mg L^{-1} in unfertilized peat and 93.40 mg L^{-1} to 108.0 mg L^{-1} in fertilized peat. Strawberry has high nutrient demands, especially for K (Tagliavini et al., 2005). A potential higher K concentration in the growing medium may have resulted in the higher production of strawberry fruits, as K has an effect on fruit quality and

quantity, and roots in the biochar treatment (Ebrahimi et al., 2012). To prove this concept however, more research is necessary in which the concentration of K and other nutrients should be measured in the strawberry plant and fruits. (2) The effect of biochar on the plant properties could be indirect through a change in the rhizosphere microbiome. The increase in PGPR bacteria from week six onwards, could have resulted in the higher production of strawberry fruits and roots in the biochar treatment. (3) Biochar could have a direct or indirect (through its effect on the rhizosphere microbiome) response on the auxin pathway of the plant. It has been shown that biochar application can induce auxin-related genes (Viger et al., 2014). Auxin is known to be connected with the plant root development, with a major role in the production of lateral roots and root hairs (Overvoorde et al., 2010). Subsequently, it is known that bacteria interacting with the plant, i.e. rhizosphere associated organisms, can produce auxin and interfere with the auxin-regulated plant developmental processes (Spaepen and Vanderleyden, 2011). Therefore, the increase in lateral root development of the plants grown in biochar-amended peat could be auxin-related.

Biochar also increased the post-harvest resistance of the strawberry fruits against *B. cinerea*. Previously, it has been shown that biochar increased disease resistance of (1) strawberry grown in peat against *B. cinerea*, *Colletotrichum acutatum* and *Podosphaera apahanis* leaf infections (Meller-Harel et al. 2012) and (2) field-grown pepper and tomato plants against to *B. cinerea* and *Leveillula taurica* leaf infections (Elad et al., 2010). Moreover, induced resistance against soilborne pathogens, including nematodes, by biochar has been reported more recently (Jaiswal et al., 2015; Huang et al., 2015; George et al., 2016). Following hypotheses are made for the mechanisms involved: (1) A nutrient addition effect of biochar (see above), as the susceptibility of plants to diseases is also known to be influenced by its nutritional status (Nam et al., 2006; Xu et al., 2013; Lecompte et al., 2010). Therefore, in further research the plant tissue nutrient content should be analyzed in the biochar-amended plants as compared to the control plants, (2) The change in the rhizosphere microbiome from week six onwards due to biochar towards bacteria involved in biological control. It is known that the susceptibility of plants to diseases is influenced by its rhizosphere microbiome (e.g., Berendsen et al., 2012), (3) Biochar could have directly or indirectly (through its effect of the rhizosphere microbiome) changed the metabolite composition of the strawberry fruits. It is shown that biochar addition to substrate alters the secondary metabolite composition of tomato fruits (Petrucelli et al., 2015). Furthermore, antioxidant and fatty acid composition of

fruits has been shown to be related to the disease resistance of fruits (Cao et al., 2014). Therefore, biochar addition to the peat, could result in a change in strawberry fruit composition, which could make them less susceptible for *B. cinerea* infection. This effect of biochar on the overall composition of the strawberry fruits will be tested in future experiments, based on the protocol described in Palencia *et al*. (2016).

We established an experiment to evaluate the effect of *B. cinerea* on the strawberry rhizosphere microbiome after leaf inoculation on plants grown in peat with and without incorporation of biochar. First we observed an increase in bacterial richness when strawberry plants grown in peat were infected with *B. cinerea*; this was not observed in plants grown in peat amended with biochar. The microbial species richness of the rhizosphere has been proposed as a predictor of the aboveground plant diversity and productivity (van der Heijden et al., 2008; Lau and Lennen, 2011; Wagg et al., 2011). A higher belowground richness and diversity could even act as an insurance for maintaining plant productivity, even under changing environmental conditions (Wagg et al., 2011). Therefore, the increase in bacterial richness could be a reaction of the plant on the aboveground infection, to maintain its productivity or counteract the pathogen. Second, the relative abundance of 34 bacterial genera in the strawberry rhizosphere was influenced by the aboveground infection in the non-biochar treatment, but was not seen in the biochar-amended peat. It is known that five factors can influence the rhizosphere microbial composition: soil type, plant genotype, addition of fertilizers, crop rotation and application of pesticides (Massart et al., 2015). Rosberg et al. (2014) already showed that inoculation of tomato with the root pathogen *Pythium* results in changes in the rhizosphere community. Nonetheless, to our knowledge the present study is the first report to reveal that an aboveground fungal infection can change the rhizosphere community composition. Additionally, this effect seemed to be neutralized once biochar is added to the peat. A comparison of the relative abundances of the bacterial genera that were significantly changed in the peat-assay compared to biochar-amended peat, reveals that after infection the relative abundance of the bacterial genera came near the levels observed in the rhizosphere of the biochar-amended peat for the same genera. This indicates that biochar already "prepared" the rhizosphere's community to the infection in three ways: (1) by increasing the richness of the bacterial community, (2) by shifting towards a higher relative abundance of genera including species acting as biocontrol agent or involved in the N-cycle

and (3) by shifting the relative abundance of the bacterial genera in the rhizosphere towards those which we obtain after infection of the plant. However, these rhizosphere microbiome effects were not accompanied with an increased resistance of the leaves against *B. cinerea*. This may be attributed to the low disease severity observed on the leaves (about 15% of the leaf area was affected in the control treatment). In Chapter 2, we showed that biochar amendment in unfertilized and non-limed peat increased the resistance of leaves against *B. cinerea*, but in that experiment a disease severity of 50% was observed in the control treatment. Using more conducive conditions for *B. cinerea* on the leaves in the present study may have shown a difference between both treatments, but further research is necessary to confirm this.

In conclusion, this chapter indicates that upon both biochar incorporation in peat and above ground pathogen attack, plants recruit rhizosphere microbes that may help them in their defence and plant growth promotion. These findings are important for a sustainable strawberry production worldwide.

Table AI3.1: **Chemical properties of peat and peat amended with 3% biochar during a 13 weeks interval.** Values are averages±standard errors for 3 replicates

	pH-H_2O		EC (µS/cm)		NO_3-N (mg/L peat)		NH_4-N (mg/L peat)		SO_4 (mg/L peat)		Cl (mg/L peat)		P in H_2O (mg/L peat)	
	Peat (a)	peat+BC (b)	peat	Peat+BC	peat	Peat+BC	Peat (a)	Peat+BC (b)	peat	Peat+BC	peat	Peat+BC	peat	Peat+BC
Optimal range	**3.8 – 6.0**		**200 - 400**		**Not applicable**		**Not applicable**		**0-100**		**0-100**		**Not applicable**	
T1	4.41±0.06	4.73±0.07	76.33±8.83	68.67±5.33	6.50±1.21	6.60±0.90	10.83±1.85	6.73±1.14	36.17±9.73	38.40±5.12	10.00±0.00	10.23±0.12	5.93±1.04	5.43±0.68
T2	4.83±0.32	4.79±0.04	91.00±27.05	70.00±8.62	9.70±3.60	6.90±0.71	16.43±7.38	5.03±0.03	56.63±30.41	30.60±0.61	10.37±0.37	10.77±0.39	8.63±3.93	4.80±0.10
T3	4.73±0.09	5.04±0.20	37.00±10.00	55.00±9.64	5.00±0.00	7.67±2.67	5.87±0.87	5.07±0.07	12.50±0.80	24.00±3.98	10.70±0.70	10.00±0.00	4.70±0.00	5.10±0.40
T6	4.35±0.05	4.90±0.03	86.33±16.68	46.67±3.17	8.97±2.18	5.93±0.66	6.97±1.97	5.00±0.00	49.93±15.86	23.17±4.4	11.40±0.84	10.00±0.00	8.50±2.06	4.70±0.00
T9	4.28±0.10	4.61±0.06	83.00±43.25	50.33±6.96	13.57±8.57	5.00±0.00	9.73±4.73	5.00±0.00	50.73±32.09	35.03±9.09	13.23±3.23	10.57±0.43	10.10±5.40	4.70±0.00
T10	4.41±0.05	5.18±0.58	80.67±7.86	59.00±14.01	6.17±1.17	7.13±2.13	6.37±1.37	5.00±0.00	45.07±12.88	34.37±8.94	18.43±6.74	13.03±2.84	6.00±1.30	5.47±0.77
T12	4.36±0.02	4.72±0.04	61.67±8.84	49.00±4.51	5.47±0.37	5.00±0.00	5.60±0.60	5.00±0.00	31.50±7.04	32.70±5.24	14.83±3.36	10.10±0.10	4.83±0.13	4.70±0.00
T13	4.36±0.02	4.75±0.09	51.68±2.91	51.67±7.36	5.00±0.00	5.23±0.27	5.60±0.00	5.00±0.00	26.63±3.70	39.47±7.91	10.00±0.00	11.03±0.61	4.70±0.00	4.70±0.00

Table AI3.2: **Chemical and physical properties of peat at the end of the experiment (week 13)** (DM: dry matter, EC: electrical conductivity). Values are averages ± standard deviations for 10 replicates.

	pH-H_2O	EC (µS/cm)	SO_4 in H_2O (mg/L peat)	Cl in H_2O (mg/L peat)	P in H_2O (mg/L peat)	Water availability (ml)	Water in plant (ml)	NO_3-N (mg/L peat)	NH_4-N (mg/L peat)
Inoculation experiment									
0% biochar + NI	4.31±0.04 (a)	76.00±17.90 (a)	47.24±14.45 (a)	10.66±0.66 (a)	8.15±3.38 (a)			8.24±2.49 (a)	6.61±1.61 (a)
0% biochar + I	4.55±0.20 (ab)	76.27±12.55 (a)	42.98±9.69 (a)	14.93±4.90 (a)	5.82±0.78 (a)			6.45±0.87 (a)	5.47±0.35 (a)
3% biochar + NI	4.86±0.05 (b)	71.73±6.88 (a)	58.09±6.15 (a)	12.15±0.72 (a)	6.30±0.69 (a)			8.14±1.67 (a)	5.12±0.08 (a)
3% biochar + I	4.84±0.05 (b)	59.58±7.28 (a)	47.07±6.98 (a)	10.93±0.43 (a)	5.48±0.81 (a)			7.51±1.67 (a)	5.00±0.00 (a)

Values within the same columns, following the same letter are not significantly different ($p < 0.05$)

Chapter 4

The lettuce rhizosphere microbiome in response to chitin and in relation to human health

Parts of this study are published in:

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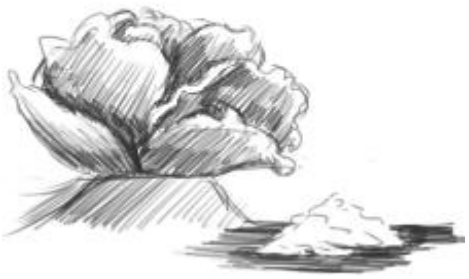


For this chapter, following analysis were perfomed by the author of this PhD dissertation:

Statistical analysis of the PLFA data
All analysis covering the amplicon sequencing data
All analysis covering the whole genome shotgun sequencing data

Utilization of organic amendments such as chitin is one of the most economical and practical options for improving soil and substrate quality, plant growth and plant resilience (De Boer et al., 1999; El Hadrami et al., 2010; Sharp, 2013). Soil treatment with chitin has been shown to decrease the infection rate of plant roots by nematodes (Sarathchandra et al., 1996; Radwan et al., 2012) and to increase disease suppressiveness against the fungal soil-borne pathogens *Verticillium dahliae* and *Rhizoctonia solani* (Cretoiu et al., 2013; Postma and Schilder, 2015). The mechanism behind this suppressiveness is often related to a change in the soil and rhizosphere microbiome (Cretoiu et al., 2013). Micro-organisms capable of hydrolyzing the chitinous cell wall of pathogenic fungi and nematodes eggs will increase in numbers and/or activities in response to the chitin amendment. In addition, also secondary responders to the added chitin confer overall pathogen suppression (El Hadrami et al., 2010). Next to a direct effect on pathogens, changes in this rhizosphere microbiology may also affect the plant physiology and its capacity to be colonised by micro-organisms, including plant and human pathogens (El Hadrami et al., 2010; Gu et al., 2013; Markland et al., 2015). Rhizosphere organisms well studied for their beneficial effects on plant growth and health, can be increased by the utilization of chitin amendment in order to enhance plant growth and resilience to plant pathogens (Dutta & Isaac, 1979; Hallman et al., 1999). In addition, chitin has also been shown to trigger plant immunity and acts as a pathogen-associated-molecular pattern (PAMP), triggering the plant defense against chitin-containing harmful organisms (de Jonge et al., 2010; Sharp, 2013). To date, no research has been done to investigate the indirect effect of chitin soil amendment on zoonotic bacterial human pathogens that can survive on fresh produce crops.

Authorities promote the consumption of fresh fruit and vegetables, but at the same time concerns have been raised about the food safety of leafy vegetables. Leafy vegetables, such as lettuce, are considered as high risk food, as various *Escherichia coli* O157:H7 and *Salmonella enterica* outbreaks have been related to the consumption of lettuce greenery that can carry these pathogens (Ward et al., 2002; Horby et al., 2003; Welinder-Olsson et al., 2004; Friesema et al., 2008; Nygård et al., 2008; Söderström et al., 2008). It is usually accepted that zoonotic bacterial pathogens enter the agricultural environment via animal faeces, which in turn may contaminate irrigation water and soil. Irrigation water is considered as the most likely key

route of dispersal of zoonotic pathogens from faeces to plants (Barak and Schroeder, 2012; Holvoet et al., 2015).

The biology of *E. coli* and *S. enterica* on lettuce leaves under various conditions has been extensively studied (e.g., Brandl and Amundson, 2008; Oliveira et al., 2012; Van der Linden et al., 2014). A recent study showed that butterhead lettuce grown in greenhouses with a sprinkle irrigation system may present a potential health hazard when the green parts are contaminated near harvest (Van der Linden et al., 2013). Reduction in the survival of zoonotic bacterial human pathogens in the preharvest environment can help prevent spread of pathogens during postharvest washing and packaging. A variety of direct control mechanisms such as disinfectants (including chlorine, hydrogen peroxide, organic acids and ozons) are being used to reduce this pre-harvest survival, but there is a need to preserve food by natural means (Oliveira et al., 2015). Hence, bacteria isolated from the rhizosphere and leaves of leafy greens have been shown to suppress human pathogens (e.g., Markland et al. 2015; Oliveira et al., 2015) and chitin derivates have been found to have antibacterial activity against zoonotic bacterial pathogens (e.g., Jeon et al., 2014). However, no studies have investigated the indirect effect of chitin addition to the growing medium on the survival on zoonotic bacterial pathogens on the leaves. Growing media that could reduce the carrier capacity of crops for these pathogens would be an interesting strategy for sustainable control.

The objectives of this chapter were twofold. First, we studied the effect of chitin mixed in potting soil on lettuce growth and on the capacity of these lettuce plants to carry two zoonotic bacterial pathogens, *E. coli* O157:H7 and *S. enterica* on their leaves. Second, changes in the microbial rhizosphere of lettuce were assessed. We hypothesize that the chitin favours chitin-degrading micro-organisms in the soil, among which important populations of plant growth promoting rhizobacteria (PGPR) and fungi (PGPF), and the stimulation of these groups in the lettuce rhizosphere could make the plant leaves less prone to colonization by the human pathogens. To assess this colonization, we used selective platings as described by Van der Linden *et al.* (2013). To assess the microbial rhizosphere dynamics, three techniques were used: phospholipid fatty acid (PLFA) analysis, 16S and ITS2 rRNA gene amplicon sequencing and shotgun metagenomics.

4.1 MATERIALS AND METHODS

Within this section, an overview of the materials and methods for the lettuce growth (4.1.1), the *E. coli* and *S. enterica* inoculation and detection assay (4.1.2) and rhizosphere microbiome community profiling (4.1.3 – 4.1.6) is provided. In the end, an overview of statistical methods is given (4.1.7).

4.1.1 Lettuce-peat bioassay

Chitin flakes purified from crab shell were obtained from BioLog Hepp Gmbh (lot: 90200705). An amount of 2% (2 g chitin / 100 g potting soil) was used in each experiment.

Pelletized butterhead lettuce seeds (*Lactuca sativa* L. var. *capitate* "Alexandria") obtained from Rijk Zwaan Distribution B.V. (De Lier, The Netherlands), were germinated on moist filter papers (Whatman filters 2) in petri dishes. The seedlings were either transplanted into a 100% peat based-potting soil or 2% chitin amended potting soil, both with a pH of 5.5-6.0 (Universal Substrate LP2B, Peltracom, Belgium). Per 1.5 L pot, one seedling was planted and placed in a growth chamber with conditions set at 19 °C during day and 12 °C at night, a relative humidity of 70-80 %, and a photoperiod of 14 h. After 55 days, five plants per treatment were sampled for PLFA analysis, five plants per treatment were sampled for high-throughput sequencing (HTS) and seven plants per treatment were inoculated with *S. enterica* sv. Thompson RM1987N or *E. coli* O157:H7 (see 3.1.2). At the end of the experiment (8 days after pathogen inoculation, see 3.1.2), the lettuce heads were harvested and weighed.

4.1.2 Bacterial strains, inoculation and detection on lettuce leaves

Two bacterial strains were used: *Salmonella enterica* sv. Thompson RM1987N and *Escherichia coli* O157:H7 MB3885 (Van der Linden et al., 2013). Both strains were streaked from a glycerol frozen stock maintained at -70 °C onto tryptone soya agar (TSA; Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 h. One colony was transferred to 10 mL of tryptone soya broth (Oxoid) and incubated at 37 °C for 18 h while shaken at 200 rpm. Cells of each strain were washed twice by centrifugation (6000xg, 15 min) in 50 mM phosphate buffered saline (PBS, pH 7.4). The optical density (OD) was measured at 595 nm using a microplate reader and concentrations were estimated based on an OD-colony-forming-unit (CFU) mL^{-1} standard curve. The appropriate amount of cells was resuspended in PBS to a concentration of $1x10^4$ CFU mL^{-1}.

The plants were inoculated at a concentration of 10^4 CFU mL^{-1} of PBS with a hand sprayer as described by Van der Linden *et al*. (2013). To count the pathogen concentrations on the lettuce leaves, individual leaves were placed in extraction bags with membrane filter (Bioreba) and weighed. PBS with 0.05% Tween 20 was added at a 1/1 (wt/vol) ratio and the samples were ground for ± 15 s at maximum speed (Homex 6, Bioreba) until a homogenous mixture was obtained. Tenfold dilutions of the resulting suspension were made in 0.1% peptone and 100 µL aliquots were spread-plated in duplicate on xylose lysine desoxycholate agar (Lab M, Bury, UK) overlaid with TSA for *S. enterica* and on cefixime–tellurite sorbitol Mac Conkey agar (Lab M, Bury, UK) overlaid with TSA for *E. coli* O157:H7 (Van der Linden et al., 2013). All plates were incubated at 37 °C for 24 h. Three randomly chosen plants from each treatment were sampled at 4 and 8 days after inoculation (dai), while one plant per treatment was sampled at day 0 (= immediately after inoculation). From each plant, three middle-aged leaves were collected in a single extraction bag and analysed for *E. coli* O157:H7 and *S. enterica* as described above. For mature lettuce, the 12^{th} to 14^{th} leaves in the head are considered as middle-aged. Leaf age is an important factor influencing the survival of both pathogens on the leaves. Middle-aged leaves were selected because Van der linden *et al.* (2013) found that the middle-aged leaves yielded the most consistent results for both pathogens, with the smallest standard deviations and smallest effect of environmental factors (which are difficult to control in the growth chamber). This was especially the case for *S. enterica*. The experiment was done twice for each pathogen. So, in total 6 leaves for 0 dai, 18 leaves for 4 dai and 18 leaves for 8 dai were analysed.

4.1.3 PLFA analysis

PLFA analysis was done on soil samples (approximately 50 g) taken from five pots per treatment from *S. enterica* inoculated plants and stored at -20 °C until they were freeze-dried. PLFA analysis was done as described in Chapter 2, section 2.1.7.

4.1.4 Rhizosphere sampling and DNA extraction

The lettuce rhizosphere was sampled according to Lundberg *et al.* (2012) of the same five pots for PLFA analysis (*S. enterica* inoculated plants). Loose soil was manually removed from the roots by kneading and shaking. We followed the established definition of rhizosphere soil as extending up to 1 mm from the root surface. Subsequently, roots with the remaining soil aggregates were placed in a sterile 50 mL tube containing 25 mL phosphate buffer. Tubes were

vortexed at maximum speed for 15 s, which released most of the rhizosphere soil from the roots and turned the water turbid. The turbid solution was then filtered through a 100 µm nylon mesh cell strainer to remove broken plant parts and large sediment. The turbid filtrate was centrifuged for 15 min at 3,200 g to form a pellet containing fine sediment and micro-organisms. Most of the supernatant was removed and the pellets were stored at -20 °C until DNA extraction. DNA was extracted from 250 mg of the pellet with the PowerSoil DNA isolation kit (Mo Bio, USA) according to the manufacturer's instructions. This DNA was used for bacterial 16S (V3-V4) and fungal ITS2 rRNA gene amplicon sequencing and shotgun metagenomics as described in sections 3.1.5 and 3.1.6 respectively.

4.1.5 Rhizosphere microbiology: amplicon sequencing

In total, five samples were used for amplicon sequencing. The bacterial and fungal rhizosphere populations were identified by sequencing the V3-V4 fragment of the 16S rRNA gene and the ITS2 gene fragment respectively, using Illumina technology (Illumina, San Diego, CA, USA). Using an amplification and dual-index PCR successively, fragments were amplified and extended with Illumina specific adaptors, which is described in detail in Chapter 2 section 2.1.8. Each PCR step was followed by a PCR product clean-up using the CleanPCR reagent kit (MAGBIO, Gaithersburg, MD, USA).

Final libraries were quality controlled using the Qiaxcel Advanced, with the Qiaxcel DNA High Resolution kit (QIAGEN, Germantown, MD, USA), and concentrations were measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The final barcoded libraries of each sample were diluted to 10 nM and pooled in a 2:1 ratio for bacterial and fungal libraries respectively. Resulting libraries were sequenced using Illumina MiSeq v3 technology (2 x 300 bp) by Macrogen, South-Korea, using 30% PhiX DNA as spike-in.

Additionally, two technical replicates for each treatment (one control and one chitin rhizosphere, so 4 samples in total) were included in the library to study the reproducibility of sequencing, with a separate DNA extraction and sequencing done on the same rhizosphere of a single plant.

Demultiplexing of the amplicon dataset and removal of the barcodes was performed by the sequencing provider. The raw sequence data is available in the NCBI Sequence Read Archive under the accession number PRJNA294362. A detailed description of the sequence read processing can be found in Chapter 2 section 2.1.8. Briefly, Trimmomatic v0.32 was used for

removing the primers (Bolger et al., 2014). Raw Illumina forward and reverse reads were merged using the program PEAR (Zhang et al., 2014). To extract the ITS2 sequences from the complete amplicon sequence, which includes parts of the neighboring, highly conserved, ribosomal genes, the ITSx program was used (Bengtsson-Palme et al. 2013). In the following steps, different programs of the Usearch software v7.0.1090 were used (Edgar, 2014). Merged sequences were quality filtered. Next, sequences of all samples that needed to be compared to each other were merged, dereplicated and sorted by size. Clustering the reads into Operational Taxonomic Units (OTUs) was done using Uparse, with an identity level of 97% for bacterial sequences and 98.5% for fungal sequences (Edgar, 2014). Chimeras were removed from the V3-V4 fragments using Uchime with the RDP Gold database as a reference (Edgar et al., 2011). Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm at 97% identity, and then converted into an OTU table (McDonald et al., 2012).

4.1.6 Rhizosphere microbiology: shotgun metagenomics

In total, four samples were selected from each treatment (no chitin addition, 2% chitin addition) for shotgun metagenomics. 1 µg DNA was sheared by ultrasonication using the covaris M220 at NxtGnt (Ghent, Belgium). Further preparation of the shotgun libraries was done by the Floodlight Genomics LLC lab (Knoxville, USA) under supervision of dr. Kurt Lamour and were sequenced on one Illumina HiSeq 2500 lane (2 x 250 bp) by Macrogen, South-Korea.

Demultiplexing of the shotgun dataset and removal of the barcodes was performed by the sequencing provider. The raw sequence data is available on the EBI metagenomics web portal under the project "ERP017180". Before sequence read processing, data quality has been checked using fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Truseq sequencing adaptors were present in 10% of the reads and were removed using the program cutadapt on the paired sequences (Martin, 2011).

Further on, reads were processed using EBI-metagenomics v3.0 (Mitchel et al., 2015). Briefly, SeqPrep v1.1 (https://github.com/jstjohn/SeqPrep) was used to merge the paired end Illumina reads. Low quality data (low quality ends and sequences with > 10% undetermined nucleotides) was trimmed using Trimmomatic (Bolger et al., 2014). Only sequences with a minimal length of 100 bp were retained. For the identification and masking of non-coding RNAs, HMMER v3.1b1 was used (Finn et al., 2011). 16S rRNA genes and mRNA genes were

filtered from the set and used for taxonomic and functional analysis respectively. To cluster the reads in OTUs and assign taxonomy, QIIME (v1.9.1) was used with greengenes (v13.8) as the reference database (DeSantis et al., 2006; Caporaso et al., 2010a). This limits the taxonomical identification to bacterial sequences. For functional gene annotation, first Fraggenescan (v1.20) was used to identify (fragmented) genes in short reads (Rho et al., 2010). Further on, InterProScan (v5.19-58.0) matches the mRNA reads toward predicted coding sequences using a subset of databases (Jones et al., 2014). As output, a list of Gene Ontology (GO) Terms is created.

4.1.7 Downstream data analysis and statistics

Lettuce growth, zoonotic pathogens enumeration and absolute PLFA concentrations were analysed with Statistica 12 (Statsoft) using a multi-factor analysis of variance with $p<0.05$. Full factorial design was performed first. If all interaction terms were not significant, a t-test was done to compare the mean of the chitin treatment with the control treatment. The factors studied for the lettuce growth were: treatment (with or without chitin) and experiment (1 and 2), with fresh weight per plant as dependent variable. For the enumeration of the zoonotic pathogens on lettuce leaves, treatment (with or without chitin), sampling time (days 4 and 8) and experiment (1 and 2) were the factors with CFU g^{-1} lettuce leaf as dependent variable.

Statistical differences in the absolute values of the PLFA's between the different treatments were determined using a MANOVA analysis.

Statistical differences of the relative abundances in PLFA were determined using ANOVA by the Statistical Analysis of Metagenomic Profiles (STAMP) program (Parks and Beiko, 2010). Correction of multiple testing was done using the Benjamini-Hochberg False Discovery Rate (FDR) method. Principal Coordinate Analysis (PCoA), in which the dissimilarity matrices were based on the Bray-Curtis index, on the PLFA data was done using the R vegan package (version 2.0-10) (Oksanen et al., 2010) with dissimilarity matrices calculated using the Bray-Curtis index.

OTU tables of the V3-V4 and ITS2 amplicon sequencing were analysed using the QIIME software package (v1.9.0) (Caporaso et al., 2010a). Representative bacterial OTU sequences were aligned to the SILVA v119 97% core set (version 119) using QIIME (Caporaso et al., 2010b; Quast et al., 2012). Taxonomy assignment was done using the uclust assignment method,

accepting maximum 3 hits for each query sequence and then assigning the most specific taxonomic label that is associated with at least 51% of the hits. Similarly, taxon assignments of fungal OTU sequences were done using the UNITE database (version 7.0) (Kõljalg et al. 2013).

Rarefaction analysis was done using the "alpha_rarefaction.py" script of QIIME. Rarefaction curves were estimated for both bacterial and fungal OTUs. Convergence was reached at 70,000 sequences for the bacterial OTUs (Figure 4.1A) and at 10,000 sequences for the fungal OTUs. Those rarefaction depths were used to determine the number of observed OTUs representing the bacterial and fungal richness. Shannon-Wiener diversity indices were calculated using the "alpha_diversity.py" script (QIIME) and used to estimate the within sample diversity. To find significant differences among mean richness and diversity indices, ANOVA analysis was done. Tukey HSD tests were used to find the mean richness and diversity indices that are significantly different from each other. Both analysis were done using the R program (version 3.1.0) (R core team, 2015).

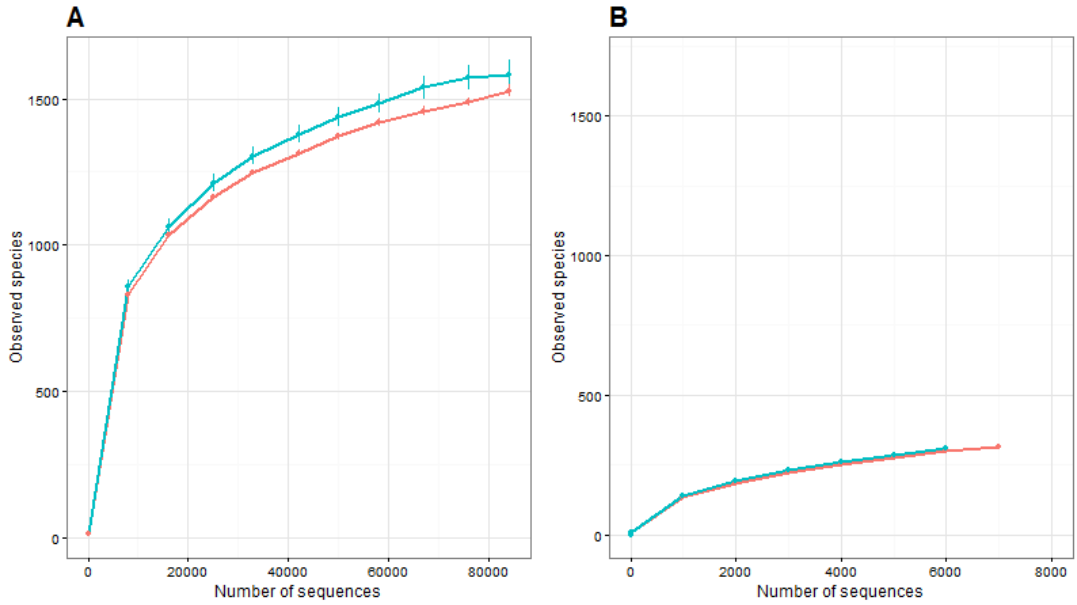


Figure 4.1 Rarefaction curves of the 16S rRNA gene data retrieved from (A) amplicon sequencing data (V3-V4), and (B) shotgun metagenomics data. For the amplicon sequencing data, saturation is reached around 70,000 sequences. For the shotgun data, saturation was not reached and compared to amplicon sequencing data, community complexity was low.

Multivariate analysis was done using the specific R package vegan (version 2.0-10) (Oksanen et al., 2010). Dissimilarity matrices (based on the Bray-Curtis dissimilarity index) were calculated from the OTU tables of the fungal and bacterial sequences obtained from amplicon sequencing and from the taxonomy OTU tables obtained from EBI metagenomics of the

shotgun metagenomics data, which did not reach saturation based on rarefaction analysis (Figure 4.1B). The OTU tables were normalized by removing those OTUs with an abundance lower than 0.01% in at least one sample. Homogeneity of the dataset was first tested using the betadisper functions. If variances were equal, effect of chitin addition on the bacterial and fungal communities was studied by doing a PERMANOVA analysis on these dissimilarity indices. To visualise the observed differences in bacterial community composition, PCoA on the dissimilarity matrices was done.

The STAMP analysis software was used to study individual differences in the bacterial or fungal taxonomic groups, both for the amplicon sequencing and shotgun sequencing data (Parks and Beiko 2010). For each experiment, ANOVA analyses were done on an OTU table clustered until genus level to determine the effect of chitin addition on the individual groups (phyla, genera). To correct for multiple testing, we used the Benjamini-Hochberg FDR. The clustering of the table until genus level was done using the QIIME software ("summarize_taxa_through_plots.py") and these tables were normalized by only keeping those genera which were present with a minimal abundance of 0.01% in minimum one sample.

For the analysis of the functional data retrieved from EBI metagenomics, R statistical software was used (R core team, 2015). First dissimilarity matrices (based on the Bray-Curtis dissimilarity index) were calculated from the GO terms tables. Homogeneity of variances was tested using the 'betadisper' function. If variances were equal, effect of chitin on the microbial functions was studied by PERMANOVA analysis on the dissimilarity indices. To study differences in functional genes (biological, cellular & molecular) a read table clustered on GO terms was used. In a filtering step GO terms with low count number in most samples were removed. GO terms with a read count less than 3 counts per million for at least four samples were removed from the dataset. Normalization is based on the trimmed mean of M-values in which we correct for effective library size of the count tables (Robinson and Oshlack, 2010). This normalization takes the sequencing depth into account and corrects for the presence of highly abundant families. The counts are modelled OTU by OTU using a negative binomial model with main effect chitin addition. The effective library size was used as an offset in the model for normalization purposes, hence, all model parameters have an interpretation in terms of changes in relative abundance. Empirical Bayes estimation of the overdispersion

parameters of the negative binomial model was adopted using the quantile-adjusted conditional maximum likelihood method by shrinking the GO term overdispersion towards the common dispersion across all GO terms. Statistical tests were adopted on the appropriate contrasts of the model parameters to assess the research questions of interest. We adopted the Benjamini-Hochberg FDR procedure to correct for multiple testing. All of these analyses were done using the edgeR package, version 3.12.0 (Robinson et al., 2010).

4.2 RESULTS

The results are divided in three parts, either describing the effect of chitin on lettuce plant growth and the survival of zoonotic pathogens (section 4.2.1) or the rhizosphere microbiome (section 4.2.2 – 4.2.3). For the rhizosphere microbiome, both the bacterial and fungal taxonomy was studied by either PLFA, amplicon sequencing and shotgun metagenomics (section 4.2.2). In addition, shotgun metagenomics also gave insight in changes in the functional genes of the microbiome by chitin addition, for which we were especially interested in chitinase activity in function of plant growth and survival of zoonotic pathogens (section 4.2.3).

4.2.1 Effect of chitin soil amendment on lettuce growth and survival of zoonotic pathogens on the leaves

Two independent experiments were performed to study the effect of chitin addition on lettuce growth and survival of the zoonotic pathogens *E. coli* O157:H7 and *S. enterica*. In terms of lettuce growth there was no interaction between the chitin treatment and the experiment, and therefore data of the two independent experiments was pooled. Addition of chitin significantly ($p < 0.01$) increased the fresh weight of the lettuce plants from 172.1 ± 17.8 g per plant grown in potting soil to 213.0 ± 18.8 g per plant grown in chitin amended potting soil.

For plants grown in potting soil without chitin, the dynamics of *E. coli* O157:H7 concentrations on the leaves were highly similar to those reported by Van der Linden *et al.* (2013) who grew lettuce plants in the same conditions and used the same *E. coli* isolate. No interaction effect between the three factors (treatment, experiment and sampling day) was observed, but an interaction effect between the sampling days (0, 4 and 8 dai) and the two experiments was noted. Therefore the survival of *E. coli* on the lettuce leaves was analysed for each day separately. For day 4, no significant effect of chitin addition on the survival of *E. coli* O157:H7 was observed in the experiments, for which the data could be pooled as there was no interaction effect between the factors treatment and experiments. On day 8 a significant reduction of *E. coli* survival in experiment 2 ($p = 0.009$) was observed, but not in experiment 1 (interaction effect treatment-experiment) (Figure 4.2A).

Also for the dynamics of *S. enterica* on the leaves in our control, we reported highly similar results as the ones obtained by Van der Linden *et al*. (2013). Data could be pooled for the two

experiments as there was no general interaction effect between the three factors (treatment, experiment, sampling day) and no interaction was observed between the factors treatment and experiment. The effect of chitin on the survival of *S. enterica* was however analysed for the sampling days separately, because an interaction between the factors treatment and sampling days was observed. At sampling day 4, no significant difference between the two treatments was found, whereas 8 days after inoculation, a significant lower survival rate of *S. enterica* on the leaves in the chitin treatment compared to the control was observed (Figure 4.2B).

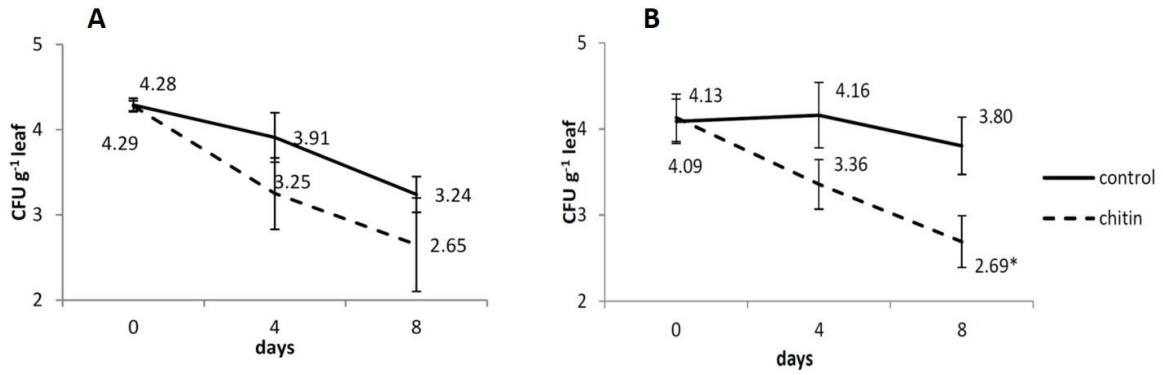


Figure 4.2 Dynamics of *Escherichia coli* O157:H7 (A) and *Salmonella enterica* sv. Thompson RM1987N (B) on middle-aged lettuce leaves at 0,4 and 8 days after spray inoculation analysed by plating as described by Van der Linden et al. (2013). Full lines and dashed lines represent control (=non-chitin treatment) and chitin treated plants respectively. The data are calculated from the log-transformed values of the pathogen per gram tissue from two independent experiments (n=2 plants or 6 leaves for day 0 and n=6 plants or 18 leaves for day 4 and 8). Asterisk means significant differences between the chitin and control treatment. Bars represent standard errors.

4.2.2 The rhizosphere microbiome: taxonomical shifts by chitin addition

For **PLFA analysis**, the soil from five individual pots of the control treatment (= without chitin) and from five individual pots of the chitin treatment was analysed from non-inoculated plants. Both the absolute (nmol g^{-1} dry soil) and the relative abundance (%) of each biomarker was assessed per treatment. All individual PLFA biomarkers and all microbial groups were significantly increased after chitin amendment (absolute abundances), resulting in a double amount of total biomass as compared to the control (Additional Information (AI) Table AI4.1). For the relative abundance, 13 of the 20 biomarkers were significantly different from the control, with a significant decrease in relative abundance for bacteria (non-specific) and Gram positive bacteria and a significant increase for the Gram negative bacteria (Table AI4.2).

To illustrate these dissimilarities in the microbial communities of the chitin supplemented soil and the control, a PCoA on the PLFA data was done (Figure 4.3). The first principal coordinate (PCo1), which represents the major variance of the dataset (94.9%) confirmed that the rhizosphere microbiome differed between potting soil with and without chitin. The second principal coordinate describes the variation between the samples in each treatment (with and without chitin). This is only a minor source of variability (2.5%), indicating a high reproducibility of the data of the 5 pots per treatment.

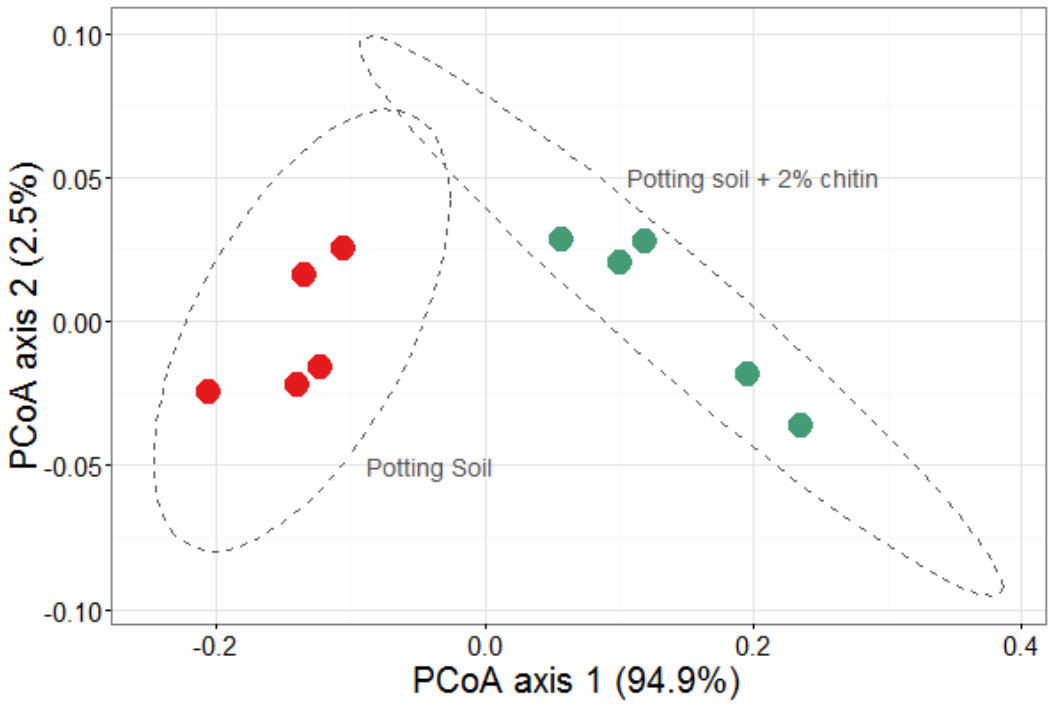


Figure 4.3 Principal Coordinates Analysis (PCoA) plot of the PLFA data. The x-axis represent 94.9 % of the variance of the dataset, the y-axis represent 2.5 % of the total variance of the dataset.

The **bacterial community composition** of the lettuce rhizosphere was analysed in first instance by **sequencing the V3-V4 region of the 16S rRNA gene**, both for plants grown in potting soil with and without chitin. The rhizospheres of five individual plants from each treatment (with and without chitin) were prepared and analysed separately. After merging of the forward and reverse reads and quality filtering, 83.8% of the sequences were retained, resulting in an average of 92,549 sequences per sample. No differences were observed between the two technical replicates per treatment, indicating reproducibility of the sequencing.

