Explorations of soil microbial processes driven by dissolved organic carbon

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Abstract

Dissolved organic carbon (DOC) is a complex, heterogeneous mixture of C compounds which, as a substrate, may influence various processes of the soil microbial community. Microbial respiration and volatile production are two such processes. These have both been linked to general disease suppression (GDS), a phenomenon in agricultural soils which inhibits pathogenic infestation in crops. The underlying hypothesis of this thesis is that the quality of DOC, via regulation of microbial processes, may be an important indicator of soil functions, including GDS. Properties of DOC quality include proportions of hydrophobic and hydrophilic fractions, and aromaticity. This thesis describes a high range in DOC fractions from various types of compost, which is often added to soil as an amendment to promote GDS. Differences in soil microbial respiration rates were attributed to differences in the composition of compost DOC added to soil in a laboratory incubation experiment. Compost DOC high in proportion of the hydrophilic (Hi) fraction promoted respiration rates. Depletion of the hydrophobic humic acid (HA) fraction was also observed. The relationship between DOC and microbial respiration was further explored in a survey of 50 arable soils. Both HA and Hi fractions of DOC that were found to be statistically, significantly related to respiration rates in these soils. Furthermore, in an assay measuring *in vitro* pathogen suppression by microbial volatile production, DOC concentration and microbial respiration were linked to growth suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* via multivariate regression modelling. This thesis provides evidence for the importance of DOC and DOC quality's influence on microbial respiration and volatile production, thus supporting the hypothesis that DOC is a microbially-relevant soil chemical parameter, and potential indicator of general disease suppression in agricultural soils.

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List of Abbreviations

CEC	cation exchange capacity
DAX-8	nonionic, macroporous, hydrophobic resin (Sigma-Aldrich)
DOC	dissolved organic carbon
DOM	dissolved organic matter
DWE	dry-weight equivalent
FA	fulvic acids
FC	fresh compost
GDS	general disease suppression
HA	humic acids
Hi	hydrophilics
HoN	hydrophobic neutrals
HY	hydrophilics
IHSS	International Humic Substances Society
IRS	Dutch Sugar Beet Research Institute
ITS	internal transcribed spacer
LMW	low molecular weight
MC	mature compost
OM	organic matter
OTU	operational taxonomic unit
PCA	principal component analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PPO	Applied Plant Research Group of Wageningen University
RDA	redundancy analysis
SFA	Segmented Flow Analyser
SIR	substrate-induced respiration
SOC	soil organic carbon
SUVA	specific ultraviolet absorption at 254 nm
TOC	total organic carbon
UPW	ultra-pure water
VOC	volatile organic compounds
WHC	water-holding capacity
WYA	water yeast agar

This thesis is dedicated to the memory of
my grandma, Catharina Straathof,
in whose garden my love for soil began.

Chapter 1

General Introduction

Angela L. Straathof

1.1 Motivation

Robust soil quality is an important factor in supporting the productivity of agricultural systems. One major threat to productivity is plant disease caused by soil-borne plant pathogens. Incidence of disease caused by these pathogens, however, is reduced in soils exhibiting general disease suppression. General disease suppression (GDS) is a naturally-occurring phenomenon whereby the soil microbial community is antagonistic towards multiple pathogens (Hoitink and Boehm, 2003). This suppression of pathogens may be a result of the non-pathogenic community producing compounds inhibitory to pathogen development, or it may be the result of competition for resources between pathogenic and non-pathogenic organisms (Termorshuizen and Jeger, 2008).

One resource that pathogenic and non-pathogenic organisms may compete for in soil is organic matter. Organic matter (OM) is a source of organic carbon (C), which fuels soil microbial processes (Haynes, 2005). Therefore, the ability of non-pathogenic organisms to consume organic C may influence both the availability of this substrate for the pathogens, and the microbial community's production of inhibitory compounds. The quality of organic C in soils, however, affects its availability as a substrate (Boyer and Groffman, 1996). If microbial community consumption of organic C with particular qualities could be linked to that community's ability to suppress pathogens, then organic C quality could be used as an indicator of a soil's capacity for GDS. An indicator of GDS would be valuable not only for informing users about a soil's current state (Janvier et al., 2007), but also for identifying management practices that may enhance desirable OM and organic C characteristics, and reduce plant disease incidence. This need for an indicator of GDS was the impetus for the research presented in this thesis, which intends to advance knowledge of soil C-driven microbial processes and contribute to the identification of a GDS indicator.

1.2 Organic Carbon in Soils: An Introduction

Soils are the largest terrestrial sink of C and almost 2/3 of the C stored in soils globally is in organic form (Lal, 2004). In arable soils alone, 167 Pg of organic C is stored (Cole et al., 1997). Soil organic carbon (SOC) is former living material in various stages of decomposition and it is found to varying degrees in every soil type. Two main pools of SOC are present in soils: stabilized SOC, and labile SOC (Haynes, 2005), which are in a constant state of flux between each other. Stabilized SOC is generally referred to as such because it is relatively resistant to decomposition, either by being composed of a recalcitrant substance such as humus, or being physically fixed to the soil mineral phase (Stevenson, 1994). Through decomposition or desorption, however, even stabilized SOC can enter the pool of labile SOC where it can be taken up by soil microorganisms or remain in solution (Haynes, 2005; Sollins et al. 1996). In solution, SOC is also known as dissolved organic carbon.

1.2.1 Soil Dissolved Organic Carbon

The term dissolved organic carbon (DOC) refers to both a phase of SOC and an operational definition as well. Carbon in soil solution that has been collected or extracted (Burford and Bremner, 1975; Curtin et al., 2011) and filtered to 0.45 µm is defined as DOC (Kalbitz et al., 2000).

Once SOC enters the pool of DOC, it has several possible fates (Figure 1.1): it may re-enter the SOC pool; it may be decomposed and remain in the DOC pool; or it may be decomposed and released as carbon dioxide (CO_2) as a result of microbial activity (Kalbitz et al., 2000). The nature of DOC as a pool in constant flux makes it difficult to study DOC dynamics and this difficulty is often exacerbated by low DOC concentrations in soils; in forest soils only up to 2% of SOC is in DOC form, and this upper limit even lower (0.4%) in agricultural soils (Haynes, 2005).
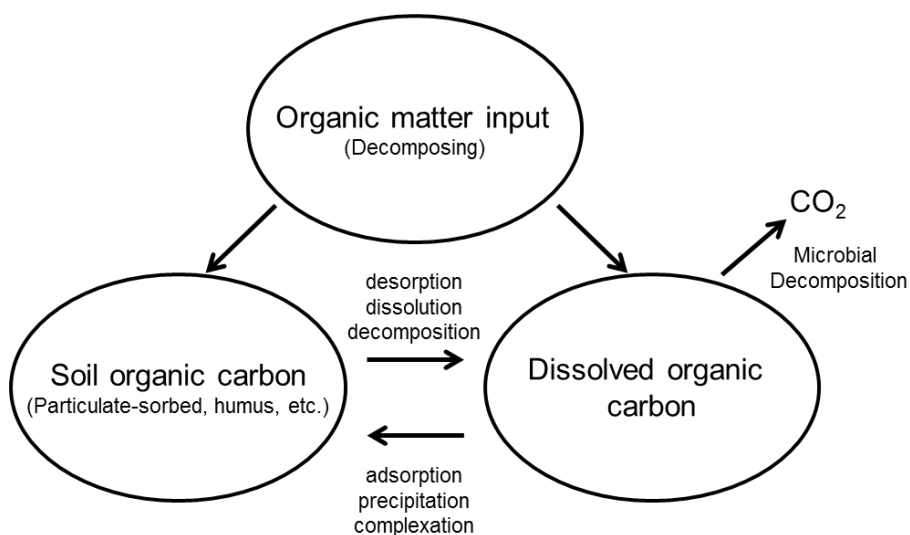
Figure 1.1: Sources and potential fates of dissolved organic carbon in soils (Adapted from Kalbitz et al., (2000)).

In addition to SOC, a potential source of DOC is OM input (Figure 1.1). Additions of OM that contribute to DOC are generally in the process of decomposition and may include plant material, decaying animal and microbial organisms, and root exudates (Kalbitz et al., 2000), although the primary source is most often plant litter (Cadisch and Giller, 1997; Paul et al., 1996). In arable soils, however, a higher degree of anthropogenic control is garnered over plant litter inputs compared to unmanaged ecosystems, and OM sources can also be imported to the system and applied as a soil amendment. These potential sources are described in further detail in the following section.