Second, **shotgun metagenomics** on four rhizosphere samples of plants grown in potting soil with and without chitin was done. Samples were analysed using the online webtool EBI-metagenomics (Mitchel et al., 2015). After quality filtering, 75.5% of the sequences were

retained with an average length of 232 bp, resulting in an average of 17,115,710 reads per sample. On average, 5,238 and 3,875 reads could be assigned to a bacterial 16S rRNA gene for unamended and 2% chitin amended potting soil rhizosphere samples respectively.

Differences in bacterial communities between treatments were analysed in two ways. First, the richness and diversity were studied in the amplicon data set, by estimating the number of observed OTUs and Shannon-Wiener diversity indices between the control and the chitin treatment (1436±35 vs. 1370±12 and 8.15±0.03 vs 8.17±0.08, respectively). Both did not differ significantly, indicating that the chitin amendment did not increase the bacterial biodiversity in the rhizosphere.

Second, the bacterial community composition was studied. PERMANOVA analysis of the amplicon sequencing data showed that chitin addition induced significant shifts in bacterial composition (taxonomic groups) (p= 0.01). This was confirmed by the shotgun metagenomics data, where the relative abundances of the reads assigned to bacterial taxonomy were significantly different due to chitin treatment (p=0.037). This is illustrated by a PCoA plot of both datasets (Figure 4.4).

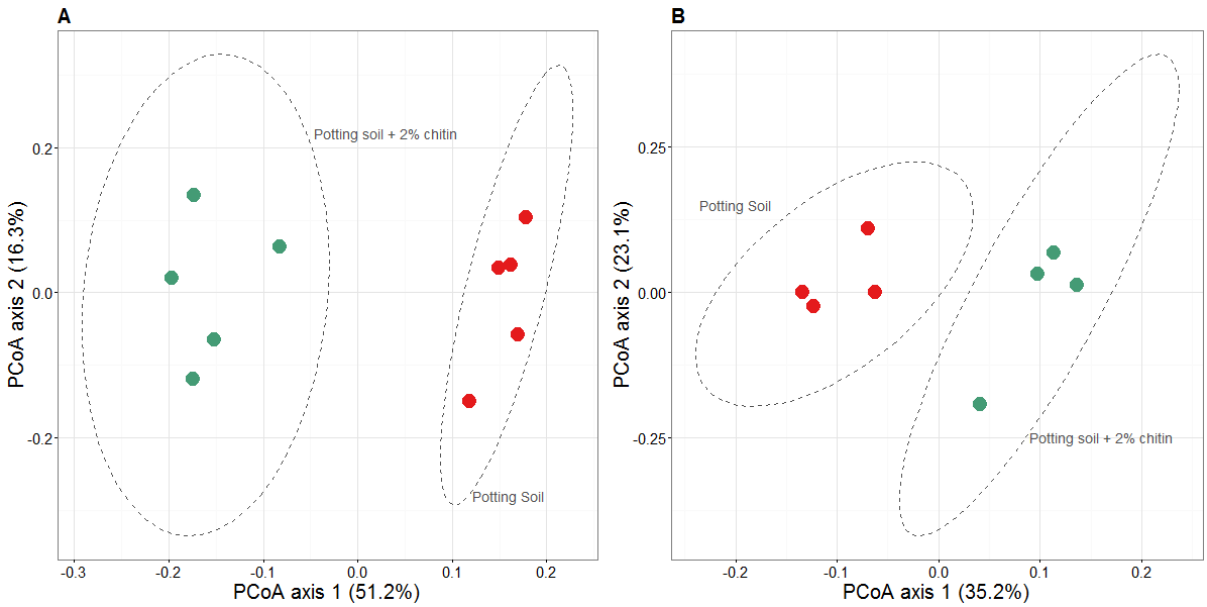


Figure 4.4 Principal Coordinates Analysis (PCoA) plot of the bacterial community. Plots are either based on amplicon sequencing of the V3-V4 16S rRNA gene (A) or on the 16S rRNA genes retrieved from a shotgun metagenomics dataset (B).

In the amplicon sequencing data, thirteen of the twenty-eight bacterial phyla showed a significant difference between the control and the chitin treatment, of which ten phyla and two candidate divisions each represented more than 1% of the community (Table 4.1). Most

importantly, the relative abundances of the *Acidobacteria* and the *Verrucomicrobia* were significantly decreased in the chitin treatment as compared to the control, whereas the relative abundance of the *Actinobacteria*, *Bacteroidetes*, and the *Proteobacteria* was significantly increased in the chitin treatment as compared to the control (Table 4.1). Analysing these 5 phyla together, it was shown that the relative abundance of the Gram negative bacteria was significantly increased in the chitin treatment. In contrast, the relative abundance of the Gram positive bacteria was not significantly different from the control (Table 4.1).

Shotgun metagenomics showed a statistically significant reduction in relative abundance of the *Verrucomicrobia*, *Acidobacteria* and *Gemmatimonadetes* and a significant increase in the relative abundance of *Bacteroidetes* and *Actinobacteria* due to the addition of chitin. With the exception of the *Proteobacteria*, the same trends in increase or decrease due to chitin addition are observed as with amplicon sequencing (Table 4.1).

Table 4.1 Bacterial composition of the lettuce rhizosphere in unamended and chitin amended potting soil. Relative abundances (%) (average±standard error) of the twelve most dominating phyla (relative abundance > 1 %) in the strawberry rhizosphere, comprising more than 95 % of the total bacterial community in the lettuce rhizosphere. Phyla that are statistically significant ($p<0.05$) in abundance for the chitin treatment compared to the control group (0% chitin) are indicated with an asterisk. Phyla that were statistically different and known to contain gram negative bacteria are indicated in **bold**, phyla containing gram positive bacterial members are underlined.

Phylum	V3-V4 Amplicon sequencing			Shotgun metagenomics		
	Control	2% chitin		control	2% chitin	
Proteobacteria (%)	47.0±0.4	49.8±0.8	*	57.1±1.0	57.2±1.8	
Bacteroidetes (%)	10.8±0.3	15.6±0.4	*	9.1±0.4	12.3±0.3	*
Verrucomicrobia (%)	10.8±0.4	7.7±0.6	*	8.9±0.5	5.9±0.9	*
Acidobacteria (%)	7.1±0.3	5.4±0.3	*	7.3±0.4	4.8±0.3	*
Actinobacteria (%)	3.8±0.1	4.8±0.2	*	5.2±0.1	6.3±0.3	*
Cyanobacteria (%)	3.2±0.5	3.0±0.6	*	3.2±0.6	3.1±1.7	
Gemmatimonadetes (%)	3.7±0.2	3.2±0.2	*	4.3±0.2	3.5±0.1	*
Planctomycetes (%)	5.9±0.4	3.2±0.2	*	1.6±0.3	1.2±0.1	
Armatimonadetes (%)	1.3±0.1	1.5±0.1	*	0.5±0.1	0.6±0.1	
Candidate divison TM7 (%)	1.0±0.2	1.7±0.1	*	0.6±0.1	0.8±0.1	
Chloroflexi (%)	1.4±0.1	1.2±0.1	*	0.8±0.1	0.5±0.1	
Candidate division OD1 (%)	1.5±0.2	1.1±0.1	*	0.6±0.1	0.4±0.1	
Gram negative bacteria	68.67	73.04	*	75.1	75.4	
Gram positive bacteria	10.98	10.19		12.5	11.1	

Amplicon sequencing revealed that chitin altered the relative abundance of 40 bacterial families, in particular 11 families of the *Proteobacteria*, 8 of the *Actinobacteria*, 6 of the

Bacteroidetes, 6 of the *Firmicutes*, 2 of the *Verrucomicrobia*, 1 of the *Acidobacteria* (unknown family of subgroup 6) and 1 of the phylum *Chlamydiae* (*Simkaniaceae*) (Figure 4.5). Next to these families, which belonged to significantly altered phyla by chitin addition, 2 families of the *Chloroflexi* (*Anaerolineaceae* and an unknown family of the *Thermomicrobia*), 2 unknown families of the *Planctomycetes* and the *Spirochaetaceae* (Phylum: *Spirochaetes*) were significantly changed in relative abundance due to chitin addition (data not shown).

The relative abundance of 38 bacterial genera was significantly different between the rhizospheres of the two treatments, of which 18 genera represented more than 0.05% of the OTUs in at least one of the two treatments. These 18 genera are reported in Table 4.2. Thirteen genera were significantly increased in the chitin treatment, including genera containing species that are reported to be involved in plant growth promotion, chitin degradation and biological control. Five genera, *Pseudolabrys*, *Alcanivorax, Candidatus solibacter, Nitrosococcus* and *Aquicella* were significantly decreased.
In the shotgun metagenomics data, eleven of these 18 genera were also identified, of which nine were also significantly increased or decreased in relative abundance due to the addition of chitin .

The **fungal microbiomes** present in rhizospheres of five plants that were grown in potting soil with or without chitin (ten rhizospheres of individual plants in total) were compared by **ITS2 sequencing**. After merging of the forward and reverse reads and quality filtering, 83.6% of the sequences were retained, resulting in an average of 50,045 sequences per sample. In total, around 21% of the sequences of the control and 17% of the sequences of the chitin amendment could not be assigned to a fungal phylum.

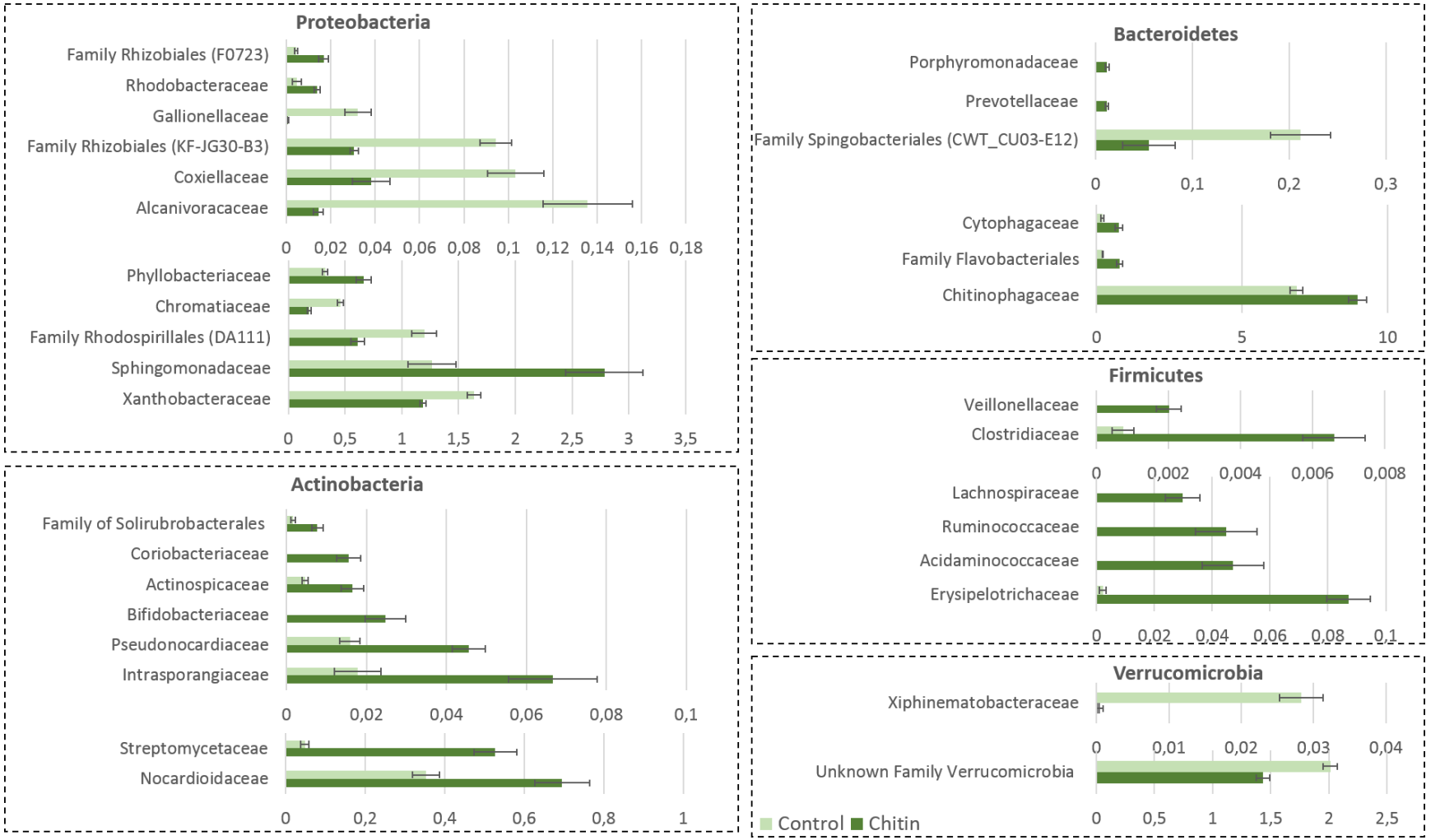


Figure 4.5 Major bacterial taxonomical changes on family level in the rhizosphere community after 2% chitin amendment to the potting soil. The graphics represent the significant differences (%) (average ± standard error) of representative families belonging to five major bacterial phyla in the lettuce rhizosphere due to the addition of chitin to the potting soil

Table 4.2 Significant differences in the relative abundance of bacterial genera (%) ± standard error between lettuce rhizospheres in potting soil with and without 2% chitin (n = 5) and the possible functions of species belonging to this genera reported in literature. PGP = Plant Growth Promotion. **Bold** means a significantly higher relative abundance in the chitin treatment as compared to the control. Underlined means a significant decrease in the relative abundance in the chitin treatment as compared to the control.

Phylum	Family	Genus	V3-V4 amplicon sequencing		Increase or decrease	Shotgun metagenomics		Increase or decrease	Possible functions (references)
			control	2% chitin		control	2% chitin		
Proteobacteria	*Pseudomonadaceae*	*Cellvibrio*	0.09±0.05	1.34±0.26	**15x**	0.08±0.04	1.08±0.36	**13x**	PGPR, chitin degradation and N-cycle (Suarez et al. 2014; Anderson and Habiger, 2012)
	Sphingomonadaceae	*Sphingomonas*	0.45±0.06	1.02±0.07	**2x**	0.38±0.02	0.49±0.07		PGP, chitin degradation and biocontrol (Wachowska et al. 2013; van Bruggen et al 2014, Zhu et al., 2007)
	Sphingobacteriaceae								
		Pedobacter	0.02±0.01	0.38±0.09	**19x**	0.03±0.01	0.33±0.07	**11x**	PGPR and biocontrol (De Boer et al. 2007)
	Rhodospirillaceae	*Azospirillum*	0.03±0.01	0.19±0.04	**6x**	0.09±0.03	0.38±0.13	**4x**	PGPR and N-cycle (Saharan & Nehra, 2011)
		Dongia	0.72±0.05	1.29±0.06	**2x**	/	/		/
	Phyllobacteriaceae	*Nitratireductor*	0.16±0.02	0.42±0.05	**3x**	/	/		N-cycle (Penton et al., 2013)
	Bradyrhizobiaceae	*Afipia*	0.38±0.02	0.58±0.04	**2x**	/	/		/
	Coxiellaceae	*Aquicella*	0.10±0.01	0.04±0.01	0.4x	0.03±0.01	0.01±0.01	0.3x	/
	Xanthobacteraceae	*Pseudolabrys*	1.53±0.07	1.13±0.03	0.7x	/	/		/
	Alcanivoracaceae	*Alcanivorax*	0.14±0.02	0.01±0.00	0.1x	0.25±0.07	0.08±0.02	0.3x	/
	Chromatiaceae	*Nitrosococcus*	0.46±0.03	0.18±0.02	0.4x	/	/		N-cycle (Juretschko et al., 1998)
Bacteroidetes	*Cytophagaceae*	*Dyadobacter*	0.02±0.0	0.33±0.07	**16x**	0.02±0.01	0.22±0.11	**11x**	/
	Chitinophagaceae	*Taibaiella*	0.30±0.07	2.14±0.42	**7x**	/	/		N-cycle (Zhang et al., 2013)
Nitrospirae	*Nitrospiraceae*	*Nitrospira*	0.24±0.05	0.90±0.10	**4x**	/	/		N-cycle (Kox & Jetten, 2015)
Actinobacteria	*Streptomycetaceae*	*Streptomyces*	0.05±0.01	0.53±0.06	**10x**	0.10±0.03	0.62±0.11	**6x**	PGP, chitin degradation and biocontrol (Saharan & Nehra, 2011; Hjort et al., 2010)
	Nocardioidaceae	*Nocardioides*	0.11±0.02	0.28±0.04	**3x**	0.08±0.02	0.18±0.03		Biocontrol (Carrer et al. 2008)
Firmicutes	*Anaeroplasmataceae*	*Asteroleplasma*	0.00±0.00	0.08±0.01	-	*0.00*±0.00	*0.01*±0.01	-	/
Acidobacteria	*Solibacteraceae*	*Candidatus*	0.52±0.03	0.17±0.01	0.3x	0.52±0.07	0.25±0.02	0.5x	/

There were no significant differences in number of observed OTUs and Shannon-Wiener diversity indices between the control and the chitin amendment (298±15 vs. 271±11 and 4.81±0.40 vs 4.65±0.10, respectively), indicating that the chitin treatment did not increase the fungal biodiversity. However, significant shifts in fungal composition (taxonomic groups) between the two treatments were observed (PERMANOVA, p=0.008). In total five fungal phyla were found across all samples, of which three phyla were significantly different between the two treatments: the *Ascomycota*, *Basidiomycota*, and the *Zygomycota* (P< 0.05, Table 4.3).

Table 4.3 Fungal composition of the lettuce rhizosphere in unamended and chitin amended potting soil. Relative abundances (%) (average±standard error) of the five fungal phyla (relative abundance > 1 %) in the strawberry rhizosphere. Phyla that are statistically significant (p<0.05) in abundance for the chitin treatment compared to the control group (0% chitin) are indicated with an asterisk.

Phylum	Treatment		
	Control	2% chitin	
Ascomycota	53.0±13.7	15.2±1.6	*
Chytridiomycota	35.2±15.0	12.3±3.6	
Basidiomycota	6.7±1.6	1.7±0.5	*
Zygomycota	4.3±2.0	70.2±0.6	*
Cercozoa	0.1±0.1	0.004±0.1	

The *Zygomycota* were significantly increased in the chitin treatment, whereas the *Basidiomycota* and *Ascomycota* were significantly decreased. The standard errors of the relative abundances of phyla over five replicates in the control treatment are quite large compared to the 2 % chitin treatment. It indicates that the chitin directs the fungal composition in a focused and consistent way. This is probably due to a high increase of the relative abundance of *Mortierella* species in the chitin treatment, compared to the control treatment (58.1% vs. 3.2%).

On family level, chitin addition altered the relative abundance of eleven fungal families significantly, in particular seven families of the *Ascomycota*, two families of the *Zygomycota* and two families of the *Basidiomycota* (Figure 4.6). Especially the *Morteriellaceae* showed a high increase, due to the genus *Morteriella* that was strongly represented and is clearly promoted by the presence of chitin in the potting soil.

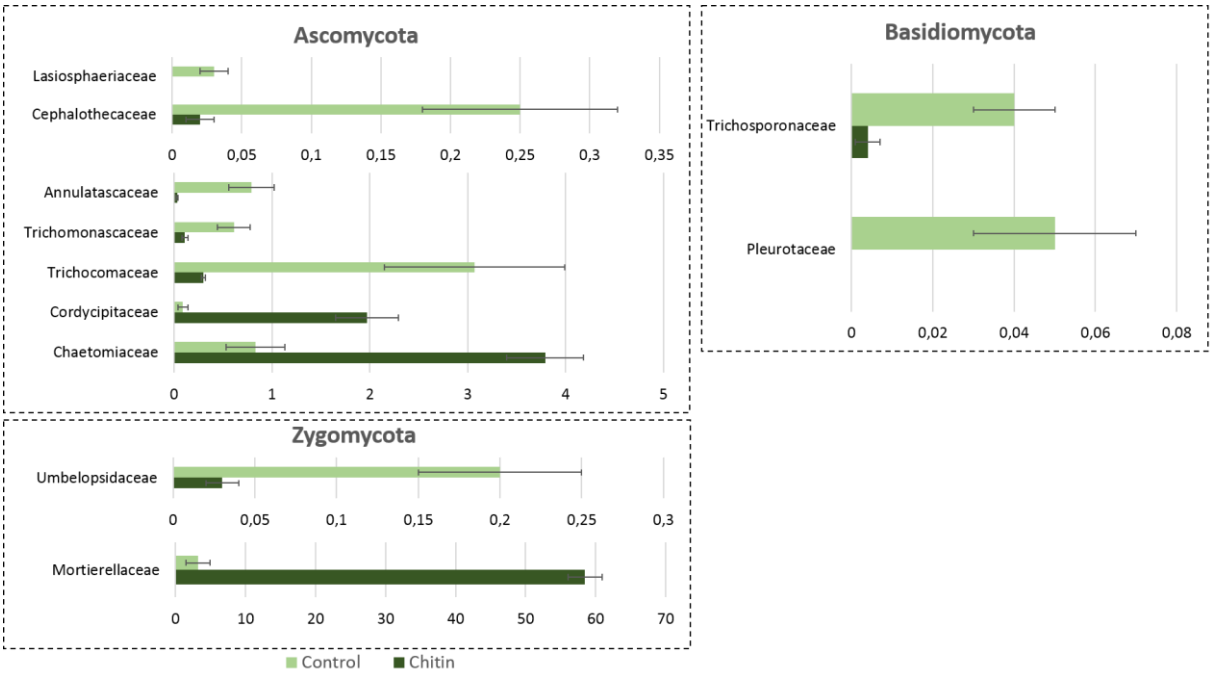


Figure 4.6 Major fungal taxonomical changes on family level in the rhizosphere community after 2% chitin amendment to the potting soil. The graphics represent the significant differences (%) (average ± standard error) of representative families belonging to three major fungal phyla in the lettuce rhizosphere due to the addition of chitin to the potting soil.

Two other fungal genera of the phylum *Ascomycota* increased in relative abundance due to the chitin treatment: *Lecanicillium* and *Pseudogymnoascus*. Additionally, only one genus decreased significantly in relative abundance: *Pseudeurotium*. All fungal genera in the rhizosphere induced by the addition of chitin to the potting soil include species reported in literature to be involved in biocontrol and/or chitin degradation (Table 4.4).

Table 4.4 Significant differences in the relative abundance of fungal species (average relative abundance(%)±standard error) between potting soil with and without 2% chitin (n = 5) and their possible functions reported in literature. PGP = Plant Growth Promotion**.** Species **in bold** mean a significant increase in the relative abundance in the chitin treatment as compared to the control treatment. Species underlined mean a significant decrease in the relative abundance in the chitin treatment as compared to the control treatment.

Phylum	Family	Genus	Treatment		**Increase** or decrease	Functions (references)
			Control	2% chitin		
Ascomycota	***Cordycipitaceae***	***Lecanicillium***	0.09±0.05	1.85±0.33	**20x**	PGP, chitin degradation, biocontrol and induced resistance (Goettel et al. 2008; Van Nam et al. 2014; Nguyen et al. 2015).
	Pseudorotiaceae	***Pseudogymnoascus***	0.96±0.30	3.46±0.26	**4x**	Biocontrol (Tagawa et al. 2010)
	Pseudorotiaceae	*Pseudeurotium*	1.81±0.42	0.12±0.02	0.07x	/
Zygomycota	***Mortierellaceae***	***Mortierella***	3.21±1.73	58.13±2.55	**18x**	Chitin degradation (Kim et al., 2008) and biocontrol (Tagawa et al., 2010)

4.2.3 The rhizosphere microbiome: functional shifts after chitin addition

Functions encoded in the metagenome were studied by clustering putative protein coding DNA fragments to GO terms. In total, around 15 % of the reads could be mapped to a functional trait. First of all, differences in microbial functional potential were studied. PERMANOVA analysis could be used on the GO term count table as there was homogeneity in the variances between microbial genes of the lettuce rhizosphere grown in potting soil and chitin amended potting soil. No significant differences in relative abundance of GO terms due to the addition of chitin could be detected ($p = 0.055$).

Nonetheless no general effect of chitin on the microbial functional genes was observed, several molecular and biological processes of the microbiome were influenced by the addition of chitin. In total, 305 of the 2611 GO terms increased or decreased in relative abundance due to the chitin amendment. From these 305 GO terms, 78 were at least doubled or reduced by half in relative abundance due to the addition of chitin (Table AI4.3). Several of these functions are involved in multiple processes, which makes it difficult to link them to chitin-related shifts. Some of the functions that increase in relative abundance due to chitin addition could be linked with e.g. microbial growth or photosynthesis. Most interesting is that several GO terms were linked with chitin-metabolic biological and molecular functions. First of all, GO terms directly linked to chitin metabolism are significantly altered due to the addition of chitin, with the exception of the chitin metabolic processes (Table 4.5). In this table, the GO term "chitinase activity" is a collective term for chitinases, 1,4-beta-poly-N-acetylglucosaminidase, chitodextrinase and poly-beta-glucosaminidases. Second, also an increase in GO terms linked with the enzymes to degrade chitin-deacetylated forms (chitosan, cellulose) is observed: chitosanase activity and cellulose catabolic process.

Table 4.5 Chitin synthase and degrading related functions. The relative abundance of reads (average ± standard error) related to chitin addition are indicated for the control (no chitin) and 2% chitin amendment potting soil, rhizosphere samples.

	Category	Description	GO term	Treatment			
				control	2% chitin		
CHITIN	Biological	Chitin biosynthetic process	GO:0006031	$3.5E^{-7}$±$0.5E^{-7}$	$28.4E^{-7}$±$1.4E^{-7}$	*	**8x**
		Chitin catabolic process	GO:0006032	$137.4E^{-7}$ ±$4.8E^{-7}$	$283.4E^{-7}$±$4.0E^{-7}$	*	**2x**
		Chitin metabolic process	GO:0006030	$3.0E^{-7}$±$0.8E^{-7}$	13.5±$2.2E^{-7}$		
	Molecular	Chitin binding	GO:0008061	$7.3E^{-7}$±$0.8E^{-7}$	$24.1E^{-7}$±$2.3E^{-7}$	*	**3x**
		Chitin synthase activity	GO:0004568	$5.0E^{-7}$±$0.8E^{-7}$	$34.2E^{-7}$±$3.8E^{-7}$	*	**7x**
		Chitinase activity	GO:0016977	$137.2E^{-7}$±$28.5E^{-7}$	$280.2E^{-7}$±$28.5E^{-7}$	*	**2.5x**
CHITOSAN	Molecular	Chitosanase activity	GO:0008811	$19.6E^{-7}$±$3.6E^{-7}$	$35.6E^{-7}$±$3.6E^{-7}$	*	**2x**
CELLULOSE	Biological	Cellulose catabolic process	GO:0030245	$145.8E^{-7}$±$17.2E^{-7}$	$295.0E^{-7}$±$32.8E^{-7}$	*	**2x**

4.3 DISCUSSION AND CONCLUSIONS

Since farmers, consumers and policy makers have become more aware of the impact of the use of chemical pesticides and fertilizers on human health and the environment, there is a renewed interest in the use of organic soil amendments, such as chitin, to improve crop yield and plant resilience. Several studies have linked these beneficial chitin amendment effects on an influence of the soil and plant rhizosphere microbiome, such as an increase in the abundance of PGPR and PGPF in the soil and/or rhizosphere of the plant (Sarathchandra et al., 1996; Radwan et al., 2012; Cretoiu et al., 2013). Although chitin addition seems to control soil-borne pathogens and enhances plant disease resistance, it was not known whether chitin addition also affects the survival of human pathogens on the plant.

In the current study, lettuce plants were grown in peat based-potting soil with and without chitin. Leafy vegetables are known to have the potential to carry human pathogens such as *E. coli* O157:H7 and *S. enterica* on their leaves (Van der Linden et al., 2013) and are thus considered high risk food. We assessed the effect of chitin addition on (i) lettuce growth; (ii) the survival of zoonotic pathogens on the lettuce leaves; and (iii) rhizosphere microbial community.

First, chitin addition to potting soil significantly increased the fresh weight of lettuce leaves by approximately 20% which is in accordance to the study of Muymas *et al.* (2015). This confirms the results of previous studies, showing the ability of chitin to promote the growth of several crops, e.g. daikon radishes, cabbage, soybean sprouts, sweet basil, grapevine (Sharp, 2013). This plant growth promotion can first be due to an enrichment in nutrients. The addition of chitin increased the number of previously reported bacterial and fungal chitin-degraders and functional genes related to the chitin metabolism. The degradation of chitin releases a substantial amount of carbon and nitrogen in the soil or substrate, which leads to an enrichment of nutrients (Hamid et al., 2013; Jacquiod et al., 2013). Second, also the increase in bacterial genera linked with the N cycle and PGPR, which can ease the uptake of nutrients for the plants and enhance the plant availability of nitrogen and phosphorus (Egamberdiyeva, 2007; Krey et al., 2013), in the rhizosphere microbiome could be related to the increased plant growth. Third, chitin can partly be deacetylated to chitosan (Beier & Bertilsson, 2013). Chitosan is known as a natural biocontrol agents with antibacterial characteristics and an enhancer of plant growth (Linden, 2000).

Second, chitin addition significantly reduced the survival of *S. enterica* on the leaves. Also the survival of *E. coli* O157:H7 seemed to be negatively affected by the chitin amendment, although this effect was not significant. To the best of our knowledge, this is the first study to show that a soil amendment can affect the survival of a zoonotic pathogen on plant leaves. It remains unclear what the exact mechanism is behind the decreased survival of *S. enterica* on the lettuce leaves. In terms of plant health, several mechanisms that occur due to chitin addition to soil have already been suggested (Sharp, 2013): (1) direct antibiosis against pests and pathogens of crops; (2) enhancement of beneficial microbes; (3) direct stimulation of plant defense responses against biotic stress; and (4) up-regulation of plant growth, development, nutrition and tolerance to abiotic stress. The three latter responses may not only explain our observed plant growth promotion effect, but could also be related to the reduced survival of *S. enterica* on the leaves. In this study, only the microbial community of the rhizosphere (2) has been studied using PLFA and HTS. The effect of chitin on the survival of zoonotic bacteria on lettuce leaves through a change in the rhizosphere microbiome can be twofold. First, the absolute and relative abundance of several fungal and bacterial groups involved in biological control increased due to the addition of chitin. Second, chitin can act as a PAMP, directly triggering the immune system of the plant (de Jonge et al., 2010). In this study not only the chitin-degrading organisms were higher abundant in relative abundance, but we also showed an increase in relative abundance of chitinase-linked genes. Chitinase activity and the production of chito-oligosaccharides will lead to a cascade reaction in the plant, triggering plant-defense genes (Langner & Göhre, 2016). This may also explain the reduced colonization of *S. enterica* on the lettuce leaves.

Several bacterial genera containing PGPR and PGPF were upregulated. However with the techniques used, it is not possible to identify the underlying mechanism of the observed plant growth promotion and reduced survival of *S. enterica*. In accordance, a recent study showed that a PGPR (*Bacillus subtilis* UD1022) applied to the roots was able to influence the survival of human pathogens *(Listeria* and *Salmonella*) on leafy greens. This was correlated with an induction of the stomata closure by the *Bacillus* strain (Markland et al., 2015). *Bacillus subtilis* well known PGPR effect is at least partly based on the production of surfactines, which induce plant immune system in a priming-like manner (Cawoy et al., 2014). In our study, no increase in the relative abundance of *Bacillus* species was seen, but other PGPR and PGPF were more

than 10-fold increased after chitin addition, including bacterial species belonging to the genera *Cellvibrio*, *Pedobacter*, *Dyadobacter*, and *Streptomyces* and fungal species belonging to the genera *Lecanicillium* and *Mortierella*. This confirms previous observations of De Boer *et al.* (1999), who showed that the rapid degradation of chitin in dune soils was most likely due to fast-growing *Mortierella* spp., whereas *Streptomyces* spp. and slow-growing fungal species (such as *Verticillium* spp., now partially re-classified as *Lecanicillium* spp.) were shown to be more involved in the degradation of chitin after prolonged incubation.

Our study addresses some limitations of previous studies and extends our knowledge about the effect of chitin on belowground microbiology. First, rhizosphere samples were studied instead of bulk field soil; second, both the fungal and bacterial community were assessed using Illumina sequencing; and third, PLFA was used as an additional technique which allows quantification of microbial biomass. In our study, amplicon sequencing showed that chitin addition to peat-based potting soil increased the relative abundance of the *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* in the rhizosphere, while those of the *Verrucomicrobia* and the *Acidobacteria* were significantly decreased. This confirms previous results that describe an increase in the relative abundance of *Proteobacteria* (Jacquiod et al., 2013; Cretoiu et al., 2014), *Actinobacteria* (Jacquiod et al., 2013) and *Bacteroidetes* (Cretoiu et al., 2014) due to chitin amendment in field soil. Shotgun metagenomics showed the same trends with the exception of the *Proteobacteria*, which were not affected by chitin treatment. Other studies comparing amplicon sequencing and shotgun metagenomics data already showed that relative abundances of the phyla can differ between the two methods. Amplicon sequencing is PCR based and both primer mismatches and the presence of multiple 16S rRNA gene copies in a bacterium can lead to false estimations (Galtier & Steel, 2007; Vetrovský & Baldrian, 2013). In a previous study, the number of *Proteobacteria* in the amplicon sequencing dataset is higher compared to the shotgun metagenomics in the Barley rhizosphere, probably due to multiple copies of the 16S rRNA gene in beta- and *gammaproteobacteria* (Bulgarelli et al., 2015). In contrast, in our study the *Proteobacteria* were higher abundant in the shotgun metagenomics dataset. This can probably be due to the reference database used. In the amplicon sequencing data, Silva was used compared to Greengenes which is used in the EBI metagenomics pipeline for taxonomic classification of shotgun metagenome data. Several databases are biased in their compositions and for Greengenes ~30% of the database are

Proteobacteria, which comprise ~5% of the database of complete microbial genomes used to assign taxonomic affiliations to metagenomics contigs (Poretsky et al., 2014). In addition, only 4,000 sequences were used of the shotgun dataset for taxonomic classification compared to 50,000 sequences in the amplicon dataset. Therefore, it could be that the sequencing depth of the shotgun set was not sufficient to cover the complete community complexity.

PLFA analyses showed a twofold increase in both fungal and bacterial biomass in the rhizosphere due to chitin amendment. Cretoiu *et al.* (2013) however showed a tenfold increase in bacterial abundance, but a tenfold decrease in fungal abundance in chitin-amended field soil compared to unamended field soil using qPCR. This increase in microbial biomass is also supported by the shotgun metagenomics data. Some functional genes which could be related to microbial growth were higher abundant when chitin was added, e. g. calcium-dependent phospholipid binding and iron ion transmembrane transport. Iron and calcium are both elements necessary for microbial growth and cell division. Therefore, increase in transport-regulated elements can be correlated with an increases uptake of the elements and a higher microbial cell division and thus biomass (Michiels et al., 2002; Symeonidis & Marangos, 2012).

Based on our results and others, chitin addition thus gives reproducible shifts in microbial community even in very different soil systems. At lower taxonomic levels, differences are more common due to the specific niche of the rhizosphere, which is expected to be different from bulk soil (e.g.., Lundberg et al., 2012; Peiffer et al., 2013). To the best of our knowledge, the presented study is the first study to use amplicon sequencing of the fungal ITS2 gene region to assess the effect of chitin soil amendment on the fungal rhizosphere microbiome. We showed that the relative abundance of the fungal genera *Lecanicillium* and *Mortierella* was highly increased, both containing species involved in plant growth promotion, chitin degradation and biological control. Additionally, *Mortierella* spp. belonging to a the complex group of the *Mortierellales* (Wagner et al., 2013) might play an important component in the phosphorus cycling of the plant (Curlevski et al., 2010).

To conclude, in this chapter we demonstrated that chitin addition to potting soil is able to increase lettuce crop growth and decrease the survival of *Salmonella* on leafy greens. Both effects were related with changes in the rhizosphere microbiome, with increases in PGPR, PGPF and previous identified chitin-degraders and chitin-related functions.

ADDITIONAL INFORMATION CHAPTER 4

Table AI4.1. Absolute concentrations (nmol g^{-1} dry soil) ± standard error of PLFA biomarkers specific for different microbial groups in potting soil with and without 2% chitin, after 55 days of lettuce cultivation in the growth chamber. Asterisks indicate a significant increase as compared to the control ($p<0.05$) by analysis of variance with n = 5.

Microbial group	PLFA biomarker	Treatment		
		Control	2% chitin	
Gram positive bacteria	i-C15:0	20.22±0.43	31.39±2.22	*
	a-C15:0	12.71±0.35	19.27±1.49	*
	i-C16:0	7.13±0.23	11.19±0.77	*
	i-C17:0	8.08±0.20	14.91±1.11	*
Actinomycetales	10Me-C16:0	4.20±0.20	5.83±0.34	*
	10Me-C17:0	4.96±0.19	8.74±0.56	*
	10Me-C18:0	0.50±0.03	3.34±1.07	*
Bacteria (non-specific)	C14:0	2.72±0.10	3.70±0.28	*
	C15:0	2.05±0.05	3.34±0.23	*
	C16:0	41.41±1.64	71.72±5.56	*
	C17:0	1.17±0.04	2.27±0.16	*
	C18:0	9.77±0.26	16.36±0.94	*
Gram negative bacteria	C16:1c9	11.14±0.74	25.18±2.72	*
	C16:1t9	4.15±0.63	8.98±0.65	*
	C17:0cy	9.48±0.53	22.56±2.27	*
	C18:1c11	9.77±0.26	16.36±0.94	*
	C19:0cy	23.56±0.78	49.23±4.32	*
Fungi	C18:1c9	15.26±0.68	38.37±4.62	*
	C18:2n9,12	22.32±3.28	31.50±1.69	*
Arbuscular mycorrhiza	C16:1c11	4.22±0.37	8.17±0.54	*
Total biomass		221.82±8.28	402.20±29.38	*

Table AI4.2. Relative abundance (%) ± standard error of biomarker PLFAs and PLFA groups in potting soil with and without 2% chitin after 55 days of lettuce cultivation in the growth chamber. Asterisk indicates a significant difference to the control ($p<0.05$) by analysis of variance with n = 5. Microbial groups and biomarkers marked **in bold** are significantly more abundant in the chitin treatment as compared to the control. Underlined microbial groups and biomarkers are significant less abundant in the chitin treatment as compared to the control.

Microbial group	**PLFA biomarker**	**Treatment**		
		Control	**2% chitin**	
Gram positive bacteria	i-C15:0	9.12±0.19	7.77±0.27	*
	a-C15:0	5.73±0.17	4.76±0.11	*
	i-C16:0	3.21±0.06	2.78±0.13	*
	i-C17:0	3.64±0.08	3.68±0.05	
Actinomycetales	10Me-C16:0	1.89±0.05	1.45±0.04	*
	10Me-C17:0	2.23±0.04	2.16±0.06	
	10Me-C18:0	0.22±0.02	0.82±0.25	*
Non-specific bacteria	C14:0	1.22±0.05	0.91±0.02	*
	C15:0	0.93±0.03	0.83±0.02	*
	C16:0	18.6±0.21	17.66±0.12	*
	C17:0	0.53±0.02	0.56±0.02	
	C18:0	4.40±0.07	4.05±0.09	*
Gram negative bacteria	**C16:1c9**	5.00±0.21	6.15±0.23	*
	C16:1t9	1.85±0.24	2.22±0.07	
	C17:0cy	4.26±0.15	5.53±0.17	*
	C18:1c11	7.77±0.23	7.30±0.27	
	C19:0cy	10.61±0.24	12.11±0.39	*
	10Me-C16:0	1.89±0.05	1.45±0.04	*
	10Me-C17:0	2.23±0.04	2.16±0.06	
	10Me-C18:0	0.22±0.02	0.82±0.25	*
				*
Fungi	C18:1c9	6.88±0.27	9.39±0.66	
	C18:2n9,12	9.95±1.32	7.83±0.35	
Arbuscular mycorrhiza	C16:1c11	1.91±0.19	2.02±0.08	

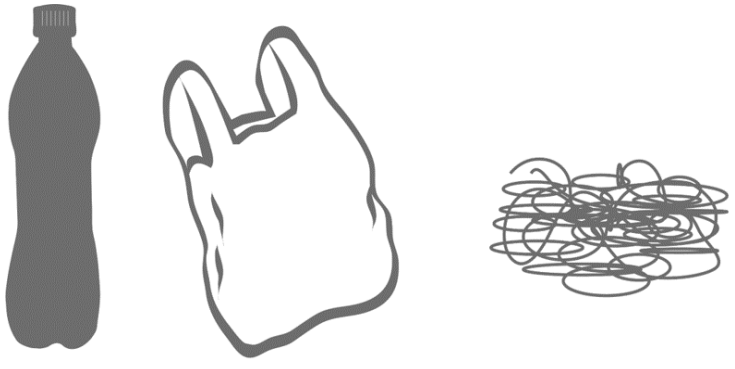
Table AI4.3 Functional GO terms that significantly increased or decreased in relative abundance due to the addition of chitin to the potting soil. Only those GO terms which were at least doubled or reduced by half due to chitin addition are represented. For several GO terms, the relation with a particular pathway or process the function is related to is given. The remark is made that these GO terms can also be related to several other processes.