1.2.2 Organic Amendments as Dissolved Organic Carbon Sources

For the purposes of this thesis, organic amendments will refer to any OM source that is deliberately and anthropogenically applied to arable soils. Organic amendments serve many purposes in their application to agricultural soils, including nutrient supplementation (Chang et al., 2007), SOC

supplementation (Gregorich et al., 1998; Smith et al., 1997), physical stabilization of the soil (Diacono and Montemurro, 2010), and suppression of plant diseases (Hoitink and Fahy, 1986; Paulitz and Bélanger, 2001). These amendments may range from animal manure (both liquid or solid forms), sawdust, and crop residues (Chantigny, 2003) to compost (Hargreaves et al., 2008) and are often integrated into field management strategies. Organic amendments influence the quantity and composition of soil DOC by either entering the soil DOC pool directly in the form of leachate (Chantigny, 2003), or decomposing further to become incorporated in the SOC pool (Kalbitz et al., 2000), and indirectly entering the DOC pool via SOC desorption or decomposition (Figure 1.1).

As a source of DOC, organic amendments can vary largely in both the absolute concentrations and chemical properties of the DOC they release, including biodegradability (Chefetz et al., 1998; Wei et al., 2014). Upon integration of organic amendment DOC into soil DOC (Figure 1.1), the new characteristics of soil DOC may shift to reflect that of the added DOC (Kalbitz et al., 2003b), with implications for soil DOC quality.

1.2.3 Dissolved Organic Carbon Quality

Soil DOC quality broadly refers to the composition of the total pool of DOC and the properties of the C compounds within the DOC pool. These compounds include soluble humic and fulvic substances (Thurman and Malcolm, 1981; Zsolnay, 1996), organic and amino acids (Amery et al., 2009), and carbohydrates ranging from mono- to poly-saccharides (Herbert et al., 1995). As DOC is a heterogeneous mixture of C compounds, characterizing DOC by the nature of these compounds provides insight into the relevance of DOC either as a substrate for microbial activity, or the capacity for feedback between pools of SOC and DOC (Figure 1.1).

Several methods are well-established for the qualification of DOC. Due to the complexity and heterogeneity of the substances, molecular identification of DOC substances only provides insight into a small proportion of total DOC

(Marschner and Kalbitz, 2003). Leenheer and Croué (2003) estimate that <10% of DOC can be identified as specific compounds and the cost increases associated with such analyses are exponential compared to identifying broader classes of compounds. Amino acids and carbohydrates are examples of low molecular weight (LMW) compound classes that have had ample methodological development (Jones, 2002; Myklestad et al., 1997). Other methods of determining physical and/or chemical DOC properties include specific UV absorbance (Amery et al., 2008) and fluorescent or infrared spectroscopy (Ellerbrock et al., 1999; Kalbitz et al., 2003) as indicators of aromaticity, size-exclusion chromatography as an indicator of molecular weight (Her et al., 2003), or fractionation based on classes of relative hydrophobicity (Guggenberger et al., 1994; Thurman and Malcolm, 1981).

1.2.4 Fractionation of Dissolved Organic Carbon

Fractionation of DOC from soil is adapted by Swift et al., (1996) from methods developed to measure humic substances in aqueous solutions (Aiken et al., 1985; Thurman and Malcolm, 1981). Hydrophobic and hydrophilic fractions of DOC are distinguishable from one another based on their solubility under acidic conditions and/or their affinity for binding to hydrophobic resin. Operational definitions of hydrophobic and hydrophilic DOC compounds have been developed and described by the International Humic Substances Society (IHSS). The advantage of this method and the employment of IHSS's operational definitions of these DOC fractions is the standardization of their measurement across experimental conditions.

The fractionation procedure is described in detail by Aiken et al. (1985) but the most important outcome of this method to emphasize here is the isolation of four fractions: humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN) and hydrophilics (Hy) (Figure 1.2). These fractions range from most hydrophobic (HA) to least (Hy) and are themselves heterogeneous mixtures of C substances, though they are considered relatively more homogeneous than total DOC (Leenheer, 1981). Hydrophobic HA molecules

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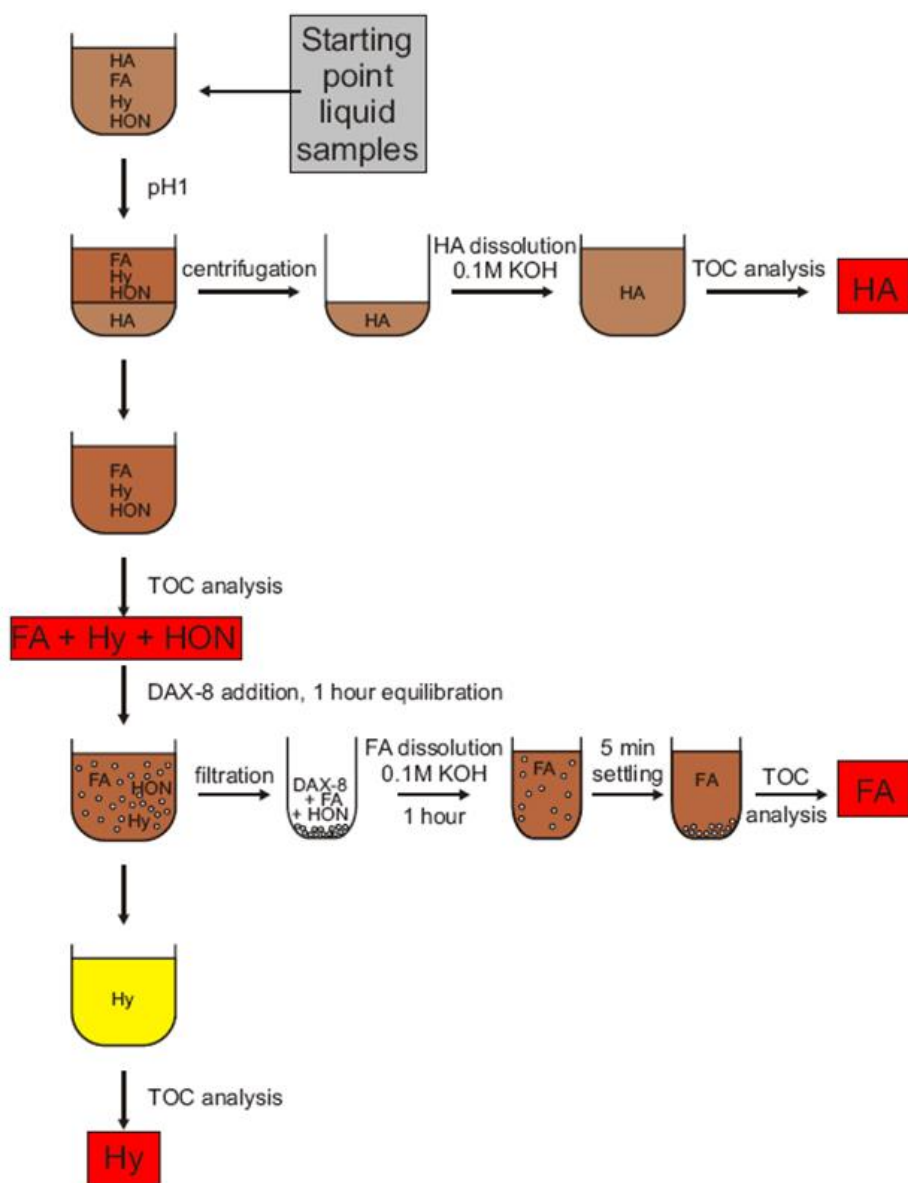**Figure 1.2:** Work-flow of the dissolved organic carbon (DOC) rapid batch fractionation method from Van Zomeren and Comans (2007). Red boxes indicate pools in solution measured for total organic carbon (TOC). Humic acids (HA), fulvic acids (FA), and hydrophilic compounds (Hy) are measured directly while hydrophobic neutrals (HON) remain adsorbed to the resin (DAX-8) after equilibration and their pool size is calculated.

are considered quite recalcitrant to microbial degradation and thus one of the most stable soil organic compounds (Kalbitz et al., 2003b), even in solution. Conversely, when measuring how specific compounds are partitioned between hydrophobic and hydrophilic pools, Amery et al. (2009) observed the majority of aliphatic, LMW C and 97% of glucose were measured in the hydrophilic pool. Guggenberger et al. (1994) have proposed that hydrophobic compounds (including FA and HON) may be in an intermediary state of decomposition between DOC source material and Hy fractions. Each fraction therefore may have properties unique to it beyond hydrophobicity.

One draw-back of the fractionation procedure has been that it is time-consuming and labour-intensive (Malcolm, 2005). These disadvantages have recently been overcome by the development of a batch-fractionation procedure (Figure 1.2) by Van Zomeren and Comans (2007) which allows for a greater number of DOC samples to be processed in a shorter amount of time with no loss of precision. Although fractionation is a widely-used and (becoming a more) accessible method for characterizing DOC, relatively little is known about the importance of the isolated fractions for soil function, particularly biological activity.