Category	GO term	Description	Increase/ decrease	Function related to
Biological process	GO:0006816	calcium-dependent cysteine-type endopeptidase activity	8.6	
	GO:0006816	calcium-dependent phospholipid binding	8.6	Microbial growth
	GO:0006031	chitin biosynthetic process	8.0	Chitin degradation
	GO:0006030	chitin synthase activity	4.5	Chitin degradation
	GO:0006030	chitinase activity	4.5	Chitin degradation
	GO:0006030	chitosanase activity	4.5	Chitin degradation
	GO:0034229	eukaryotic translation initiation factor 3 complex	4.0	
	GO:0006338	chromatin remodeling	3.7	
	GO:0016255	attachment of GPI anchor to protein	3.5	
	GO:0051016	barbed-end actin filament capping	3.0	
	GO:0006820	anion transport	2.9	
	GO:0006887	exocytosis	2.9	
	GO:0006887	exodeoxyribonuclease I activity	2.9	
	GO:0007059	chromosome segregation	2.7	
	GO:0015743	malate transport	2.7	
	GO:0016573	histone acetyltransferase activity	2.7	
	GO:0016573	histone binding	2.7	
	GO:0000077	DNA helicase activity	2.6	
	GO:0006691	L-fucose isomerase activity	2.6	
	GO:0034755	iron ion transmembrane transport	2.5	Microbial growth
	GO:0006888	ER to Golgi vesicle-mediated transport	2.5	
	GO:0006075	(1->3)-beta-D-glucan biosynthetic process	2.5	Cell wall / microbial growth
	GO:0030261	chromosome condensation	2.3	
	GO:0007016	cytoskeleton	2.3	
	GO:0006488	dolichol-linked oligosaccharide biosynthetic process	2.2	
	GO:0034314	Arp2/3 complex-mediated actin nucleation	2.1	
	GO:0006032	chitin catabolic process	2.1	Chitin degradation
	GO:0034314	Arp2/3 protein complex	2.1	
	GO:0006914	autophagy	2.0	
	GO:0030245	cellulose catabolic process	2.0	Chitin degradation
	GO:0006869	lipid transport	2.0	
	GO:0006869	lipid transporter activity	2.0	
	GO:0006869	lipid-A-disaccharide synthase activity	2.0	
Molecular function	GO:0017022	myosin complex	15.8	
	GO:0003721	telomere maintenance	7.1	
	GO:0016286	small GTPase mediated signal transduction	6.3	
	GO:0030570	pectate lyase activity	4.3	
	GO:0030570	pectin catabolic process	4.3	
	GO:0030247	polysaccharide binding	3.8	

GO:0030247	polysaccharide biosynthetic process	3.8	
GO:0016296	p-aminobenzoyl-glutamate transmembrane transport	3.8	
GO:0019904	protein domain specific binding	3.6	
GO:0019904	protein glycosylation	3.6	
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	3.2	
GO:0019887	protein kinase regulator activity	3.1	
GO:0019799	type II protein secretion system complex	3.0	
GO:0031369	translation initiation factor binding	2.8	
GO:0019901	protein kinase binding	2.6	
GO:0019901	protein kinase CK2 complex	2.6	
GO:0004864	protein phosphatase type 2A complex	2.6	
GO:0004652	polynucleotide adenylyltransferase activity	2.5	
GO:0015558	p-aminobenzoyl-glutamate uptake transmembrane transporter activity	2.4	
GO:0001104	RNA polymerase II transcription cofactor activity	2.4	
GO:0004843	thiol-dependent ubiquitin-specific protease activity	2.4	
GO:0035091	phosphatidylinositol binding	2.3	
GO:0008536	Ran GTPase binding	2.3	
GO:0003980	UDP-glucose:glycoprotein glucosyltransferase activity	2.3	
GO:0035091	phosphatidylinositol metabolic process	2.3	
GO:0009881	photosynthesis	2.3	Photosynthesis
GO:0009881	photosynthetic electron transport chain	2.3	Photosynthesis
GO:0009881	photosynthetic electron transport in photosystem II	2.3	Photosynthesis
GO:0009881	phytochelatin biosynthetic process	2.3	
GO:0009881	photosystem	2.3	Photosynthesis
GO:0009881	photosystem I	2.3	Photosyntheis
GO:0008017	microtubule binding	2.2	
GO:0016730	oxidoreductase activity, acting on iron-sulfur proteins as donors	2.2	
GO:0008017	microtubule cytoskeleton organization	2.2	
GO:0003777	microtubule motor activity	2.1	
GO:0005200	structural constituent of cytoskeleton	2.1	
GO:0043130	ubiquitin binding	2.1	
GO:0003777	microtubule nucleation	2.1	
GO:0003777	microtubule-based movement	2.1	
GO:0003777	microtubule-based process	2.1	
GO:0003777	microtubule organizing center	2.1	
GO:0043130	ubiquitin ligase complex	2.1	
GO:0016307	phosphatidylinositol phosphate kinase activity	2.0	
GO:0034450	ubiquitin-ubiquitin ligase activity	2.0	
GO:0047487	oligogalacturonide lyase activity	0.5	
GO:0047487	oligopeptide transport	0.5	
GO:0004846	urea transmembrane transport	0.3	

Part II

The Plastisphere



Chapter 5

Bacterial community profiling of marine plastic debris in the North Sea

Modified from:

De Tender CA, Devriese LI, Haegeman A, Maes S, Ruttink T, Dawyndt P. (2015). Bacterial community profiling of plastic litter in the Belgian part of the North Sea. Environmental Science and Technolology, 49, 9629-9638.

&

De Tender C, Schlundt C, Devriese LI, Mincer TJ, Zettler ER, Amaral-Zettler LA. A review of microscopy and comparative molecular-based methods to characterize "Plastisphere" communities. Analytical methods 9,2132-2143.

Plastic debris, an inevitable consequence of living the 'Plastic Age', is dominating our oceans and seas and poses a worldwide threat to aquatic wildlife (Thompson et al., 2009). It provides novel aquatic vehicles for a wide range of rafting species, such as microalgae, Bryozoa, insects and even macrobenthos, posing a threat to introduce invasive species (Aliani & Molcard, 2003; Barnes & Miller, 2005; Gregory, 2009). However, not only rafting species are able to colonise plastic as a transport vehicle, but also bacteria live the 'Plastic Age'. The presence of micro-organisms on plastic debris in the marine environment was first documented in 1972, when diatoms of the Sargasso Sea were identified on plastic fragments and rod shaped Gram-negative bacteria were isolated of polystyrene spherules (Carpenter & Smith, 1972; Carpenter et al., 1972). Microbial colonization of these plastic particles in a marine environment occurs relatively fast, varies with substrate type and is influenced by the season and geographical location (Lobelle & Cunliffe, 2011; Zettler et al., 2013; Harrison et al., 2014; Oberbeckmann et al., 2014).

It has been shown that the bacterial community composition of marine plastic litter (MPL) is significant different from the surrounding seawater, which suggests that plastic debris is a distinct microbial habitat, further referred to as "the plastisphere" (Zettler et al., 2013). Still, the microbial community may reflect its direct environment (seawater, sediment) and research is needed to define the origin and preferences of bacterial families. The characterization of the microbial communities on plastic is essential for several reasons. First, comparison of the microbial community and its surrounding environment (seawater, sediment) is needed to define the origin of bacterial colonization. Second, the response of a bacterial community to environmental factors may help elucidate the drivers of colonization. Third, the capacity to metabolize plastic or plastic-associated chemical compounds as a nutritional source could give certain species adaptive advantages, thus selecting for specific bacterial communities (Harrison et al., 2011). Fourth, plastic may serve as a transport vehicle for bacteria, including pathogens, that become associated with the biofilm and can be transported to novel environments where they do not normally occur (Osborn & Stojkovic, 2014).

Previous studies of the bacterial community on MPL focused predominantly on particles floating near the sea surface. However, the vast majority of plastic debris accumulates in the sediment, particularly in coastal areas (Moore, 2008; Claessens et al., 2011; Harrison et al.,

2014). For instance, it is estimated that the vast majority of debris entering the North Sea area will eventually sink to the seafloor (70%) while only a minor part keeps floating (15%) or is deposited on beaches (15%) (UNEP, 2005). Moreover the major part of the sunken debris in the Belgian part of the North Sea (95%) is comprised of plastic (Van Cauwenberghe et al., 2013).

In this chapter, we studied the bacterial diversity and community composition of plastic debris samples at the seafloor at five locations across the Belgian part of the North Sea. These plastics are further referred to as Marine Plastic Litter (MPL). The bacterial diversity was investigated using 16S rRNA gene amplicon sequencing and compared with those of resin pellets found on the beach (here called beach pellets) and bacterial communities of the surrounding (sediment, seawater) and broad (seawater) environment. In addition, we compared the bacterial communities of MPL of the North Sea with those sampled in the North Atlantic (Zettler et al., 2013; Amaral-Zettler, 2015) and the Pacific (Amaral-Zettler et al., 2015) making use of the Visual Analysis of Microbial Population Structure (VAMPS) website (Huse et al., 2014).

5.1 MATERIALS AND METHODS

Within this section, an overview is given of the collection and characterisation of plastic, sediment and seawater in the Belgian part of the North Sea (5.1.1-5.1.2), the bacterial community profiling of the samples (5.1.3), the statistical analysis of the amplicon sequencing data (5.1.4), and the usage of the VAMPS website (5.1.5).

5.1.1 Sample collection

All samples were collected in 2014 at the Belgian part of the North Sea. In March, three sites (NP (51.16°N; 2.71°E), OO (51.22°N; 2.85°E), ZB (51.33°N; 3.13°E)) near the mouths of the Belgian coastal harbours were selected for plastic, sediment and seawater sampling. In August, sampling at these locations was repeated for plastic and sediment collection, and two additional locations were added for plastic collection (OObis (51.45°N; 3.23°E), ZBbis (51.45°N; 2.61°E)). Seasonal variation will not be studied here, because only two sampling dates were used for sampling, and the samples can thus be seen as independent. Additionally, seawater was sampled in June at fourteen different locations to create a broader environmental context (Figure 5.1).

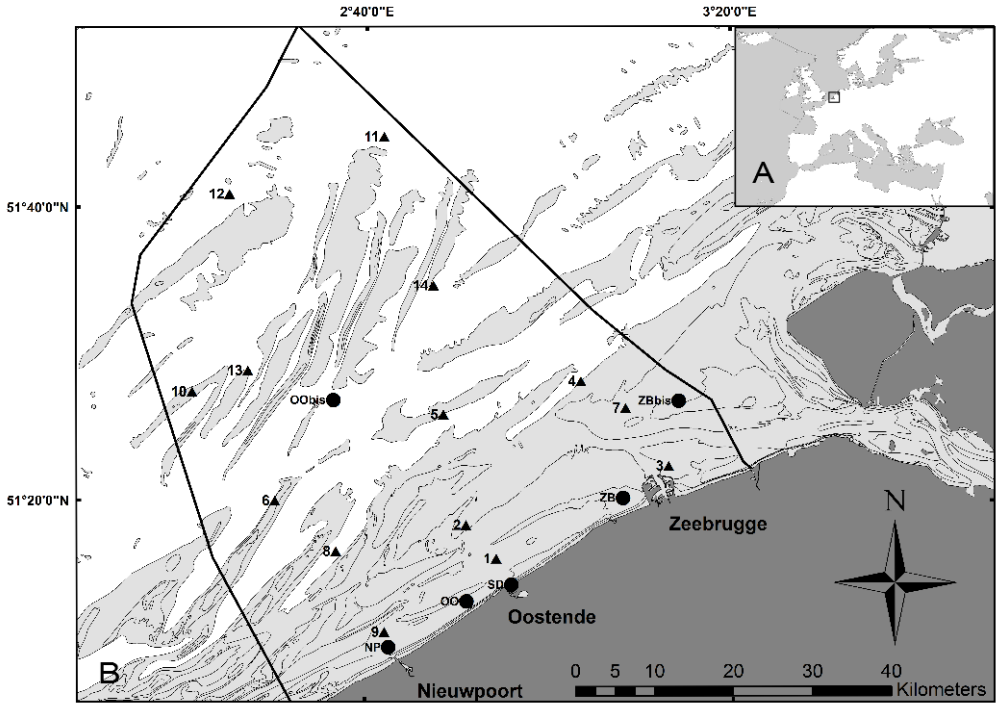


Figure 5.1 Sample collection sites at the Belgian part of the North Sea. A) Inset overview of Europe and the European waters. The Belgian Part of the North Sea (BPNS) is indicated by a square box. B) Map of the Belgian coastline and the Belgian part of the North Sea. Plastic, sediment and seawater were sampled at the coastal areas near Nieuwpoort (NP), Oostende (OO) and Zeebrugge (ZB). Plastics were sampled at two, more off-shore, additional locations (OObis, ZBbis). Beach pellets were sampled at the Spinoladijk (SD). Sample sites for community profiling of the Belgian seawater in June are numbered 1 to 14.

Plastic fragments located on top of the sediment were collected using a beam trawl equipped with a fine-meshed shrimp net with mesh size of 12 mm and a width of 3 m. Individual plastic pieces (> 25 mm) were sorted with sterile forceps, individually placed in a sterile 15 mL falcon tube and immediately frozen at -20 °C.

Per location, sediment samples were collected using three replicate Van Veen grabs. The upper (0 to 5 cm) layer of the Van Veen grab content was collected and 40 mL of this sediment was sampled in a sterile 50 mL falcon tube.

Water samples were taken 1 m below the water surface and on the seafloor using a carrousel of six 4 L Niskin bottles. Per replicate, 1 L seawater was filtered through a 0.22 µm Millipore membrane filter (Merck Millipore, Billerica, MA, USA; samples March), or through a sterivex filter (Merck Millipore, Billerica, MA, USA; samples June). Per location two surface water and two seafloor water samples were collected. When samples were not immediately used for DNA extraction, sediment samples and membrane filters were stored at -20 °C.

Resin pellets (< 5 mm) found on the beach (here called beach pellets) were collected at the Spinoladijk (SD) in Oostende (Figure 5.1). The beach pellets were picked up with sterile forceps and stored per two (based on colour) in a sterile 15 mL falcon tube at -20 °C until further use. Pellets stored together were combined as one sample for further processing.

5.1.2 Sample characteristics

Physicochemical characteristics for sediment and seawater samples were recorded per location (Additional Information (AI) Table AI5.1). Sediment samples were categorised per sampling location and date. Sediment organic matter or total organic carbon (TOC) on the upper sediment layer (0-5 cm) was measured using the 'dichromate method' (Mebius et al., 1960). Carbonate content was measured on the same sediment fraction as 'loss on ignition' (Dean, 1974). Grain size distribution was estimated using laser diffraction particle sizing and expressed as median grain size. All samples were analysed using a Malvern Mastersizer 2000G hydro version 5.40 (Malvern, Operators guide MAN0247, issue 2.0. 1999). Grain size fractions were determined as volume percentages according to the Wentworth scale (Wentworth, 1922). Throughout this study, the clay and silt fractions have been combined as clay/silt (<63 µm) (Table AI5.1).

Water samples were categorized per sampling location and date. Environmental properties were measured using the CTD SBE-19plus (Table AI5.1).

Plastic properties were categorized based on sampling location or date, plastic shape (monofilament, sheets or beach pellets) and colour (Table 5.1). Raman spectra for polymer identification were recorded using a Bruker Optics 'Senterra' dispersive Raman spectrometer with a BX51 microscope by the Raman Spectroscopy Research Group (UGent, Belgium). Measurements were performed using a red diode laser (785 nm), an aperture of 50 µm and the 20x objective lens with a spot size of approximately 10 µm on the sample. The system uses a thermoelectrically cooled CCD detector, operating at -65 °C. The instrument is controlled by OPUS® software, version 7.2. The power of the laser can be set up to 37 mW at the sample for the 785 nm laser. Number of accumulations, measuring time and laser power were set at 60 times, 30 seconds and 15.4 mW, respectively, to obtain good signal to noise ratio.

In addition, the bacterial communities of the MPL samples sampled at the North Sea were compared with those of the North Atlantic and the Pacific, sampled by the group of Amaral-Zettler (Zetller et al., 2013; Amaral-Zettler et al., 2015) (section 5.1.5). An overview of the metadata of plastic debris of all projects is given in Table AI5.2.

Table 5.1 Plastic properties of samples collected at different locations across the Belgian part of the North Sea. Macroplastic particles are indicated as marine plastic litter (MPL), microplastics as beach pellets (BP).

Location	Number	Sampling Date	Polymer type	Litter type	Colour	Detected pigment	Depth (m)
SD	BP 1	10/03/'14	PE	Beach pellet	Blue	/	0.0
	BP 2	10/03/'14	PE	Beach pellet	Yellow	/	0.0
	BP 3	10/03/'14	PE	Beach pellet	White	/	0.0
	BP 4	10/03/'14	PE	Beach pellet	Black	/	0.0
OO	MPL 1	05/03/'14	PE	Monofilament	Blue	PB15	8.5
	MPL 2*	05/03/'14	PE	Sheet	Blue	/	8.5
	MPL 3	05/03/'14	PE	Monofilament	Orange	/	8.5
	MPL 4	01/09/'14	PE	Monofilament	Blue	PB15	8.5
	MPL 5*	01/09/'14	PE	Monofilament	Blue	/	8.5
	MPL 6*	01/09/'14	/	Monofilament	White	/	8.5
	MPL 7*	01/09/'14	/	Monofilament	Black	/	8.5
NP	MPL 8	05/03/'14	PE	Monofilament	Blue	PB15	6.5
	MPL 9	05/03/'14	PE	Monofilament	Orange	/	6.5
	MPL 10	05/03/'14	PE	Sheet	Transparant	/	6.5
	MPL 11	28/08/'14	PE	Monofilament	Blue	PB15	7.2
	MPL 12*	28/08/'14	PE	Monofilament	Blue	PB15	7.2
ZB	MPL 13	06/03/'14	PE	Monofilament	Blue	PB15	6.5
	MOL 14	29/08'14	PE	Monofilament	Blue	PB15	6.7
	MPL 15	29/08/'14	PE	Monofilament	Blue	PB15	6.7
	MPL 16	29/08/'14	PE	Monofilament	Orange	/	6.7
	MPL 17	29/08/'14	PE	Monofilament	Orange	/	6.7
	MPL 18	29/08/'14	PE	Sheet	Transparant	/	6.7
ZBbis	MPL 19	29/08/'14	PE	Monofilament	Blue	/	6.5
	MPL 20	29/08/'14	PE	Monofilament	Orange	/	6.5
OObis	MPL 21	01/09/'14	PP	Monofilament	Blue	/	31.3
	MPL 22	01/09/'14	PE	Monofilament	Orange	/	31.3

**Samples excluded from analysis due to too low sequencing depth;*

"/" indicates "no information was available for this piece of plastic"

5.1.3 "Plastisphere" microbiology: high throughput sequencing

DNA of sediment and plastic samples was extracted using the Powersoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Due to a relatively low yield (< 5 ng/µL), DNA retrieved from the plastic samples was eluted in 50 µL elution buffer instead of 100 µl. If plastic particles were large, a piece of 15 cm (monofilament) or with a surface of 16 cm^2 (sheet) was cut off and used for DNA extraction. Otherwise the total sample was used and only a small fragment was kept for polymer type determination. 250 mg sediment was used for DNA extraction.

DNA extraction of the Millipore filters (containing the microbial community of seawater) was done according to the protocol of Amaral-Zettler (2015a). The Gentra Puregene kit (QIAGEN, Germantown, MD, USA), lytic enzyme (QIAGEN, Germantown, MD, USA) and proteinase K (QIAGEN, Germantown, MD, USA) were used for DNA extraction, comprising two incubation steps of 37 °C for 30 minutes and 65 °C for one hour.

16S rRNA gene amplicon sequencing

Illumina amplicon sequencing of the bacterial "plastisphere" communities was done on the V3-V4 fragment of the 16S rRNA gene using Illumina technology (Illumina, San Diego, CA, USA). Using an amplification and dual-index PCR successively, fragments were amplified and extended with Illumina specific adaptors, which is described in detail in Chapter 2 section 2.1.8. Each PCR step was followed by a PCR product clean-up using the CleanPCR reagent kit (MAGBIO, Gaithersburg, MD, USA).

Quality control of the final library samples was done using the Qiaxcel Advanced using the Qiaxcel DNA High Resolution kit (QIAGEN, Germantown, MD, USA). Concentration was measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The final barcoded libraries of each sample were diluted to 10 nM and pooled in equal amounts. The resulting libraries were sequenced using Illumina MiSeq v3 technology (2 x 300bp, paired-end) by the Nucleomics Core, Leuven, Belgium using 30% PhiX DNA as spike-in.

Sequence reads processing

The dataset was demultiplexed by the sequencing provider and barcodes were clipped off the reads. The raw sequence data is available in NCBI's Sequence Read Archive under the accession number PRJNA272679. Primers were removed using Trimmomatic v0.32 (Bolger et al., 2014). Different programs of the USEARCH software v7.0.1090 were used for the following

steps (Edgar, 2010). Forward and reverse reads were merged using a minimum overlap length of 40 bp (with a maximum of 15 bp differences) and a minimum resulting length of 350 bp using the "fastq_mergepairs" program. The resulting sequences were quality filtered using "fastq_filter" with a maximum expected error of 3. Next, sequences of all samples that needed to be compared to each other were merged, dereplicated ("derep_fulllength") and sorted by size ("sortbysize"). UPARSE ("cluster_otus") was used for clustering the reads into Operational Taxonomic Units (OTUs) at 97% identity level (Edgar, 2013). Chimeras were removed using UCHIME ("uchime_ref") with the RDP Gold database as a reference (Edgar et al., 2011). Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm (97% identity) and converted to an OTU table using "biom convert" (McDonald et al., 2012). This procedure resulted in an average of 59,962 sequences per sample with an average length of 420 bp (112 samples in total).

5.1.4 Downstream data analysis and statistics

OTU tables were analysed with the QIIME software package (v1.8.0) (Caporaso et al., 2010a). Representative OTU sequences were aligned to the Greengenes (DeSantis et al., 2006) 97 % core OTU set (v13_8) with a minimum percent identity of 97 % using the PyNast algorithm (Caporaso et al., 2010b) with QIIME default parameters. Rarefaction analyses were done using an upper rarefaction depth of 20,000 sequences, indicating that only samples with a minimal sequence count of 10,000 were retained for further analyses (Figure 5.2). Based on this criterion, data of five seawater samples (location 7 and 10; Figure 5.1) and five plastic samples (MPL2, MPL5, MPL6, MPL7, MPL12, Table 5.1) were not used in the downstream analyses.

Throughout this study, only OTUs representing at least 0.01% of the total community in at least one sample were used for the downstream analysis.

The number of observed OTUs and the Shannon-Wiener indices, which represents the bacterial richness and diversity, were determined on the rarefaction depth of 20,000 sequences. ANOVA analyses of the Chao1 richness and Shannon-Wiener diversity indices were done and differences between sampling groups were analysed using the Tukey HSD test. These analyses and the construction of segmented bar charts were done with the basic R program version 3.1.0 (R core team, 2015).

The multivariate analysis of the data was done using the R package vegan (version 2.0-10). To study multivariate spread, the betadisper function was used (Oksanen et al., 2010). If

multivariate homogeneity of group dispersions was fulfilled, differences between community types were analysed using PERMANOVA analysis on 4 a priori defined groups according to the sample origin: beach pellets, MPL, seawater and sediment. These significances were further visualized by constructing non-Metric Multidimensional Scaling (nMDS) plots, using the Bray-Curtis index as dissimilarity index. The vegan package was also used to fit environmental variables to the ordination plot (function envfit). P-values of the environmental variables were calculated by permutation and only the variables with a significant difference ($p<0.05$) were fitted on the plot.

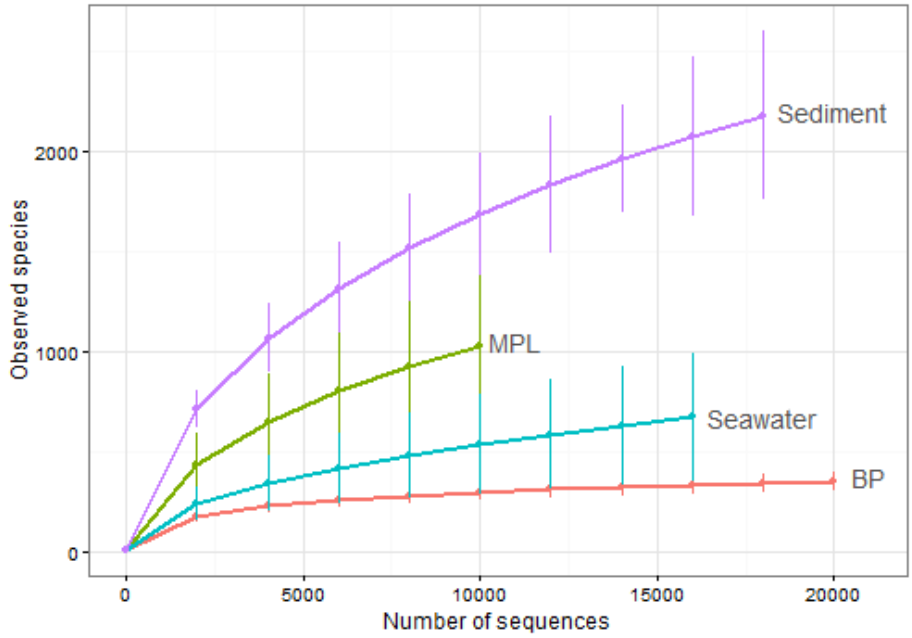


Figure 5.2 Rarefaction curves of microbial communities on beach pellets (indicated as BP) (red), seawater (surface and seafloor; blue), plastics (green) and sediment (purple) (mean ± standard error). Analysis was done using an upper rarefaction depth of 20,000 sequences.

To measure similarity in OTU presence between the MPL samples, the Jaccard similarity index was calculated. These Jaccard overlaps in pairwise comparisons were displayed as a heatmap (Jaccard, 1912).

To search for core sets of OTUs that are shared across a number of plastic samples we used Corbata (Li et al., 2013). Core members are identified as the OTUs needed to be present in at least 95% of the samples. In parallel, QIIME was used to define a core microbiome and the OTUs present in each sample, where we varied the minimum abundance. Results of both methods were similar.

5.1.5 The VAMPS Plastisphere Portal

To compare the bacterial communities of MPL sampled in the Belgian part of the North Sea with those of other regions, samples were uploaded at the VAMPS website. VAMPS provides an intuitive and interactive platform for the study of microbial communities and relationships between communities. Over 16,915 open-access datasets are already available on VAMPS, including sequences retrieved from marine (micro)plastic debris. Here we compared our dataset with two recently uploaded datasets of the North Atlantic and Pacific Ocean (Zettler et al., 2013; Amaral-Zettler et al., 2015b). These data were differentially sequenced targeting either V3-V4 (see section 4.1.3) or the V6 (Zettler et al., 2013; Amaral-Zettler et al., 2015) hypervariable regions of the 16S rRNA gene. In addition, either high-throughput Illumina (section 4.1.3; Amaral-Zettler et al., 2015) or amplicon pyrotag sequencing (Zettler et al., 2013) on the Roche 454 platform was used. Since the projects differed in many methodological repects, the comparison between bacterial communities was restricted to taxonomy based comparisons and community membership alone.

The uploaded sequences are first quality checked and then run through Global Alignment for Sequence Taxonomy (GAST) (Huse et al., 2014) to assign taxonomy basd on a curated database of rRNA sequences drawing primarily from the SILVA SSU rRNA database (Quast et al., 2013) and associated taxonomy (Yilmaz et al., 2014) prior to availability on the VAMPS platform. Using VAMPS, the microbial communities were analysed at multiple taxonomic and abundance levels. For each selected taxon, VAMPS also returned the underlying sequence data. For visualisation and comparison, the different taxa of each sample were normalized by percent in relation to the entire community abundance. The data on the VAMPS portal is located under the project code “LAZ_DET_Bv3v4”.

5.2 RESULTS

In this section, first the bacterial community of MPL sampled at the Belgian part of the North Sea, compared to seawater and sediment communities, is studied (5.2.1). This study revealed a high diversity in bacterial community composition between plastics, by which we proposed that three factors possibly influence the colonization: environmental properties (5.2.2), plastic-related properties (5.2.3) and biofilm formation stages (5.2.4). In a last part, these bacterial communities of MPL of the North Sea were compared with those of the North Atlantic and Pacific Ocean sampled by the Amaral-Zettler group (5.2.5).

5.2.1 Bacterial community structure

Both the bacterial community complexity and composition of the plastics (beach pellets and MPL), seawater and sediment were analysed. Bacterial community complexity was studied by estimating the total number of observed species and by estimating the Chao1 and Shannon-Wiener indices, representing the bacterial richness and diversity. At 10,000 sequence counts, rarefaction curves showed an average of 295, 535, 1,031 and 1,688 different OTUs of beach pellets, seawater, MPL and sediment, respectively (Figure 5.2; Table 5.2), indicating variance in the number of unique species between sample types. Significant differences in the Chao1 richness (ANOVA, p-value: 1.22e-06) and Shannon-Wiener diversity (ANOVA, p-value: 2.47e-08) indices, proved community complexity differences between the four sample types. Bacterial richness and diversity were significantly different between all sample types, with the exception of seawater communities, which showed similar diversity as those of MPL (Tukey range test, p-value: 0.92) and beach pellets (Tukey range test, p-value: 0.08) and similar richness values (p-value: 0.98) as sediment communities. The highest community richness and diversity was measured in the sediment, after which MPL contained the second most diverse community (Table 5.2).

Table 5.2 Estimation of the Chao1 richness and Shannon-Wiener diversity indices for the different sample types.

Sample Type	Observed OTUs	Chao 1 Richness index	Shannon-Wiener Diversity Index
Beach pellet	295±48 (a)	572±156 (a)	5.24±1.06 (a)
MPL	1031±370 (b)	2535±853 (b)	6.99±1.56 (b)
Sediment	1688±421 (c)	3952±990 (c)	8.87±0.51 (c)
Seawater	535±321 (ab)	3819±1621 (c)	6.74±0.73 (ab)

Significant differences in Bray-Curtis dissimilarity indices (PERMANOVA, $p<0.001$) showed differences in bacterial community composition between the different sample types, although these differences in dissimilarity could partly be caused by a considerable difference in multivariate spread (permutation based, $p<0.001$). This was confirmed in a nMDS plot, where beach pellets, MPL, seawater and sediment bacterial communities showed a separate clustering, with no observed overlap between the sample types (Figure 5.3). In addition, the cluster width of the bacterial communities of MPL was large compared to other sample types, indicating a high variability between MPL bacterial communities. In comparison, the bacterial communities of seawater sampled in June, representing the 'broad' environmental scan across the Belgian part of the North Sea, clustered together with bacterial communities of seawater sampled at the time and location of plastic sampling (surrounding environment).

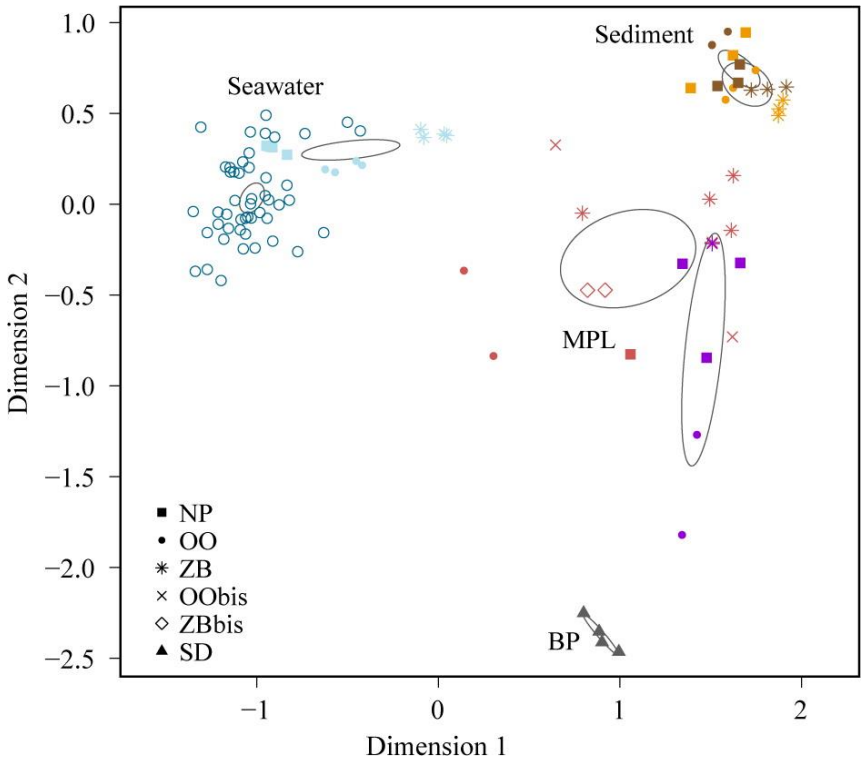


Figure 5.3 Non-Metric Multidimensional Scaling (nMDS) profile of pairwise community dissimilarity (Bray–Curtis) indices of 16S rRNA gene sequencing data of samples collected across the Belgian part of the North Sea (Dimensions: 4; Stress: 0.095). 95% confidence ellipses were constructed for each sample type. Shape represents different sampling locations. Seawater samples are indicated in light (sampled in March) and dark blue (sampled in June). Sediment samples are indicated in yellow (light: March, dark: August). MPL sampled in March and August are coloured in red and purple, respectively. BP: Beach pellets.

To define which bacterial groups determine these differences in MPL bacterial profiles compared to the environment (sediment, seawater), the taxonomic profiles on phylum and

family level between the sample types were studied (Figure 5.4 and Figure 5.5). A high variability between plastic bacterial communities of MPL and beach pellets was observed (Figure 5.3), by which we decided to visualize the taxonomic profiles of individual MPL and beach pellets. Community profiles of seawater and sediment samples were grouped per sampling location and date. MPL, seawater and sediment samples mainly consist of *Proteobacteria* and *Bacteroidetes*. Beach pellets on the other hand had a quite different composition compared to MPL, seawater and sediment. This is mainly caused by a high abundance of *Actinobacteria* on beach pellets, whereas *Proteobacteria* dominated the other sample types (Figure 5.4).

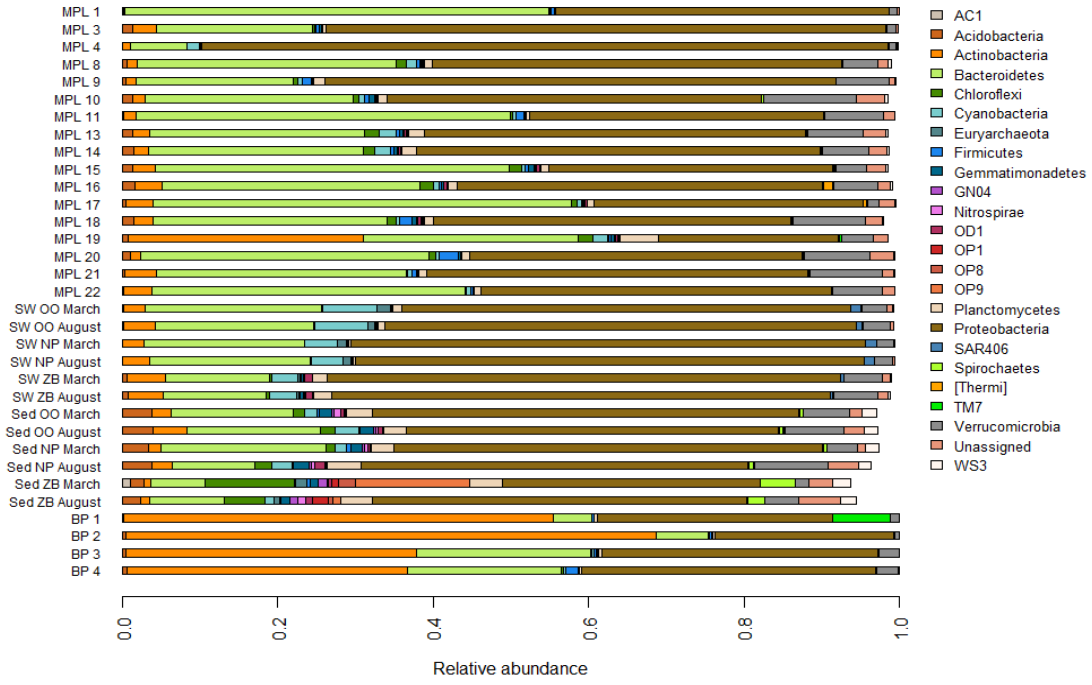


Figure 5.4 Bacterial and archaeal phyla of the plastic samples compared to the bacterial profiles of water, sediment and beach pellets. Phyla that represent at least 1% in at least one sample are shown. OTUs that could not be assigned to a specific taxon were bundled in the group "Unassigned".

On family level, especially a high abundance of *Flavobacteriaceae* was noticed on MPL, which was less prominent for sediment and seawater communities. Strikingly, most of the bacterial families found on MPL were also found in seawater and/or sediment, but with clear differences in relative abundances (Figure 5.5), causing accordingly part of the separation in sample types in the nMDS plot. Besides those shared bacterial families between MPL and their surrounding environment, certain bacterial groups, e.g. *Vibrionaceae* or *Pseudo-*

alteromonadaceae, were commonly detected on MPL but barely observed in seawater and sediment communities (Figure 5.5).

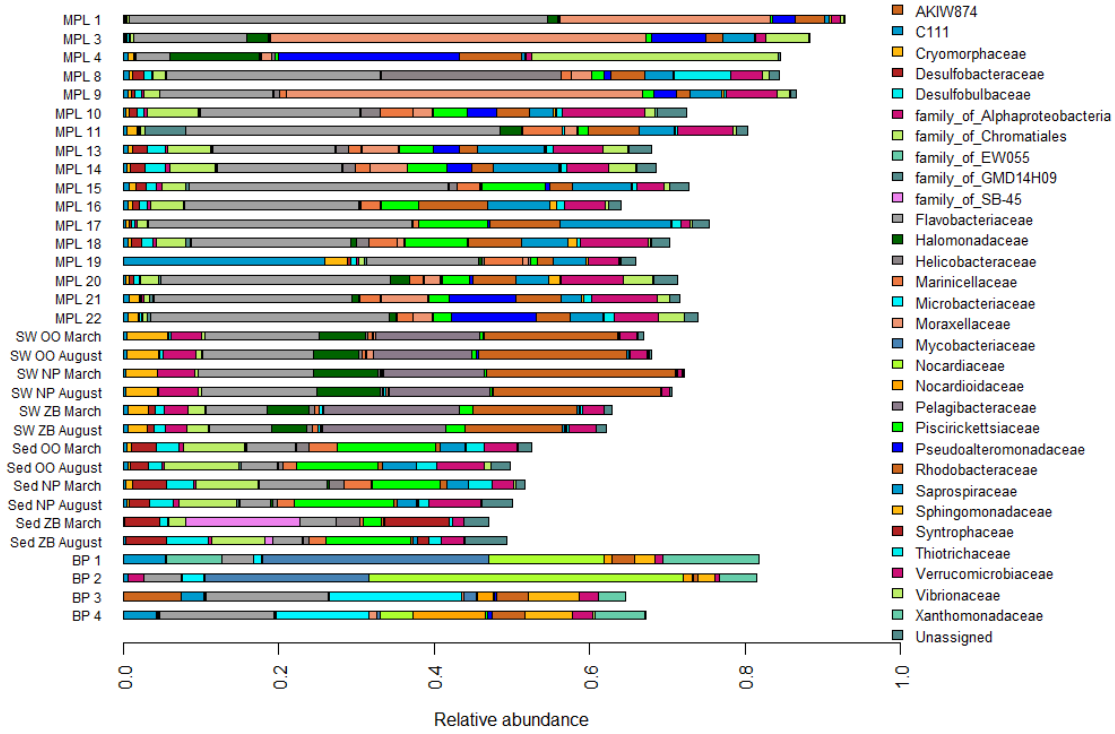


Figure 5.5 Bacterial families of the plastic samples compared to the profiles of seawater, sediment and beach pellets. Only the 25 most dominant families are included by selecting the families having a relative abundance of at least 1 % in at least one sample. OTUs that could not be assigned to a specific taxon were bundled in the group "Unassigned".

Based on the community composition on family level, similarities between MPL bacterial communities seemed to be present. Therefore, we tried to establish a core microbiome based on the OTU table. However, to obtain core members, minimum OTU abundances need to be set at a very low percentage (0.01%), resulting in thirty-six OTUs that could be defined as "core organisms" (Table 5.3). Most of these OTUs could not be classified until genus level and remained unclassified. Conversely, together these only represent approximately 18 percent of the relative abundance of all OTUs, showing that the vast majority of the OTU`s is not common.

5.2.2 Correlation of environmental properties and MPL bacterial communities

To study if any spatio-temporal structure is correlated with bacterial community composition, relatedness between the taxonomic profiles of the 17 MPL samples was visualized in a heatmap, using the Jaccard similarity index for pairwise comparisons. Samples were ordered

according to sampling location to visualize whether samples with a high fraction of shared OTUs were derived from the same location (Figure 5.6).

Table 5.3 "Core" organisms of MPL samples sampled in the Belgian part of the North Sea. In total, 36 OTUs could be defined as "core" organisms, with a minimum cut-off value of 0.01% in which the OTUs should be present in at least 95% of the samples. For each OTU the miniman and maximum relative abundance (%) it was present on one plastic sample is given.

OTU	Phylum	Class	Family	Genus	Minimum (%)	Maximum (%)
28	Actinobacteria	Acidimicrobiia	C111	Unassigned	0.02	0.32
178	Bacteroidetes	Flavobacteriia	Flavobacteriaceae	Unassigned	0.03	0.86
250				*Lutimonas*	0.05	3.02
33				Unassigned	0.02	2.44
388				*Gramella*	0.02	15.6
440				*Polaribacter*	0.02	5.30
559				Unassigned	0.02	3.90
5760				*Lutimonas*	0.03	0.69
7892				Unassigned	0.03	3.33
92				*Robiginitalea*	0.01	1.41
6385	Cyanobacteria	Chloroplast	Unknown	Unassigned	0.02	0.82
64				Unassigned	0.03	1.54
109	Proteobacteria	Alphaproteobacteria	Rhodobacteraceae	Unassigned	0.06	3.01
12				Unassigned	0.02	2.78
1503				*Phaeobacter*	0.04	1.54
35				Unassinged	0.02	0.35
3551				unassigned	0.03	4.09
113			Phyllobacteriaceae	unassigned	0.01	1.52
500		Deltaproteobacteria	Desulfobulbaceae	Unassigned	0.01	0.21
37			Desulfuromonadaceae	Unassigned	0.03	1.02
570			Unknown	Unassigned	0.03	0.35
306		Epsilonproteobacteria	Helicobacteraceae	Unassigned	0.03	22.68
282		Gammaproteobacteria	OM60	Unassigned	0.01	1.46
95				Unassigned	0.02	0.31
1782			Unknown	Unassinged	0.01	0.85
2399			Unknown	Unassigned	0.06	1.94
56			Unknown	Unassigned	0.01	0.23
228			Marinicellaceae	Unassigned	0.01	1.55
543				Unassigned	0.02	1.40
100			Piscirickettsiaceae	Unassigned	0.06	2.78
166				Unassigned	0.05	2.58
3907				Unassigned	0.02	0.81
1147	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiaceae	*Persicirhabdus*	0.03	2.40
3175				*Persicirhabdus*	0.01	1.07
50				*Persicirhabdus*	0.05	1.36
829				*Rubritalea*	0.02	4.51

The highest number of OTUs per sample and the highest number of shared OTUs between samples were mainly detected in the samples of ZB, indicating that location-related properties influence the bacterial composition of MPL. However, for the other locations, similarities between samples was limited, except for OObis (Figure 5.6).