1.2.5 The Biological Relevance of Dissolved Organic Carbon (Fractions)

Due to its soluble nature and ability to permeate the soil matrix, DOC has been presumed a relatively bioavailable source of C for soil microorganisms (Kalbitz et al., 2000; Marschner and Kalbitz, 2003). The microbial processes shown to be influenced by soil DOC (quality) include: nutrient mineralization (Haynes and Beare, 1997; Janzen et al., 1997), biomass accumulation, and activity (respiration) rates (Boyer and Groffman, 1996; Brooks et al., 1999; Janzen et al., 1997; Marschner and Noble, 2000). However, in reviewing many studies on the biodegradability of DOC, Haynes (2005) determined a range of only 10-40% of DOC that is available for decomposition to CO_2 by the soil microbial community. Since biological activity of soils is frequently proposed

as a potential indicator of overall soil quality (Gregorich et al., 1994; Janvier et al., 2007), this 10-40% of DOC may be critical for many processes.

The remaining proportion of DOC considered "non"-bioavailable is suggested to be composed of recalcitrant humic-like substances (Haynes, 2005). Indeed, Jandl and Sollins (1997) and Qualls and Haines (1992), in some of the few studies on the biodegradability of specific DOC fractions, confirmed that hydrophilics were the fraction of DOC most rapidly depleted by soil microorganisms. However, the recalcitrant nature of HA has been challenged (Boyer and Groffman, 1996) and it may be more useful to think of HA molecules as a potential reservoir of LMW C that is intermittently adsorbed to HA surfaces (Sutton and Sposito, 2005). The biological relevance of DOC may not only support microbial activity and thus soil CO_2 production (Figure 1.1), but the activity may in turn influence concentrations and quality of DOC (Magill and Aber, 2000). Figure 1.3 is a conceptual representation of the feedback between the components of the soil microbial biomass and the pool of total DOC. As different functional groups of soil microorganisms have different
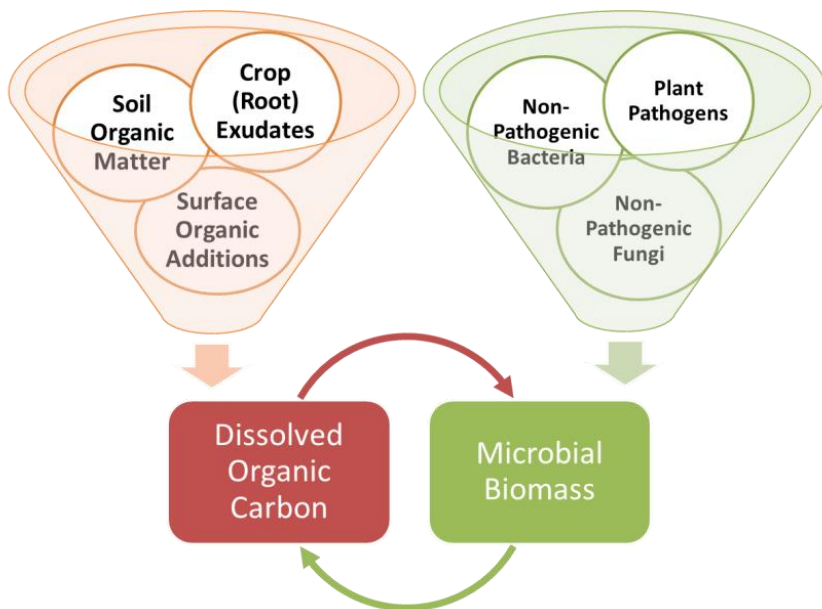

Figure 1.3: Conceptual framework of dissolved organic carbon and microbial biomass feedback loop in agricultural soils.

C substrate preferences (Fierer et al., 2008; Kramer and Gleixner, 2008), and secondary (non-CO_2) products of decomposition remain in solution as DOC (Kalbitz et al., 2003), the feedback between properties of the microbial community and DOC pools in soils may be intricate and rapid. However, much remains to be explored about this feedback, including the role of DOC fractions as indicators of DOC quality as a substrate, and the implications of this for various microbial processes.

1.2.6 Predicting Disease Suppressiveness of Agricultural Soils

One microbially-mediated soil process that may also be influenced by DOC quality is GDS. This thesis is one outcome of the Dutch Technology Foundation-funded project *Predicting Disease Suppressiveness of Agricultural Soils*. The objective of this project is to identify indicators of a soil's capacity for GDS and develop management recommendations for farmers to increase GDS. The research presented in this thesis was conducted in close collaboration with that conducted for the thesis *Suppression of Soil-Borne Plant Pathogens*, by Maaike van Agtmaal (2015), which the reader is also encouraged to refer to. Together, these two theses investigate the hypotheses of *Predicting Disease Suppressiveness of Agricultural Soils*, which are that:

1) GDS is determined by soil microbial community activity, including decomposition of the substrate DOC, and production of pathogen-inhibiting volatile compounds;

2) The activity rates of the soil microbial community are reflected in the quantity and composition of the soil DOC pool, any organic amendment DOC pool, and/or subfractions of these pools;

3) OM management is a tool in management of DOC and consequently of GDS.

1.3 Research Objectives

The three hypotheses developed for the project *Predicting Disease Suppressiveness of Agricultural Soils* were the foundation of the research objectives of this thesis. The broad objectives of this thesis were to determine how DOC and DOC fractions ranged among soils, and how DOC properties were related to various processes of the soil microbial community. Addressing these broad objectives was approached by sequentially addressing the following more specific research objectives:

1) To investigate differences in the DOC characteristics of organic materials used for soil amendment, and relate these differences to how the materials were processed (Chapter 2);

2) To measure how differences in DOC from different organic amendments influenced fractions of soil DOC (Chapter 3);

3) To measure how changes in fractions of soil DOC were related to changes in soil microbial respiration (Chapter 3);

4) To determine how DOC properties ranged among different agricultural soil types, and to what degree these properties could be used to explain microbial respiration (Chapter 4);

5) To relate DOC properties among different agricultural soil types to the microbial production of pathogen-suppressing volatiles (Chapter 5);

6) To consider DOC in a new context relative to soil microbial functions, especially GDS, and develop hypotheses for future research consideration (Chapter 6).

1.4 Thesis Outline and Experimental Approach

The research objectives outlined in this thesis were investigated using a variety of experimental approaches. Many were exploratory and aimed to gather information about DOC properties from sources that we previously had little information for (Objectives 1, 4 and 5), while others used a systematic application of treatments (Objectives 2 and 3) that built on earlier experiments.

In Chapter 2, I characterized DOC from various compost types that had received different input material and undergone different processing conditions, thereby addressing Objective 1. Based on vast variation reported in the literature about how soil microbial functions may respond to compost application, I hypothesized that the range in DOC fraction concentration may be the mechanism behind these differences, so I measured this potential range. While previous studies had investigated changes in DOC quality in one compost type over time, or differences in total DOC among compost types, this would be the first study to perform an in-depth comparison of DOC quality among many compost types. The high range measured suggested that these differences may indeed be significant enough to result in differences in soil microbial response.

The results of Chapter 2 thus prompted our experimental treatments in Chapter 3. In Chapter 3 I aimed to determine if the differences in compost DOC quality found in Chapter 2 had discernible effects on soil DOC fractions when that compost DOC was added to soil. To this end, I selected two of the composts measured in Chapter 2 that had different DOC fraction profiles. I hypothesized that not only would the differences in compost DOC quality affect soil DOC quality upon addition (Objective 2), but that these differences would be further reflected in the rates of soil microbial activity measured from this soil over a period of incubation (Objective 3). In addition to confirming both of these hypotheses, Chapter 3's experiment produced some surprising results: humic acid DOC fractions that were expected to remain stable over the incubation period were actually very dynamic. The results of Chapter 3 were unique and important, but I wondered how representative this one soil type was of what may be measured in other agricultural soils.

Therefore, in follow-up to Chapter 3, a much broader survey of agricultural soils was conducted in another exploratory experimental approach. The 50 soils sampled for Chapters 4 and 5 came from a variety of arable soil types undergoing several different management regimes. All 50 soils were measured in collaboration with BLGG Agroexpertus and the Netherlands Institute of Ecology to determine the broadest set of physical, chemical, microbial and DOC parameters. In both Chapters 4 and 5, using a statistical approach, I determined the influence of DOC properties on the soil microbial processes of respiration and suppressive-volatile production, respectively.

Chapter 4 describes both the general patterns of variability in indicators of DOC quality and the (lack of) influence of field management practices on those indicators. These DOC properties were then analysed separately and together as independent variables in a model explaining variation in soil microbial respiration rates, which is motivated by Objective 4. By comparing performances of models with various DOC properties as independent variables, more insight is provided into the biological relevance of DOC fractions and their aromaticity, which supplements the findings of Chapter 3. Peripheral to Objective 4, the results of this experiment revealed interesting relationships between the properties of DOC aromaticity and proportions of DOC hydrophilic fractions. The survey I performed in Chapter 4 is the most detailed study conducted to date on the properties of DOC (fractions) among agricultural soils.

Succeeding my analysis of DOC's influence on microbial respiration is Chapter 5's analysis of pathogen-suppression rates. This final experiment builds on the statistical approach of Chapter 4, but includes DOC properties in conjunction with a wider range of soil chemical and microbial community properties as independent variables, and considers the production of pathogen-suppressing microbial volatiles as the dependent variable. Identifying the nature of this relationship is the research priority of Objective 5. The importance of DOC for pathogen-suppression *in vitro* is confirmed by the significance of both DOC and microbial respiration as model parameters for the overall suppression of three soil-borne plant pathogens. These results subsequently lead to the

recommendations I make for future research, which are proposed in Chapter 5 and elaborated on in Chapter 6.

Chapter 6 is the final chapter in this thesis, in which I interpret the results of each chapter not only relative to one another, but to the broader body of literature on soil organic carbon quality and microbial function. Furthermore, as per Objective 6, I go on to describe the implications of this thesis for development of future research priorities in such pertinent fields as soil OM turnover and general disease suppression.

Chapter 2

Input materials and processing conditions control compost dissolved organic carbon quality

Angela L. Straathof, Rob N.J. Comans

Abstract

Dissolved organic carbon (DOC) has been proposed as an indicator of compost maturity and stability. Further fractionation of compost DOC may be useful for determining how particular composting conditions will influence DOC quality. Eleven composts ranging in input materials and processing techniques were analysed; concentrations of DOC ranged from 428 mg kg^{-1} to 7300 mg kg^{-1}. Compost DOC was qualified by fractionation into pools of humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN), and hydrophilic (Hi) compounds. The range in proportion of DOC pools was highly variable, even for composts with similar total DOC concentrations. Longer composting time and higher temperatures consistently corresponded with a depletion of hydrophilics, suggesting a preferential turnover of these compounds during the thermophilic composting phase. Qualification of DOC pools through fractionation may be an informative tool in predicting the effects of a processing technique on compost quality and, ultimately, soil functional processes.

2.1 Introduction

Composts are produced using many different processing techniques and with a broad range of input materials, resulting in physical and chemical heterogeneity between compost types (Lannan et al., 2012). This heterogeneity makes it difficult to predict how a soil's biogeochemical properties are influenced by compost application. Dissolved organic carbon (DOC) concentration is associated with many indicators of both compost and soil quality, including maturity and microbial activity, respectively. Therefore, DOC has been proposed as an indicator of compost maturity and stability (Bernal et al., 1998; Zmora-Nahum et al., 2005). Between compost types, however, DOC quality may range broadly and independently of total DOC, depending on input materials (Wei et al., 2014) and duration of composting (Said-Pullicino et al., 2007).

Qualification of DOC is often performed by isolating and quantifying the pools of hydrophobic and/or hydrophilic DOC in a solution extracted from solid-phase materials (Aiken et al., 1985), such as compost. Hydrophobic compounds include humic acids, fulvic acids and hydrophobic neutrals. These compounds tend to be more aromatic and higher in molecular weight than hydrophilic compounds (Aiken et al., 1985). These various pools of DOC also function distinctly from one another; the size and hydrophobicity of compost humic acids facilitate their ability to complex contaminants such as trace metals, polycyclic aromatic hydrocarbons, and pesticides (Semple, 2001). Meanwhile, hydrophilics extracted from compost have been found to influence rates of microbial activity in soils (Straathof et al., 2014) which may subsequently impact nutrient availability and turnover. As compost's application purposes range from remediation to nutrient supplementation to pathogenic disease suppression (Termorshuizen et al., 2006), the characterization of the respective pools that influence these processes may be valuable information for end users.

The objective of this experiment was to investigate the range in quality of dissolved organic carbon from a variety of composts. It was hypothesized that composts sourced from different organic input materials and composted

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under different processing conditions would have different DOC quality profiles independent of total DOC concentration. Furthermore, it was hypothesized that increasing the length of maturation time and/or increasing proportions of woody input material would result in higher concentrations of hydrophobic compounds relative to hydrophilic compounds. This will provide insight into how input materials and processing conditions of a particular compost influence the DOC quality of the final product.

2.2 Materials and Methods

2.2.1 Compost Collection and Characterization

Eleven compost types were collected from different composting facilities in The Netherlands between March 2012 and June 2013. Each compost was collected on-site from the commercial composting facilities (Table 2.1) except MW6, which was received from the supplier in commercial packaging. Composts collected on-site were shoveled from a minimum depth of 30 cm below the surface of the compost heap, from multiple points in the heap, and homogenized manually into one sample. All composts were collected at the stage of readiness for commercial distribution (*i.e.* a final product). The input materials and/or processing conditions (Table 2.1) were different for each compost, to better obtain a broad range in properties. Composts were transported to the laboratory and refrigerated (4°C) for a maximum of one week before pretreatment and analysis.

Prior to chemical analysis (Table 2.2), composts were air dried at 40°C and ground (<1mm) for homogeneity of each sample. For pH, total soluble N and PO_4-P, samples were equilibrated for 1h in a 1:10 ratio of dry material to 0.01M $CaCl_2$, and filtered to 0.45 µm. Nitrogen and P were measured on a San^{++} 6 channel segmented flow analyser (SFA) (Skalar, The Netherlands). Calcium carbonate content on each compost was determined using the

Table 2.1: Composts included in this study, source material ingredients of the compost, and processing procedure.

Compost code	Composting facilities	Source material	Peak T[a] °C	Time at peak T d	Additional
MW-1	Van Iersel Compost, Biezenmortel, NL	55% shredded wood, 25% grass litter, 20% leaf litter	80	52	Sieved to 10 mm. 10 d in windrow (turned 5x), 42 d on tablebed (turned 4x)
MW-2	Van Iersel Compost, Biezenmortel, NL	55% shredded wood, 25% grass litter, 20% leaf litter	80	52	Sieved to 15 mm. 10 d in windrow (turned 5x), 42 d on tablebed (turned 4x)
FL	Van Iersel Compost, Biezenmortel, NL	Forest (leaf) litter	70	28	Sieved to 30 mm. Windrow, turned 6x
SG	Van Iersel Compost, Biezenmortel, NL	75% soil sieved from woody municipal waste + 25% grass litter	75	14	Sieved to 20 mm. Windrow, turned 7x
MW-3	Van Iersel Compost, Biezenmortel, NL	65% shredded wood, 17.5% grass litter, 17.5% leaf litter	80	52	Sieved to 15 mm. 10 d in windrow (turned 5x), 42 d on tablebed (turned 4x)
FG-1	Van Iersel Compost, Biezenmortel, NL	70% shredded wood, + 30% clay, grass litter & compost as inoculum	70	84	Windrow, turned 15x
FG-2	Orgaworld, Lelystad, NL	Grass clippings + fungal-inoculated mulched wood	>65	21	Turned 7-10x. Inoculated with *Trichoderma spp.*
MW-IW	Orgaworld, Zeeasterweg, NL	80% municipal organic waste + 20% industrial organic waste	50-65	1	Turned 2x
MW-4	Orgaworld, Lelystad, NL	Municipal organic waste[b]	50-65	1	Turned 2x
MW-5	Orgaworld, Drachten, NL	Municipal organic waste	65-70	10	Turned 3x
MW-6	Comgoed, Dirksland, NL	Municipal organic waste	55-60; >50	3; 14	Turned 4-5x. 3 d at peak T then 14 d slightly below peak T.

[a]Internal maximum temperature of compost heap
[b]Municipal organic waste is a mixture of vegetable, fruit and garden waste collected from curb-side municipal programs

Scheibler method (ISO10693) and total elemental C and N measurements were performed on a LECO Truspec CHN analyser (LECO Corporation, St. Joseph, MI, USA) (Table 2.2). Organic matter (OM) content was determined by loss on ignition from 105 to 550°C (Table 2.2).

Extraction of DOC was performed on dry-weight-equivalent fresh compost material in ultra-pure water (1:10). Due to heterogeneity of the fresh compost materials, four suspensions of 10 g compost were equilibrated, filtered, and subsequently pooled. Equilibration for 1 h via end-to-end shaking preceded 0.45 μm filtration of the solution. A subsample of the pooled solution was taken for measuring total DOC concentration on a TOC-5050A analyser (Shimadzu Corporation, Kyoto, Japan). The remainder of the total DOC sample was fractionated.

Table 2.2: Chemical properties of 11 composts analysed. Data presented is based on dry matter. Extractions (pH, total soluble N and P) were performed in 1:10 solutions of dry, ground compost in 0.01M $CaCl_2$, except dissolved organic carbon (DOC) concentration, which was extracted from 1:10 solutions of fresh compost (dry-matter equivalent) in ultra-pure water. OM= organic matter content.