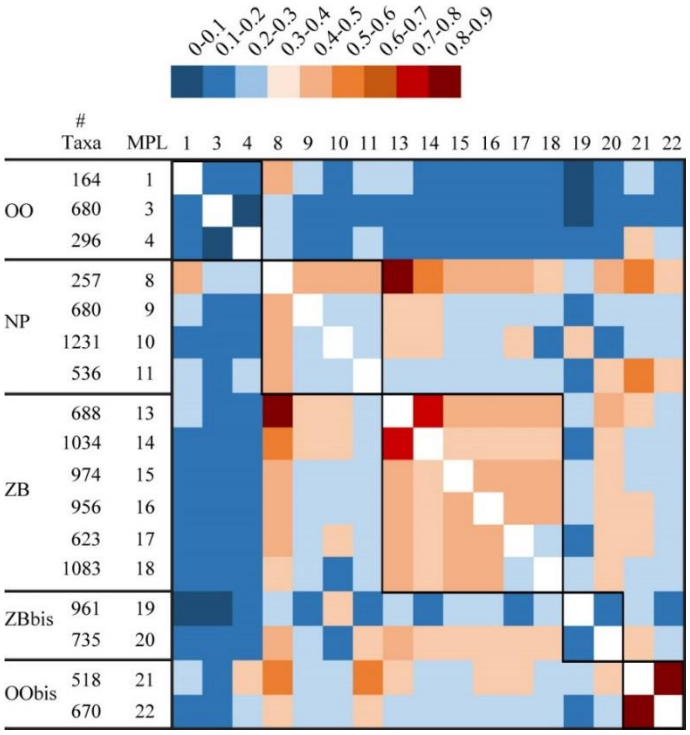


Figure 5.6 Heatmap construction of MPL samples (Table 5.1). Jaccard similarity indices were calculated for all plastic pairs, representing the fraction of shared OTUs between MPL samples. Similarity in OTUs between samples is indicated by a colour scheme (blue: low amount of shared OTUs, red: high amount of shared OTUs). Location of sampling and total number of OTUs that represents more than 0.01% of the sample are indicated next to the heatmap.

For each location, several environmental parameters were measured and considered as possible factors influencing the bacterial colonization of plastic (Table AI5.1). We constructed a nMDS plot of the MPL samples, to which the correlation with significant (p-value <0.05) environmental data of seawater and sediment was fitted (Figure 5.7; Table AI5.1). Differences in salinity, temperature, oxidation reduction potential, turbidity, oxygen content and density of the seawater, and the total organic carbon and inorganic carbon content of sediment appear to be correlated to the diversity in MPL bacterial communities. In this plot, it seems that a higher salinity is negatively correlated with the MPL samples of ZB (13-18; Figure 5.7). In addition, bacterial communities of MPL sampled in March were negatively correlated with temperature.

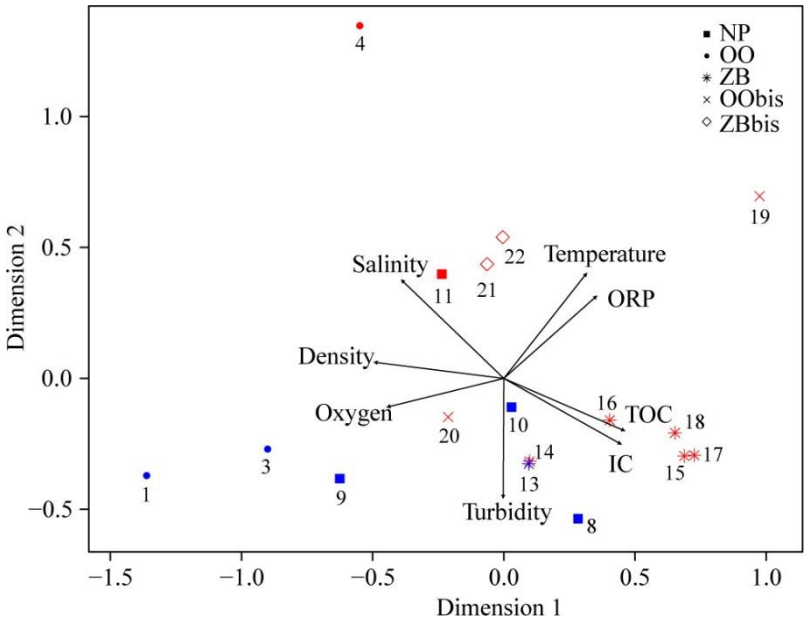


Figure 5.7 Correlation of environmental variables and the nMDS profile of pairwise community dissimilarity (Bray–Curtis) indices of 16S sequencing data of the MPL samples (Dimensions: 4; Stress: 0.053). Only those environmental parameters of seawater and sediment that were significantly different (p-value <0.05) between samples were fitted to the plot, where the length of the arrow is proportional to the correlation (ORP: oxidation reduction potential, TOC: total organic carbon content, IC: inorganic carbon content).

5.2.3 Correlation of plastic-related factors and MPL bacterial communities

We investigated whether factors inherent to the plastic, such as polymer type (e.g. polyethylene (PE) or polypropylene (PP)), plastic shape (monofilament, sheet) and presence of pigment dyes could explain part of the diversity of microbial colonization of plastic particles (Table 5.1). Because sixteen PE samples but only one PP sample were obtained, the influence of polymer type on the bacterial community profile could not be separated from the other factors (Table 5.1; Figure AI5.1; Figure AI5.2). No clear difference in bacterial profile was observed between the PP sample and the PE samples and even a high number of shared OTUs between the PP sample (MPL21) and the most closely related PE sample (MPL22) was observed (Figure 5.4; Figure 5.6). Next, the other plastic properties (shape, pigment dyes) were fitted to the nMDS plot as been done for the environmental factors in 5.2.2. No correlation of any of these parameters was found with the variation in MPL bacterial communities.

In contrast, on beach pellets, colour could be correlated with bacterial community composition. A different taxonomic profile was observed on coloured beach pellets (blue, yellow) compared to uncoloured (black, white) (Figure 5.5). This is at least in part due to a high relative abundance of one species on the coloured resin pellets: *Mycobacterium*

frederiksbergense (21-29%), whereas this genus was rather absent on white and black beach pellets and could not be found on MPL.

5.2.4 Correlation of biofilm formation stages and MPL bacterial communities

MPL community profiles showed a dominance of *Proteobacteria* and *Bacteroidetes* (Figure 5.4). Previous studies have shown that *Alpha*- or *Gammaproteobacteria* are characteristic for primary biofilm colonization in the marine environment and *Bacteroidetes* act as secondary colonizers, as their abundance increases over time (Hörsch et al., 2005; Lee et al., 2008; Elifantz et al., 2013). It is important to note that the actual age of the biofilm on our plastic samples could not be established, due to the unknown history of randomly sampled free-floating plastics. Instead, we estimated the relative abundances of *Alpha*- and *Gammaproteobacteria* and *Bacteroidetes* for each sample, and used these as putative signatures of biofilm formation stages (Figure 5.8).

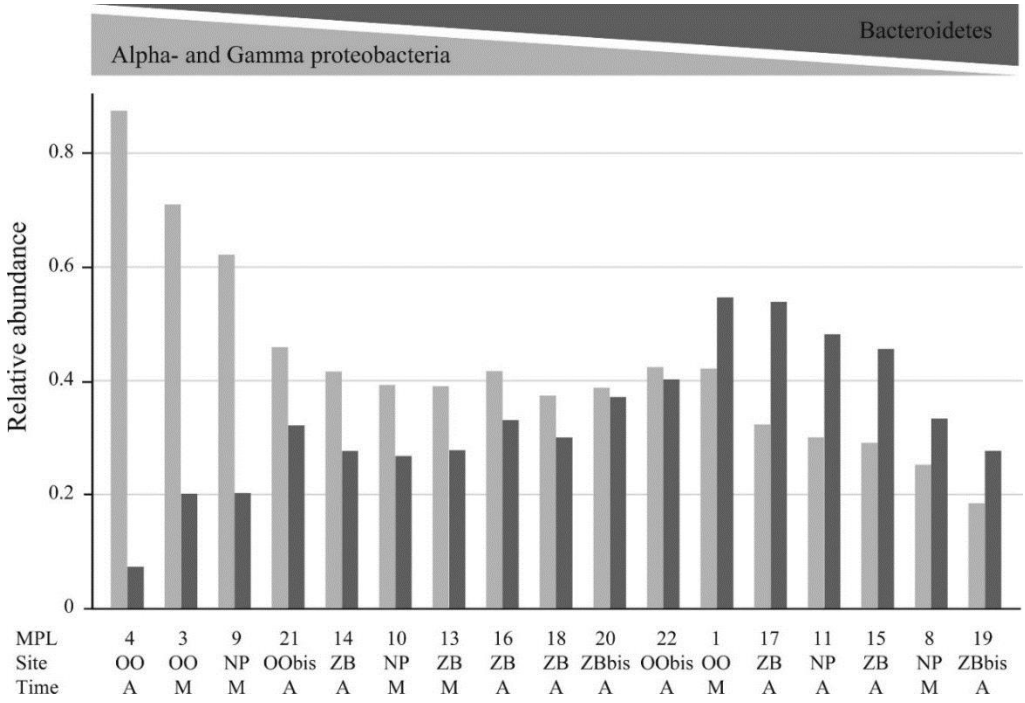


Figure 5.8 Representation of the primary (*Alpha*- and *Gammaproteobacteria*) and secondary (*Bacteroidetes*) biofilm colonizers in a marine environment. MPL were ordered according the relative abundance of the *Proteobacteria* and *Bacteroidetes* classes. (A) Dominance of *Proteobacteria* classes, (B) Similar amounts of *Proteobacteria* classes and Bacteroidetes, and (C) Dominance of *Bacteroidetes* (Time: A = August, M = March).

Three different groups could be discriminated in the sample set: MPL samples where *Proteobacteria* classes dominated, samples where the abundance of *Proteobacteria* classes and *Bacteroidetes* were similar and samples where *Bacteroidetes* dominated (Figure 5.8). With the exception of MPL1, the plastics with a low number of OTUs (MPL3, MPL4, MPL9) had the

highest number of *Proteobacteria* classes (Figure 5.6; Figure 5.8), suggesting that these plastics display characteristics of early stages of biofilm formation, whereas the others could represent later stages of biofilm formation.

5.2.5 Comparative analysis using the Plastisphere portal on the VAMPS platform

The bacterial community of MPL sampled in the Belgian part of the North Sea was compared with those sampled in the North Atlantic (Zettler et al., 2013; Amaral-Zettler et al., 2015) and the Pacific Ocean (Amaral-Zettler et al., 2015). In total, the bacterial communities of 98 samples of plastic debris were compared. Across all datasets, *Proteobacteria*, *Bacteroidetes* and *Cyanobacteria* dominated the plastic litter samples. *Proteobacteria* constituted the most common phylum in each dataset and usually dominated the datasets with an abundance range between 23 to 88% (with an average of 52%) (Figure 5.9; Figure 5.10). *Bacteroidetes* were less abundant (2 to 58%, average 13%) but also occurred in each sample. *Cyanobacteria* were observed on 85% of the analysed PD and constituted 1 to 60% (18% on average) of the community.

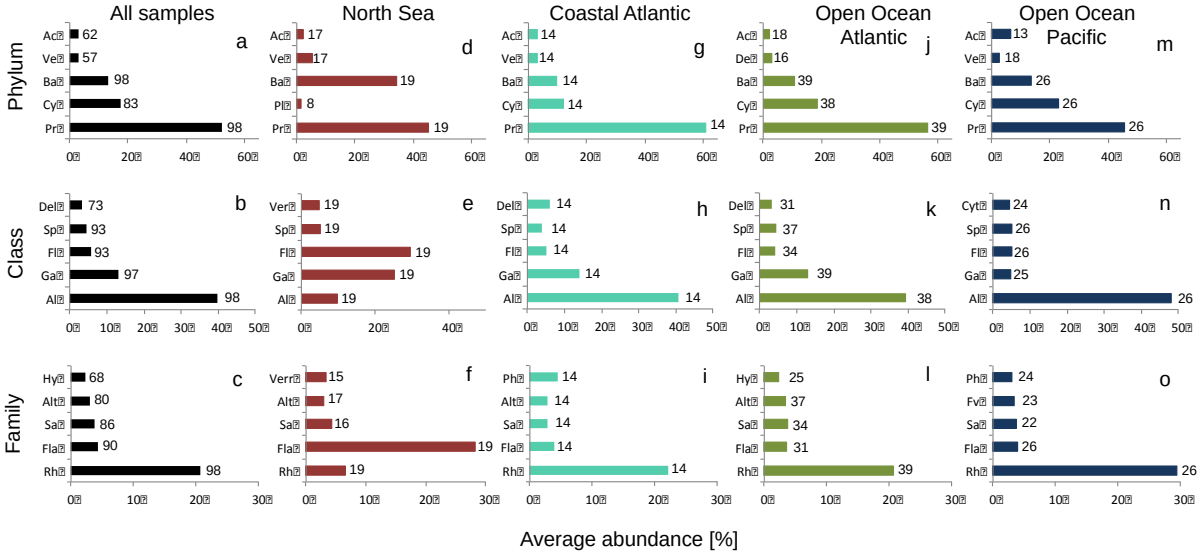


Figure 5.9 An overview of the five most abundant taxa on PE samples in the different marine regions at different taxonomic levels. Numbers adjacent to the bars represent the numbers of samples of the 98 examined that included these taxa. Of the 98 datasets, 19 were from the North Sea, 14 from the coastal Atlantic, 39 from the Atlantic open ocean and 26 from the Pacific open ocean. The following abbreviations apply: **Phylum:** Ac *Actinobacteria*; Ve *Verrucomicrobia*; Ba *Bacteroidetes*; Cy *Cyanobacteria*; Pr *Proteobacteria*; Pl *Planctomycetes*; De *Deferribacteres*. **Class:** Del *Deltaproteobacteria*; Sp *Sphingobacteria*; Fl *Flavobacteria*; Ga *Gammaproteobacteria*; Al *Alphaproteobacteria*; Ver *Verrucomicrobiae*; Cyt *Cytophagia*. **Family:** Hy *Hyphomonadaceae*; Alt *Alteromonadaceae*; Sa *Saprospiraceae*; Fla *Flavobacteriaceae*; Rh *Rhodobacteraceae*; Verr *Verrucomicrobiaceae*; Ph *Phylobacteriaceae*; Fv *Flammeovirgaceae*.

The most common class in our datasets was the *Alphaproteobacteria* representing 5 to 67% (40% on average) of the relative abundance of the entire community, followed by *Gammaproteobacteria* (range 1.6 to 80%; on average 13%) and *Flavobacteria* (range 1 to 52%; on average 6%) *(*Figure 5.9; Figure 5.10).

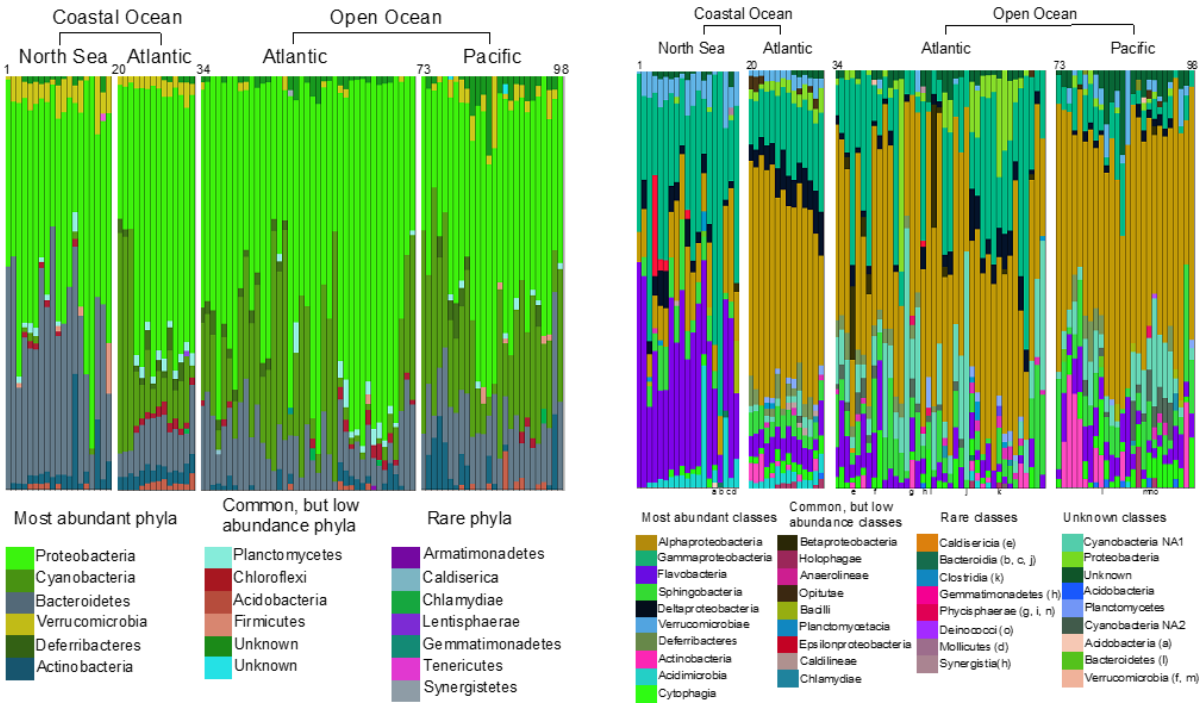


Figure 5.10 Overview of the relative abundance and taxonomic breakdown of bacterial phyla (left) and classes (right) on plastic debris sampled in different marine regions. Only phyla or classes that contributed ≥1% relative abundance are shown. The sample order in the figure is the same as provided in Table AI5.2, numbers above bar charts helps for orientation.

In our VAMPS meta-analysis, the most common bacterial family was the *Rhodobacteraceae* occurring at 20% relative abundance on average in each sample (Figure 5.11). Within our datasets, *Flavobacteriaceae* and *Saprospiraceae* were the second most abundant families occurring on 90 and 86 samples, respectively, but were low in relative abundance (both around 4% on average) on PE.

To identify possible "core" plastisphere microbiome members for different marine regimes, we compared the five most abundant and common taxa of four different marine regions (Figure 5.9) against each other: offshore versus coastal regions and Atlantic versus Pacific Ocean versus North Sea. Clear differences could be observed between the North Sea and all other regions. For the Atlantic and Pacific Oceans, all datasets exhibited similar dominance structures with *Proteobacteria*, *Bacteroidetes* and *Cyanobacteria* as the most abundant taxa

(representing >80% of the relative abundance), without showing differences between the coastal versus open ocean. In comparison to these regions, the North Sea samples varied in composition. *Cyanobacteria* were observed in only one quarter of the samples, and next to *Proteobacteria* and *Bacteroidetes*, the *Verrucomicrobia* were the third most dominant fraction.

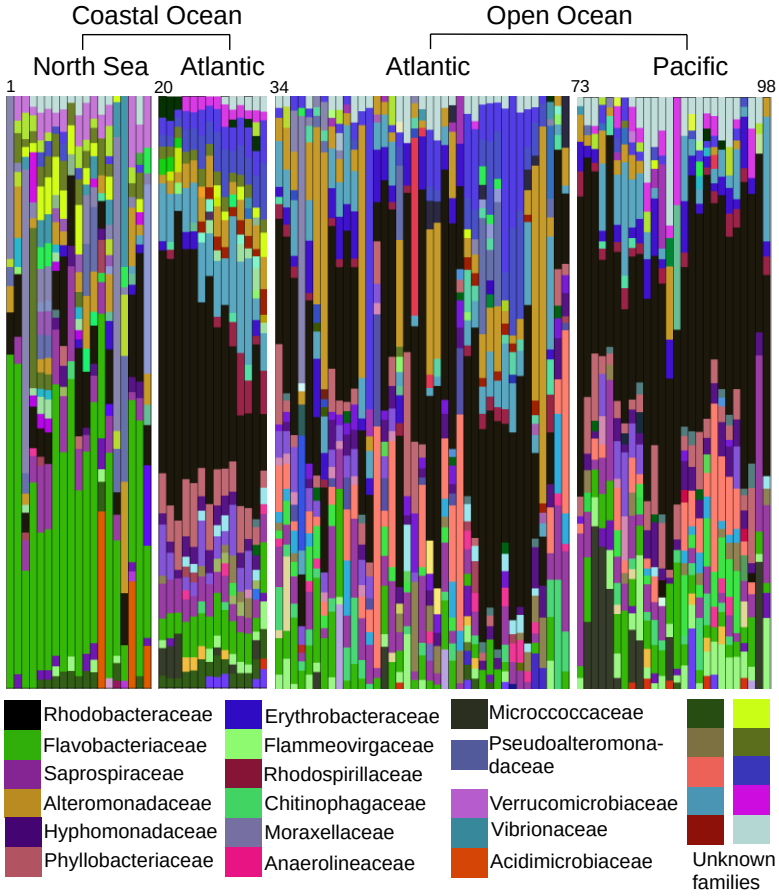


Figure 5.11 Overview of the relative abundance and taxonomic breakdown of bacterial families on plastic debris sampled in different marine regions. Only families that contributed ≥1% relative abundance are shown. The sample order in the figure is the same as provided in Table AI 5.2, numbers above bar charts helps for orientation.

At the class level, *Alphaproteobacteria* was the most abundant class in the Atlantic and Pacific Oceans, whereas *Flavobacteria* and *Gammaproteobacteria* were the most abundant classes in the North Sea (Figure 5.10). The same trend was also observed at the family level, where *Rhodobacteraceae* were most abundant in all regions except for the North Sea where *Flavobacteriaceae* dominated on average (Figure 5.11).

5.3 DISCUSSION AND CONCLUSIONS

To investigate the diversity in microbial communities on plastic and to identify drivers of bacterial colonization, we sampled plastic (MPL and beach pellets) at the seafloor at five locations at the Belgian part of the North Sea. Initially, we aimed to compare them to the bacterial communities of their surrounding environment, i.e. seawater and sediment sampled at the same location and time, which could be in contact with the plastic during the period of sampling and could serve as potential sources of bacteria. However, plastic is mobile and can be transported through ocean currents over longer distances, but also over smaller areas, like the North Sea (Galgani et al., 2013; Neumann et al., 2014; UNEP, 2014). Therefore, the surrounding environment could be of limited relevance, or even inappropriate, as reference, and we expanded the comparison of plastic and environment communities by sampling seawater at fourteen different locations across the Belgium part of the North Sea.

Differences in observed number of species and community composition show a clear distinction between bacterial communities of MPL, beach pellets, sediment and seawater. This indicates that bacterial communities colonizing plastic substrates are markedly different from the bacterial communities of seawater and sediment, both in the surrounding as 'broad' environment, and irrespective of the time of sampling. The substantial variation observed within sample types may still be caused by spatio-temporal dependent factors, such as environmental parameters, as discussed further below. Taken together, our results denote MPL as a distinct microbial niche in the marine environment, called the "plastisphere", and further confirm previous results of Zettler *et al.* (2013) who showed a difference in community composition between floating plastic debris and the surrounding seawater.

Most of the bacterial families found on MPL were also found in seawater and/or sediment, indicating the role of the marine environment serving as a bacterial source for plastic colonization. Besides those shared bacterial families certain bacterial groups, e.g. *Vibrionaceae* or *Pseudoalteromonadaceae*, are commonly detected on MPL but barely observed in seawater and sediment communities. It is expected that foreign bacteria well-adapted to MPL properties could attach to the surface and travel with the plastic particle (Osborn & Stojkovic, 2014). In addition, plastic can originate from different land- and sea-based sources (Galgani et al., 2013; Neumann et al., 2014; UNEP, 2014), each with their natural occurring bacterial communities. We expect that if the affinity for the plastic material is high

enough, micro-organisms could stay attached on MPL despite changing environments. In that way not only the marine or aquatic environment (and changes thereof) can shape the community, but also the plastic's transportation history is important in the bacterial colonization process. This role is further illustrated by the distinct bacterial profile found on beach pellets. Bacterial community assemblage of beach pellets was mainly different from the MPL samples due to a relatively high abundance of *Actinobacteria* on beach pellets, compared to a dominance of *Proteobacteria* on the other sample types. Contrary to MPL, beach pellets were sampled on the beach, i.e. an intertidal environment with influences of both aquatic and terrestrial environments, which could explain the high abundance of *Actinobacteria* (Elifantz et al., 2013).

Because we found similar families on the different MPL samples, we tried to establish a core microbiome, hypothesizing that these species play a major role in the plastic colonization process and/ or in plastic degradation. On phylum, class and family level, similarities between plastic sample microbial communities were observed, however this seems to be correlated to location. Plastics samples in the North Sea region seemed to posses a different microbial community composition compared to those of Atlantic and Pacific Oceans. Strikingly, the high diversity in community profiles across the set of plastics sampled in the Belgian part of the North Sea precludes the identification of core microbiomes on OTU level, in contrast to previous studies on fewer samples (Zettler et al., 2013). Therefore, we investigated three aspects that potentially drive bacterial colonization and may explain the observed microbial diversity on MPL: (i) environmental parameters such as physicochemical properties (temperature, oxygen, salinity); (ii) physicochemical properties of plastic; and (iii) stages of biofilm formation, as biofilm formation is a dynamic process.

First we established whether any spatio-temporal structure could explain the diversity in bacterial communities, and whether this structure overlaps with variance in environmental parameters at the location and time of sampling. The plastic bacterial community composition was correlated with variation in salinity, temperature, oxidation reduction potential, turbidity, oxygen content and density of the seawater, and the total organic carbon and inorganic carbon content of sediment to MPL bacterial communities. Temperature, oxygen content and ORP are correlated with sampling date, because higher temperature and ORP and a lower oxygen content were measured in August compared to March. To be sure that a seasonal

effect on MPL bacterial communities is present, multiple sampling times should be tested. In addition, we observed a difference in profile of samples taken at ZB, a sampling point near the harbour of Zeebrugge, compared to other regions. This could be explained in two ways. First, the influx of freshwater from the Rhine/Meuse and the Scheldt Estuary by horizontal dispersion, lowers the seawater salinity in the Zeebrugge region, which could explain the observed correlation of salinity to the bacterial structure, and the discrimination between locations (Lacroix et al., 2004). Second, in marine environments, the median grain size of the sediment, together with other factors such as phytoplankton blooms, amount of suspended organic material and marine snow, determine the turbidity. A smaller median grain size and a high rate of dredged material deposition will lead to more cloudy water. Turbidity was therefore highest in coastal areas, especially ZB, which contains a lot of sludge and dredged material. In addition, the small median grain size and high organic content measured in ZB, makes these sediments more susceptible to environmental pollution (De Witte et al., 2014; Gauquie et al., 2015), which could have a complimentary effect on the bacterial profile and provide an alternative explanation of the clustering of the ZB samples.

The correlation of bacterial colonization and physicochemical properties of plastic was studied second. In this study, no effect of polymer type was observed as no clear difference in bacterial profile was observed between the PP sample and the PE samples. This is in contrast with previous observations of Zettler *et al*. (2013), who showed differences in microbial communities between three PE and three PP samples, which could be due to our small sampling size of PP items. No other correlations with bacterial community profile and plastic physicochemical properties were observed for samples obtained from the North Sea.

Third, differences in biofilm formation stages could indicate differences in MPL bacterial community composition. The hydrophobic surface of plastics promotes microbial colonization and biofilm formation. Biofilm formation is a dynamic process and the taxonomic composition changes over time (Zettler et al., 2013; Harrison et al., 2014). Therefore, variation in biofilm formation stages may explain at least part of the variation in MPL bacterial composition. We showed that the MPL could be classified in groups containing a large amount of previously identified primary biofilm colonizers and groups colonised by a higher abundance of previously identified secondary colonizers (Lee et al., 2008; Elifantz et al., 2013). This implies that at least part of the large variation in bacterial composition across our plastic samples could be explained by differences in stages of biofilm formation, possibly due to varying

exposure times of the plastic to the marine environment. To confirm this observation, and to be able to 'map' bacterial profiles onto a microbial biofilm developmental timescale, a controlled exposure experiment using long-term time series at a fixed location to study formation and maintenance of microbial biofilms should be performed (Chapter 6).

On the beach pellets, one species of the Mycobacteriaceae, *Mycobacterium frederiksbergense*, caught our attention, because its high abundance on the yellow and blue coloured beach pellets, compared to other pellets and plastics. Notably, *M. frederiksbergense* is known for its degrading capacity of diverse polycyclic aromatic hydrocarbons like anthracene (Willumsen et al., 2001; Wick et al., 2003), a building block for the production of anthraquinone (a precursor for dye synthesis) (Bien et al., 2000). Several patents describe the use of anthraquinone derivatives for colouring resin pellets (US patent 3441536, 6593483, 6635350), which suggests the presence of anthracene derivates on the blue and yellow beach pellets. While black and white pellets were abundant at the time and location of sampling at the Spinoladijk, blue and yellow pellets were rather rare and the material collected was sufficient for bacterial taxonomic profiling, but not for chemical profiling. White and black pellets however, were also used for chemical profiling, revealing very low levels of anthracene on these beach pellets, as expected for uncoloured resins (Hörsch et al., 2005). For now, the observation of *M. frederiksbergense* on blue and yellow beach pellets, the documented use of anthraquinone derivatives as pigments for blue and yellow resin pellets, taken together with the putative capacity of *M. frederiksbergense* to metabolize anthracene derivates as carbon source, indeed suggest that presence of dyes and adsorbed chemicals, or perhaps pollutants, could influence MPL bacterial colonization. Parallel studies in our laboratory identified more than 250 different chemical compounds on plastic debris (synthetic rope and sheets) of the Belgian part of the North Sea (Gauquie et al., 2015), indicating that the relationship between chemical profile and bacterial colonization may be quite complex and requires large numbers of samples to capture both the chemical and taxonomic diversity.

When comparing the bacterial communities of the North Sea region with those of the North Atlantic and Pacific Ocean, distinct profiles were noticed. Nonetheless striking patterns do emerge in the data (e.g. similarities Atlantic and Pacific Ocean) that suggest there may in fact be a Plastisphere "core microbiome" within given marine regions. The North Sea region showed a high abundance of *Flavobacteriaceae* (Oberbeckmann et al., 2016) which was also

the dominant group in our study. We advocate that a more powerful approach would employ a standardized protocol, used by all research groups studying Plastisphere communities.

To this end, agreeing upon the following aspects of a standard operating procedure would facilitate comparisons of Plastisphere communities. Therefore, we propose to set-up a standardized procedure based on the following recommendations:

(1) Standardised sample preservation. We recommend storing plastic samples in lysis buffer at -20 °C to preserve DNA. We tested several options for storing plastics (in seawater, in seawater at -20°C, at -80 °C, at -20°C and at -20°C in lysis buffer) in the laboratory. We determined that storage at -20°C in lysis buffer yielded the highest DNA concentration after extraction (De Tender et al., unpublished results).

(2) Standardised DNA extractions. A recent study of Debeljak *et al.* (2017) tested different DNA extraction methods used in the literature for plastisphere communities. The Qiagen Puregene Tissue kit yielded the highest DNA concentrations for most sizes and amounts of plastics at relatively low cost and short processing time.

(3) Standardized 16S rRNA gene hypervariable target region for amplification. Since most studies have employed part of or the entire V6 hypervariable region, continuing to include this region would provide the highest comparative potential. Although the VAMPS plastisphere portal shows that comparison between datasets comprising sequences from different hypervariable targets is possible, the comparison is limited to taxonomic information.

One could also recommend using the same sequencing platform across studies, however since sequencing technology evolves so rapidly, this may not be a feasible recommendation.

To conclude, in this chapter we showed that bacterial communities on MPL are different in composition compared to seawater and sediment. The surrounding environment can thereby serve as a bacterial source, but also distinct bacterial groups were found on MPL of the North Sea, indicating that plastic can act as a transport vehicle. In addition, the diversity in bacterial communities between plastic items can be explained by at least three factors: differences in environmental parameters, plastic-related factors and stages in biofilm formation. More research however is needed to disentangle all separate influences on the microbial population of plastic. In addition, we showed that compared to other regions, the plastisphere community of MPL sampled in the North Sea is more or less distinct. To make comparison possible between datasets however, there is a need for a standard operating procedure.

ADDITIONAL INFORMATION CHAPTER 5

Table AI5.1: Physicochemical characteristics of water and sediment sampled at different locations across the Belgian part of the North Sea.

Seawater	Sampling Date	Depth (m)	Temperature (°C)	Salinity (PSU)	Oxygen (%saturation)	Pressure (db)	Conductivity (µS/cm)	Turbidity (NTU)	Oxidation reduction potential (ORP) (mV)	Density (kg/m³)	Sound Velocity (m/s)
OO	05/03/'14	8.5	7.8	33.5	84.0	8.07	34 704	204.0	218.70	1026.18	1480.20
NP	05/03/'14	6.5	8.0	33.5	78.0	6.05	34 756	154.0	218.47	1025.86	1480.34
ZB	06/03/'14	6.5	7.5	30.5	82.0	6.05	31 012	379.0	188.43	1023.37	1474.31
1	25/06/'14	9.0	18.6	30.8	106.9	9.08	42 835	43.9	/	1023.25	1513.13
2	25/06/'14	11.0	18.3	32.8	129.2	/	/	/	/	/	/
3	25/06/'14	7.0	18.5	32.3	123.0	/	/	/	/	/	/
4	24/06/'14	21.0	17.8	31.7	104.9	22.19	44 052	44.2	133.57	1024.17	1514.33
5	24/06/'14	22.0	17.3	30.0	103.9	22.19	44 055	32.9	130.54	1024.71	1513.09
6	24/06/'14	6.6	17.2	34.3	78.4	/	/	/	/	/	/
7	25/06/'14	10.0	18.3	30.9	108.9	10.09	43 475	36.6	143.03	1023.50	1514.18
8	24/06/'14	27.0	17.9	33.6	88.5	27.24	44 136	35.3	142.80	1024.37	1514.22
9	24/06/'14	13.0	18.7	33.0	124.7	13.11	43 860	51.8	134.20	1023.65	1514.83
10	24/06/'14	38.0	15.4	35.0	99.9	38.34	43 358	35.9	124.40	1026.08	1508.58
11	24/06/'14	36.0	15.8	35.0	97.5	/	/	/	/	/	/
12	24/06/'14	36.0	15.8	35.0	100.0	/	/	/	/	/	/
13	24/06/'14	32.0	15.9	34.9	101.2	32.28	43 720	36.7	157.50	1025.84	1509.93
14	24/06/'14	33.0	16.8	34.5	99.4	33.29	44 121	32.9	145.20	1025.36	1512.08

Sediment	Sampling date	TOC (%)	Inorganic Carbon (%)	Median grain size (µm)
OO	05/03/'14	0.34	1.72	180.39
	01/09/'14	1.18	2.04	108.65
NP	05/03/'14	0.22	2.10	186.95
	28/08/'14	1.02	1.76	199.15
ZB	06/03/'14	2.15	3.80	33.41
	29/08/'14	8.97	15.47	29.06

Table AI 5.2 Metadata of the 98 plastic debris samples collected in the North Sea, coastal and open Atlantic Ocean, and open Pacific Ocean. The order of the metadata corresponds to the order of the samples shown in Figures 4.9 Untill 4.11 . The sample ID's listed below correspond to the sample ID's in the VAMPS "Plastiphere" portal. PE = Polyethylene, PP = Polypropylene, HDPE = High Density Polyethylene.

Sample order	Sample ID	sampling date (year-month-day)	Lati-tude	Longi-tude	Poly-mer Type	experimental factor	Samp-ling depth [m]	Salinity	Tem-pera-ture [°C]	Sequencing method	Target gene
North Sea											
1	LAZ_DET_Bv3v4_MPL1	2014-3-5	51.16	2.71	PE	free floating plastic	8.5	33.5	8	Illumina Miseq 2x 300bp	16S rRNA v3v4
2	LAZ_DET_Bv3v4_MPL11	2014-8-28	51.16	2.71	PE	free floating plastic	7.2	33.6	17.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
3	LAZ_DET_Bv3v4_MPL8	2014-3-5	51.16	2.71	PE	free floating plastic	6.5	33.5	8	Illumina Miseq 2x 300bp	16S rRNA v3v4
4	LAZ_DET_Bv342_MPL9	2014-3-5	51.16	2.71	PE	free floating plastic	6.5	33.5	8	Illumina Miseq 2x 300bp	16S rRNA v3v4
5	LAZ_DET_Bv3v4_MPL13	2014-3-6	51.33	3.13	PE	free floating plastic	6.5	30.5	7.5	Illumina Miseq 2x 300bp	16S rRNA v3v4
6	LAZ_DET_Bv3v4_MPL14	2014-8-29	51.33	3.13	PE	free floating plastic	6.7	31.2	17.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
7	LAZ_DET_Bv3v4_MPL15	2014-8-29	51.33	3.13	PE	free floating plastic	6.7	31.2	17.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
8	LAZ_DET_Bv3v4_MPL16	2014-8-29	51.33	3.13	PE	free floating plastic	6.7	31.2	17.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
9	LAZ_DET_Bv3v4_MPL17	2014-8-29	51.33	3.13	PE	free floating plastic	6.7	31.2	17.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
10	LAZ_DET_Bv3v4_MPL18	2014-8-29	51.33	3.13	PE	free floating plastic	6.7	31.2	17.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
11	LAZ_DET_Bv3v4_MPL19	2014-8-29	51.45	3.24	PE	free floating plastic	6.5	31.2	18.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
12	LAZ_DET_Bv3v4_MPL20	2014-8-29	51.45	3.24	PE	free floating plastic	6.5	31.2	18.7	Illumina Miseq 2x 300bp	16S rRNA v3v4
13	LAZ_DET_Bv3v4_MPL21	2014-9-1	51.45	2.61	PP	free floating plastic	31.3	34.6	18.1	Illumina Miseq 2x 300bp	16S rRNA v3v4
14	LAZ_DET_Bv3v4_MPL22	2014-9-1	51.45	2.61	PE	free floating plastic	31.3	34.6	18.1	Illumina Miseq 2x 300bp	16S rRNA v3v4
15	LAZ_DET_Bv3v4_MPL3	2014-3-5	51.22	2.86	PE	free floating plastic	8.5	33.5	7.8	Illumina Miseq 2x 300bp	16S rRNA v3v4
16	LAZ_DET_Bv3v4_MPL4	2014-9-1	51.22	2.86	PE	free floating plastic	8.5	33.8	17.8	Illumina Miseq 2x 300bp	16S rRNA v3v4
17	LAZ_DET_Bv3v4_MPL5	2014-9-1	51.22	2.86	PE	free floating plastic	8.5	33.8	17.8	Illumina Miseq 2x 300bp	16S rRNA v3v4
18	LAZ_DET_Bv3v4_MPL6	2014-9-1	51.22	2.86	PE	free floating plastic	8.5	33.8	17.8	Illumina Miseq 2x 300bp	16S rRNA v3v4
19	LAZ_DET_Bv3v4_MPL7	2014-9-1	51.22	2.86	PE	free floating plastic	8.5	33.8	17.8	Illumina Miseq 2x 300bp	16S rRNA v3v4
Coastal Atlantic Ocean											
20	LAZ_SEA_Bv6-WHD_0016_2013_07_17_Bv6	2013-7-17	41.53	-70.67	HDPE	incubation	0.5	30	23.8	Illumina HiSeq	16S rRNA v6
21	LAZ_SEA_Bv6-WHD_0017_2013_07_17_Bv7	2013-7-17	41.53	-70.67	HDPE	incubation	0.5	30	23.8	Illumina HiSeq	16S rRNA v6
22	LAZ_SEA_Bv6-WHD_0018_2013_07_17_Bv8	2013-7-17	41.53	-70.67	HDPE	incubation	0.5	30	23.8	Illumina HiSeq	16S rRNA v6
23	LAZ_SEA_Bv6-WHD_0028_2013_07_24_Bv9	2013-7-24	41.53	-70.67	HDPE	incubation	0.5	30	24	Illumina HiSeq	16S rRNA v6
24	LAZ_SEA_Bv6-WHD_0029_2013_07_24_Bv10	2013-7-24	41.53	-70.67	HDPE	incubation	0.5	30	24	Illumina HiSeq	16S rRNA v6

25	LAZ_SEA_Bv6-WHD_0040_2013_07_31_Bv11	2013-7-31	41.53	-70.67	HDPE	incubation	0.5	30.4	23.5	Illumina HiSeq	16S rRNA v6
26	LAZ_SEA_Bv6-WHD_0041_2013_07_31_Bv12	2013-7-31	41.53	-70.67	HDPE	incubation	0.5	30.4	23.5	Illumina HiSeq	16S rRNA v6
27	LAZ_SEA_Bv6-WHD_0052_2013_08_07_Bv13	2013-8-7	41.53	-70.67	HDPE	incubation	0.5	29.3	22.9	Illumina HiSeq	16S rRNA v6
28	LAZ_SEA_Bv6-WHD_0053_2013_08_07_Bv16	2013-8-7	41.53	-70.67	HDPE	incubation	0.5	29.3	22.9	Illumina HiSeq	16S rRNA v6
29	LAZ_SEA_Bv6-WHD_0054_2013_08_07_Bv14	2013-8-7	41.53	-70.67	HDPE	incubation	0.5	29.3	22.9	Illumina HiSeq	16S rRNA v6
30	LAZ_SEA_Bv6-WHD_0065_2013_09_06_Bv16	2013-9-6	41.53	-70.67	HDPE	incubation	0.5	29.6	21.5	Illumina HiSeq	16S rRNA v6
31	LAZ_SEA_Bv6-WHD_0066_2013_09_06_Bv15	2013-9-6	41.53	-70.67	HDPE	incubation	0.5	29.6	21.5	Illumina HiSeq	16S rRNA v6
32	LAZ_SEA_Bv6-WHD_0076_2013_10_02_Bv16	2013-10-2	41.53	-70.67	HDPE	incubation	0.5	29	18.7	Illumina HiSeq	16S rRNA v6
33	LAZ_SEA_Bv6-WHD_0088_2013_11_06_Bv17	2013-11-6	41.53	-70.67	HDPE	incubation	0.5	29.7	12.9	Illumina HiSeq	16S rRNA v6
Open Atlantic Ocean											
34	LAZ_SEA_Bv6-SEA_0029_20120518_Bv6	2012-5-18	21.52	-64.89	HDPE	free floating microplastic	0	36.4	25.8	Illumina HiSeq	16S rRNA v6
35	LAZ_SEA_Bv6-SEA_0035_20120519_Bv6	2012-5-19	22	-65.18	HDPE	free floating microplastic	0	36	25.9	Illumina HiSeq	16S rRNA v6
36	LAZ_SEA_Bv6-SEA_0036_20120519_Bv6	2012-5-19	22	-65.18	HDPE	free floating microplastic	0	36	25.9	Illumina HiSeq	16S rRNA v6
37	LAZ_SEA_Bv6-SEA_0042_20120520_Bv6	2012-5-20	23	-65.08	HDPE	free floating microplastic	0	36	26	Illumina HiSeq	16S rRNA v6
38	LAZ_SEA_Bv6-SEA_0049_20120521_Bv6	2012-5-21	25	-64.58	HDPE	free floating microplastic	0	36.07	26.2	Illumina HiSeq	16S rRNA v6
39	LAZ_SEA_Bv6-SEA_0063_20120523_Bv6	2012-5-23	27	-63.57	HDPE	free floating microplastic	0	36.5	24.5	Illumina HiSeq	16S rRNA v6
40	LAZ_SEA_Bv6-SEA_0064_20120523_Bv6	2012-5-23	27	-63.57	HDPE	free floating microplastic	0	36.5	24.5	Illumina HiSeq	16S rRNA v6
41	LAZ_SEA_Bv6-SEA_0094_20120527_Bv6	2012-5-27	31.65	-64.26	HDPE	free floating microplastic	0	36.4	22.7	Illumina HiSeq	16S rRNA v6
42	LAZ_SEA_Bv6-SEA_0095_20120527_Bv6	2012-5-27	31.65	-64.26	HDPE	free floating microplastic	0	36.4	22.7	Illumina HiSeq	16S rRNA v6
43	LAZ_SEA_Bv6-SEA_0107_20120607_Bv6	2012-6-7	35.55	-65.66	HDPE	free floating microplastic	0	36.52	22.4	Illumina HiSeq	16S rRNA v6
44	LAZ_SEA_Bv6-SEA_0108_20120607_Bv6	2012-6-7	35.55	-65.66	HDPE	free floating microplastic	0	36.52	22.4	Illumina HiSeq	16S rRNA v6

45	LAZ_SEA_Bv6-SEA_0122_20120608_Bv6	2012-6-8	36.34	-68.01	HDPE	free floating microplastic	0	36.46	22.7	Illumina HiSeq	16S rRNA v6
46	LAZ_SEA_Bv6-SEA_0128_20120609_Bv6	2012-6-9	37.43	-68.01	HDPE	free floating microplastic	0	36.34	23.2	Illumina HiSeq	16S rRNA v6
47	LAZ_SEA_Bv6-SEA_0136_20120610_Bv6	2012-6-10	39.14	-67.83	HDPE	free floating microplastic	0	34.18	18.9	Illumina HiSeq	16S rRNA v6
48	LAZ_SEA_Bv6-SEA_0137_20120610_Bv6	2012-6-10	39.14	-67.83	HDPE	free floating microplastic	0	34.18	18.9	Illumina HiSeq	16S rRNA v6
49	LAZ_SEA_Bv6-SEA_0142_20120611_Bv6	2012-6-11	39.98	-68.88	HDPE	free floating microplastic	0	33.76	16.6	Illumina HiSeq	16S rRNA v6
50	LAZ_SEA_Bv6-SEA_0358_5_14_2013_Bv6	2013-5-14	17.75	-64.70	PE	free floating plastic	0	36.2	29.6	Illumina HiSeq	16S rRNA v6
51	LAZ_SEA_Bv6-SEA_0365_2013516_Bv6	2013-5-16	17.98	-64.57	PE	free floating microplastic	0	36.43	27.8	Illumina HiSeq	16S rRNA v6
52	LAZ_SEA_Bv6-SEA_0380_5_19_2013_Bv6	2013-5-19	22.87	-64.47	PE	free floating microplastic	0	36.25	26.8	Illumina HiSeq	16S rRNA v6
53	LAZ_SEA_Bv6-SEA_0435_5_26_2013_Bv6	2013-5-26	32.11	-64.36	PE	free floating microplastic	0	36.73	22.6	Illumina HiSeq	16S rRNA v6
54	LAZ_SEA_Bv6-SEA_0439_5_26_2013_Bv6	2013-5-26	32.09	-64.46	PE	incubation	0	36.73	22.8	Illumina HiSeq	16S rRNA v6
55	LAZ_SEA_Bv6-SEA_0440_5_26_2013_Bv6	2013-5-26	32.09	-64.46	PE	incubation	0	36.73	22.8	Illumina HiSeq	16S rRNA v6
56	LAZ_SEA_Bv6-SEA_0449_6_02_2013_Bv6	2013-6-2	32.38	-64.68	PE	free floating macroplastic	0	NAN	NAN	Illumina HiSeq	16S rRNA v6
57	LAZ_SEA_Bv6-SEA_0450_6_02_2013_Bv6	2013-6-2	32.38	-64.68	PE	free floating macroplastic	0	NAN	NAN	Illumina HiSeq	16S rRNA v6
58	LAZ_SEA_Bv6-SEA_0462_6_05_2013_Bv6	2013-6-5	33.87	-65.94	PE	free floating microplastic	0	36.69	23.3	Illumina HiSeq	16S rRNA v6
59	LAZ_SEA_Bv6-SEA_0477_6_07_2013_Bv6	2013-6-7	35.70	-65.91	PE	incubation	0	36.3	23.5	Illumina HiSeq	16S rRNA v6
60	LAZ_SEA_Bv6-SEA_0478_6_07_2013_Bv6	2013-6-7	35.70	-65.91	PE	incubation	0	36.3	23.5	Illumina HiSeq	16S rRNA v6
61	LAZ_SEA_Bv6-SEA_0485_6_07_2013_Bv6	2013-6-16	41.38	-70.88	HDPE	incubation	0	31.86	16.7	Illumina HiSeq	16S rRNA v6
62	LAZ_SEA_Bv6-SEA_0486_6_07_2013_Bv6	2013-6-16	41.38	-70.88	HDPE	incubation	0	31.86	16.7	Illumina HiSeq	16S rRNA v6
63	LAZ_SEA_Bv6-SEA_0487_6_07_2013_Bv6	2013-6-16	41.38	-70.88	PS	incubation	0	31.86	16.7	Illumina HiSeq	16S rRNA v6
64	LAZ_SEA_Bv6-SEA_0528_201367_Bv6	2013-6-7	35.58	-66.13	HDPE	incubation	0	36.5	22.3	Illumina HiSeq	16S rRNA v6
65	LAZ_SEA_Bv6-SEA_0529_201367_Bv6	2013-6-7	35.58	-66.13	LDPE	incubation	0	36.5	22.3	Illumina HiSeq	16S rRNA v6

66	LAZ_SEA_Bv6-SEA_0530_201367_Bv6	2013-6-7	35.58	-66.13	LDPE	incubation	0	36.5	22.3	Illumina HiSeq	16S rRNA v6
67	LAZ_SEA_Bv6-SEA_0537_2013527_Bv6	2013-5-27	32.04	-64.48	HDPE	incubation	0	36.3	22.7	Illumina HiSeq	16S rRNA v6
68	LAZ_SEA_Bv6-SEA_0539_2013521_Bv6	2013-5-21	25.26	-64.46	HDPE	incubation	0	36	25.7	Illumina HiSeq	16S rRNA v6
69	LAZ_SEA_Bv6-SEA_0540_2013521_Bv6	2013-5-21	25.26	-64.46	HDPE	incubation	0	36	25.7	Illumina HiSeq	16S rRNA v6
70	LAZ_SEA_Bv6v4--SEA_0002_2010_07_07_Bv6v4	2010-6-21	31.63	-41.42	PE	free floating microplastic	0	36.91	24.6	Illumina HiSeq	16S rRNA V6-V4
71	LAZ_SEA_Bv6v4--SEA_0008_2012_05_20_Bv6v5	2012-5-20	23.29	-65.08	PE	free floating microplastic	0	36.01	26	Illumina HiSeq	16S rRNA V6-V4
72	LAZ_SEA_Bv6v4--SEA_0011_2012_05_22_Bv6v5	2012-5-22	26.08	-64.20	PE	free floating microplastic	0	36.07	26	Illumina HiSeq	16S rRNA V6-V4
Open Pacific Ocean											
73	LAZ_SEA_Bv6-SEA_0173_20121006_Bv6	2012-10-6	31.81	-121.57	PE	free floating microplastic	0	33.19	18.3	Illumina HiSeq	16S rRNA v6
74	LAZ_SEA_Bv6-SEA_0178_20121008_Bv6	2012-10-9	33.02	-125.58	PE	free floating microplastic	0	33.17	18.9	Illumina HiSeq	16S rRNA v6
75	LAZ_SEA_Bv6-SEA_0208_20121013_Bv6	2012-10-13	33.48	-132.45	PE	free floating microplastic	0	33.5	21.2	Illumina HiSeq	16S rRNA v6
76	LAZ_SEA_Bv6-SEA_0209_20121013_Bv6	2012-10-13	33.48	-132.45	PE	free floating microplastic	0	33.5	21.2	Illumina HiSeq	16S rRNA v6
77	LAZ_SEA_Bv6-SEA_0219_20121014_Bv6	2012-10-14	33.70	-133.46	PE	free floating microplastic	0	33.32	20.9	Illumina HiSeq	16S rRNA v6
78	LAZ_SEA_Bv6-SEA_0236_20121016_Bv6	2012-10-16	33.56	-135.43	PE	free floating microplastic	0	33.74	21.7	Illumina HiSeq	16S rRNA v6
79	LAZ_SEA_Bv6-SEA_0239_20121017_Bv6	2012-10-17	33.26	-136.21	PE	free floating microplastic	0	34.39	22.1	Illumina HiSeq	16S rRNA v6
80	LAZ_SEA_Bv6-SEA_0243_20121018_Bv6	2012-10-18	32.90	-137.23	PE	free floating microplastic	0	34.67	22.2	Illumina HiSeq	16S rRNA v6
81	LAZ_SEA_Bv6-SEA_0244_20121018_Bv6	2012-10-18	32.90	-137.23	PE	free floating microplastic	0	34.67	22.2	Illumina HiSeq	16S rRNA v6
82	LAZ_SEA_Bv6-SEA_0249_20121019_Bv6	2012-10-19	32.26	-138.57	PE	free floating microplastic	0	34.9	23	Illumina HiSeq	16S rRNA v6
83	LAZ_SEA_Bv6-SEA_0253_20121020_Bv6	2012-10-20	32.05	-139.10	PE	free floating microplastic	0	34.87	23.2	Illumina HiSeq	16S rRNA v6
84	LAZ_SEA_Bv6-SEA_0254_20121020_Bv6	2012-10-20	32.05	-139.10	PE	free floating microplastic	0	34.87	23.2	Illumina HiSeq	16S rRNA v6
85	LAZ_SEA_Bv6-SEA_0268_20121022_Bv6	2012-10-22	31.91	-139.58	PE	free floating microplastic	0	34.8	23.1	Illumina HiSeq	16S rRNA v6

86	LAZ_SEA_Bv6-SEA_0273_20121023_Bv6	2012-10-23	30.26	-140.68	PE	free floating microplastic	0	35.04	23.2	Illumina HiSeq	16S rRNA v6
87	LAZ_SEA_Bv6-SEA_0291_2012_10_25_Bv6	2012-10-25	30.02	-142.31	PE	free floating microplastic	0	35	23.3	Illumina HiSeq	16S rRNA v6
88	LAZ_SEA_Bv6-SEA_0292_2012_10_25_Bv6	2012-10-25	30.02	-142.31	PE	free floating microplastic	0	35	23.3	Illumina HiSeq	16S rRNA v6
89	LAZ_SEA_Bv6-SEA_0295_20121026_Bv6	2012-10-26	30.03	-143.97	PE	free floating microplastic	0	35.4	23.7	Illumina HiSeq	16S rRNA v6
90	LAZ_SEA_Bv6-SEA_0296_20121026_Bv6	2012-10-26	30.03	-143.97	PE	free floating microplastic	0	35.4	23.7	Illumina HiSeq	16S rRNA v6
91	LAZ_SEA_Bv6-SEA_0302_2012_10_27_Bv6	2012-10-27	30.07	-145.06	PE	free floating microplastic	0	35.41	24.1	Illumina HiSeq	16S rRNA v6
92	LAZ_SEA_Bv6-SEA_0306_20121028_Bv6	2012-10-28	30.42	-145.75	PE	free floating microplastic	0	35.4	24.2	Illumina HiSeq	16S rRNA v6
93	LAZ_SEA_Bv6-SEA_0307_2012_10_28_Bv6	2012-10-28	30.42	-145.75	PE	free floating microplastic	0	35.4	24.2	Illumina HiSeq	16S rRNA v6
94	LAZ_SEA_Bv6-SEA_0318_2012_10_29_Bv6	2012-10-29	29.89	-145.79	PE	free floating microplastic	0	35.4	24.2	Illumina HiSeq	16S rRNA v6
95	LAZ_SEA_Bv6-SEA_0334_2012_11_01_Bv6	2012-11-1	25.30	-147.62	PE	free floating microplastic	0	35.57	25	Illumina HiSeq	16S rRNA v6
96	LAZ_SEA_Bv6-SEA_0341_20121102_Bv6	2012-11-2	23.91	-149.11	PE	free floating microplastic	0	35.32	25.6	Illumina HiSeq	16S rRNA v6
97	LAZ_SEA_Bv6-SEA_0345_20121103_Bv6	2012-11-3	22.52	-150.02	PE	free floating microplastic	0	35.34	25.2	Illumina HiSeq	16S rRNA v6
98	LAZ_SEA_Bv6-SEA_0350_20121104_Bv6	2012-11-4	21.39	-152.02	PE	free floating microplastic	0	35.09	25.7	Illumina HiSeq	16S rRNA v6

Figure AI5.1: Raman spectra of a plastic sampled at OO (MPL1) and identified as polyethylene (PE). One pigment of the Phthalo Blue family (PB) was detected.

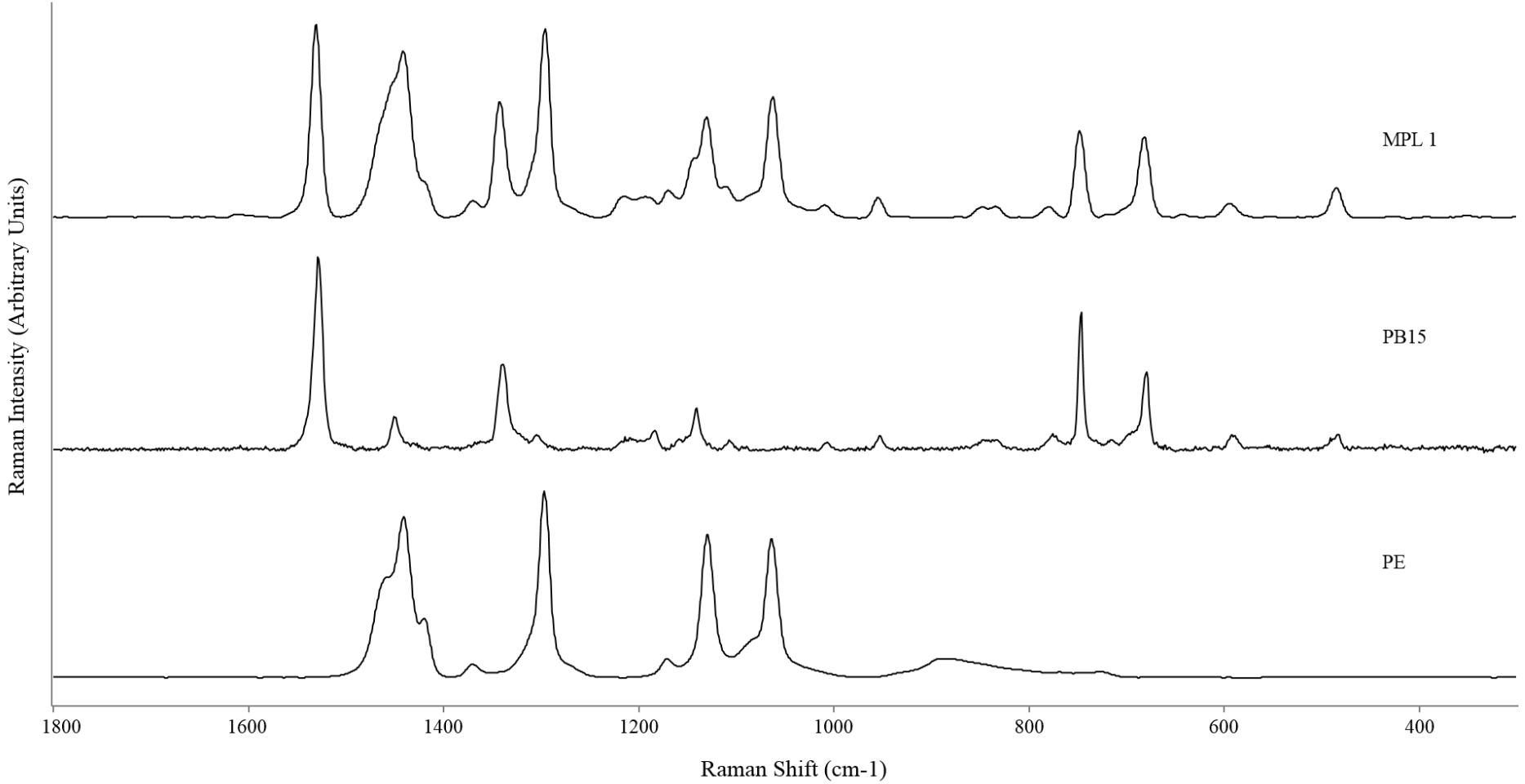
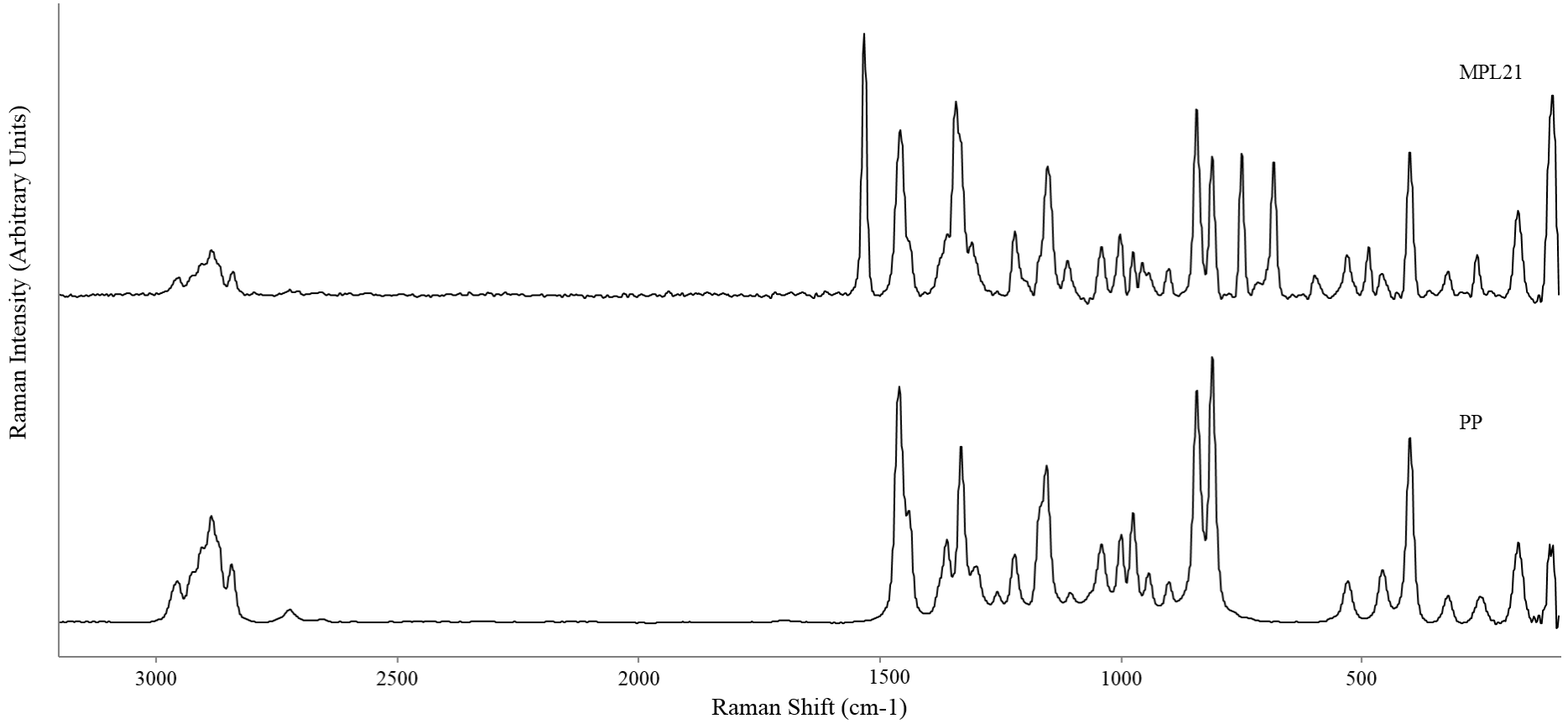


Figure AI5.2: Raman spectra of a plastic sampled at OObis (MPL21) and identified as polypropylene (PP).



Chapter 6

Microbial biofilm dynamics of the plastisphere

Modified from:

De Tender C, Devriese LI, Haegeman A, Maes S, Vangeyte J, Cattrijsse A, Dawyndt P, Ruttink T. The temporal dynamics of microbial colonization on plastic debris in the North Sea. Submitted to Environmental Science and Technology

In Chapter 5 we showed that the hard, hydrophobic surface of plastic debris is an ideal environment for colonization by bacteria. Previous studies have shown that merely one week`s exposure to the marine environment is sufficient for microbial biofilm formation on plastic debris (Lobelle & Cunfliffe, 2011), which is influenced by polymer type, environmental conditions and season (Oberbeckmann et al, 2014; Mincer et al, 2016).

As for most thermoplastics, degradation of PE is extremely slow, and it is therefore expected to persist in the marine ecosystem (Gewert et al., 2015). Micro-organisms may contribute to the degradation of PD in the marine environment. So far, only a few marine bacterial strains have been identified as potential PE degraders. *Arthrobacter* sp. and *Pseudomonas* sp. were isolated from high-density PE (HDPE) debris from the Gulf of Mannar, a marine coastal area (Balasubramanian et al., 2010). Furthermore, *Kocuria palustris*, *Bacillus pumilis* and *Bacillus subtilis* strains were isolated from low-density PE debris originating from the Arabian Sea (Harshvardhan & Bhavanath, 2013). Recently it was shown that also a marine fungus, *Zalerion maritimum*, has the potential to actively degrade PE (Paço et al., 2017). Furthermore, several microbial strains, including bacteria and fungi, were isolated from PD in different types of soil environment, and described as potential PE degraders (Restrepo-Flórez et al., 2014). Most studies on PE degradation are based on growth on medium with plastic polymers as the sole carbon source, PE mass loss and size reduction, and the screening of changes in functional groups by FT-IR. Only few studies described the actual degradation, e.g. based on enzyme production by the bacterial strain (Yoshida et al., 2016).

The formation of a biofilm, a structured system which facilitates metabolic interaction between cells (Davey & O'Toole, 2000), can be important in terms of biodegradation. Biofilm formation can increase degradation efficiency of pollutants such as diesel oil, and destruction of the biofilm architecture can disrupt interspecies cooperation and interfere with degradation efficiency (Laniczak et al., 2010; Verhagen et al., 2011). To date, little is known both about the temporal dynamics of colonization and biofilm formation on PD and about the microbial interactions underlying the resulting biodegradation process. Previous studies on biofilm formation on plastics under controlled conditions focused on short-term processes only (< 8 weeks) (Lobelle & Cunliffe, 2011; Harrison et al., 2014; Oberbeckmann et al., 2014; Eich et al., 2015; Oberbeckmann et al., 2016) or did not include taxonomic classification of the bacterial communities (Sudhakar et al., 2007; Artham et al., 2009; Webb et al., 2009; O'Brine & Thompson, 2010; Nauendorf et al., 2016). Most of those studies focused on floating plastic

litter, whereas the predominant part of PD is located on the seafloor (Barnes et al., 2009; Sherrington, 2016). Reconstructing the temporal dynamics of microbial colonization based on randomly sampled PD proved difficult by comparing independent pieces of plastic with unknown history, travel pattern, and duration of exposure to the marine environment to each other (see Chapter 5).

We established a long-term exposure time-series experiment in which two types of PE were exposed to the Belgian part of the North Sea at two different locations. First, plastics were exposed and sampled at the harbour of Ostend, a semi-enclosed environment, with low influence of currents. The presence of anthropogenic activity, e.g. waste-pipes, land run-off and oil discharges, and the small median grain size of the sediment makes this environment more susceptible to environmental pollution (Gauquie et al., 2015). Second, plastics were exposed and sampled at the Thornton windmill park, which we will further refer to as the "offshore" environment. In this area, currents are stronger, but pollution is less pronounced.
The aims of this chapter are fourfold. First, an in-depth study of the biofilm formed on plastic exposed to the marine environment on fixed locations was done. Previous research focused on bacterial communities (Chapter 5; Zettler et al., 2013; Harisson et al., 2014; Oberbeckmann et al., 2014; Oberbeckman et al., 2016), whereas the fungal community has been less intensively studied (Oberbeckman et al., 2016). Therefore, we used 16S rRNA gene and ITS2 metabarcoding in parallel to study the taxonomic composition of bacterial and fungal communities, respectively. To study possible factors that affect biofilm formation, we compared two types of plastic (sheet or dolly rope) in two different environments (harbour or offshore). Second, the temporal dynamics of bacterial and fungal colonization of PD are reconstructed, and we identified signature species for early, intermediate or late phases of long-term exposure in the harbour environment. This series of microbial colonization in the harbour was used to evaluate biofilm formation stages in the offshore samples of the exposure series. In addition, these were compared to bacterial communities of previously described4 randomly collected samples exposed to similar offshore conditions. Third, possible sources of microorganisms were studied by comparing taxonomic profiles of plastic to those of sediment and seawater. Fourth, we investigated if bacterial and fungal species previously identified as potential PE degraders were also present in the biofilm to thus assess whether microbial biodegradation in the marine environment may take place.

6.1 MATERIALS AND METHODS

Within this section, an overview is given of the experimental design of the plastic exposure experiment and sample collection (6.1.1), the assay used to measure biofilm formation (6.1.2), the bacterial and fungal community profiling of the samples (6.1.3) and the statistical analysis of the amplicon sequencing data (6.1.4).

6.1.1 Experimental design and sample collection

From September 2015 until July 2016, PE fragments were exposed to the marine environment at two different locations in the Belgian part of the North Sea: at the harbour of Ostend (51°13'N, 2°56'E) and offshore, at the Thornton windmill park (51° 34'N, 2° 58' E) (Figure 6.1). These locations are characterised by different features (Additional Information (AI) Table AI6.1). Environmental properties (seawater temperature, conductivity, pH, oxygen, salinity and density) were measured using the CTD SBE-19plus on each sampling date. Sediment organic matter or total organic carbon (TOC) of the upper sediment layer (0-5 cm) was measured at the first sampling date for both locations, using the "dichromate method" (see Chapter 5). In addition, concentrations of pollutants in the sediment were compiled from previous studies and are listed in Table AI6.1 (De Witte, 2013; VLIZ, 2015).

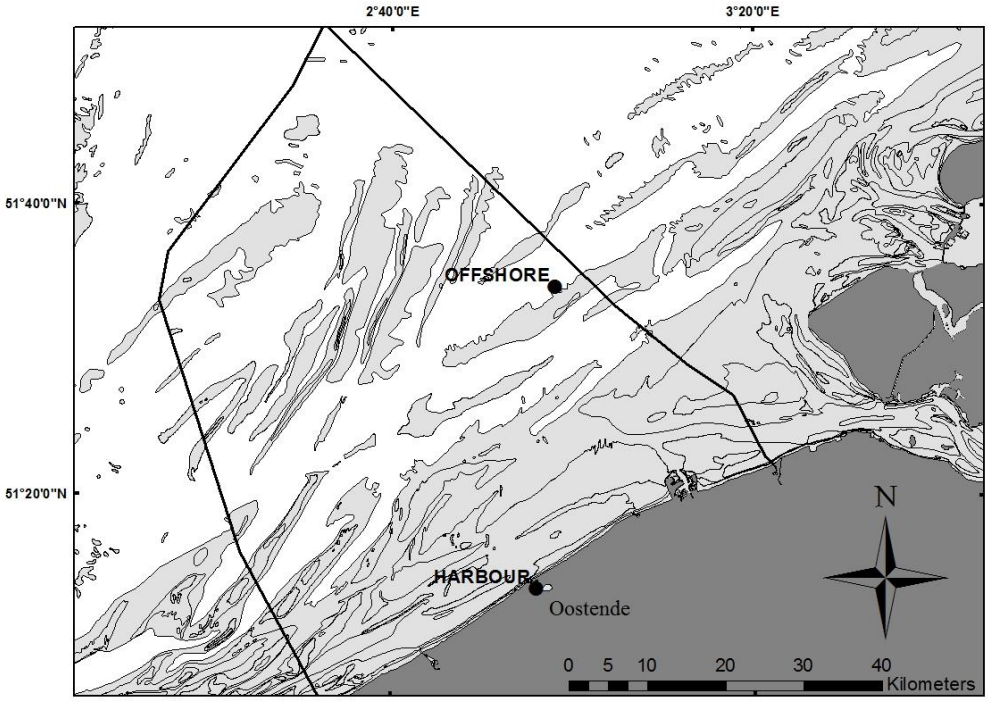


Figure 6.1 Map of the Belgian coastline and the Belgian part of the North Sea. The two experimental sites Harbour and Offshore are located at the harbour of Ostend and the Thornton windmill park.

Two types of PE, with different colours and shapes, were exposed to the two environments: transparent plastic sheets (A4 size) (RKW Hyplast, Hoogstraten, Belgium) and orange-coloured dolly ropes (ø 1 cm, length 20 cm; ø single monofilament 1 mm). Three pieces of each type of plastic, representing three biological replicates, were attached to a wooden block, which was secured in a construction (total length: +/- 60 m, weight +/- 75 kg) comprising a buoy, ropes (ø 16 mm), chains and an anchor and concrete weights (Figure AI6.1). At the start of the experiment, sets of thirteen and five identical constructions, respectively, were placed on the seafloor in the harbour and offshore. Handling of these constructions was always done with plastic gloves to avoid contamination. In addition, from three randomly chosen constructions a piece of PE sheet and dolly rope were cut with sterilized scissors to study the microbial load of plastics at the onset of the experiment, using metabarcoding. This was done for both locations.

At the harbour, one construction per week was pulled up and removed during the first month, and from then on one construction per month. This led to thirteen collection dates: 1 (September 2015), 2, 3, 4, 9, 14, 18, 22, 27, 31, 35, 40 and 44 (July 2016) weeks after placing the constructions. Offshore, the constructions were brought up and removed on four collection dates: 4 (October 2015), 14, 18 and 22 (February 2016) weeks after placing them. Upon collection of a construction, which was done with sterile forceps, scissors and gloves, half of the plastic was immediately stored at -20 °C for DNA extraction and the other half was air-dried and stored at room temperature for the biofilm assay. Offshore, three replicate seawater and sediment samples were collected on the same date as the constructions were sampled, as described in Chapter 5. Per replicate, 1 L seawater was filtered through a 0.22 µm Millipore membrane filter (Merck Millipore, Billerica, MA). After collection, sediment samples and the membrane filters were stored at -20 °C until further use.

6.1.2 Biofilm assay

The quantitative biofilm assay developed by Lobelle and Cunliffe (2011) was used to measure biofilm formation on the plastic sheets. Briefly, plastic samples (4x5 cm, n=2 per time point x location x replicate, resulting in n=6 per time point x location) were rinsed three times with sterile water and air-dried for at least 45 min in sterile Petri dishes. These plastics were stained with crystal violet (1% w/v) for 45 min and washed three times with sterile seawater. Stained samples were air-dried for another 45 min, cut into four pieces of similar size and placed into

a 2 mL Eppendorf tube to which 1 mL ethanol (95% v/v) was added. The ethanol was then diluted 100-fold in ethanol and transferred to a cuvette to measure the optical density at 595 nm using an UV-VIS spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Brussel, Belgium). The optical density is directly proportional to the amount of biofilm per surface area on the plastic.

6.1.3 "Plastisphere" microbiology: high-throughput sequencing

DNA was extracted from the sediment and plastic samples using the Powersoil DNA isolation kit (MOBIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. In total, 250 mg of sediment, a piece of 2 cm by 2.5 cm (total surface area of ± 10 cm2) of the plastic sheet, or 10 individual monofilaments with a length of 2.5 cm of the dolly rope (surface area of ± 10 cm2) were used for DNA extraction. Before extraction, plastic was rinsed three times with sterile water to remove sediment particles and loosely attached organisms. DNA was extracted from the Millipore filters containing the micro-organisms of seawater as described in Chapter 5 section 5.1.3. The DNA extracts of all samples were stored at -20 °C until further use for amplicon sequencing.

<u>16S rRNA gene and ITS2 amplicon sequencing</u>

Amplicon sequencing of the V3-V4 fragment of the 16S rRNA gene and the ITS2 gene fragment using Illumina technology (Illumina, San Diego, CA, USA) was done to study both the bacterial and fungal communities on plastic debris. DNA fragments were amplified and extended with Illumina specific index adaptors using an amplification PCR followed by a dual-index PCR, as described in detail in Chapter 2 section 2.1.8. Each PCR reaction product was purified using the CleanPCR reagent kit (MAGBIO, Gaithersburg, MD, USA). Libraries were quality-controlled using the Qiaxcel Advanced, with the Qiaxcel DNA High Resolution kit (QIAGEN, Germantown, MD, USA), and concentrations were measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The indexed libraries of each sample were diluted to 10 nM and pooled in a 2:1 ratio for bacterial and fungal libraries, respectively. Resulting libraries were sequenced using Illumina MiSeq v3 technology (2 x 300 bp) by Macrogen, South-Korea, using 30% PhiX DNA as spike-in.

Sequence reads processing

Demultiplexing of the amplicon dataset and removal of the barcodes was performed by the sequencing provider. The raw sequence data is available in the NCBI Sequence Read Archive under the accession number Bioproject ID PRJNA360358 for the bacterial sequences and PRJNA356487 for the fungal sequences. Processing the sequence reads to Operational Taxonomic Unit (OTU) tables was done as described in detail in Chapter 2 section 2.1.8.

The bacterial load of three PE sheets and three ropes was checked at the onset of the experiment before exposure to the marine environment. Samples were analysed by metabarcoding in parallel to all other samples. Because the PCR product concentration was low, all DNA was used for sequencing. For each sample, the number of reads was < 100.

6.1.4 Downstream data analysis and statistics

OTU tables of the 16S V3-V4 rRNA gene region and ITS2 amplicon sequencing of the samples of the exposure experiment and the 16S V3-V4 rRNA gene region amplicon sequences of plastic samples of Chapter 5 were analysed using the QIIME software package (v1.9.0) (Caporaso et al, 2010a). Taxonomy was assigned with the script “assign_taxonomy.py” using the uclust method considering maximum 3 database hits, with the Silva v119 97% rep set (as provided by QIIME) as reference for the bacterial sequences and UNITE v7 (dynamic) for fungal sequences (Caporaso et al, 2010b; Quast et al, 2012; Kõljalg et al, 2013).

A part of the fungal sequences could not be classified using the UNITE database. These sequences were extracted from the total data set and their taxonomy was assigned using Basic Local Alignment Search Tool (BLAST) for sequence comparison with the non-redundant nucleotide database of NCBI (Altschul et al, 1990). We kept the best hit per query using an e-value cut-off of 1e-5 and a minimal percent identity of 98.5%.

Rarefaction analysis was done using the “alpha_rarefaction.py” script of QIIME. A plateau was reached at 10,000 sequences for the bacterial and fungal OTUs. Richness of the bacteria and fungi was determined on rarefied data, for which the number of sequences was set on the reached plateau.

A core microbiome was calculated separately for the bacterial and fungal communities. Only plastics exposed for at least four weeks were considered to calculate a core microbiome to account for some lag-time for biofilm build-up. This core microbiome was calculated separately for the two environments. OTUs were denoted as core organisms if their relative

abundance contributed at least 0.1% to the total community of a sample in at least 90% of the plastic items per environment. Calculations for the core microbiome were done in R (R core team, 2015). In addition, the genera that were most abundant at the last time point of sampling were calculated by selecting those genera with a minimal mean abundance of 1% over the replicates for sheets or dolly ropes.

The multivariate analysis was done using the specific R package vegan (version 2.0-10) (Oksanen et al, 2010). The OTU tables of bacterial and fungal sequences, as generated by Usearch, were normalized by calculating relative abundances. Next, OTUs with a low count number were removed by only retaining the OTUs which had a minimal relative abundance of 0.01 % in at least three samples. The dissimilarity matrix, based on the Bray-Curtis dissimilarity index, was calculated from this normalized and filtered OTU table, for both the bacterial and fungal sequences. The homogeneity of the variances was checked on this dissimilarity matrix using the betadisper function. The significance of the factors environment, type of plastic and time, and their various interaction effects were analysed using PERMANOVA analysis (number of permutations = 1,000) using the Bray-Curtis dissimilarity index matrix as input. Factors were considered significantly different if p-value < 0.05.

6.2 RESULTS

In this section, the biofilm formation is discussed first by looking at the bacterial and fungal community of plastics exposed at the harbour (6.2.1) and offshore environment (6.2.2) Further on, factors which influence the colonization of plastic and the bacterial and fungal sources of colonization are investigated (6.1.3). In addition, we set out to investigate if previously identified bacterial and fungal species can potentially act as biodegraders (6.1.4).

6.2.1 Bacterial and fungal colonization in the harbour environment

From the first week of exposure onwards, a coating comprising a microbial biofilm, sediment particles, algae and macro-fouling (e.g. mussels) was formed on the plastic sheets at the harbour (Figure 6.2). Using a quantitative biofilm formation assay (Lobelle & Cunliffe, 2011), at least part of the coating on the plastic sheets could be attributed to a microbial biofilm (Figure 6.3). This biofilm was already detected after one week of exposure, and increased slightly until week 27, followed by a period of stronger growth until week 40.

The taxonomic composition of the bacterial and fungal community on plastic at the harbour was analyzed in detail by 16S and ITS2 metabarcoding. Richness of the samples was studied by estimating the number of observed OTUs and the Chao1 index. Both measures showed that the richness of sheets is similar to that of dolly ropes; both for bacterial OTUs (Figure AI6.2 and AI6.3 - Harbour) and for fungal OTUs (Figure AI6.2 and AI6.3 - Harbour). The number of bacterial OTUs is only slightly higher on dolly ropes compared to sheets in the first few weeks of exposure to the harbour environment. At each time point, the bacterial richness of plastic (mean around 1500 OTUs) was markedly higher than fungal richness (mean around 500 OTUs). The bacterial community of plastic sheets and dolly ropes at the harbour displayed a gradual change in taxonomic composition during the period of exposure (Figure 6.4A and Figure 6.4B). This temporal gradient, more evident on the plastic sheets, is, at least in part, caused by shifts in abundance of particular bacterial classes: an increase in the relative abundance of *alpha*- and *betaproteobacteria* and *flavobacteria*, and a decrease in the relative abundance of gammaproteobacteria (Figure 6.4A and Figure 6.4B). *Alpha*- and *gammaproteobacteria* are characteristic for primary biofilm colonization, while *bacteroidetes* are known secondary biofilm colonizers in the marine environment (Hörsch et al., 2005; Lee et al., 2008; Elifantz et al., 2013).

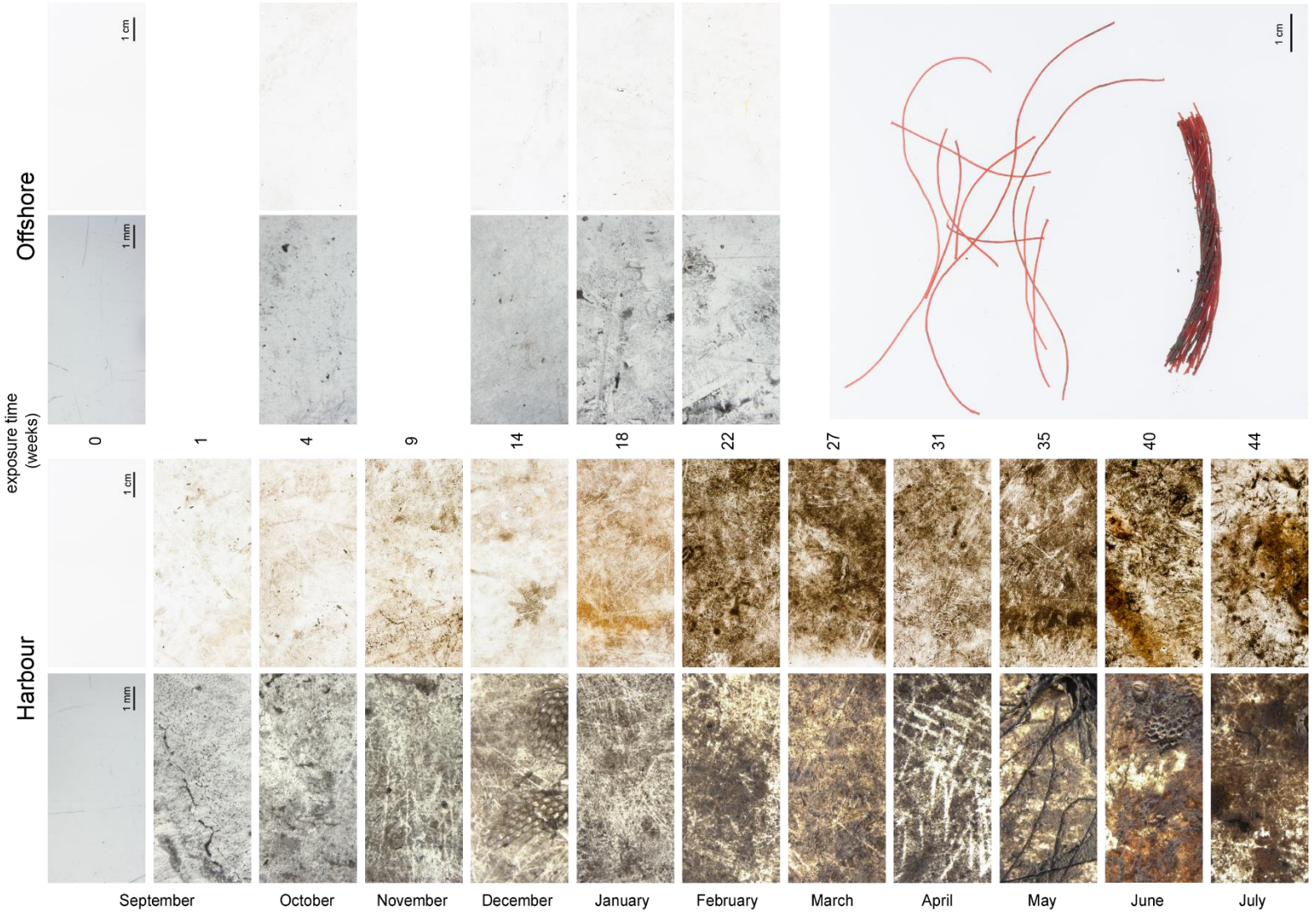


Figure 6.2 Biofilm formation on plastic after exposure at two locations in the Belgian part of the North Sea. Offshore, plastics were exposed for 5 months (22 weeks), while at the harbour exposre time was 10 months (44 weeks). Both macroscopic (top) and microscopic (bottom) images are given for both environments. Fouling of the plastic sheets consists of a small fraction of sediment particles, macrofouling and the formation of a microbial biofilm.