Compost	pH	N – total soluble	P-PO$_4$	CaCO$_3$	DOC	Total C	Total N	OM
		mg kg^{-1}	mg kg^{-1}	%	mg kg^{-1}	g kg^{-1}	g kg^{-1}	%
MW-1	6.74	226	15.1	0.54	663	101.0	8.1	18.6
MW-2	7.13	218	13.4	0.69	997	102.6	7.7	20.6
FL	6.91	59	89.9	0.41	903	182.2	8.4	42.5
SG	7.30	88	10.9	0.34	904	74.2	4.9	18.5
MW-3	6.92	101	31.8	0.65	1085	151.2	10.7	26.7
FG-1	7.39	79	2.1	0.62	428	83.4	5.1	17.9
FG-2	7.14	248	11.9	0.96	527	97.0	7.3	25.3
MW-IW	7.07	381	48.4	2.33	4674	258.1	19.1	37.0
MW-4	7.16	1102	238	3.95	7307	194.0	15.3	45.3
MW-5	7.18	649	52.6	2.86	6753	165.4	14.6	35.9
MW-6	7.0	N/A	N/A	2.90	505	183.3	11.7	31.6

2.2.2 Fractionation of DOC

After extraction, the total DOC sample was fractionated using the operational definitions set by the International Humic Substances Society (IHSS) and described by Aiken et al. (1985). Hydrophobic humic acid (HA), fulvic acid (FA) and neutral (HoN) compounds were physically separated from hydrophilic (Hi) compounds through pH changes to the solution and equilibration with a resin. In this experiment, the batch fractionation procedure (Van Zomeren and Comans, 2007) was used; first, the total DOC sample was acidified to pH 1 with 6 M HCl and allowed to stand overnight. This precipitated humic acids out of solution. The acidified solution was then centrifuged (15 min, 3000 g), separating the HA from the supernatant containing FA+HoN+Hi. Next, the HA pellet was resuspended in 0.1 M KOH (pH 12) and the HA DOC concentration was determined on a TOC-5050A analyser (Shimadzu Corporation, Kyoto, Japan). The supernatant (FA+HoN+Hi) was then added in a 1:10 resin to solution ratio to the resin DAX-8 (Sigma-Aldrich). DAX-8 is a non-ionic, macroporous resin. The resin was prepared for use by extracting organic impurities in a 24 h Soxhlet extraction with acetonitrile and then methanol, as described by Van Zomeren and Comans (2007), and rinsed with ultra-pure water. The resin was then equilibrated with FA+HoN+Hi for 1 h using horizontal shaking. This pulled the hydrophobic FA and HoN compounds out of the solution by binding them to the surface of the resin. The Hi compounds do not bind to the resin but remain in the solution, which was separated from the resin after equilibration. The DOC concentration of Hi was measured on the San^{++} 6 channel SFA (Skalar, The Netherlands). Finally, the resin with the adsorbed FA+HoN pools was equilibrated in several wash steps with 0.1 M KOH. Each wash step re-dissolved part of the FA pool from the resin surface and they were repeated until the concentration of the wash solution was not higher than a blank sample (4 to 6 wash steps per sample, depending on the FA concentration in the starting total DOC solution). The DOC concentration of FA was also measured on the SFA. The DOC concentration of the HoN pool is calculated by determining the

proportion of the DOC which was not re-dissolved from the resin (as it remains bound to the resin even under alkaline conditions). The percent DOC return from the beginning to the end of the fractionation procedure was 85 to 105%.

2.3 Results and Discussion

2.3.1 Compost chemical properties

The compost samples selected for this study were found to encompass a broad range of chemical properties (Table 2.2). pH was the least variable parameter measured, with all composts having near-neutral pH. Total soluble N was positively correlated with Total N (r=0.68, P=0.03), although compost MW4 had relatively high soluble N to total N (Table 2.2). This sample also had a very high DOC concentration (7307 mg kg^{-1}) relative to its total C concentration (194 g kg^{-1}). Organic matter (OM) had a broad range between samples (18.5 to 45%) but did not seem to correspond with any particular input materials or processing procedure (Table 2.1).

The most nutrient-rich composts in terms of both total and total soluble N, PO$_4$, and total C were MWIW, MW4 and MW5. These were also the composts with the shortest periods (1 to 10 d) spent at peak internal composting temperature (Table 2.1) and all were sourced from municipal organic waste household collection programs. These three composts also had the highest DOC concentrations, which were 6 to 10 times higher than the mean of the other eight composts. While they did have relatively high % OM, there were composts with comparably high % OM and much lower DOC (See FL and MW6, Table 2.2). These three high-DOC composts (MWIW, MW4 and MW5) had reached maturation according to the manufacturer's internal standards, but did not meet the DOC level of 4000 mg kg^{-1} recommended by Zmora-Nahum et al. (2005) as the upper limit of DOC concentration for a compost to be considered mature. This high DOC concentration is most likely because of C preservation from the compost spending less time at peak composting temperature (Table 2.1). During

the composting process, the most biodegradable forms of C are mineralized (Bernal et al., 1998; Paré et al., 1998) along with other nutrients (*i.e.* N and P), fuelling microbial activity. While all other composts spent 14 to 42 d at maximum internal temperature, MWIW, MW4, MW5 and MW6 had shorter opportunity for C turnover (1 to 10 d). Paré et al. (1998) describe a compost with maximum loss of C as CO_2 during days 4 to 11 of a 59 d composting period. Decreases in DOC concentration during the thermophilic composting phase are well reported in the literature (Benito et al., 2003; Paré et al., 1998; Raj and Antil, 2011). Therefore, it is likely that the conservation of total DOC in these three composts is a result of reduced cumulative microbial activity which, as described below, subsequently alters the composition of the remaining DOC as well.

2.3.2 Compost DOC quality

Variability was found between samples not only in total DOC concentrations, but also in the composition of the DOC quality profiles (Figure 2.1). When considering HA, FA, HoN and Hi pool sizes relative to one another, FA nearly consistently had the highest proportion of total DOC. Humic acids had low variability relative to other pools (10 to 27%). Of the hydrophobic compounds (HA+FA+HoN), HoN tended to have the lowest concentrations across the compost types (except MW6). These HoN compounds are highly aliphatic in compost (Chefetz et al., 1998), including quinones (Amery et al., 2009), which are known to be released by microbial turnover (Paul, 2006). The Hi pool, typically comprised of low-molecular weight sugars and amino acids (Amery et al., 2009), had both the highest absolute concentrations and proportion of total DOC under conditions of <10 d in thermophilic phase.

Three composts had both the highest absolute DOC concentrations and the highest proportions of hydrophilic (Hi) DOC: MWIW, MW4 and MW5. These three composts underwent the shortest durations at peak composting temperature (Table 2.1) and all three were sourced from municipal household
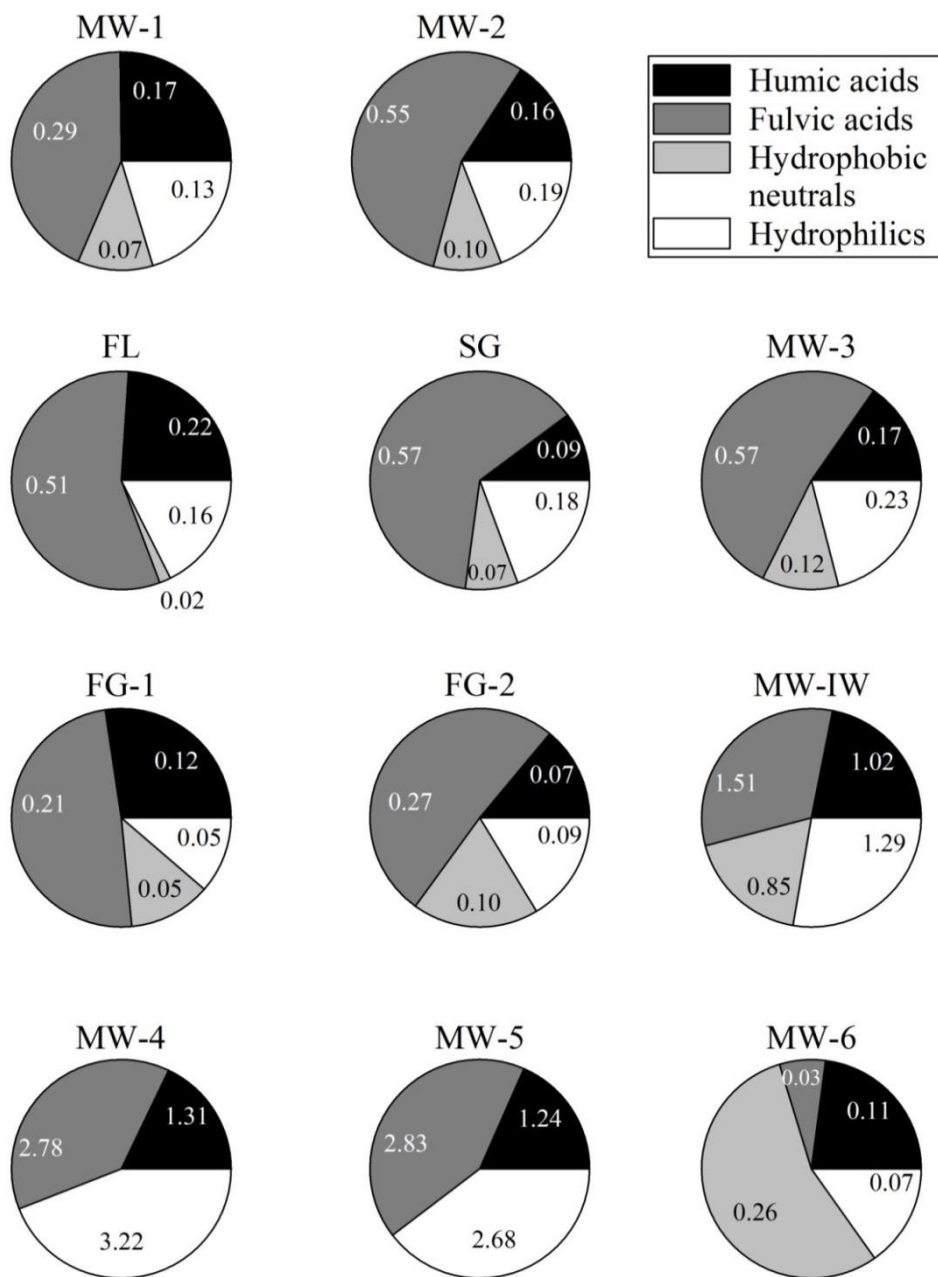
23