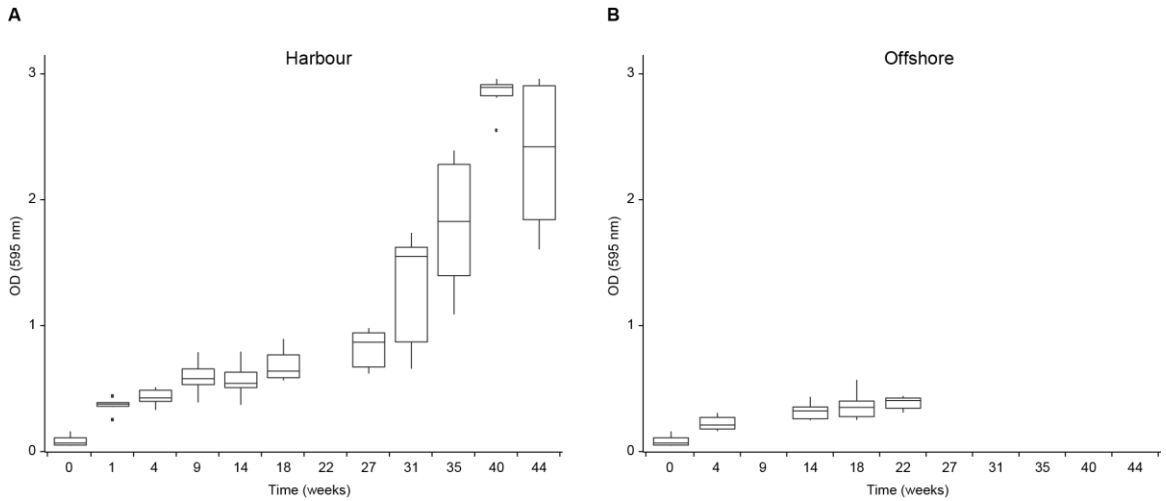


Figure 6.3 Boxplots of the optical density of crystal violet staining representing biofilm formation on plastic sheets. A) Plastic exposed at the harbour of Ostend, B) Plastic exposed offshore. The amount of biofilm is measured as optical density after crystal violet staining according to the methods described in Lobelle & Cunliffe (2011), (n=6).

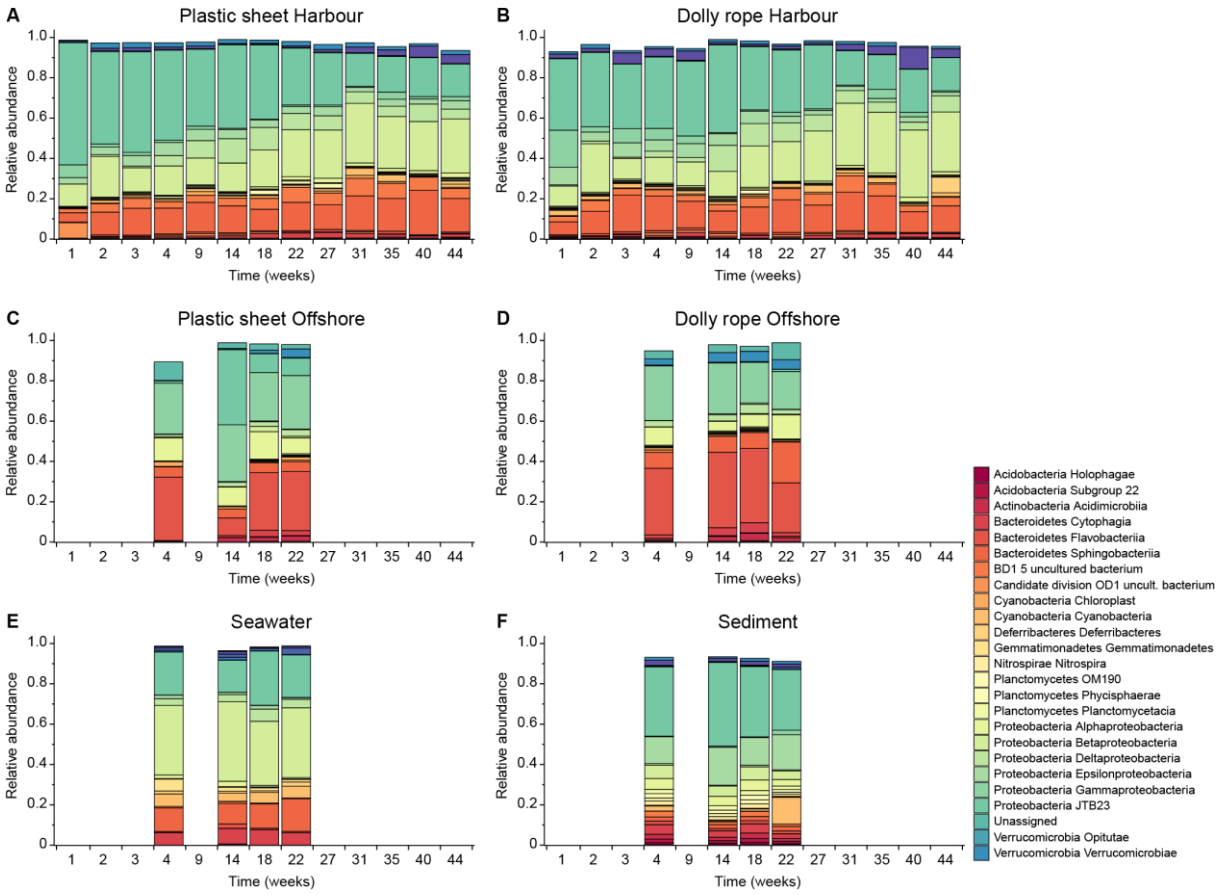


Figure 6.4 Bacterial community composition of plastics exposed to the marine environment, sediment, and seawater over time. Mean relative abundances of the different bacterial classes (16S V3-V4 region) on plastic items (n=3), sediment (n=3) and seawater (n=3) in the North Sea region. Only classes representing at least 1% of the community are shown.

On the plastic sheets, a gradual decrease in the relative abundance of these primary colonizers and an increase in secondary colonizers was observed (Figure 6.5A), suggesting that subsequent time points reflect progressive stages of biofilm formation. This shift from primary to secondary colonizers, however, was not as clearly discernible for the dolly ropes (Figure 6.5B).

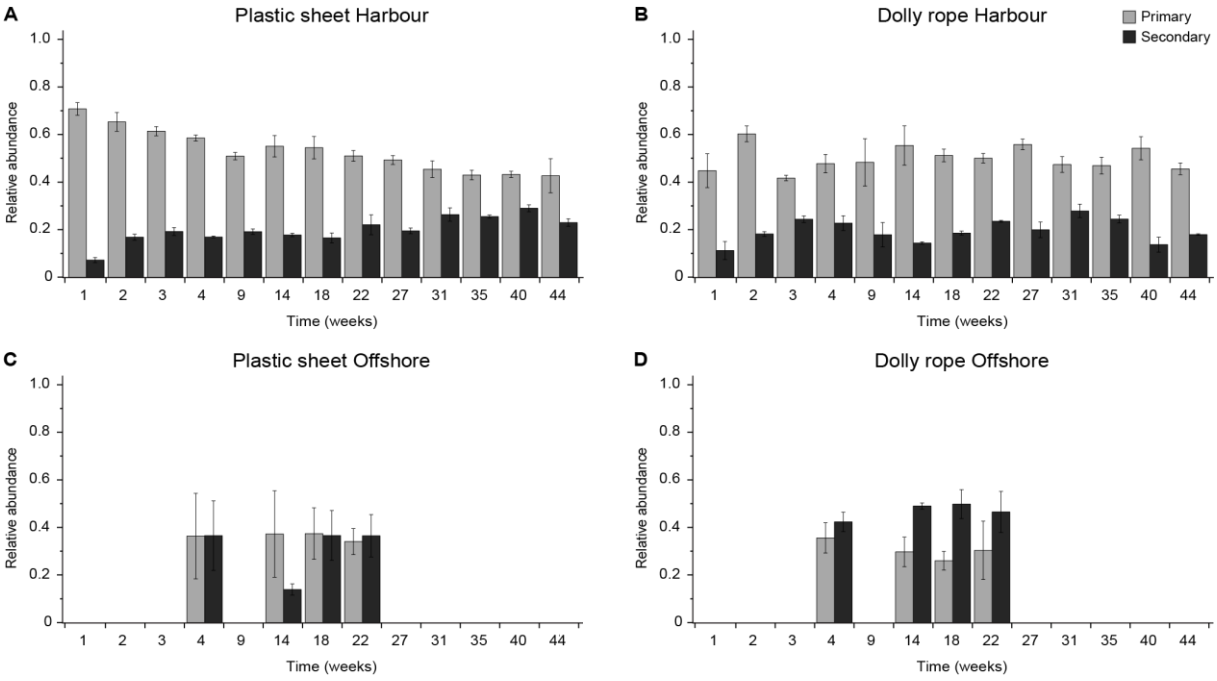


Figure 6.5 Representation of the primary (alpha-and gammaproteobacteria) and secondary (bacteroidetes) colonizers on plastics during the exposure period. A) plastic sheets exposed at the harbour (44 weeks), B) dolly ropes exposed at the harbour (44 weeks), C) plastic sheets exposed offshore (22 weeks), D) dolly ropes exposed offshore (22 weeks).

Next, we defined a core bacteriome of plastic samples (see 6.1.4). In total, 25 bacterial core OTUs were identified both on plastic sheets and dolly ropes (Table 6.1). Based on their temporal profile, these core members were classified into four groups: (1) OTUs without a clear period of high relative abundance (neutral), e.g. *Arenicella*, *Methylotenera*; (2) OTUs with higher abundance in the beginning (early stage; week 1-14) of the exposure period, e.g. *Sulfurovum*, *Maritimimonas*; (3) OTUs with higher abundance in the middle (intermediate; week 14-35) of the exposure period, e.g. *Robiginitomaculum*, and (4) OTUs with highest abundance at the end (late stage; week 35-44) of the exposure period, e.g. *Sulfitobacter*, *Psychroserpens* (Table 6.1).

Next, the fungal community on the plastic sheets and dolly ropes in the harbour was studied. Strikingly, the majority of the fungal sequences (28% to 97% of the reads per sample) could not be assigned using the UNITE database (Figure 6.6A and Figure 6.6B). Using NCBI Blast,

some of those reads could be assigned to fungi, others to other members of the eukaryotes, e.g. *Paramoeba permaquidensis, Paramoeba aestuarina, Pleurobrachia pileus, Sugiura chengshanense, Sagartia elegans,* and *Rhizostoma pulmo*, but the vast majority remained unassigned.

Table 6.1 Core OTUs of plastics sampled at the harbour or offshore. OTUs are defined as a core member if they represent at least 0.1% of the sample in at least 95% of all samples per environment.

HARBOUR

OTU	Phylum	Family	Genus	Peak moment (weeks)	Colonizer
27	*Proteobacteria*	*Helicobacteraceae*	*Sulfurovum*	1-14	Early
15	*Proteobacteria*	*Ectothiorhodospiraceae*	*Acidiferrobacter*	1-14	Early
57	*Proteobacteria*	*JTB255 marine benthic group*	*uncultured*	1-14	Early
87	*Proteobacteria*	*JTB255 marine benthic group*	*uncultured*	1-14	Early
28	*Proteobacteria*	*JTB255 marine benthic group*		1-14	Early
62	*Bacteroidetes*	*Flavobacteriaceae*	*Eudoraea*	1-14	Early
19	*Bacteroidetes*	*Flavobacteriaceae*	*Maritimimonas*	1-14	Early
1508	*Bacteroidetes*	*Flavobacteriaceae*		1-14	Early
51	*Bacteroidetes*	*Flavobacteriaceae*		1-14	Early
9	*Proteobacteria*	*Hyphomonadaceae*	*Robiginitomaculum*	14-35	Intermediary
591	*Proteobacteria*	*Rhodobacteraceae*		14-35	Intermediary
567	*Proteobacteria*	*Rhodobacteraceae*		14-35	Intermediary
35	*Proteobacteria*	*Erythrobacteraceae*		14-35	Intermediary
16	*Proteobacteria*	*IheB2-23*	*uncultured*	14-35	Intermediary
43	*Proteobacteria*	*Unkown Thiotrichales*		14-35	Intermediary
5	*Bacteroidetes*	*Flavobacteriaceae*	*Maritimimonas*	14-35	Intermediary
84	*Proteobacteria*	*Rhodobacteraceae*	*Profundibacterium*	31-45	Late
335	*Proteobacteria*	*Rhodobacteraceae*	*Sulfitobacter*	31-45	Late
11149	*Proteobacteria*	*Rhodobacteraceae*	*uncultured*	31-45	Late
63	*Bacteroidetes*	*Flavobacteriaceae*	*Psychroserpens*	31-45	Late
10		*Verrucomicrobiaceae*	*Persicirhabdus*	31-45	Late
107	*Proteobacteria*	*Rhodobiaceae*	*Anderseniella*	None	Neutral
2	*Proteobacteria*	*Methylophilaceae*	*Methylotenera*	None	Neutral
74	*Proteobacteria*	*Unknown Family*	*Arenicella*	None	Neutral
3	*Proteobacteria*	*Unkown Methylococcales*		None	Neutral

OFFSHORE

OTU	Phylum	Family	Genus	Peak moment	Colonizer
14	*Bacteroidetes*	*Flavobacteriaceae*	*Lacinutrix*	None	Neutral
1508	*Bacteroidetes*	*Flavobacteriaceae*		None	Neutral
30	*Bacteroidetes*	*Flavobacteriaceae*	*Tenacibaculum*	None	Neutral
54	*Bacteroidetes*	*Flavobacteriaceae*	*Polaribacter*	None	Neutral
12	*Bacteroidetes*	*Flavobacteriaceae*	*uncultured*	None	Neutral
63	*Bacteroidetes*	*Flavobacteriaceae*	*Psychroserpens*	None	Neutral
59	*Proteobacteria*	*Unknown Caulobacterales*		None	Neutral
442	*Proteobacteria*	*Hyphomonadaceae*	*Hellea*	None	Neutral
11149	*Proteobacteria*	*Rhodobacteraceae*	*uncultured*	None	Neutral
189	*Proteobacteria*	*Alcanivoracaceae*	*Kangiella*	None	Neutral

Within the share of the fungal sequences that were assigned to a certain taxonomy, the *Ascomycota* were highly abundant, followed by a smaller fraction of *Basidiomycota* (Figure

6.6A and 6.6B). *Zygomycota* were also identified, but only represented a minor fraction. In addition, genera that were highly abundant on sheets or dolly ropes (> 1%) sampled at the last time point (44 weeks) were studied (Table 6.2). Especially members of the *Lecanoromycetes*, e.g. *Physconia*, *Candelariella*, and *Caloplaca* were abundant. No clear temporal profile characterised by early, intermediate, and late stage abundance peaks could be identified, essentially because the fungal community profile varied considerably, even between successive time points (Figure 6.6A and Figure 6.6B). In addition, no core group of fungal organisms could be identified, illustrating the variability of the fungal community through time.

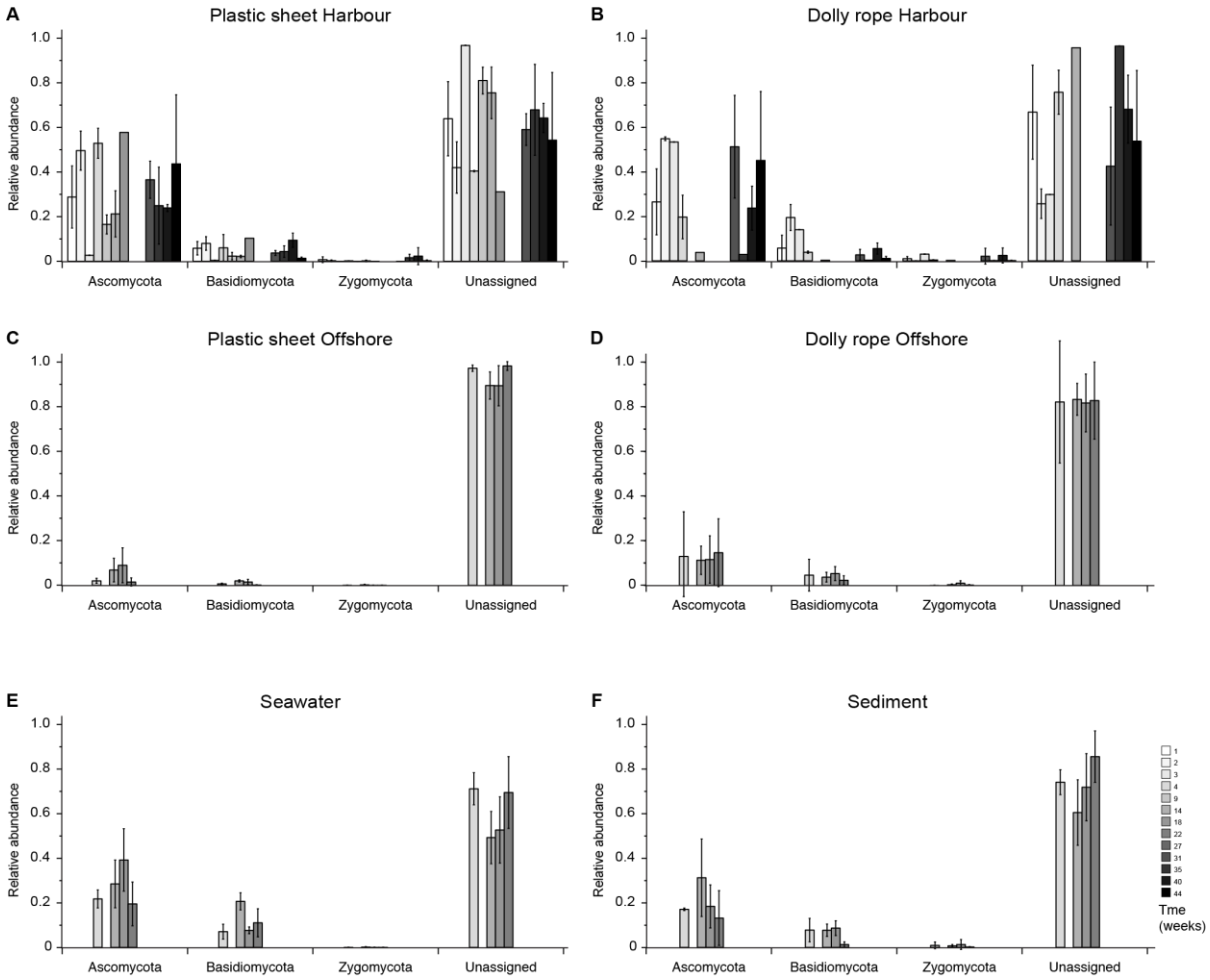


Figure 6.6 Fungal community composition of plastic during exposure to the marine environment, sediment, and seawater. Mean relative abundances of the different fungal classes (ITS2 region) on plastic items (n=3), sediment (n=3) and seawater (n=3) in the North Sea region. Only classes representing at least 1% of the community are shown.

Table 6.2 Fungal genera present on plastic sampled at the harbour at the end of the exposure experiment (t=44 weeks). Only genera with abundance of at least 1% on at least 1 type of plastic (sheet or dolly rope) are given (mean relative abundance (%) ± standard error).

Phylum	Class	Family	Genus	Sheet	Dolly rope
Ascomycota	*Dothideomycetes*	*Davidiellaceae*	*Cladosporium*	3.44±0.38	5.59±2.45
		Incertae sedis	*Unidentified*	2.61±1.06	2.85±1.26
			Other	4.20±2.73	5.00±2.13
		Pleosporaceae	*Alternaria*	1.28±0.69	2.82±2.45
			Other	1.03±0.45	12.93±7.03
		Unidentified	*Unidentified*	1.20±0.85	1.06±0.54
		Other	*Other*	1.79±0.17	0.51±0.13
		Other	*Other*	6.38±3.74	16.34±9.03
	Lecanoromycetes	*Candelariaceae*	*Candelariella*	14.34±8.44	1.91±1.26
		Physciaceae	*Physconia*	6.54±2.63	5.87±4.44
		Teloschistaceae	*Caloplaca*	18.64±7.02	18.72±8.86
			Unidentified	9.76±8.67	2.37±1.23
			Other	5.12±2.22	5.35±1.91
	Sordariomycetes	*Nectriaceae*	*Fusarium*	2.18±0.29	3.74±1.90
			Other	1.20±0.56	0.47±0.13
Basidiomycota	*Tremellomycetes*	*Incertae sedis*	*Cryptococcus*	1.62±0.49	1.48±0.65

6.2.2 Bacterial and fungal colonization in the offshore environment

Biofilm formation occurred on plastic exposed to offshore conditions, but was much less pronounced compared to the harbour environment as described above. For instance, the biofilm layer was hardly visible by the naked eye even after 22 weeks of exposure (Figure 6.2), and the amount of biofilm that had accumulated after 22 weeks of exposure offshore was similar to the amount that had already accumulated at the harbour after 1 week of exposure (Figure 6.3). Until week 18 of the exposure period, the number of unique bacterial OTUs and the Chao1 index, both representing the richness of the samples, on the plastic sheets sampled offshore remained low (< 1000 OTUs; Chao1: <1200 OTUs) compared to plastic sheets and dolly ropes exposed to the harbour environment (Figure AI6.2 and AI6.3). In contrast, the number of unique fungal OTUs varied between 300 and 500 (Chao1: 400 – 700 OTUs) for most points in time, and this was observed for both types of plastic and in both environments (Figure AI6.2 and AI6.3). Offshore, the bacterial community on plastic sheets and dolly ropes was dominated by flavobacteria and gammaproteobacteria (Figure 6.4C and Figure 6.4D). No temporal gradient could be observed (Figure 6.4C and Figure 6.4D), and the proportion of primary and secondary colonizers remained fairly stable throughout the time series for both types of plastic sampled offshore (Figure 6.5C and Figure 6.5D). A core bacteriome consisting of 10 bacterial OTUs was identified for the plastics sampled offshore (Table 6.1). In contrast

to the abundance profiles observed in the harbour, the offshore core OTUs did not display a clear temporal change in abundance.

Next, the fungal community on the plastic exposed to offshore conditions was studied. Between 81% and 99% of fungal sequences remained unassigned, which is an even higher share compared to the fungal communities on plastics from the harbour (Figure 6.6). *Ascomycota* and *Basidiomycota* were the most prominent of the assigned sequences, which was similar to plastic sampled at the harbour and seawater and sediment fungal communities (Figure 6.6E and Figure 6.6F). The most common fungal genera on plastic sheets and dolly ropes sampled at the last time point (22 weeks) are listed in Table 6.3. Like for the harbour environment, no fungal core members could be detected on offshore samples.

Table 6.3 Fungal genera present on plastic sampled offshore at the end of the exposure experiment (t=44 weeks). Only genera with abundance of at least 1% on at least 1 type of plastic (sheet or dolly rope) are given (mean relative abundance (%) ± standard error).

Phylum	Class	Family	Genus	Sheet	Dolly rope
Ascomycota	*Dothideomycetes*	*Davidiellaceae*	*Cladosporium*	9.59±2.15	0.84±0.38
		Incertae sedis	*Other*	2.58±2.20	0.00±0.00
		Phaeosphaeriaceae	*Phaeosphaeria*	0.10±0.10	2.04±2.00
		Pleosporaceae	*Alternaria*	1.84±1.60	1.47±0.49
			Other	13.43±10.20	3.96±1.63
		Other	*Other*	4.80±2.42	2.28±1.18
		Other	*Other*	2.32±2.18	2.27±1.78
	Eurotiomycetes	*Trichocomaceae*	*Penicillium*	1.16±1.10	0.08±0.08
	Lecanoromycetes	*Candelariaceae*	*Candelariella*	1.34±1.12	3.52±1.84
		Lecanoraceae	*Lecanora*	0.78±0.60	1.21±0.84
		Physciaceae	*Physconia*	3.77±2.52	5.29±2.66
		Teloschistaceae	*Caloplaca*	3.36±1.84	8.94±2.61
			Other	4.70±2.36	15.63±3.45
	Leotiomycetes	*Erysiphaceae*	*Blumeria*	1.86±1.66	0.00±0.00
		Sclerotiniaceae	*Other*	5.97±2.32	0.02±0.02
	Saccharomycetes	*Incertae sedis*	*Cyberlindnera*	1.57±1.50	0.00±0.00
			Debaryomyces	2.22±2.22	0.02±0.02
		Saccharomycetaceae	*Saccharomyces*	2.22±2.20	0.12±0.10
	Sordariomycetes	*Nectriaceae*	*Other*	1.53±1.20	0.00±0.00
		Lulworthiaceae	*Lulwoana*	0.00±0.00	3.27±1.64
		Chaetomiaceae	*Other*	2.22±2.20	4.28±4.02
		Other	*Other*	4.92±2.69	0.83±0.53
		Incertae sedis	*Microdochium*	2.22±2.20	0.00±0.00
	Unidentified	*Unidentified*	*Unidentified*	1.47±1.47	3.56±1.16
	Other	*Other*	*Other*	1.08±0.96	14.84±1.65
Basidiomycota	*Agaricostilbomycetes*	*Kondoaceae*	*Kondoa*	3.56±1.84	0.71±0.46
	Incertae sedis	*Malasseziaceae*	*Malassezia*	2.13±2.01	3.50±1.52
	Microbotryomycetes	*Incertae sedis*	*Rhodotorula*	0.42±0.40	2.38±2.03
			Sporobolomyces	0.00±0.00	2.69±0.87
	Tremellomycetes	*Incertae sedis*	*Dioszegia*	5.60±3.99	0.21±0.21
Zygomycota	*Incertae sedis*	*Mortierellaceae*	*Mortierella*	0.00±0.00	1.26±0.55

6.2.3 Factors that affect biofilm formation

PERMANOVA analysis revealed that the three factors: sample type (plastic sheet, dolly rope, seawater or sediment; PERMANOVA, $p<0.001$); environment (harbour or offshore) (PERMANOVA, $p<0.001$); and time of exposure (PERMANOVA, $p<0.001$), significantly affect the bacterial community composition, with highly significant interaction effects (all interaction effects $p<0.001$) between all three factors. It should be noted that these differences in community profiles could partly be caused by a considerable difference in multivariable spread (permutation based, $p < 0.001$), as variances were heterogeneous.

Next, Principal Coordinate Analysis (PCoA) (Figure 6.7) was used to visualise similarities and dissimilarities of the bacterial communities of plastics sampled in the harbour and offshore.

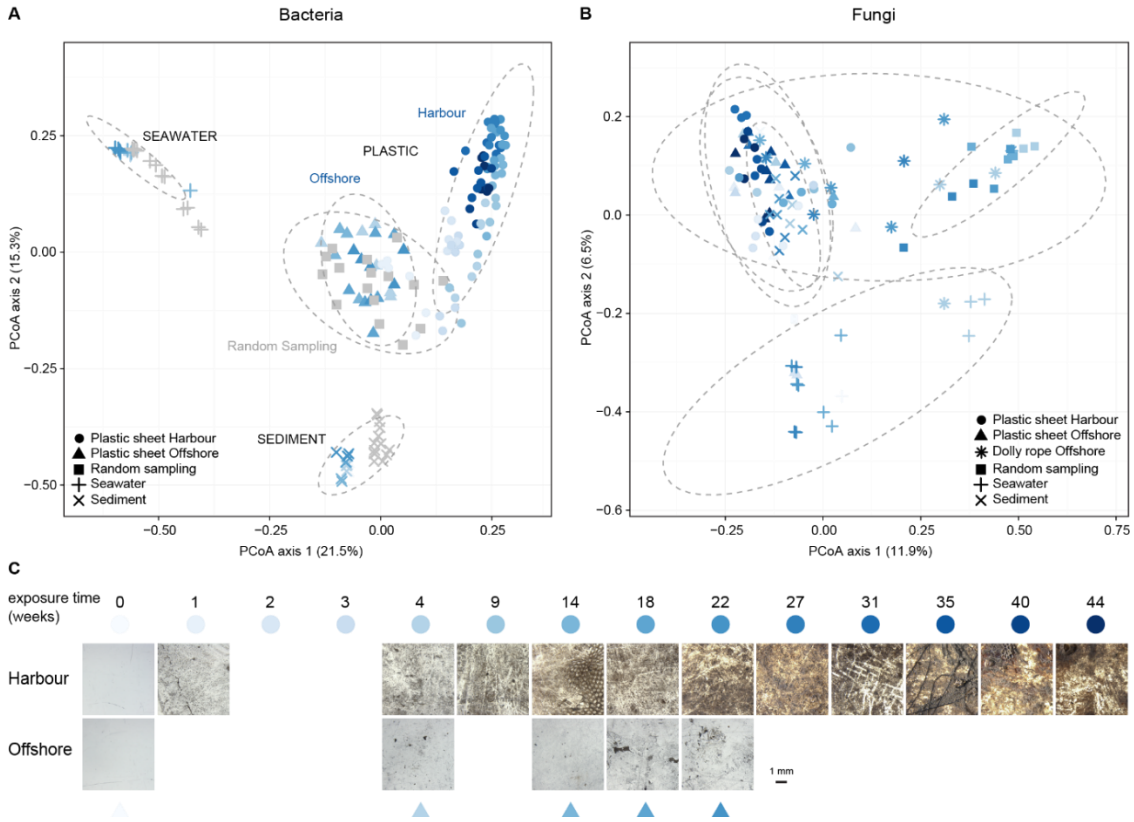


Figure 6.7 PCoA profile of pairwise community dissimilarity indices (Bray-Curtis), calculated from the OTU table of the microbial community on plastic, seawater and sediment samples. Samples of the exposure series are shown in blue. Colour intensities indicate the time of exposure. The shape represents the different sample types (plastic at the harbour, plastic offshore, seawater or sediment). Grey dotted ovals indicate the 95% confidence intervals for each sample type. A) bacterial communities. Samples of a broad sampling campaign in 2014 (Chapter 5) are represented as grey boxes. B) fungal communities. **Bottom panel**: Macro- and microscopic images of the coating formed on plastic sheets during prolonged exposure to harbour (left panel) and offshore (right panel) conditions.

First, bacterial communities of plastic sampled at the harbour noticeably shifts away from the earlier time points (Figure 6.7A; Figure 6.4B; Figure 6.4B), indicating that plastic sampled in the harbour displayed a gradual change in bacterial community composition. Together, this temporal profile of the harbour plastic was used to evaluate biofilm formation stages in the offshore plastic samples. Strikingly, the bacterial community composition on the plastics exposed to offshore conditions at any time, resembled those at the earliest time points (first two weeks) on plastics from the harbour. Conversely, bacterial signatures of intermediate or late stage biofilm as observed in the harbour were not observed on any of the plastics, during five months of exposure to offshore conditions. Eight of the 10 OTUs of the offshore core set were not identified in the harbour core set. The other two OTUs of the offshore core set, classified as *Psychroserpens* and an uncultured Rhodobacteriaceae bacteria, were also found in the harbour core set. Under offshore conditions these OTUs presented 1-1.5% of the bacterial community, while they were slightly more abundant in late stage biofilms of the harbour (2%).

Second, we used PCoA to compare samples of our new exposure time series to the previously described dataset of Chapter 5, comprising bacterial communities of independently collected, randomly sampled pieces of plastic of the North Sea. The bacterial communities of these randomly collected pieces of plastic cluster with those of the plastic samples which were exposed offshore, even though these plastic samples originate from different locations and were sampled in different seasons. Late stage bacterial community profiles as identified in the harbour exposure series, were also not observed in any of our previously described, randomly collected pieces of plastic (Figure 6.7A). This further shows that the variation in biofilm composition observed is similar in 'free-moving' plastic and plastic fixed to the seafloor in our constructions.

Third, we compared the taxonomic composition of plastic to that of seawater and sediment to investigate if these environments could serve as a bacterial source for plastic biofilm organisms. Sediment and seawater were sampled offshore in our new series, and were also compared to sediment and seawater samples of our previously published plastic survey (Chapter 5). Seawater and sediment bacterial communities were quite similar for both sample sets and they consistently clustered separately from all plastic samples (Figure 6.7A). Detailed comparison at the bacterial class level revealed that several of the major classes present in seawater (*alphaproteobacteria* and *flavobacteria*) and sediment (*gammaproteobacteria*)

were also detected on plastic sampled offshore and at the harbour (Figure 6.4E; Figure 6.4F). Conversely, all bacterial OTUs observed on the plastics of the exposure series were also present in sediment or seawater. This indicates that taxonomic profiles overlap, and that the separate clustering in the PCoA resulted from shifts in the relative abundance of the bacteria (Figure 6.4; Figure 6.7).

PERMANOVA analysis on the fungal community composition showed significant effects of sample type ($p<0.001$), environment ($p<0.001$), and exposure time ($p<0.001$). In contrast to the consistent patterns observed for the bacterial communities, PCoA analysis showed the high variability in fungal communities for both types of plastic, even between biological replicates (Figure 6.7B). No clear temporal shifts in the fungal community composition could be observed across successive sampling points under harbour or offshore conditions, as illustrated by a broad clustering of individual samples within the PCoA plots (Figure 6.7B).

6.2.4 Screening for potential PE degraders

After three months of exposure to offshore conditions we observed rips at several places on the plastic sheets (Figure 6.2). However, due to the experimental setup, we were neither able to quantify the physical degradation of the plastic, nor the loss of plastic mass due to biological degradation and/or microbial metabolic activity. Instead, we searched for the presence of micro-organisms with a previously identified potential for PE biodegradation (Restrepo-Florez et al., 2014). Three fungal OTUs identified at species level were detected on plastic samples, especially at the harbour, which had previously been identified as potential PE biodegraders in a soil environment: *Cladosporium cladosporioides*, *Fusarium redolens* and at lower abundance *Mortierella alpina* (Table 6.4) (Restrepo-Florez et al., 2014). The relative abundance of *C. cladosporioides* and *F. redolens* increased towards the end of the exposure period. In contrast, none of the currently known PE biodegrading bacterial genera were identified on our plastic samples exposed to the North Sea environment.

Table 6.4 Temporal dynamics in relative abundance of three previously identified fungal PE biodegraders: *M. alpina*, *F. redolens* and *C. cladosporioides*. Relative abundances are given for A) plastic sheets at the harbour, B) dolly ropes at the harbour, C) plastic sheets offshore and D) dolly ropes offshore. The mean relative abundances of other species within the same genera (indicated as "other"), are given for each time point within the exposure period. Time points consisting of less than 3 biological replicates were present, due to a low number of sequence reads in one or more of the replicates, are excluded from the table.

HARBOUR SHEET														
Genus	**Species**	**t1**	**t2**	**t3**	**t4**	**t9**	**t14**	**t18**	**t22**	**t27**	**t31**	**t35**	**t40**	**t44**
Mortierella	*alpina*	0,00±0,00	0,02±0,02	0,00±0,00	0,09±0,07	0,00±0,00	0,00±0,00	0,00±0,00	/	/	0,00±0,00	0,02±0,02	0,00±0,00	0,00±0,00
	other	0,57±0,57	0,24±0,18	0,02±0,00	0,06±0,04	0,02±0,01	0,20±0,20	0,01±0,01	/	/	0,02±0,02	1,16±0,42	1,84±1,75	0,24±0,15
Fusarium	*redolens*	0,00±0,00	0,14±0,12	0,04±0,01	0,07±0,06	0,00±0,00	0,01±0,00	0,00±0,00	/	/	0,00±0,00	0,00±0,00	0,53±0,44	0,99±0,46
	other	0,15±0,15	0,00±0,00	0,03±0,00	0,00±0,00	0,07±0,05	0,01±0,00	0,00±0,00	/	/	0,00±0,00	0,00±0,00	0,27±0,27	0,10±0,09
Cladosporium	*cladosporioides*	0,14±0,11	0,96±0,73	0,10±0,02	0,16±0,09	0,07±0,01	0,36±0,34	0,02±0,01	/	/	0,13±0,23	0,73±0,49	0,16±0,07	0,83±0,74
	other	2,80±0,97	5,22±0,47	0,11±0,02	1,15±0,26	0,42±0,29	0,41±0,34	1,18±0,10	/	/	0,52±0,13	1,25±0,42	3,66±2,00	0,72±0,11
HARBOUR DOLLY ROPE														
Genus	**Species**	**t1**	**t2**	**t3**	**t4**	**t9**	**t14**	**t18**	**t22**	**t27**	**t31**	**t35**	**t40**	**t44**
Mortierella	*alpina*	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00	/	0,00±0,00	/	/	/	0,03±0,03	0,00±0,00	0,00±0,00	0,00±0,00
	other	0,96±0,55	0,00±0,00	2,98±0,15	0,40±0,02	/	0,22±0,00	/	/	/	2,04v2,02	0,13±0,02	2,40±1,96	0,11±0,07
Fusarium	*redolens*	0,01±0,01	0,00±0,00	0,00±0,00	0,00±0,00	/	0,00±0,00	/	/	/	0,05±0,05	0,00±0,00	0,00±0,00	1,33±1,00
	other	0,00±0,00	0,04±0,04	0,00±0,00	0,00±0,00	/	0,08±0,02	/	/	/	0,11±0,11	0,00±0,00	0,01±0,01	0,10±0,07
Cladosporium	*cladosporioides*	0,76±0,40	9,63±1,84	0,89±0,12	0,19±0,05	/	0,08±0,03	/	/	/	0,27±0,17	0,01±0,01	0,36±0,30	0,10±0,04
	other	2,21±0,99	9,24±1,15	1,30±0,56	0,80±0,16	/	0,02±0,01	/	/	/	0,48v0,35	0,15±0,02	1,49±0,73	1,59±0,53
OFFSHORE SHEET														
Genus	**Species**	**t1**	**t2**	**t3**	**t4**	**t9**	**t14**	**t18**	**t22**	**t27**	**t31**	**t35**	**t40**	**t44**
Mortierella	*alpina*	/	/	/	0,00±0,00	/	0,00±0,00	0,00±0,00	0,00±0,00	/	/	/	/	/
	other	/	/	/	0,00±0,00	/	0,23±0,16	0,00±0,00	0,00±0,00	/	/	/	/	/
Fusarium	*redolens*	/	/	/	0,00±0,00	/	0,00±0,00	0,00±0,00	0,00±0,00	/	/	/	/	/
	other	/	/	/	0,00±0,00	/	0,00±0,00	0,00±0,00	0,00±0,00	/	/	/	/	/
Cladosporium	*cladosporioides*	/	/	/	0,02±0,02	/	0,09±0,07	0,40±0,04	0,00±0,00	/	/	/	/	/
	other	/	/	/	0,19±0,08	/	0,41±0,14	0,00±0,00	0,11±0,06	/	/	/	/	/
OFFSHORE DOLLY ROPE														
Genus	**Species**	**t1**	**t2**	**t3**	**t4**	**t9**	**t14**	**t18**	**t22**	**t27**	**t31**	**t35**	**t40**	**t44**
Mortierella	*alpina*	/	/	/	0,00±0,00	/	0,00±0,00	0,00±0,00	0,00±0,00	/	/	/	/	/
	other	/	/	/	0,26±0,13	/	0,26±0,13	0,77±0,67	0,18±0,09	/	/	/	/	/
Fusarium	*redolens*	/	/	/	0,00v0,00	/	0,00±0,00	0,00±0,00	0,00±0,00	/	/	/	/	/
	other	/	/	/	0,00±0,00	/	0,00±0,00	0,00±0,00	0,00±0,00	/	/	/	/	/
Cladosporium	*cladosporioides*	/	/	/	0,55±0,48	/	0,55±0,48	0,03±0,03	0,00±0,00	/	/	/	/	/
	other	/	/	/	0,69±0,48	/	0,69±0,48	0,65±0,36	0,09±0,07	/	/	/	/	/

6.3 DISCUSSION AND CONCLUSIONS

This chapter describes the temporal aspects of bacterial and fungal colonization of PD located on the seafloor in the harbour of Ostend and offshore in the North Sea. In the semi-enclosed environment of the harbour, the formation of a microbial biofilm was observed after merely one week, which is in accordance with previous studies (Lobelle & Cunliffe, 2011; Harrison et al., 2014). Progressive stages of biofilm formation on plastics sampled at the harbour could be discriminated based on the increase of microbial biomass over time, a high bacterial richness in the first months, a gradual change in the taxonomic composition, and a shift towards more secondary colonizers at later stages in the harbour. A core group of 25 bacterial OTUs were detected across all plastic samples from four weeks onwards until the end of the exposure period at the harbour. Those core organisms could be divided into four groups depending on the timing of their peak abundance. The gradual change in relative abundance of those organisms revealed two important features. First, individual pieces of plastic show highly reproducible taxonomic compositions, although they are collected in a time-series from independent constructions sunk to the seafloor sharing a history of exposure to the same environment. This shows that at least part of the biofilm formation process is sufficiently stable to reconstruct its temporal dynamics. Second, our strategy can be used to identify indicator species for distinct biofilm formation stages in natural environments where PD effectively accumulates. We are aware that some indicator species identified in this study may be specific for the environment of the harbour of Ostend, e.g. members of the *flavobacteriaceae* family (Chapter 5), and may not be found in other locations. However, we suggest that repeating a similar setup in many other locations and conditions will ultimately reveal common and unique patterns of biofilm formation on PD. It is also possible that the core organisms could be commonly found on other types of hard surfaces and may not be uniquely found on plastic surfaces. To identify plastic-specific components of biofilms, these should be compared to a range of other substrata, such as ropes, wood, and metal or other types of marine debris, as has been done by Oberbeckmann *et al.* (2016). As we only reconstructed one cycle of biofilm formation starting in September, temporal changes in the taxonomic composition probably reflect the colonization process itself but are confounded with seasonal variation of environmental factors. To delineate the contribution of seasonal and environmental effects on the colonization process staggered-start experiments should be performed by starting the exposure period at different times during the year, or on different

locations. For instance, the composition of the bacterial community of plastics sampled in the North Sea environment has been shown to be different compared to other marine environments, e.g. the Pacific Ocean (Chapter 5).

We further showed that all bacterial and fungal groups attached to the plastic surface could be detected in the surrounding seawater or sediment, thus serving as a source of micro-organisms to colonize the plastic surface. So, while the species compositions overlap, the microbial species abundance profiles between seawater and sediment and the surface substrata are different. We further show that large variation exists within the series of communities found on the surface of plastic substrata. For instance, different microbial communities develop on plastic sheets and dolly ropes, even though the process occurred in the same environment and both plastic types were PE. Furthermore, the temporal aspect of biofilm formation is an important factor that defines the community found at a given moment. The most striking of our observations is that samples taken offshore, either with known history at a fixed location or from randomly sampled pieces of 'free-moving' plastic in the North Sea environment (Chapter 5), are most similar to early phase biofilms observed on plastics sampled in the harbour. Taken together, these observations either suggest that biofilm formation is stimulated in the nutrient-rich and semi-protected harbour conditions, or that under offshore conditions it may be subject to environmental influences that either hamper or disturb biofilm formation, or may result in erase-and-restart scenarios. For instance, the biofilm formation process can be set back to an early stage due to the seawater-sand emulsion moving over the surface of the plastic and scraping off the biofilm. In addition, the ropes and sheets were fixed on the same wooden block, and tidal currents may move ropes over the sheets, which could result in partly removal of the biofilm. Interestingly, the fungal profiles of plastics exposed offshore or at the harbour display rather discontinuous shifts in the communities that do not follow the sampling order, and are thus unlikely to reflect successive stages of a developmental process. At least in the harbour environment, this does not seem to be related to any impact on the biofilm itself, as the bacterial profiles of the same samples display clear and well-structured temporal dynamics. This suggests that mechanical forces may not be the only factor that affects biofilm formation. If erase-and-restart scenarios would occur, this may explain the large variation and the lack of 'late' stage taxonomic signatures, but it would also have several consequences for future studies. First, understanding long-term

processes underlying biofilm formation and metabolic activity, as studied under controlled conditions, may be of limited help to understand the processes as they occur offshore. Second, previous research on free-moving plastic has identified the presence of certain bacterial families (i.e. Pseudoalteromonadaceae and Vibrionaceae) on randomly sampled PD that were not detected in the surrounding seawater or sediment at the time and location of sampling (Chapter 5). This suggests that free-moving PD can act as a vector and transport micro-organisms through the marine environment. Our results suggest that the stability of the biofilm is a further aspect that needs to be taken into account when assessing the risk of plastic as vector for invasive and/or pathogenic species.

In contrast to the large body of evidence accumulating for bacterial colonization of PD (Chapter 5; Zettler et al., 2013; Harisson et al., 2014; Oberbeckmann et al., 2014; Oberbeckman et al., 2016), only few studies have been published on fungal colonization of PD. For instance, Oberbeckmann and coworkers studied the eukaryotic colonization of PD in the North Sea environment through 18S rRNA gene amplicon sequencing, and showed the presence of Ascomycota and Basidiomycota on PD (Oberbeckman et al., 2016). We found members of the *Lecanoromycetes*, e.g. *Candelariella*, *Physconia*, and *Lecanora* on plastics sampled at the harbour and offshore. These lichens are commonly detected in coastal and maritime zones (Clayden et al., 2010).

While fewer studies focus on the fungal community than on the bacterial community on PD, we believe that it is important to study these simultaneously for two main reasons. First, several soil-related bacterial and fungal species able to degrade PE have previously been described (Restrepo-Florez et al., 2014) and these could also play a role in plastic degradation in marine environments, provided that they would occur there. Second, bacterial attachment to hyphal surfaces can enhance synergistic action of bacteria and fungi, which is necessary to break down highly complex substrates, such as PE polymers (Characklis et al., 1990). A large number of fungal OTUs was identified showing the complexity and diversity of the fungal community on PD, but little is known about the biological functions that they can perform. Our study, as well as future research into the functioning of fungal species in marine biofilms, suffer from a prominent gap in current taxonomic reference databases. For instance, the UNITE database, which is currently the largest reference database for fungal taxonomic profiling, is predominantly based on fruiting bodies, rhizosphere, and soil samples, but

contains only a limited number of marine sequences (Kõljalg et al., 2013). Consequently, the majority of fungal sequences in our study was assigned to soil organisms, or could not be assigned at all. Additionally, marine fungi are not functionally characterised, in contrast to soil fungi. Therefore, a comprehensive marine fungal database should be established for future studies on the fungal communities that colonize PD.

One of the main reasons for studying microbial communities on PD in the marine environment is to identify plastic biodegrading micro-organisms. As nature's cleaners, micro-organisms may have the potential to degrade plastics accumulating in the marine environment, but so far there is no hard evidence for such biodegradation (Osborn & Stojkovic, 2014). In general, the substrate degradation efficiency increases when a microbial biofilm is formed on the substrate (Davey & O'Toole, 2000). Microbial populations that form biofilms display higher metabolic activity than microbes in planktonic mode of growth (Gilan et al., 2004). We did not find any bacterial species that had previously been associated with plastic degradation, but did detect three fungal OTUs representing previously identified PE degraders in late stage biofilms: *Cladosporium cladosporioides*, *Fusarium redolens*, and at lower abundance *Mortierella alpine.* Because phylogeny assignment based on rRNA gene sequences is not necessarily linked to biodegradation potential, isolated strains should now be tested in PE biodegradation assays to further verify biodegradation activity.

ADDITIONAL INFORMATION CHAPTER 6

Table AI6.1: Properties of the harbour and offshore environment. Pollutant concentrations of the harbour are achieved from the "Vlaamse Milieu Maatschappij", which measure the pollutants on a yearly base. Pollutant concentrations offshore is obtained from De Witte et al. (2016). Measured properties are obtained from Flanders Marine Institute (VLIZ), Belgium (2015).

GENERAL FEATURES	**Harbour**
Sedimenttype	Silt, by which it can adsorb organic pollutants (see pollutant concentration, sediment characteristics)
Pollutants	Presence of organic pollutants, POPs
Sampling depth	6 m
Anthropogenic activities	land run-off ship discharges pollution through waste pipes
Currents	relatively weak
Tides, currents and waves	
Wave height (cm)	65,0
Polutant concentrations (mg/ kg DM)	
Zn (µg g-1)	750
Cd (µg g-1)	5
Pb (µg g-1)	230
Cu (µg g-1)	179
Cr (µg g-1)	102
Ni (µg g-1)	30
Hg (µg g-1)	900
polyaromatic carbohydrates (PAK) (µg g-1)	6,09
polychloorbifenyl (PCB) (ng g-1)	102
Sediment characteristics	
Total organic carbon (TOC) (% OC)	3,33
Median grain size (µm)	106,8

MEASURED PROPERTIES	**sep/'15**	**oct/'15**	**nov/'15**	**dec/'15**	**jan/'16**	**feb/'16**	**mar/'16**	**apr/'16**	**may/'16**	**june/'16**	**july/'16**
Temperature (°C)	/	12,3	10,6	10,9	7,7	6,6	6,5	9,9	10,5	14,6	20,6
Conductivity (µS/cm)	/	37.600	37.000	38.000	34.900	31.600	33.300	37.000	34.500	37.500	47.100
pH	/	7,9	8,0	8,2	8,0	7,9	8,1	8,3	8,5	7,9	8,3
Oxygen (mg/L)	/	6,3	/	8,6	7,1	/	12,5	13,3	7,9	9,2	10,1
Salinity (PSU)	/	32,4	33,3	34,1	33,9	31,3	33,4	34,0	30,9	30,4	31,7

Density (kg/m^3)	/	1.024,5	1.025,5	1.026,2	1.026,5	1.024,6	1.026,3	1.026,2	1.023,7	1.022,5	1.023,7

GENERAL FEATURES	**Offshore**
Sedimenttype	Sand
Pollutants	Sedimentype is almost inert; hard to adsorb pollutants
Sampling depth	26 m
Anthropogenic activities	Activities covering the wind farm Fisheries
Currents	Strong
Tides, currents and waves	
Wave height (cm)	182,1
Polutant concentrations (mg/ kg DM)	
Zn (µg g-1)	124,9
Cd (µg g-1)	0,4
Pb (µg g-1)	34,7
Cu (µg g-1)	16,1
Cr (µg g-1)	73,5
Ni (µg g-1)	22,4
Hg (µg g-1)	173
polyaromatic carbohydrates (PAK) (µg g-1)	0,6
polychloorbifenyl (PCB) (ng g-1)	5,3
Sediment characteristics	
Total organic carbon (TOC) (% OC)	0,09
Median grain size (µm)	678,4

MEASURED PROPERTIES	**sep/'15**	**oct/'15**	**nov/'15**	**dec/'15**	**Jan/'16**	**feb/'16**	**mar/'16**	**apr/'16**	**may/'16**	**june/'16**	**july/'16**
Temperature (°C)							/				
	16,3	13,1	11,3	10,4	6,5	7,7		9,4	12,7	15,8	19,8
Conductivity (µS/cm)							/				
	42.876	38.760	38.274	37.646	32.481	35.463		34.968	38.657	39.331	44.847
pH							/				
	8,1	7,9	8,0	8,2	7,9	8,0		8,4	8,4	8,1	8,2
Oxygen (mg/L)							/	/			
	7,7	8,0	8,3	8,8	7,3	7,0			7,3	10,0	9,0
Salinity (PSU)							/				
	33,8	32,8	34,0	34,2	32,4	34,5		32,3	33,1	31,1	32,7
Density (kg/m^3)							/				
	1.024,8	1.024,8	1.026,0	1.026,3	1.025,5	1.027,0		1.025,1	1.025,1	1.022,9	1.023,1

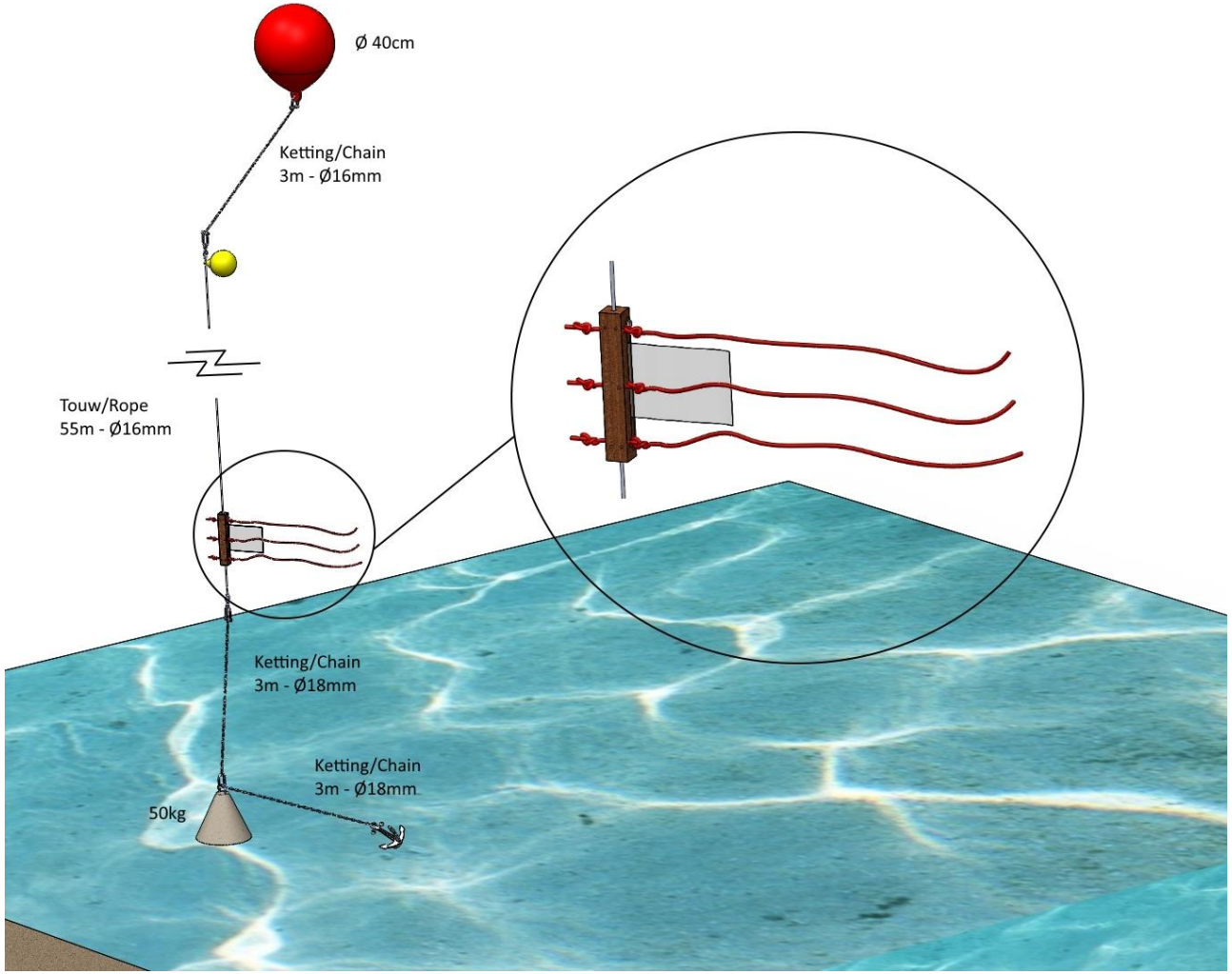


Figure AI6.1 Technical plan of the construction used to expose plastics in the Belgian part of the North Sea. Three pieces of each type of plastic were attached to a wooden block, which was secured in a construction comprising a buoy, ropes (ø 16 mm), chains and an anchor and concrete weights.

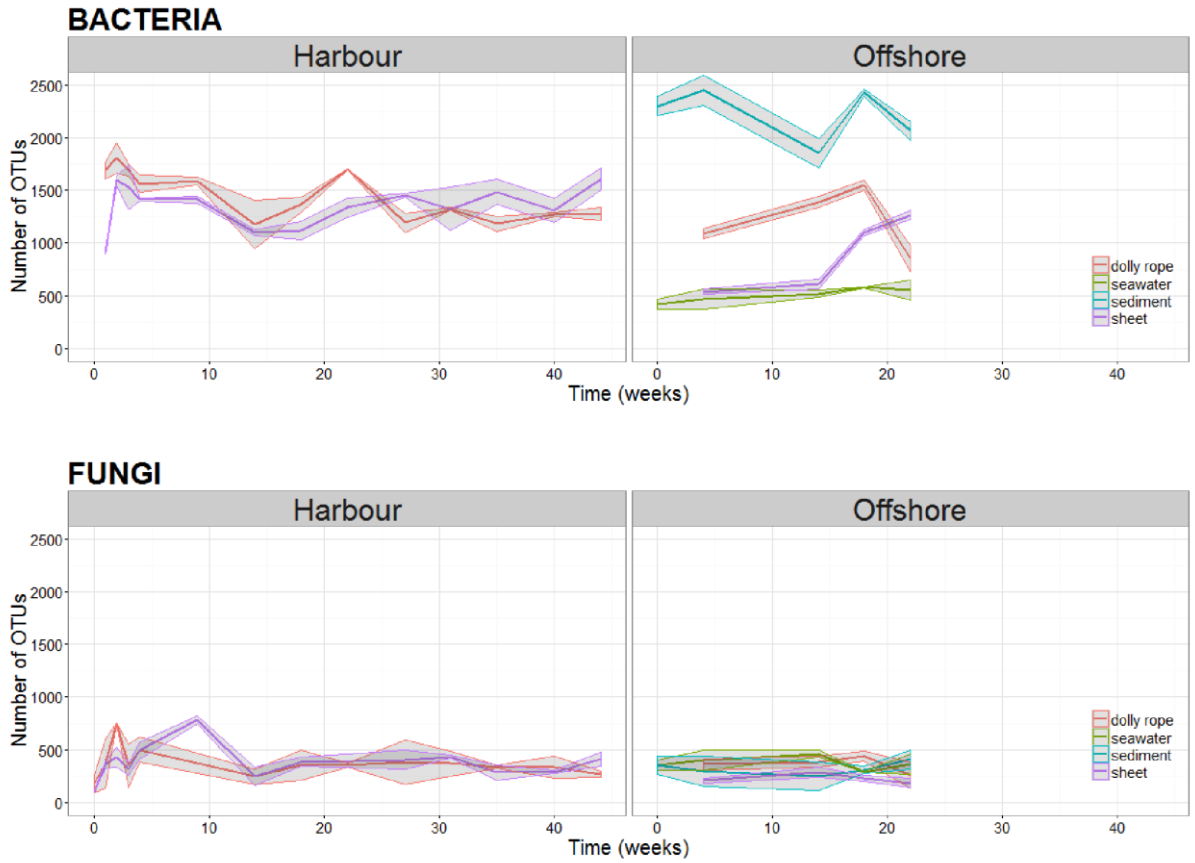


Figure AI6.2. Richness of the bacterial and fungal communities on plastics, seawater, and sediment during 44 weeks (harbour) or 22 weeks (offshore) of exposure. The richness of plastic sheets and dolly ropes are shown in purple and red, respectively. The number of unique OTUs in seawater and sediment are indicated in green and blue, respectively. The shaded grey area represents the 95% confidence bands. Top panels: bacterial community richness. Bottom panels: fungal community richness.

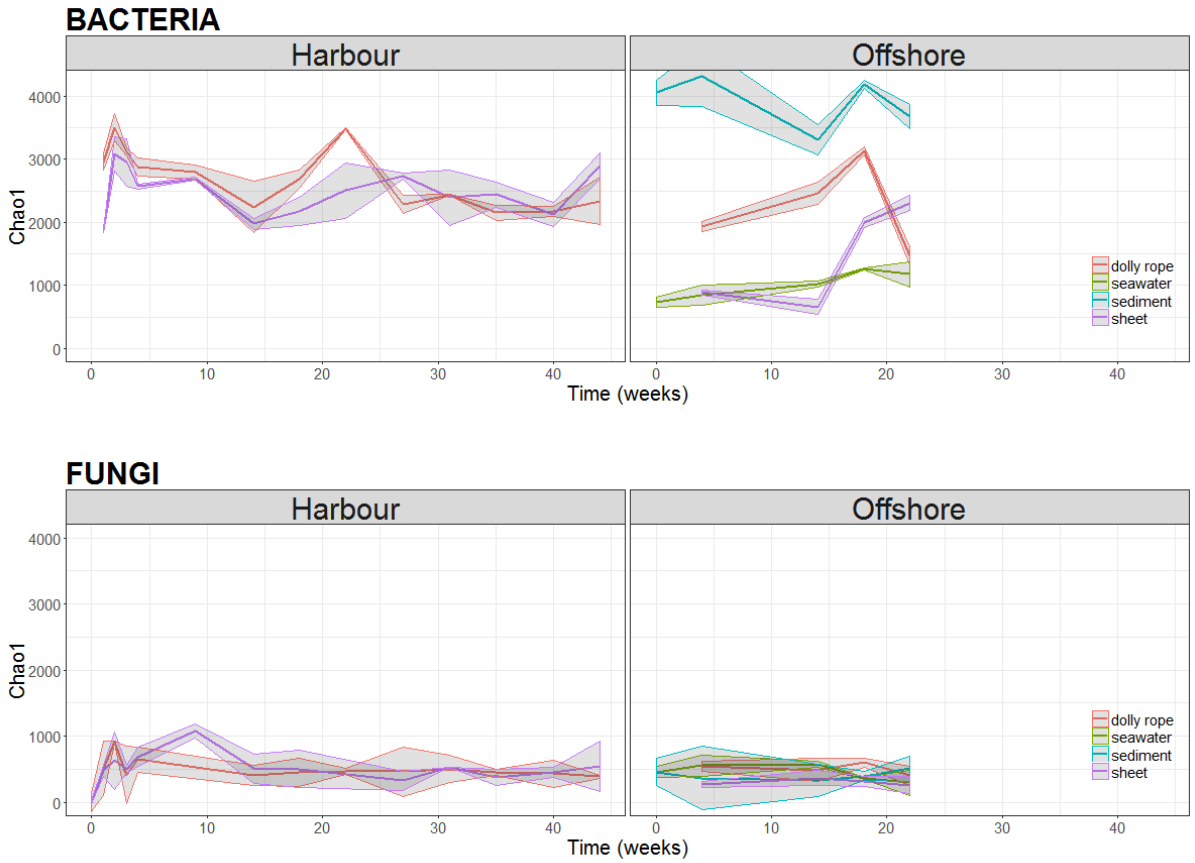


Figure AI6.3. Chao1 indices, a richness estimation of microbial communities, of the bacterial and fungal communities on plastics, seawater, and sediment during 44 weeks (harbour) or 22 weeks (offshore) of exposure, calculated at a rarefaction depth of 10,000 sequences. The chao1 indices of plastic sheets and dolly ropes are shown in purple and red, respectively. The number of unique OTUs in seawater and sediment are indicated in green and blue, respectively. The shaded grey area represents the 95% confidence bands. Top panels: bacterial community richness. Bottom panels: fungal community richness.

Chapter 7

General discussion and future perspectives

In this PhD, high-throughput sequencing (HTS) techniques have been successfully applied in either a soil (rhizosphere) or marine ("plastisphere") context (Figure 7.1).

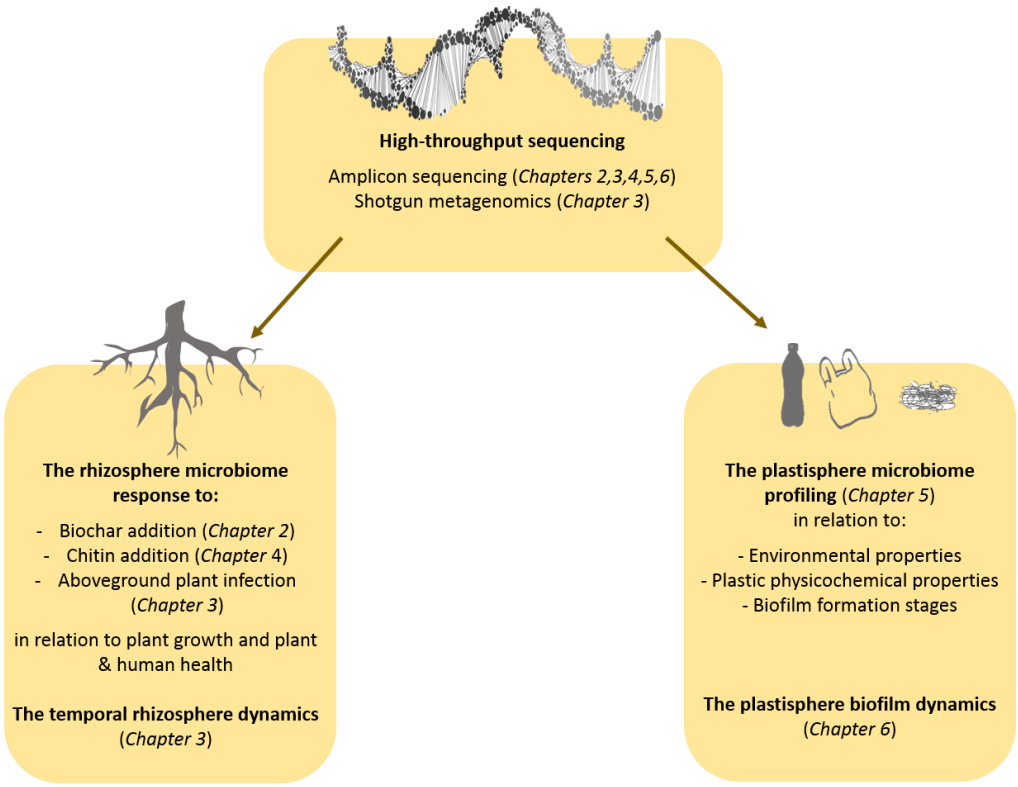


Figure 7.1 Schematic overview of the four research chapters of this PhD (source DNA strand: https://research.medicine.umich.edu).

This chapter discusses the major research findings of this work, its implications, and possible future perspectives. For this purpose, the discussion is divided in three main topics:

1) **The possibilities and drawbacks of amplicon sequencing and shotgun metagenomics in agricultural and marine environmental contexts**.
2) **Evaluation of the effects of two soil/substrate amendments on the rhizosphere microbiome.** Biochar and chitin addition can change the rhizosphere microbiome, which can be related to a positive effect on the plant growth and a negative effect on plant and human pathogens on the leaves. Both amendments are therefore good candidates to be used in agri- or horticultural practices.
3) **Characterization of the bacterial and fungal community composition and dynamics of "the plastisphere".** Plastics exposed to the North Sea will be colonised by bacteria and fungi. This colonization is influenced by environmental and plastic related factors. This microbial load on plastics may be a potential threat (pathogens) or provide a solution to the plastic pollution problem (degradation).

7.1 HIGH THROUGHPUT SEQUENCING

In this PhD research, amplicon sequencing is mainly used to study the taxonomic composition of bacterial (V3-V4 variable region of the 16S rRNA gene) and fungal communities (ITS2 variable region). The technique has proven to be applicable in both soil and marine environments and has been used to characterize microbial communities and their temporal dynamics (Figure 7.1***Figure***).

In Part I, the rhizosphere microbiome is studied by using a combination of amplicon sequencing and phospholipid fatty acids (PLFA) analysis (Chapter 2, Chapter 3 ,Chapter 4), and/or amplicon sequencing and shotgun metagenomics (Chapter 4).

PLFA analysis and amplicon sequencing have proven to be complementary techniques to study biomass, microbial diversity and shifts in relative abundances in microbial communities (Table 7.1). After amplicon sequencing, sequences are classified in Operational Taxonomic Units (OTUs). The number of unique OTUs gives an estimation of the richness of a sample (Chapter 2 and 4). To compare samples, absolute number of OTU are normalised for differences in sampling depth by dividing the counts by the total number of sequences in a sample. This generates relative abundances for each OTU. In contrast, PLFA analysis provides complementary data on the total microbial biomass and the biomass per microbial group, but cannot be used to calculate community richness. To make a comparison between both techniques, one can calculate the relative abundances of the PLFA biomarkers as done in Chapter 4 of this dissertation. It was shown that both amplicon sequencing and PLFA analysis indicate an increase in relative abundance of Gram negative-bacteria in the lettuce rhizosphere due to chitin addition to potting soil, but the decrease of relative abundance of Gram-positive bacteria in the PLFA analysis was not confirmed by amplicon sequencing. It needs to be noted, that it is not possible to classify all groups or group members to either gram-negative or gram-positive, as some groups contain species of both cell wall types, which can bias the comparison. Therefore, comparisons of both techniques based on relative abundances is rather difficult, and we conclude that based on taxonomical identification, both techniques are rather complementary. PLFA is therefore a good technique for studying the biomass of microbial groups, and amplicon sequencing for indicating the richness of the sample and shifts in relative abundances in the OTUs, which are generally classified to the genus level (Table 7.1).

Table 7.1 Comparison of the characteristics of PLFA analysis, amplicon sequencing and shotgun metagenomics based on taxonomic and functional information. The microbial groups for PLFA analysis or defined on the classification of phospholipids to: Gram positive bacteria, non-specific bacteria, gram negative bacteria, actinomycetes, AM Fungi and fungi.

	PLFA analysis	Amplicon sequencing	Shotgun metagenomics
Taxonomic information	✓	✓	✓
Richness/diversity	×	✓	✓
Relative abundances	✓	✓	✓
Level of classification	Microbial groups	Genera	Genera/Species
Microbial biomass	✓	×	×
Functional information	×	×	✓

Amplicon sequencing and shotgun metagenomics are complementary for taxonomic and functional microbial characterization (Table 7.1). In Chapter 4, both techniques are used to study the rhizosphere microbiome influenced by the addition of chitin. Both amplicon sequencing and shotgun metagenomics have proven to be appropriate to study the bacterial community composition of the rhizosphere and results are comparable. A relatively higher relative abundance of *Proteobacteria* is observed in the shotgun dataset compared to the amplicon sequencing data. This is in contrast to previous research that studied the Barley rhizosphere and found a higher amount of *Proteobacteria* in the amplicon sequencing dataset, probably due to the presence of multiple copies of the 16S rRNA gene in *beta*- and *gammaproteobacteria* (Bulgarelli et al., 2015). To assign taxonomy to the sequence reads, several non-redundant rRNA sequence databases are available for which Silva, Greengenes and Ribosomal Database Project (RDP) are the most popular. In our study, a different reference database is used for the amplicon sequencing data (Silva) and the shotgun metagenome data (Greengenes), which was taxonomically classified by EBI metagenomics. Several databases are biased in their composition and for Greengenes around 30% of the database are *Proteobacteria*, which comprise around 5% of the database of complete microbial genomes and thus could explain the difference (Poretsky et al., 2014). In addition, the sequencing depth in the shotgun metagenome data showed to be insufficient to cover the complete bacterial diversity in the samples.

Moreover, it is generally assumed that the PCR amplification in amplicon sequencing introduces a bias and that not all sequences are amplified with the same efficiency (Schirmer et al., 2015). Based on the results of Chapter 4, this bias seems to be limited as similar

conclusions can be made with the amplicon sequencing data compared to shotgun metagenomics. Therefore, we can assume that sequencing the V3-V4 variable region of the 16S rRNA gene after PCR amplification is a robust method to analyse bacterial communities in the rhizosphere.

For the fungal rhizosphere community, a similar comparison cannot be made as EBI-metagenomics does not provide taxonomic information on the fungal sequences in the shotgun metagenomics dataset. Also for other open-source web-applications, the taxonomic information of microbial groups other than bacteria is limited. Metagenome abundance estimates are based on taxonomic classification using a reference-based method. Therefore, database-related biases likely will play a role in the community characterisation in favour of bacterial taxa (Bulgarelli et al., 2015). The use of alternative markers, such as the ITS or 18S rRNA gene region, to target fungal communities through the application of amplicon sequencing is therefore the favoured option so far.

Shotgun metagenomics is however an important technique as it provides information on the functional genes in the metagenome of an environment, e.g. the rhizosphere. In our study (Chapter 4), 15 % of the genes are mapped to a functional trait, which is in line with another study in the rhizosphere (Yan et al., 2017). Therefore, caution should be taken as more than 80 percent of reads cannot be annotated. In contrast to the taxonomic classification where the number of OTUs that significantly differed due to the addition of chitin are limited (Chapter 4), more than 300 functional genes significantly differed in relative abundance in the rhizosphere due to chitin addition. Therefore it is extremely important that before performing a shotgun metagenomics study, knowledge is available on the specific functional traits and pathways one wants to study. In Chapter 4, this was done for the functions involved in chitin degradation.

Despite several benefits of HTS techniques, the analysis of these large volumes of data remains challenging. Based on the two research topics in this PhD, the main challenging aspects were the biology, bioinformatics and statistics.

Biology: Amplicon sequencing of the rhizosphere microbiome (Part I) has given a thorough understanding of the community composition and the influence of biochar (Chapter 2, 3) and chitin (Chapter 4) on the bacterial and fungal communities. In Part II, amplicon sequencing has proven to be a good technique to study the bacterial “plastisphere” community. However, the

fungal community of marine plastic debris is typified by many OTUs that cannot be assigned to a certain known taxon (Chapter 6). This is at least in part due to a lack in current taxonomic reference databases. The UNITE database, which is currently the largest reference database for fungal taxonomic profiling, is predominantly based on fruiting bodies, rhizosphere and soil samples (Kõljalg et al., 2013). In addition, marine fungi are less extensively studied compared to soil fungi. Therefore, a comprehensive marine fungal database needs to be established if one wants to study fungal communities on marine plastic debris and in the marine environment in general.

In addition, a major limitation of HTS techniques is that it is extremely difficult to learn new gene and pathway functions from the pure sequence data. To do so, cultivation of organisms is required, nonetheless most organisms are “uncultivable”. Recent advances in growing these species include co-culture with other bacteria, recreating the environment in the laboratory, and combining these approaches with micro-cultivation technology to increase throughput and access rare species. Therefore, whereas HTS techniques have a lot of potential, we need to keep in mind that cultivation remains necessary (Zengler, 2009; Stewart, 2012).

Bioinformatics: In contrast to amplicon sequencing data, which is commonly analysed by using the QIIME software (Caporaso et al., 2010a), the analysis of shotgun metagenomics data (Chapter 4) remains challenging for bioinformaticians. This is at least in part due to the size of the datasets and the lack of non-redundant databases. Several open-source bioinformatics tools are available, such as MG-Rast and EBI-metagenomics, which ease the data analysis for non-bioinformaticians (Meyer et al., 2008; Mitchell et al., 2015). However web-based analysis is often related to a long execution time and the data visualization and output these tools provide are limited. As an example, most open-source methods focus on bacterial taxonomic classification, whereas we have seen that in certain cases also other groups, e.g. fungi (Chapter 4, Chapter 6) can be important to study. However, new methods become available each year which will overcome these problems. As an example, the Unipept pipeline (Mesuere et al., 2016), currently used for metaproteome data analysis, is being extended for the analysis of metagenomics data. DNA sequences will be translated into a series of peptide and protein sequences, solving the latter using existing Unipept functionality and map the results back to the metagenomics context. This approach makes Unipept unique compared to existing pipelines such as MG-Rast and EBI metagenomics, where DNA sequences are mapped towards rRNA databases to retrieve taxonomic information, especially on bacterial sequences

(unpublished, Cizar Almalak & Bart Mesuere). This has the advantage that all sequences are used for taxonomic classification and not only those of the 16S gene, which proved to be insufficient to cover the complete community complexity due to a low sequencing depth for 16S sequences (Chapter 4).

Statistics: The statistical analysis of both amplicon sequencing and shotgun metagenomics data is notoriously difficult. The reads in meta-'omics sequencing applications typically exhibit a high variability, huge fluctuations in sequencing coverage and contain many zeros as compared to transcriptome data, containing mRNA reads. Therefore, the tools that are currently used for differential expression analysis in transcriptome studies, e.g. EdgeR (Robinson & Oshlack, 2010) and DESeq2 (Love et al., 2014), are currently the best option for studying differential differences in amplicon and shotgun sequencing data. However for these sequencing techniques the methods are currently suboptimal and should be adapted to fit for the data. New pipelines are produced each year, and probably these limitations will be overcome in time.

In conclusion, both amplicon sequencing and shotgun metagenomics are important tools to study microbial communities in both an agricultural context and marine environment (plastic debris, sediment, seawater). The combination of both techniques is optimal to retrieve as many information on the microbial community as possible, and when studying a soil (or sediment)–related community, the combination with PLFA analysis can be made to gain knowledge on the absolute microbial biomass. The remark should be made however that only making use of HTS techniques will not be sufficient and culture-dependent methods will still be essential to expand our basic taxonomic and functional knowledge of micro-organisms.

7.2 THE RHIZOSPHERE IN RELATION TO PLANT GROWTH, PLANT HEALTH & INTERACTION TO HUMAN PATHOGENS

In Chapters 2, 3 and 4, the addition of two soil or substrate amendments, biochar and chitin, is proven to affect the rhizosphere microbiology, plant growth and diseases, but also survival of human pathogens on the leaves (chitin).

Biochar addition changed the physicochemical properties of soil and substrate. It also induced major changes in the rhizosphere microbiology of the strawberry plants and in plant development and health (Chapter 2, Chapter 3). Based on the results of this thesis, we can conclude that biochar works best under nutrient-limiting conditions to improve plant health and disease resistance, such as in an unfertilized and unlimed peat growing medium. Additional fertilization of peat reduces the effects and when lettuce plants were grown in nutrient-rich soil, biochar effects on plant properties, plant health and rhizosphere microbiology were even absent.

In nutrient-poor substrate conditions, biochar increased the strawberry plant and root growth, the fruit production and the plant resistance towards *Botrytis cinerea* infection on fruits and leaves. Several hypotheses have been suggested regarding the mode of action of biochar in this condition, in which two are the most prominent (Figure 7.2).

First, biochar can serve as a fertilizer. The higher nutrient stock in the substrate supplemented with biochar, supplies necessary nutrients for the plant and the microbial community. Also in terms of fruit production, strawberry has a high nutrient demand. In terms of plant health, potassium deficiency in plants is amongst other related to less pest resistance. The remark should be made however that a higher nutrient stock in the substrate will not directly enhance plant growth or disease resistance, as nutrients should also be taken up by the plant. Therefore in future experiments, the plant tissue and strawberry fruit nutrient content should be analysed in plants grown in biochar-amended and unamended peat. In addition, despite the significant increase in nutrient composition due to biochar addition in the substrate, the values remain extremely low compared to the reference values for appropriate plant growth.

Second, plant properties and plant health can be affected through a change in the rhizosphere microbiome due to the addition of biochar. Biochar addition to substrate increased the relative abundance of bacteria involved in the microbial N cycling, Plant Growth Promoting Rhizobacteria (PGPR) and biocontrol agents from week six of plant growth onwards. These

organisms can ease the uptake of nutrients for the plants and enhance the plant availability of nitrogen and phosphorus (Egamberdiyeva, 2007; Krey et al., 2013). On the other hand, PGPR can promote the induced systemic resistance (ISR) of the plant (Mehari et al., 2015). This interplay of biochar-mediated processes correlated with a change in nutrient availability and bacterial community composition in the rhizosphere, are schematically represented in Figure 7.2.

Previous studies have shown that five factors can influence the rhizosphere microbial composition: soil type, plant genotype, addition of fertilizers, crop rotation and application of pesticides (Massart et al., 2015). This PhD research has shown that an aboveground fungal infection can also alter the rhizosphere community composition (Chapter 3). The presence of pathogens can induce a higher production and leakage of root exudates, which provide nutrients for micro-organisms and are therefore able to change the rhizosphere microbiology (Doornbos et al., 2012). Interestingly, the change in bacterial community composition of the rhizosphere induced by the *B. cinerea* infection of a strawberry leaf was comparable to the rhizosphere community composition of biochar-amended substrates.

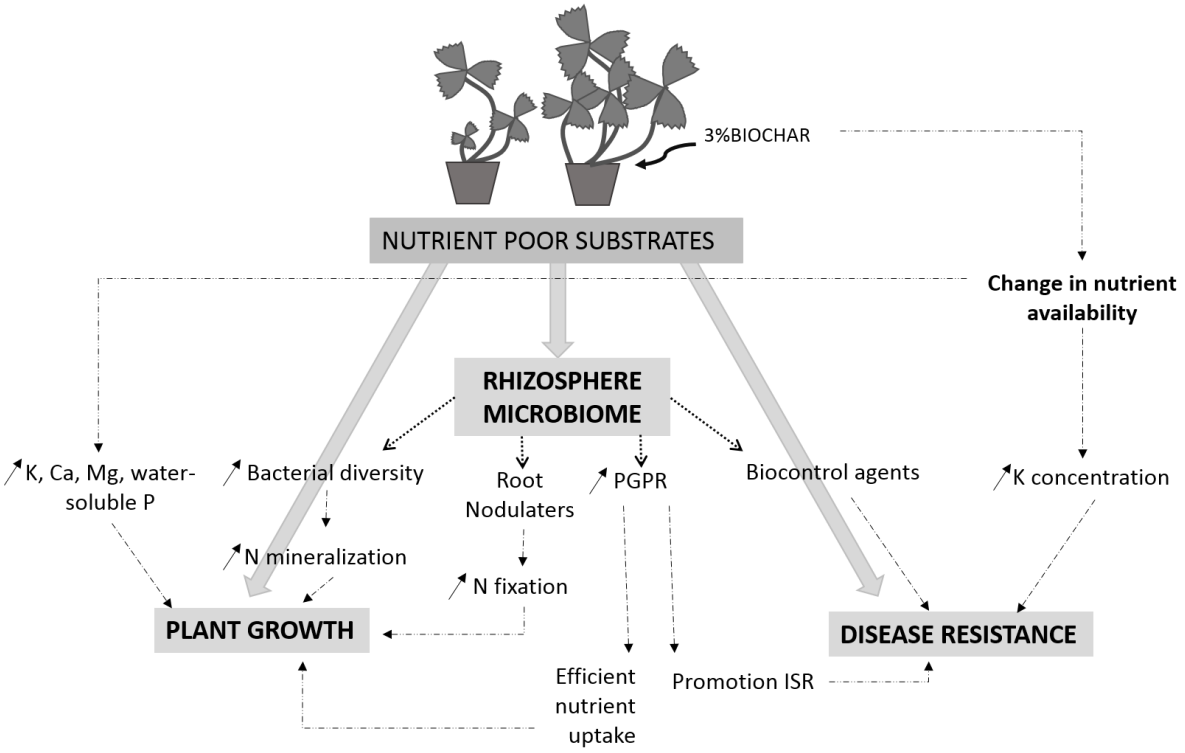


Figure 7.2 Schematic overview of the biochar-mediated processes in nutrient-poor conditions and the interplay between these processes (case study: strawberry, graphical abstract from De Tender et al., 2016). The full grey arrows represent the main effects observed due to biochar addition. The dotted lines represent the interactions of the several main factors.

Chitin addition to potting soil has shown to be able to alter the lettuce rhizosphere microbiome, increase lettuce growth and reduce the survival of a zoonotic bacterial pathogen, *Salmonella enterica*, on the leaves (Chapter 4) (Figure 7.3).

Chitin addition increased the growth of the lettuce plants by 20%. This can be mediated through a change in the soil and rhizosphere microbiome. First, chitin degradation by the microbial community can release a substantial amount of carbon and nitrogen in the potting soil, providing necessary nutrients for the plant (Hamid et al., 2013). The remark should be made however that in this study, the nutrient composition of potting soil was not measured and thus the observed growth effects by a change in the nutrient stock are hypothetical. Second, several bacterial genera linked with the N cycle and PGPR increased in relative abundance due to the chitin addition, which can ease the uptake of nutrients for the plant and enhance plant availability of nitrogen and phosphorus (Egamberdiyeva, 2007; Krey et al., 2013). Third, part of the chitin could be deacetylated to chitosan, a component known to serve as a natural biocontrol agent and able to enhance plant growth (Linden, 2000).