Figure 2.1: Relative composition of dissolved organic carbon (DOC) extracted from 11 compost types. Each pool is presented as a proportion of total DOC. Numbers in pie charts are the absolute concentrations (g DOC kg-1 compost) of the corresponding DOC pools.

organic waste, *i.e.* low prevalence of woody materials. All other composts underwent a longer composting period (>10 d), higher composting temperature (≥70 °C (except MW6)), and had lower proportions of Hi, which indicates turnover of these compounds during composting. The Hi pool may be preferentially used as a substrate by microorganisms throughout the composting process (Said-Pullicino et al., 2007), subsequently contributing a lower proportion to the composition of the remaining DOC. Furthermore, when added to soil, compost DOC high in Hi proportion has been found to promote short-term (<2d) microbial activity rates more so than a compost with a higher proportion of hydrophobic compounds (Straathof et al., 2014). Measurement of Hi DOC may therefore be a more informative indicator of the effect compost addition may have on soil microbial activity rates than measurements of OM or even total DOC. Alternatively, HA DOC and other hydrophobic pools (FA+HoN) dominated composts that underwent ≥28 d at peak composting temperature (Figure 2.1; Table 2.1). These hydrophobics are aromatic and more resistant to turnover than hydrophilic compounds and thus, as hydrophilics are depleted, they constitute a larger proportion of total DOC. The aromatic and recalcitrant nature of the hydrophobics may also contribute to their ability to bind pollutants when applied to soil (Semple, 2001). Particularly the HA in this hydrophobic fraction may contribute to the formation of soil aggregates and, hence, to the remediation of soil structure through HA's ability to bind strongly to reactive mineral surfaces (Weng et al., 2006).

Composts with the most similar DOC profiles were those with both similar inputs and similar lengths of time at peak temperature. Composts MW2 and MW3 both had a DOC pool breakdown of approximately 55% FA, 20% Hi, 15% HA and 10% HoN. As both composts were processed similarly in terms of temperature, time, and sieving (Table 2.1), this suggests these treatments are reflected in the DOC composition. The two samples varied slightly in input materials (Table 2.1), but by <10%. Compost MW1 also underwent the same processing treatment and had a similar quality composition as MW2 and MW3. However, it was sieved to a smaller size (10 mm vs. 15 mm), which probably resulted in a larger proportion of its material dominated by highly decomposed

materials. This is reflected in its more dominant proportion of HA (Figure 2.1), a highly transformed DOC pool. These three composts' processing similarities are reflected in their shared DOC quality profiles, validating the consistency of this fractionation method as an indicator of compost input materials and processing treatments.
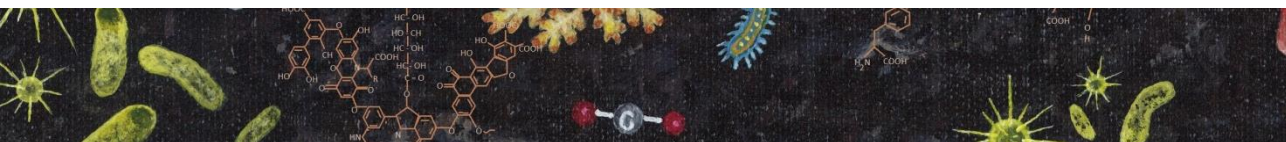
2.4 Conclusions

The DOC fractionation method showed consistency between composts with similar input materials and processing conditions. Composts with the shortest thermophilic periods ($\leq$10 d) and lower composting temperatures ($\leq$70 °C) had the highest DOC concentrations and the highest proportion of hydrophilic compounds. This suggests preferential turnover of hydrophilics during composting, while hydrophobics remain relatively conserved. Based on the variability of these pools between composts, and the consistent influence of processing conditions on DOC composition, these results suggest that DOC quality via fractionation is a valuable parameter to consider when assessing the suitability of a compost for application to soil.

Acknowledgements

Chapter 3

Dynamics of soil dissolved organic carbon pools reveal both hydrophobic and hydrophilic compounds sustain microbial respiration

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Abstract

The quality of dissolved organic carbon (DOC) released from soil organic amendments may influence soil microbial activity and the quality of the soil's DOC pools. Measurements of total DOC are often considered in relation to microbial activity levels but here we propose that quantification of DOC fractions is a more informative alternative. In a laboratory incubation, soil received DOC that was extracted from three organic matter sources: fresh compost, mature compost, and a mixture of the two. Soil microbial respiration (CO_2 emission), and concentrations of hydrophobic (humic acids (HA), fulvic acids (FA) and neutrals (HoN)) and hydrophilic (Hi) DOC fractions were measured throughout the 35 d incubation. The A_{254} specific UV absorption of total and HA DOC were measured at the start and end of the incubation as an indicator of aromaticity. Microbial respiration rates were highest in soils amended with fresh compost DOC, which had a higher proportion of Hi compounds. Concentration of Hi was significantly and positively correlated with soil respiration, explaining 24% more variation than total DOC. Humic acid concentrations significantly decreased over 35 d, including a 33% reduction in HA from an unamended control soil. Compost treated soils' HA pools increased in aromaticity, suggesting preferential mineralization of the least aromatic HA molecules. A decrease in $SUVA_{254}$ values in other HA pools may be the result of HA degradation in the absence of low-aromatic HA. Our observation of depletion of hydrophobic compounds from the HA fraction provides evidence that humic substances can be a relatively reactive pool, which can provide, together with hydrophilic compounds, a readily available C source to the microbial community.

3.1 Introduction

Dissolved organic carbon (DOC) plays a key role in sustaining soil microbial activity due to its solubility and lability (Kalbitz et al., 2000; Chantigny, 2003) and thus may be a pertinent indicator of soil quality. Soil DOC is both a substrate for microbial activity and a byproduct of the subsequent microbial metabolic processes (Marschner and Kalbitz, 2003; Van Hees et al., 2005; Bolan et al., 2011). The quality of DOC influences the variability of several soil factors, such as microbial community composition (De Graaff et al., 2010), nutrient availability and leaching (Gerard H. Ros et al., 2010), and the rate of soil C turnover (Boddy et al., 2007; Jandl and Sollins, 1997). As a substrate, DOC may originate from plant residues, root exudates, decomposing litter, and, in agricultural soils, from applied organic amendments (Chantigny, 2003). The range in potential sources results in a biochemically heterogeneous DOC solution, which exacerbates uncertainty in predicting how a particular amendment impacts soil microbial activity and C turnover rates.

Several studies have measured the influence of various qualities of C additions on soil C turnover (Guggenberger et al., 1994; De Nobili et al., 2001; Kalbitz et al., 2003a; Boddy et al., 2007). Those describing rapid evolution of CO_2 from soil (<1h after C addition) attributed this to the turnover of low molecular weight (LMW) C compounds (De Nobili et al., 2001; Boddy et al., 2007), such as short-chain polysaccharides and amino acids. Van Hees et al. (2005) compared forest soil respiration rates between LMW and high molecular weight DOC compounds and concluded that the latter drives only 14% of CO_2 emissions. This suggests that DOC containing high proportions of LMW compounds would stimulate microbial activity relatively more than DOC with a smaller proportion of LMW C. Qualifying DOC into proportions of labile, LMW C vs more recalcitrant, aromatic C compounds may therefore provide a more powerful indication of C turnover rates and microbial activity potential than measurements of DOC concentration alone.