To my knowledge, this is the first study to show that chitin addition in potting soil has the potential to intervene in the aboveground interaction of the plant with bacteria, and in this case with zoonotic bacterial pathogens for which the plant can function as a carrier of human disease. The mechanism behind this decreased survival of the zoonotic pathogen is currently unknown, however it is co-occurring with a change in the rhizosphere microbiome. Chitin and chitosan are both known to be involved in plant health and similar mechanisms could be related to the decrease in survival of *S. enterica* on the lettuce leaves. First, as stated above, chitin promotes bacterial and fungal groups involved in plant growth promotion and in biological control. Second, chitin is also known as a microbe/pathogen-associated-molecular pattern (MAMP/PAMP) which can trigger plant immunity (Langner & Göhre, 2016), which may also influence the survival of *S. enterica* on lettuce leaves.

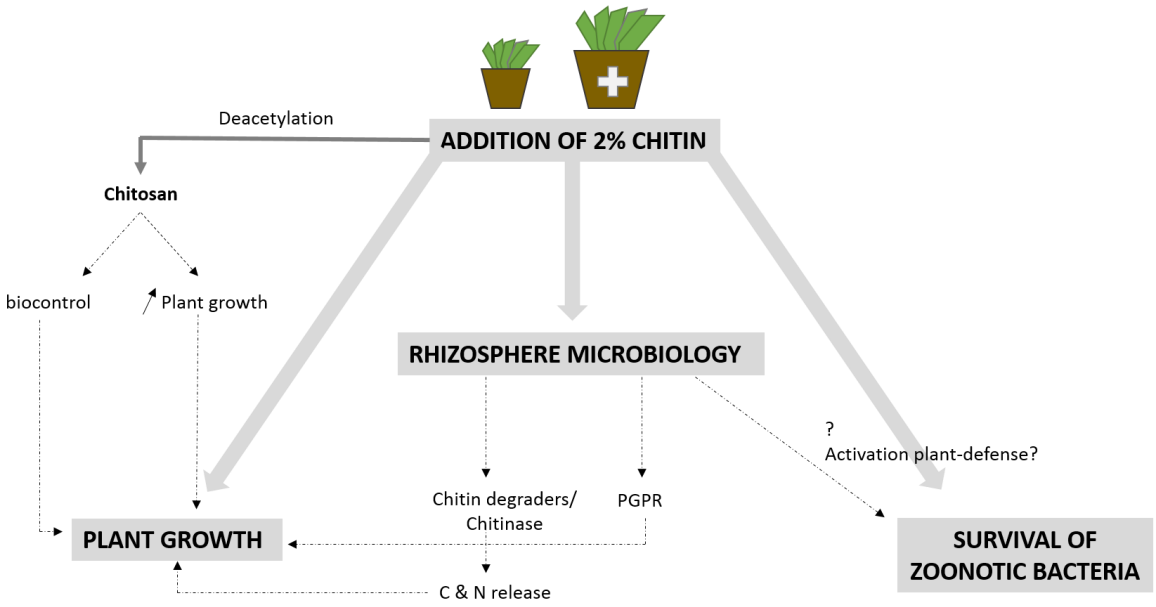


Figure 7.3 Schematic overview of the chitin-mediated processes and the interplay between these processes (case study: lettuce). The full grey arrows represent the main effects observed due to biochar addition. The dotted lines represent the interactions of the several main factors. Also the effect of chitosan, the deacetylated product of chitin, on plant growth has been added.

7.2.1 Futures studies

Based on the observations in Part I, biochar and chitin amendment are both able to alter the rhizosphere microbiome of plants. Despite different plant-growing media systems were studied, it seems that their mode of action is different**.** Biochar mainly influences the bacterial community composition towards higher relative abundances of PGPR and biocontrol agents (Chapter 2, Chapter 3). In contrast, chitin influences both the bacterial and fungal community composition, in which especially chitin degraders, often related to plant growth promotion, are promoted (Chapter 4).

To unravel the mode of action of biochar and chitin on the rhizosphere microbiology further, I suggest to perform following studies (Figure 7.4):

(1) To gain knowledge regarding the functional genes that are enriched due to the addition of chitin or biochar, a combination of shotgun metagenomics and metatranscriptomics can be used. For biochar, to our knowledge no information is available regarding enrichment of specific functions. Chitin addition is in a first instance linked to chitinases, and chitin deacetylases, which can have a broad range of downstream effects. In Chapter 4, we already show that chitin addition increases the bacteria and fungi that harbour these functions and the abundance of genes linked to chitin-metabolism. To ascertain differences in chitinase production by the rhizosphere microbiome, one can also look to

the microbial activity. Metatranscriptomics can be used to identify enzymes that are differentially expressed in response to chitin treatment. Similar, the use of shotgun metagenomics and metatranscriptomics can identify genes and functions influenced by the addition of biochar, for which one can focus on disease-related functions (plant disease susceptibility) and functions related to the N- and C-cycle (plant growth).

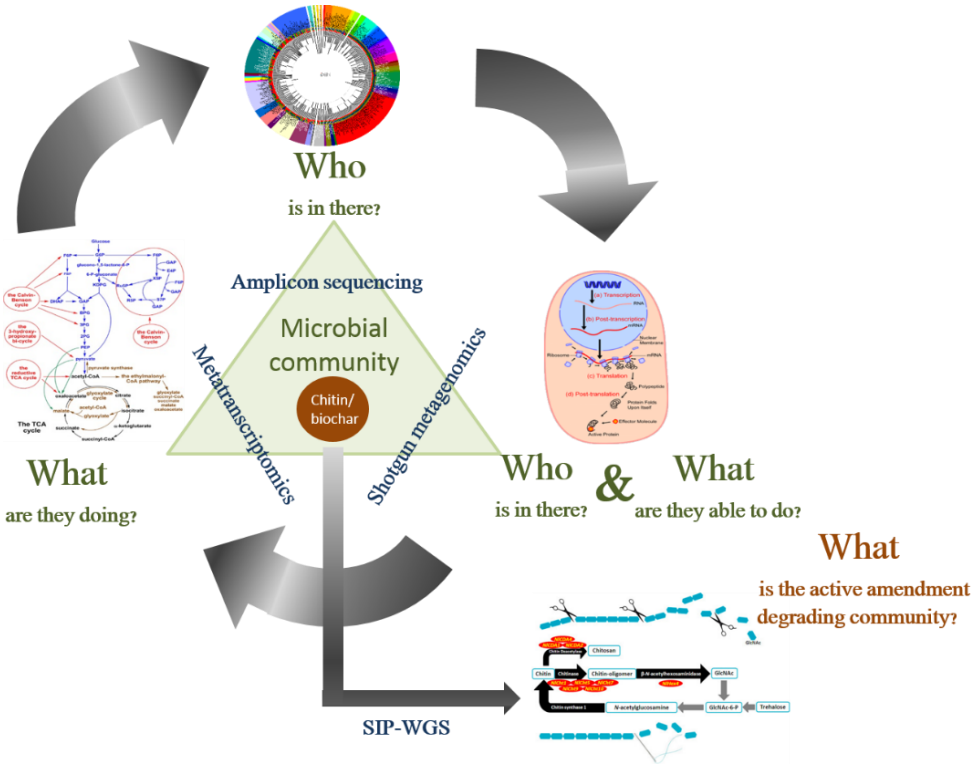


Figure 7.4 Techniques to study the microbial community in a given environment, here typically the substrate or the plant rhizosphere. Taxonomic classification of a specific target group (16S, ITS, etc.) can be done using amplicon sequencing. To identify the complete metagenome and look into the functions they encode, whole genome shotgun (WGS) sequencing (DNA) can be used. The active part of the metagenome can be studied using metatranscriptomics (RNA). Finally, a combination of Stable Isotope Probing (SIP) and WGS can identify the specific part of the community that actively metabolizes the amendment.

(2) DNA Stable Isotope Probing (DNA-SIP) can be used to identify those micro-organisms capable of metabolizing biochar or chitin. Stable isotope (^{13}C) labelled biochar or chitin can be added to the substrate. Subsequently, organisms that actively metabolize the labelled biochemical component will incorporate the stable isotope into their DNA, which will be referred to as ^{13}C-DNA or “heavy DNA”. By isopycnic ultracentrifugation, this “heavy” DNA will be separated from the “light” ^{12}C-DNA (Neufeld et al., 2007). To identify the organisms capable of metabolizing biochar or chitin, this technique can be combined with large-scale amplicon sequencing. In addition, one could combine the technique with shotgun

metagenomics, allowing to identify amendment-related specific functions (Chen & Murrell et al., 2010; Bell et al., 2011).

This thesis mainly focuses on the identification and characterisation of bacterial and fungal community members influenced by the application of biochar and chitin, and to a lesser extent in the functional genome composition influenced by chitin addition. By expanding the research towards shotgun metagenomics, metatranscriptomics and DNA-SIP, one can even get a more thorough understanding of the effect of chitin and biochar addition on the rhizosphere microbiome activity, the relation with plant growth and create new perspectives to enhance plant resistance and resilience towards plant diseases and pests, but also food safety by implementing plant growth systems with reduced risk of contamination with zoonotic pathogens.

In this thesis, we focused on the rhizosphere microbiome composition. Complementary, the response of the plant on the addition of chitin or biochar has been studied in terms of plant growth, yield and disease resistance. Here I propose two more plant analysis that would be very interesting to do in order to understand the plant response to biochar and chitin better. First, I already proposed to measure also the nutrient content on plant leaves and fruits (see above). Second, the plant defense should also be monitored. Although we have not studied this for chitin in this PhD, chitin is already well known to trigger plant immunity by acting as a MAMP/PAMP (Langner & Göhre, 2016). For biochar however, this is largely unknown and was subject of only two recent studies. Mehari *et al.* (2015) showed that the systemic resistance of tomato against *B. cinerea* induced by biochar amendment was related to priming of defense responses particularly in ethylene and jasmonic acid related genes, which are known to be crucial in resistance against *B. cinerea.* Huang *et al.* (2015) tested the same biochar as used in Chapter 2 and 3 of this dissertation and showed that the increased resistance against nematode development in rice roots was associated with biochar-primed H_2O_2 accumulation as well as with the transcriptional enhancement of genes involved in the ethylene signalling pathway. The increased susceptibility of a rice line deficient in ethylene signalling, further confirmed that biochar-induced priming acts at least partly through ethylene signalling. In Chapter 2 of this thesis, we have shown that biochar amendment to nutrient-poor growing media is able to reduce the infection rate on strawberry plants. To understand the relationship between the addition of biochar or chitin and plant defense, the expression of defense genes

in the plant can be studied. To reveal differences in plant defense gene expression by the addition of biochar (or chitin), quantitative reverse transcription PCR (RT-qPCR) can be used, which measures the amount of gene transcript amplified in the sample while the reaction occurs. During my PhD dissertation, two master thesis were conducted at ILVO in collaboration with UGhent (Prof. Dr. Ir. Tina Kyndt) in order to (1) optimize a protocol for RNA extraction from strawberry plants and (2) Identify relevant strawberry defense genes and corresponding primers for the RT-qPCR (Van Nuffel, 2015; Shein Lee Diaz, 2016). These protocols will be used in future research at ILVO to understand the effect of biochar and chitin substrate amendment on the strawberry plant defense response.

7.2.2 Economic, ecologic and human health benefits

Further studies should be undertaken to unravel the mode of action of biochar and chitin on the plant-substrate-microbiome interplay because both amendments seem to have promising use in agri- and/or horticulture. Crop cultivation in Europe often relies on high pesticide and fertilizer use, especially for soilless cultivation such as in peat based systems. In Belgium, strawberry cultivation is economically important with an annual production of about 50 million kg of fruits and a turnover of € 130 million (VILT, 2013; Appeltans, 2014). Strawberry is however almost solely cultivated in peat-based substrates, and peat is in general nutrient-poor, very conducive to diseases and not easily colonised by biocontrol agents (Hoitink et al., 2001). In addition, strawberry fruits demand a high nutrient supply as one culture of strawberries per ha in soilless cultivation takes up 125 kg N, 18 kg P (or 40 kg P_2O_5), 160 kg K (or 190 kg K_2O) and 56 kg Ca (Lieten & Misotten, 1993). The plants are also very susceptible to diseases, resulting in 8 to 14 pesticide residues on 500 g of strawberry fruits (2011, veiling Haspengouw). The application of biochar or chitin in such cultivation system can thus be a good alternative strategy to improve crop productivity and disease resistance of plants. This can reduce the application amount of fertilizers and pesticides, whereas the crop productivity or crop yield is retained. So far, for this purpose separate biocontrol agents are sometimes introduced to soils or substrates for the promotion of plant growth or to increase disease resistance. However, many of these microbial strains do not survive in the soil or substrate, or cannot establish high densities in the rhizosphere that are necessary to control pathogens (Raaijmakers et al., 2009). Therefore it has already been suggested to apply consortia of different micro-organism which are complementary or synergistic to have a more effective

effect (Bakker et al., 2012). Finding the right micro-organisms in these consortia is however difficult and thus chitin and biochar addition can be a good alternative as they change the natural rhizosphere community towards plant-beneficial organisms.

Application of chitin or biochar in substrates can therefore be beneficial in three ways: from an economic point of view, an ecological point of view and in terms of human health.

Economic point of view. The economical benefits of chitin and biochar addition can be twofold. First of all, chitin has proven to increase the lettuce yield, while biochar has proven to increase the strawberry fruit yield. In case of strawberry this is only seen in nutrient-limiting circumstances and effects diminish once fertilizer is added, so in normal strawberry cultivation this yield increase may not be seen. Second, both amendments make it possible to reduce the amount of fertilizer and pesticides applied in agri- and horticulture, which reduces the purchase costs of both products. In return, biochar and chitin need to be bought by the farmer. To compare the costs and benefits, we can take the strawberry cultivation as an example. In general, around € 4,500 euro per ha of pesticides is bought for strawberry cultivation (VILT, 2013). Typically, three plants per m^2 are planted (GFActueel, 2005), which result in a pesticide cost of € 0.15 per strawberry plant. In addition, organic fertilizer for strawberries can be bought at a price of € 6.80 per 1.5 kg, which can be applied for 75 plants (www.FransAgro.be). This results in a mean cost of € 0.091 per strawberry plants. Based on these prices, the cost of fertilization and pesticide application of a strawberry plant is estimated on € 0.24. This is off course a rough estimation and prices can differ by soil/substrate type, weather circumstances, purchase amount, etc.

In comparison, the cost of chitin is dependent on the purity of the product. The purchase cost of a relatively pure product obtained by chemical extraction is estimated at € 12 per kg (Roberts, 2008). Application of 2% chitin is comparable to a dose of 30 g of chitin per plant (based on the bioassay of Chapter 4), which estimates the cost on € 0.366 per plant. In contrast, the estimated cost of pig manure biochar is € 394 per ton (Kuppens et al., 2015). For a 3% biochar concentration, we added 54 g of biochar, which results in an estimated cost of € 0.021 per plant. Based on these estimations, application of chitin will be more expensive compared to fertilizer and pesticide application, while biochar application will reduce costs. This is however only an very rough estimation towards the economic consequences and these estimations are based on only a few prices. Also in terms of chitin production, this is calculated for chemical-extracted chitin and costs can be severely reduced if a less-pure form retrieved

from crab shells could be used. In addition, different biochars can be applied, each with its own purchase cost. Also the optimal application concentration of biochar and chitin can change according to soil and plant type, the type of biochar and the purity of the chitin. Therefore, several market studies should be done before a general conclusion can be made.

Ecological point of view: The use of pesticides and fertilizers affects the environment in several ways. Several implications of pesticide use have been reported such as loss in species diversity, honey bee mortality, reduction of nitrogen fixation in soil and substrates, and even bird and fish kills through the spread of pesticides through the food chain and contamination of aquatic systems (Zacharia, 2011). Also some fertilizers may contain toxic substances that can spread through the environment through erosion and the groundwater (UNEP, 1998). Reduction of fertilizers and pesticides in agri- and horticulture through the use of biochar or chitin will thus be beneficial for the soil and aquatic environment.

Human health. In Chapter 4, a direct correlation between the survival of the human pathogen *S. enterica* and chitin application has been shown. Leafy vegetables such as lettuce are considered as high risk food, as various *E. coli* and *S. enterica* outbreaks have been related to the consumption of these vegetables (e.g. Ward et al., 2002; Friesema et al., 2008). Considering food safety, chitin can thus be a promising soil or substrate amendment. In addition, application of pesticides in agriculture is accompanied with public health effects. On strawberry, pesticides are sprayed with a risk for residues on the fruits. Through strawberry consumption, these pesticides residues can thus be taken up in the human body (Zacharia, 2011). These chemicals will bio-accumulate in the body over time and can result in mild skin irritation and birth defects to even tumours, genetic changes and blood and nerve disorders after a long-term exposure (Miller et al., 2004). Therefore, a reduction in pesticide application through the application of biochar can be beneficial regarding human health. Also for chitin, several studies have shown beneficial effects in terms of plant defense against plant pathogens, (Cretoiu et al., 2013; Postma and Schilder, 2015), which may also results in a reduced use of pesticides.

7.3 THE PLASTISPHERE: PLASTIC AS A SUBSTRATE FOR MICROBIAL ATTACHMENT

In 2014, Osborn and Stojkovic (2014) reviewed the knowledge concerning microbial colonization of plastic debris in the marine environment. They concluded that five major research questions and challenges regarding "the plastisphere" should get attention (Figure 7.5). In this part of the discussion, the research described in Chapters 5 and 6 concerning "the plastisphere" will be discussed in relation to these five research questions and how the output of this thesis can contribute in providing an answer to these questions. These inquiries can be divided in two topics: items influencing the plastic microbial colonization (I, II and III) and ecological consequences of plastic microbial colonization (III, IV, V) (Figure 7.5).

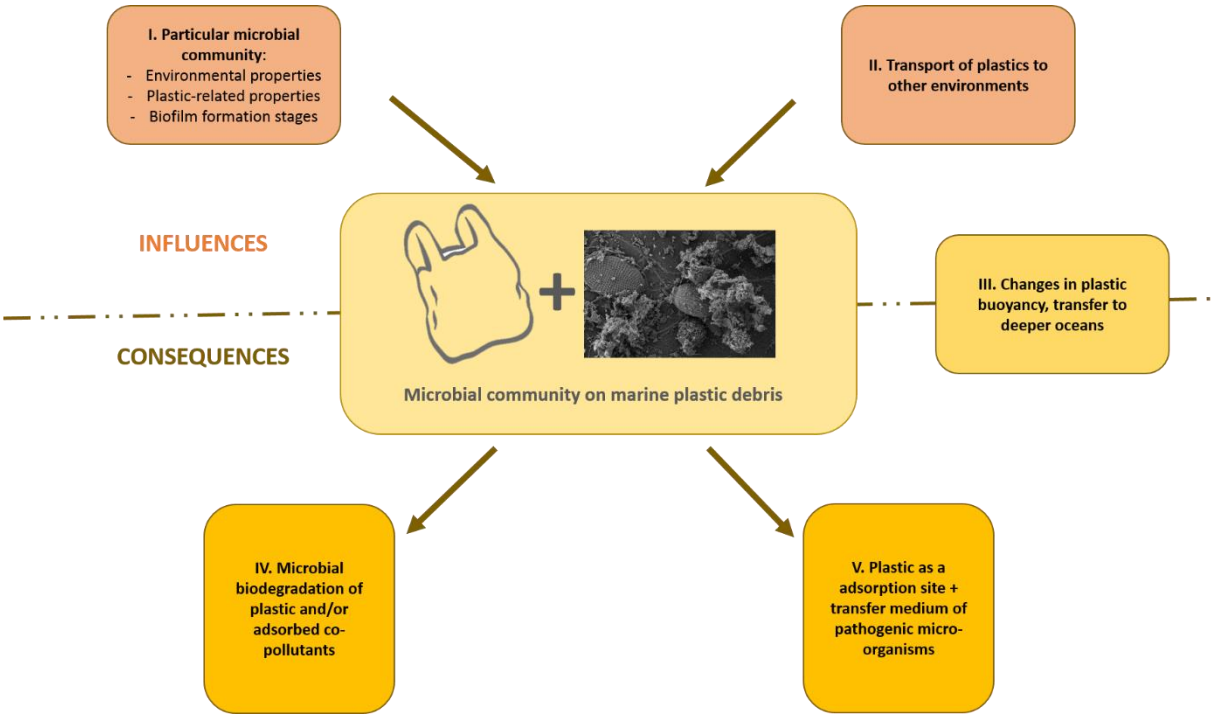


Figure 7.5 Five major research questions concerning the microbial community on marine plastic debris according to Osborn and Stojkovic (2014). These questions can be divided in two main topics: (1) external influences capable of changing the microbial community composition; and (2) consequences on the environment and animal and human health of the microbial colonization of plastic debris.

I. Do plastic surfaces select specifically for particular microbial species and/or alternatively, are plastic surfaces just primarily a convenient substrate for colonization?

In 2013, Zettler *et al*. have shown differences in bacterial community composition between floating plastic litter collected in the North Atlantic and seawater. This conclusion is confirmed and strengthened by the results of this thesis. The bacterial community of plastic debris collected in the North Sea, both by random sampling (Chapter 5) and during a controlled exposure experiment (Chapter 6), is markedly different from seawater and sediment bacterial

communities. In addition, the fungal community of plastic debris was studied under controlled conditions, which display a significantly different composition compared to sediment and seawater communities (Chapter 6).

Interestingly, a high variation in composition of the microbial communities of randomly sampled plastics in the North Sea was observed (Chapter 5). The microbial communities on plastic were even so diverse that it was hard to constitute a "core" microbiome could be constituted on OTU level, comprising organisms with a high affinity for plastics or substrates in general. In this thesis, three aspects have been proposed that potentially drive the bacterial colonization and explain the observed microbial variability on marine plastic debris (Chapter 5): environmental-related physicochemical properties, plastic-related properties and differences in microbial biofilm formation stages between plastic items.

First, differences in physicochemical properties of the environment (seawater, sediment), dependent of the location and time of sampling, are correlated with changes in bacterial community composition (Chapter 5). An effect of sampling location was especially observed for plastic debris sampled near the harbour of Zeebrugge. The bacterial communities of these plastic samples were quite similar. In contrast, compared to plastic debris sampled at other locations, a high diversity was observed. Based on the physicochemical properties of the environments, two explanations can be given. First, the seawater of the Zeebrugge region has a lower seawater salinity as a result of the influx of freshwater from the Rhine/Meuse and Scheldt Estuary (Lacroix et al., 2004). Second, in marine environments, the median grain size of the sediment and factors such as phytoplankton blooms, amount of suspended organic material and marine snow, determine the turbidity. In coastal areas, such as the harbour of Zeebrugge, turbidity is high due to a small median grain size and a high level of dredged material, which makes these sediments more susceptible to environmental pollution (De Witte et al., 2014; Gauquie et al., 2015). Both the lower salinity and higher susceptibility of environmental pollution, linked with the location the plastic was sampled, is thus probably linked to a difference in microbial community composition of plastic debris compared to other regions. Other studies confirm that geographical location, is one of the major determinants of the bacterial community composition of marine plastic debris (Eich et al., 2015; Oberbeckmann et al., 2016). This is also confirmed by the comparison of the plastics sampled at the North Sea compared to those of the Pacific and Atlantic, where differences in microbial

community composition are seen between plastics sampled at the different locations (Amaral-Zettler et al., 2015b).

Second, differences in physicochemical factors of plastic can influence the bacterial community (Chapter 5). In our study, we investigated if polymer type, plastic shape and/or plastic colour is correlated with a change in bacterial community. Previous studies (Zettler et al., 2013; Hoellein et al., 2014) showed that polymer type affect the bacterial profile of plastic debris, which could not be studied here due to insufficient sampling. However, we still predicted that colour or adsorbed chemicals can affect the bacterial community composition of plastic. The relationship of the adsorbed chemicals and bacterial community profiling will however be quite complex, as it has been shown previously that in the North Sea, more than 250 different chemical compounds can be found on plastic debris (Gauquie et al., 2015).

Third, variation in biofilm formation stages may explain part of the variation in plastic debris bacterial communities (Chapter 5). To confirm this observation, a controlled exposure experiment was done in which PE samples are exposed to the North Sea for a long-term period on two fixed locations: the harbour of Ostend and offshore (Chapter 6). At the harbour, the bacterial community of plastics showed progressive stages of biofilm formation. In contrast, this was not seen for the plastics sampled offshore, which represent the “natural environment” and are thus related to the random sampled plastics collected in Chapter 5. This suggests that in an offshore condition, biofilm formation is either repeatedly reset, permanently kept at an early stage or occurs much slower compared to the harbour environment. This retardation in biofilm formation could be due to an external mechanical force such as sand carried by currents. Based on these observations, we assume that differences in biofilm formation stages can explain variation in bacterial communities between plastic debris in specific environments, e.g. the harbour of Ostend. In offshore environments, this temporal profile is however not observed. To be sure this effect is not a retardation of biofilm formation, a study should be performed over a longer time frame.

Based on these observations, the conclusion can be made that the microbial community of plastic debris in a marine environment selects for particular microbial species and can be described as a new microbial habitat, referred to as “the plastisphere”.

II. How does the structure and function of plastisphere microbial communities change during transport from terrestrial environments, via freshwater, into marine waters and additionally into benthic environments?

Based on the "transport" history, the plastic debris used for microbial community profiling in Part II can be divided in three main groups. First, the resin pellets sampled at the Spinoladijk probably travelled from a marine environment (accidental losses by transport; North Sea) to an intertidal environment with influences of both aquatic and terrestrial environments (beach). Second, the travel history of plastic debris sampled at five locations in the North Sea (Chapter 5) in March and August is unknown. These samples can both be derived from a land-based or ocean-based source, could have travelled through rivers to the ocean and/or could be translocated already a long time in the marine environment. Third, the plastics sampled in the exposure experiment (Chapter 6) were "sterile" when entering the marine environment and thus were only influenced by the marine environment at the specific location (harbour, offshore). Therefore, they do not have a travel history.

After manufacturing, resin pellets are transported to a converting facility where the plastic is compounded and processed into useful products. During the transport of these pellets, a potential for accidental losses is always present due to mishandling of cargo or accidental spills from ships. These losses result in the appearance of resin pellets in freshwater and marine habitats, and is one of the major routes of plastic pollution in aquatic environments. Eventually, these resin pellets will drift ashore and end up on beaches (GESAMP, 2016). The resin pellets sampled at the Spinoladijk (beach in Ostend) have probably followed a similar route. The bacterial community assemblage of these resin pellets was particularly different from the marine plastic litter samples collected in the North Sea (Chapter 5). The predominant groups of marine plastic litter samples, *Proteobacteria* and *Bacteroidetes*, were also found on the resin pellets, although they were less abundant, indicating marine influences on the bacterial community composition. However, a high abundance of *Actinobacteria* dominated the resin pellets, a phylum commonly found in coastal environments (Elifantz et al., 2013) and barely detected on the plastic debris samples retrieved from the Belgian part of the North Sea.

The travel and exposure history of the plastic debris sampled in the North Sea in Chapter 5 is not known. The bacterial community of these plastics mainly consisted of bacterial families

found in the seawater or sediment. However, some particular groups, e.g. *Vibrionaceae* and *Pseudoalteromonadaceae*, were detected on plastics and not found in seawater and sediment, indicating that the surrounding environment did not serve as a source for these bacterial groups. In comparison, in an exposure experiment in the North Sea (Chapter 6) microbe-free plastics were brought in the marine environment on a specific location and were not moved until collection for microbial biofilm determination. The bacterial community members found on these plastics were also detected in either the surrounding seawater or sediment. Based on these observations, it seems that the randomly sampled plastics acquired bacterial communities originating from a different environment, which could be either land-based or marine-based. Therefore, we expect that if the affinity for the plastic material is high enough, micro-organisms can stay attached on the plastic debris while travelling through the marine environment. Given that those "random sampled" plastics are "free-moving", it is possible that they originate from a different region, and that plastic debris can act as a transport vector to transfer species through environments.

To conclude, we hypothesize that based on our observations, part of the microbial composition of plastic debris can change during transport. However to know in which extend this changes, another experiment should be set up in which microbial communities of plastics are studied during transport. A major fraction of plastics is transferred from rivers and estuaries to the marine environment (Li et al., 2016). Therefore, in terms of the Belgian part of the North Sea it would be interesting to study the microbial communities of plastics from the Scheldt estuary travelling to the North Sea.

III. How does microbial biofilm formation drive reductions in plastic buoyancy leading to plastic transport to the deeper ocean and into sediments?

A recent study in False Bay (South Africa) has shown that biofouling of floating PE samples is sufficient to let the plastics sink to the seafloor, within a period of 17 to 66 days (Fazey and Ryan, 2016). Ye and Andrady (1991) hypothesized that most plastic materials will foul sufficiently to sink in a relatively short period of exposure (7-9 weeks). The sinking rate depends on plastic size, with smaller plastics sinking quicker to the seafloor compared to bigger pieces (Fazey and Ryan, 2016). In our study, we only determined microbial biofilm formation on plastics at the seafloor either in a "natural" environment (Chapter 5) or a controlled exposure experiment (Chapter 6). The plastics in the controlled experiment were

fixed at the seafloor because they were embedded in a construction. The "randomly" sampled plastics (Chapter 5) however, were sampled at the seafloor under "natural" conditions. Most of the sampled plastic debris was PE. The density of PE is estimated at 970 kg m^{-3}, whereas those of seawater is estimated at 1027 kg m^{-3} at 10 °C (www.soortgelijkgewicht.com). Under normal conditions, the plastics sampled in Chapter 5 should thus be floating and not be found on the seafloor. This translocation can be due to the appearance of a microbial biofilm, however also other factors such as exposure to sunlight and sorption of organic pollutants to the plastic surface are known to reduce plastic buoyancy (Gewert et al., 2015). In addition, we showed that the bacterial community of these plastics resembled those of the controlled experiment, where biofilm formation seemed to be hampered. It could also be however, that an initial biofilm was present on these plastics, but due to external forces such as tides and currents, resulting in scraping due to sand emulsion, this biofilm has been removed. More research concerning the ability of microbial biofilm in terms of reduction of plastic buoyancy is therefore necessary and should be tested for several regions and plastic types to determine the relationship between fouling rates and plastic sinking rates (Fazey and Ryan, 2016).

IV. Does microbial degradation of plastic and of adsorbed co-pollutants occur in marine environments and if so over what timescale?

One of the main reasons to study microbial communities on plastic debris in the marine environment is to identify and isolate plastic degrading micro-organisms. The primarily studied polymer in this PhD, PE, is hard to degrade and expected to persist for centuries in the marine environment (Kiessling et al., 2015). So far, there is no hard evidence for PE biodegradation and only a few marine bacterial species have been proposed as potential PE degraders: *Arthrobacter* sp. and *Pseudomonas* sp. isolated from high-density PE debris in the Gulf of Mannar; and *Kocuria palustris*, *Bacillus pumilis* and *Bacillus subtilis* species isolated from low-density PE debris originating from the Arabian Sea (Balasubramanian et al., 2010; Harshvardhan and Bhavanath, 2013). In addition, several bacterial species in soil are described that have potential for PE degradation (Restrepo-Flórez et al., 2014). Although biodegradation cannot be proven using amplicon sequencing, a taxonomic screening for these organisms based on the V3-V4 16S rRNA gene region can be done. For both studies described in Chapters 5 and 6 however, none of these previous identified marine bacterial PE degraders can be detected.

Previously reported studies making use of HTS techniques to characterize the microbial community of plastics, mainly focus on the bacterial community composition (Zettler et al., 2013; Oberbeckmann et al., 2016). In soil environments however, it has been shown that several fungal species have potential to degrade PE (Restrep-Flórez et al., 2014). In addition it was recently shown that also a marine fungus, *Zalerion maritimum*, has the potential to actively degrade PE (Paço et al., 2017). Therefore, we hypothesized that to maximize the chance of identifying PE degrading micro-organisms in a marine environment, the fungal community of plastic debris should be studied in parallel with the bacterial community. In this PhD study, the fungal community composition of plastic debris in a marine environment is studied for the first time by sequencing the ITS2 variable region (Chapter 6). Based on taxonomic classification, we identified three fungal OTUs representing previously identified PE degraders (Restrepo-Flórez et al., 2014): *Cladosporium cladosporioides*, *Fusarium redolens* and *Mortierella alpine*. However it should be considered that phylogeny based on rRNA gene sequences is not necessarily linked to biodegradation potentials and strains should be isolated and tested in PE biodegradation assays to further verify biodegradation activity (see below).

Instead of looking to the degradation of the plastic polymer, one could also be interested in the degradation of adsorbed co-pollutants as it has already been shown that more than 250 different chemical compounds can be adsorbed on plastic debris (Gauquie et al., 2015). In Chapter 5, one species of the Mycobacteriaceae, *Mycobacterium fredriksberegense*, is highly abundant on yellow and blue coloured resin pellets, whereas it is barely detected on other pellets and plastics. *M. frederiksbergense* is typically known for its degrading capacity of diverse polycyclic aromatic hydrocarbons, like anthracene and anthraquinone derivates (Willumsen et al., 2001; Wick et al., 2003). Anthraquinone is typically used as precursor for dye synthesis and used for colouring resin pellets, which suggests the presence of anthracene derivates on the blue and yellow beach pellets (Bien et al., 2000; US patents 3441536, 6593483, 6635350). This can be an explanation for the high abundance of *M. frederiksbergense* on coloured resin pellets while the taxon is almost absent on other plastic debris sampled in the North Sea and uncoloured resin pellets. This not only suggest that presence of dyes, or even adsorbed chemicals or pollutants can influence the colonization of plastic debris, but also indicates that adsorbed co-pollutants can be degraded by micro-organisms.

To conclude, no biodegradation of plastics nor adsorbed co-pollutants was studied in this PhD study, but several assumptions are made indicating that degradation can be possible. To prove biodegradation of plastic, one can do several experiments of which two are highlighted here:

(1) Possible biodegraders, either pure cultures or by studying an entire microbial community, can be detected through biodegradation assays. Several techniques are available to study biodegradation, however most can be only used in *in vitro* conditions. ASTM standard test methods D6691-09 and D7991-15 can be used to study aerobic biodegradation of plastic materials in the seawater column or sediment respectively. For both methods, the amount of biogass (CO_2) produced during the plastic exposure to the environment will be measured and compared with the theoretical amount of CO_2 produced in case of total conversion of the organic carbon present in the plastic. Another method to actually indicate degradation of plastic is by studying enzyme production by microbial strains (Yoshida et al., 2016).

    Through the application of Fourier-transformed infrared spectra (FT-IR) the changes in the chemical properties of plastic can be measured and formation or disappearance of functional groups can be determined (Pometto et al., 1993). In addition, through the use of microscopy techniques, such as scanning electron microscopy (SEM), surface changes of the polymer can be checked. SEM makes it possible to identify for example microcracks made by micro-organisms (Arutchelvi et al., 2007). These techniques however only give an indication of the degradation of plastics, while the production of enzymes and CO_2 measurement prove the biodegradation of the plastic.

(2) Techniques described in (1) are interesting to see if biodegradation occurs. However, if a microbial community is used instead of pure cultures, these techniques are not able to discriminate between plastic-metabolizing organisms and secondary metabolizers, which metabolise compounds provided by the true plastic-consuming organisms. Similar as described in 7.2.1, DNA-SIP can be used to detect plastic metabolizers through the incorporation of ^{13}C-DNA in their DNA. The major drawback of the technique is however that stable isotope labelled material is quite expensive and so far ^{13}C-labelled PE is solely available as a powder (http://www.sigmaaldrich.com). Despite these drawbacks, the use of this technique can provide information on PE biodegraders and may be used in future studies.

V. Are plastic surfaces a potential site for accumulation of pathogenic micro-organisms that can be ingested by and impact upon marine fauna?

No specific attention has been paid in this PhD to potential pathogenic micro-organisms on plastic debris. However, in accordance to Zettler *et al.* (2013), we detected several members of the *Vibrionaceae* family, a family known to contain several pathogenic organisms, on plastic debris sampled in the North Sea (Chapter 5). In 2016, the presence of potentially pathogenic *Vibrio* sp. on marine microplastics has been confirmed (Kirstein et al., 2016). The large quantities of plastic debris released in the marine environment thus provides opportunities for the dispersal of pathogens as some species seem to be able to stay attached to the plastic debris (see 7.3 section III). This can have several important ecological and animal/human health implications. Especially microplastics are easily mistaken as food by animals and taken up (Van Cauwenberghe, 2015). Pathogenic organisms adhered on the microplastic can then contaminate the marine organism or be transferred to other organisms through the food chain. To date, concentrations of these pathogenic agents remain very low at sea and may not be relevant in terms of an animal and human risk assessment (GESAMP, 2016). Future studies on the presence of pathogenic organisms, the abundance of these organisms and their impacts are therefore necessary.

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ISO TS14256-1:2003	Determination of nitrate, nitrite and ammonium by extraction potassium-chloride solution (soil)
ISO 10694	Determination of organic and total carbon (soil)
EN 13037	Determination of pH in peat systems
EN 13038	Determination electrical conductivity peat s
EN 13039	Determination organic matter in peat .
EN 13040	Soil improvers and growing media
EN 13652	Extraction of water soluble nutrients in peat

Curriculum Vitae

PERSONAL DATA

Name	Caroline Arthur Diana De Tender
Date of birth	2 September 1990
Place of birth	Sint-Niklaas
Nationality	Belgian
Address	Koutermolenstraat 7 B4, 9111 Belsele
Mail	caroline.detender@hotmail.com

EDUCATION

2011-2013	Master of Science in Bioscience Engineering option Cell and Gene Biotechnology (Ghent University, Ghent): Magna cum Laude *Master thesis: Localisation and interaction of the* Arabidopsis thaliana *Euonymus-like lectine and arabinogalactans*
2008-2011	Bachelor of Science in Bioscience Engineering, option Cell and Gene Biotechnology (Ghent University, Ghent): Cum Laude

DOCTORAL TRAINING COURSE

Specialist courses

2015-2016	Statistical Genomics (Prof. Lieven Clement, Manama statistical data analysis)
2014-2015	N2N Multidisciplinary seminar series on bioinformatics
2013-2014	Unix for bioinformatics (prof. Lieven Sterck, master biotechnology)

Transferable skills

2015-2016	Applying for a postdoctoral job (Carreer management)
2014-2015	Summer School Science communication “Zeg ‘t eens” (Communication skils)
2013-2014	Introduction to high-performance computing (Research & Valorisation)
2013-2014	Getting started with high performance computing part 2 – HPC basics (Prof. Peter Dawyndt; Research & Valorisation)
2013-2014	Getting started with high performance computing Part 1 – Unix command line (Prof. Peter Dawyndt; Research & Valorisation)

PUBLICATIONS

A1

De Tender C, Schlundt C, Devriese LI, Mincer TJ, Zettler ER, Amaral-Zettler LA. (2017). A review of microscopy and comparative molecular-based methods to characterize "Plastisphere" communities. Analytical Methods, 9, 2132-2143.

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CONGRESS ABSTRACTS

Devriese L, De Backer A, Maes S, Van Hoey G, Haegeman A, Ruttink T, Wittoeck J, Hillewaert H, De Tender C, Hostens K. (2017). A DNA (meta)barcoding approach to assess changes in seabed ecosytems related to human-induced pressures. DNAqua Kick-off event, Essen, Germany.

Devriese L, Haegeman A, Maes S, Ruttink T, De Backer A, Van Hoey G, Wittoeck J, Hillewaert H, De Tender C, Hostens K. (2016). A DNA (meta)barcoding approach to tackle marine benthic biodiversity. North Sea Open Science Conference, Ostend, Belgium.

De Tender C, Devriese L, Haegeman A, Maes S, Ruttink T, Dawyndt P. (2016). The dynamic bacterial colonization on plastic: an exposure experiment at sea. North Sea Open Science Conference, Ostend, Belgium.

De Tender C, Debode J, Vandecasteele B, Cremelie P, Haegeman A, Ruttink T, Dawyndt P, Maes M. (2016). Dynamic changes in the strawberry rhizobiome in response to biochar. SETAC/iEOS Joint Focused Topic Meeting: Environmental and (eco)toxicological omics and epigenetics: science, technology and regulatory applications, Ghent, Belgium.

De Tender C, Devriese L, Haegeman A, Cattrijsse A, Vangeyte J, Maes S, Ruttink T, Dawyndt P. (2016) Temporal dynamics of bacterial colonization of plastic debris in the North Sea. SETAC/iEOS Joint Focused Topic Meeting: Environmental and (eco)toxicological omics and epigenetics: science, technology and regulatory applications, Ghent, Belgium.

De Tender C, Devriese L, Haegeman A, Maes S, Ruttink T, Robbens J, Dawyndt P. (2016). Living in the Anthropocene: plastic debris as a new bacterial habitat. ISME 2016, Montréal, Canada.

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De Tender C, Devriese L, Haegeman A, Maes S, Ruttink T, Dawyndt P, Robbens J. (2015). Plastic debris: a distinct niche in the marine environment. Bageco 13, Milan, Italy.

De Tender C, Devriese L, Maes S, Haegeman A, Ruttink T, Robbens J, Dawyndt P. (2015). Metagenomics @ sea: a floating house in a marine environment. VLIZ Young Marine Scientist's day.

Debode J, De Tender C, Cremelie p, Vandecasteele B, Maes M. (2015). Effect of biochar amendment on the rhizosphere microbiome of lettuce and strawberry. Rhizosphere 4, The Netherlands

Debode J, Wesemael W, Ebrahimi N, De Tender C, Cremelie P, D'Hose T, Viaene N, Vandecasteele B, Maes M. (2014). The effect of biochar on plant pathogens and rhizosphere microbiology. The biochar effect along the soil-rhizosphere-plant-atmosphere continuum.

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SUPERVISED DISSERTATIONS

2016-2017	Master dissertation Ana Shein Lee (Faculty of Bioscience engineering Ghent University & Universidad Complutense de Madrid). *"Plant microbiome interactions on strawberry grown in sustainable substrates"*
2016-2017	Internship Ana Shein Lee (ILVO & Universidad complutense de Madrid) *"Studying the fungal community on plastic debris in the marine environment"*
2014-2015	Internship Giang Thu Mai (ILVO & Faculty of Sciences-Ghent University) "*Methods to study whole genome shotgun data"*
2013-2014	Master dissertation Sidney Vandenabeele (Faculty of Bioscience engineering – Ghent University) *"Studie van de bacteriële diversiteit van de rhizosfeer van* Lactuca sativa *en* Fragaria x ananassa *in functie van ziekteweerbaarheid"*
2013-2014	Bachelor dissertation Jelle Verborgh (Faculty of Bioscience engineering – Ghent Univerisy) *"Next-generation sequencing als identificatietechniek voor micro-organismen op microplastics in zee"*