One method of qualifying DOC is to partition the total pool into operationally defined fractions based on their solubility and relative

hydrophobicity (van Zomeren and Comans, 2007). This is a rapid-batch procedure based on the International Humic Substances Society's standard method for isolation of humic substances from organic matter in soils and natural waters (Swift, 1996; Thurman and Malcolm, 1981). By equilibrating a DOC solution with an absorbent hydrophobic resin (DAX-8), hydrophobic compounds are pulled out of solution and physically separated from hydrophilic compounds. The isolated pools resulting from this fractionation are distinguishable as aromatic, hydrophobic fraction (humic (HA) and fulvic acids (FA), hydrophobic neutrals (HoN)) and a hydrophilic fraction (Hi). Despite the relative ease of the method and its pervasiveness in environmental chemistry applications, only a few studies have looked at the dynamics of DOC quality over time using these fractions as a proxy. Ros et al. (2010) quantified fractions in three different soils after 35 d of incubation at different temperatures. They observed a decrease in the absolute concentration of a sandy soil's HA fraction at 10 and 20°C, which resulted in this pool contributing a smaller proportion of the total DOC. At the same time, this study observed a stable Hi concentration at the end of incubation, which seems counterintuitive in contrast with other studies describing a high degradability of LMW compounds (Boddy et al., 2007; Bolan et al., 2011; De Nobili et al., 2001; Lannan et al., 2012a) which would be present in the Hi pool. In a study of how known compounds in homogenous, prepared solutions are partitioned between hydrophobic and hydrophilic pools, Amery et al. (2009) observed the majority of aliphatic C and 97% of a fractionated glucose solution was measured in the hydrophilic pool. This pool contains the largest proportion of LMW compounds (<500 Da) (Thurman et al., 1982). Conversely, hydrophobic HA molecules are considered a source of C quite recalcitrant to microbial degradation and thus one of the most stable soil organic compounds (Kalbitz et al., 2003b). This HA pool also contains larger, more aromatic molecules, in the range of 500-10000 Da (Thurman et al., 1982). Fulvic acids tend to be less aromatic and lower in molecular weight (500-2000 Da (Thurman et al., 1982)) than humic substances. Guggenberger et al. (1994) have proposed that hydrophobic compounds may be in an intermediary state of decomposition between DOC source material and Hi

fractions, but the authors do not identify a mechanism behind this flux or explore the implications for soil quality.

The biological relevance of each of these DOC fractions in soil remains unclear, especially for agricultural soils (Haynes, 2005)**,** as research has yet to link fractionation in real-time with measurements of microbial activity. Although Chantigny (2003) describes the effect of organic amendments on soil DOC concentration, their impact on soil DOC quality over time may be highly variable depending on the amendment's composition and their effect on C turnover rates. The aim of this study was to investigate the dynamics of soil DOC quality using fractionation and specific UV absorbance, in parallel with measurements of soil microbial respiration, in a soil amended with compost-derived DOC. Due to increased bioavailability, we hypothesized that soils receiving DOC with a higher proportion of hydrophilic compounds would have respiration rates stimulated more so than those receiving more aromatic hydrophobic compounds. We expected DOC extracted from soil to reflect the characteristics of the added DOC with respect to prevalence of certain fractions and aromaticity measurements. These quality measurements and soil microbial respiration rates were carried out simultaneously, in order to elucidate the influence of hydrophobic vs hydrophilic DOC fractions on soil C turnover rates and better understand the contribution of DOC to soil activity levels.

3.2 Materials and Methods

3.2.1 Amendment Characterization

Two different composts were used as a source of DOC to add to soil. To obtain DOC varying in quality, the composts were selected based on their different source materials and maturation treatments. They were collected in October 2012 from two different commercial compost facilities in the Netherlands. The first compost, hereafter referred to as *fresh compost,* was collected from the Orgaworld Biocel fermentation facility in Lelystad, The

Netherlands. The input material of the fresh compost consisted of municipally collected household fruit, vegetable and garden residues. These materials were composted for 1 d above 65°C (internal temperature of the compost heap) and 6 d above 45°C, with two instances of the material being turned. The second compost, hereafter referred to as *mature compost*, was collected from the Van Iersel compost facility in Biezenmortel, The Netherlands. It consisted, by mass, of 75% soil sieved from woody compost inputs and 25% municipal grass cuttings. The composting period for the mature compost lasted for 2 weeks at an internal temperature of 60-70°C, and the compost heap was turned twice a week. After this period, the mature compost was cured at ambient outdoor temperatures for 6-8 weeks. Both composts were collected at the stage of being a final, commercial product and their chemical characteristics are described in Table 3.1.

The compost DOC was obtained via a 1:2 compost to ultra-pure water (UPW) extraction: after 1 h equilibration by horizontal shaking, 20 min centrifuging at 3000 g and 10 min ultra-speed centrifuging at 11700 g, the supernatant was vacuum-filtered through a 0.2 μm cellulose nitrate membrane, which had been pre-rinsed with 100 ml UPW to avoid C release from the membrane itself (Khan and Subramania-Pillai, 2006). The filter size was selected to exclude any microbes from the solution (Norris and Ribbons, 1969). This extraction was performed on both fresh and mature compost, as well as a 50/50 mass mixture of the two (hereafter referred to as *mix compost*) so that three DOC amendment solutions ranging in quality were prepared. The solutions were then freeze-dried for stable storage and to allow for re-dissolution at equal DOC concentrations.

3.2.2 Soil Collection and Characterization

The soil was collected in October 2012 from the Wageningen UR Applied Plant Research site in Vredepeel, Netherlands, from the 0-20 cm layer of an agricultural field. It is classified as non-calcareous loamy sand soil. It was suitable to assess DOC dynamics, since a high level in carbonates and clay

minerals strongly affects DOC concentration and adsorption (Chantigny, 2003; Kalbitz et al., 2000), and contribute to abiotic soil CO_2 release; this soil was less than 0.01% $CaCO_3$ (Table 3.1) and only 1% clay. Roots and crop residues were manually removed and the soil was air dried at 22±1°C for 8 hours until 30% water holding capacity was reached.

Table 3.1: Initial characteristics of the soil and composts used in the study. Numbers in brackets are SE for dry matter (n=2; except organic matter (OM), where n=3). Humic acid (HA), fulvic acid (FA), hydrophobic neutral (HoN) and hydrophilic (Hi) dissolved organic carbon (DOC) are presented as a percent of total DOC.

Source material	pH	OM	CaCO₃	Total DOC	HA	FA	HoN	Hi
		%	%	mg kg^{-1}	%	%	%	%
Soil	6.68	3.6 (0.1)	0.01	38.2	6.3	18.6	41.4	33.7
Fresh Compost	8.12	38.7 (1.2)	3.95	1624 (270.1)	23.5 (0)	31.2 (3.1)	10.1 (4.6)	35.3 (1.4)
Mature Compost	8.35	17.5 (0.5)	0.34	559 (30.1)	16.1 (3.8)	37.3 (5.0)	23.8 (0.6)	22.9 (0.6)

3.2.3 Soil Incubation and Respiration Measurements

An incubation experiment was established in November 2012 to measure DOC turnover in soil to which the extracted compost DOC had been added. The incubation was conducted in 575 ml glass bottles with 150 g dry-weight-equivalent soil at 20°C in the dark for 35 d. After soil was placed in the bottles but before DOC addition, a pre-incubation period of 5 d occurred. This was to avoid measurement of CO_2 that may have been produced as a result of physical or chemical changes caused during sampling and transfer of the soils to the bottles. The compost DOC was resuspended in UPW and solutions were added at rates of 70 or 30 μg C g^{-1} dry soil (high (H) and low (L) rates,

respectively), which adjusted the final soil moisture content to 60% water holding capacity. The control treatment received only UPW. These rates correspond with a compost application of 50 or 20 Mg ha^{-1} compost. Thus, three DOC source types were added at two rates of DOC addition, making six treatments. Five replicates per treatment and for the control were established (3 DOC sources x 2 DOC rates x 5 replicates + 5 controls = 35 jars for CO_2 measurements). Soil moisture in each bottle was adjusted to 60% water holding capacity using UPW as needed, at least once per week.

The soil's CO_2 emission was measured from each bottle's headspace by sealing the jar with a rubber-septum for a time duration which increased from 6-24 h throughout the 35 d incubation period, depending on the emission concentration at that point in the incubation. Headspace CO_2 concentrations were measured using an INNOVA 1412 Photoacoustic field gas-monitor (LumaSense Technologies, Ballerup Denmark). This procedure was repeated on days 1, 2, 4, 7, 10, 14, 21, 28 and 35. The CO_2 emission rate (μg C g^{-1} soil h^{-1}) from each replicate was calculated as:

$$CO_2 = \frac{\left(\frac{V_{tot}*[CO2hs]-V_{gm}*[CO2air]}{V_{hs}}\right)*\left(\frac{V_{hs}}{W_{soil}*22.4}*12\right)}{t}$$

where V_{tot}, V_{gm} and V_{hs} are the total, gas monitor's and bottle's headspace volumes (l), $CO2_{hs}$ and $CO2_{air}$ are the measured CO_2 concentrations (μl CO_2 l^{-1}) of the bottle's headspace and the ambient air, W_{soil} is the soil dry weight (g), t is the duration of the sealing (h), 22.4 is the molar volume of CO_2 (l mol^{-1}) at STP, and 12 the molar mass of C (g mol^{-1}). Respiration rates on days between measurements points were calculated using linear interpolation. Respiration related to the addition of DOC (*i.e.* independent of basal respiration) was calculated by subtracting the mean CO_2 emission rate of the unamended control soil from the rates of soils with added DOC measured at the same time point.

The cumulative curve of C mineralized as a percent of total C added was calculated after linear interpolation between measurements as:

$$\%C\ mineralized = \Sigma \frac{(rate(i)*t(i-i0)+t(j-i)*(rate(i)*0.5+rate(j)*0.5)}{Cadded} * 100$$

where $rate_{(i)}$ and $rate_{(j)}$ are the CO_2 emission rates on different days ($\mu g\ C\ g^{-1}$ soil h^{-1}) after subtraction of basal respiration rates, $t_{(i-i0)}$ and $t_{(j-i)}$ are the time differences between consecutive measurements (h) and C_{added} is the C rate applied at the experiment's beginning ($\mu g\ C\ g^{-1}$ of compost DOC solution).

3.2.4 DOC Extraction and Fractionation

To measure DOC dynamics over the course of the 35 d incubation, 12 additional microcosms for each treatment were established and incubated in parallel to the five CO_2-measured replicates. Three of these additional microcosms from each treatment were destructively harvested on days 2, 6, 13 and 35. On these days, DOC was extracted from each replicate in 1:2 soil to UPW solution ratio in the same method as the aforementioned compost extraction. The filtered DOC solution was then qualified using a rapid-batch fractionation procedure described by Van Zomeren and Comans (2007). This fractionation method uses precipitation and dissolution properties established by the International Humic Substances Society (IHSS) to operationally isolate four DOC pools: humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN), and hydrophilics (Hi), the concentrations of which are either measured directly or calculated by differences before and after isolation. Briefly, the starting solution of the total extracted DOC was acidified to pH 1 using 6 M HCl, which results in the precipitation of any humic acids that were in solution. The acidified solution was then centrifuged, separating the HA from the supernatant. The pellet of HA was resuspended in a base solution of 0.1 M KOH and the concentration determined on a Shimadzu total organic carbon analyzer (5000A). The supernatant was then equilibrated with the resin DAX-8 (Sigma-Aldrich) for 1 h at 220 rpm horizontal shaking at a 1:5 resin to solution ratio. This equilibration step pulled hydrophobic FA and HoN compounds out of solution

by binding them to the surface of the resin. The compounds that remained in solution are operationally defined as hydrophilic and their concentration was measured as DOC on a Segmented Flow Analyzer (SFA). Finally, the resin separated from the Hi fraction was equilibrated in 0.1 M KOH, re-dissolving the FA pool. The concentration of FA was also measured as DOC on an SFA while the concentration of the HoN pool was calculated by determining the proportion of the DOC which was not re-dissolved from the resin (as it remains bound to the resin even under alkaline conditions). We deemed 90-110% mass balances of the sum of the individual fractions, relative to the original total DOC concentration, as an acceptable return.

To test for the adsorption of added DOC to the soil solid phase throughout the incubation time, an acid and base extraction and fractionation of the total soil matrix was also performed, as described by Van Zomeren and Comans (2007).

3.2.5 Aromaticity of DOC

To assess aromaticity of the DOC solutions, specific ultraviolet absorbance (SUVA) was measured in total DOC and HA fractions extracted after 2 and 35 d of incubation. Three replicates of 1.5 ml soil-extracted DOC from each treatment were analyzed with a spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham MA, USA) and UPW was used as a blank. Three replicates of 1.5 ml isolated and redissolved HA from each treatment were also analyzed with 0.1M KOH used as a blank. Specific UV absorbance (SUVA, $1\ g^{-1}\ cm^{-1}$) at 254 nm was calculated through the following equation, as described by Weishaar et al. (2003) and adapted by Amery et al. (2008):

$$SUVA = \frac{A_{(254)}*1000}{b*[DOC]}$$

where A_{254} is absorbance at 254 nm (dimensionless), b is the length path (cm) and DOC the dissolved organic carbon concentration ($mg\ l^{-1}$) of the solution.

3.2.6 Data Analysis

Statistical analyses were performed with GenStat 15th Edition (VSN International, Hemel Hempstead UK). Homogeneity of variance was tested through a Bartlett's test and normality through a Shapiro-Wilk's test; all data met the requirements. A three-way repeated measures ANOVA, with time as the third variable, was run to test significance of compost DOC type and rate of addition on soil respiration, DOC concentrations and SUV absorption. Tukey's multiple means comparison test was employed to avoid Type I errors where $P<0.05$ was the significance level selected. Linear regression was performed on log soil respiration rates (independent variable) and total DOC and all DOC pool concentrations. Sigma Plot 11.0 (Systat Software Inc., 2008, Chicago IL) was used for curve fitting of C mineralization rates.

3.3 Results

3.3.1 Carbon mineralization dynamics

The CO_2 emission rates from incubated soils decreased throughout the incubation time for all treatments (Table 3.2), most rapidly between day 1 and day 7. No significant interaction was found between DOC compost source and DOC addition rate ($P>0.05$), therefore only main effects are presented here. The data of all treatment effects may be found in the Supplementary Materials Table S1 (http://dx.doi.org/10.1016/j.soilbio.2014.09.004). Treatment effects of DOC compost source and DOC addition rate resulted in significant differences ($P<0.05$) only until 4 d after the start of the incubation. Respiration measurements taken on 7, 14, 21 and 35 d after the start of incubation indicated no differences between treatments. This corresponded with trends in DOC concentrations, which did not differ significantly among treatments after day 6 (Table 3.2, $P>0.05$). Thus, Table 3.2 presents days 1-6 and day 35 of soil respiration values and soil DOC concentrations. The results of a three-way ANOVA to test the main effects of DOC compost source and DOC addition rate

Table 3.2: Mean respiration rates (CO_2 µg C g^{-1} h^{-1}) (n=5) and dissolved organic carbon (DOC) (n=3) concentrations from incubated soil over a 35 d period. Humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN) and hydrophilic compounds (Hi) were fractionated from DOC. Different letters *within* a column indicate significantly different least squared means after Tukey's adjustment ($P<0.05$). Main effects were significant for compost source ($P<0.001$) and addition rate ($P<0.001$) as DOC treatments added to soils.

Main Effect		Time d	CO_2 µg C g^{-1} h^{-1}	DOC mg kg^{-1}	HA mg kg^{-1}	FA mg kg^{-1}	HoN mg kg^{-1}	Hi mg kg^{-1}
Compost source of DOC	Fresh	1	0.157a					
		2	0.103b	22.7b	8.6bcd	3.7bcd	1.6cd	8.7b
		4	0.057d					
		6		10.4de	7.3cde	2.1de	0.4e	7.4bc
		35	0.002e	7.6e	1.7f	1.7e	2.4abc	1.0d
	Mix	1	0.143a					
		2	0.092bc	24.9b	13.6b	5.0b	0.5de	11.2a
		4	0.050d					
		6		15.9c	10.7bc	1.4e	2.6abc	5.7c
		35	0e	11.9cde	4.1def	1.8e	2.7abc	0.4d
	Mature	1	0.099b					
		2	0.076c	41.9a	20.7a	7.4a	3.3a	11.2a
		4	0.050d					
		6		22.4b	12.37bc	4.4bc	1.9bc	7.9b
		35	-0.003e	12.6cd	2.6ef	3.0cde	2.9ab	1.0d
Addition Rate of DOC	High	1	0.170A					
		2	0.12B	35.0A	14.7A	7.4A	2.3AB	11.1A
		4	0.068D					
		6		18.3C	10.4B	3.8B	1.6BC	7.8C
		35	-0.003F	10.9DE	2.2C	2.5BC	2.8A	1.1E
	Low	1	0.095C					
		2	0.061D	24.7B	13.9AB	3.3B	1.3C	9.6B
		4	0.036E					
		6		14.1D	9.9B	1.5C	1.7BC	6.1D
		35	0.002F	10.3E	3.4C	1.8C	2.6A	0.5E

indicated that on days 1-2 the soils receiving fresh compost DOC (which had the highest starting proportion of Hi (Table 3.1)) respired significantly more CO_2 than soils receiving mature compost DOC (Table 3.2, *P*<0.001). Soils with additions of mix compost respired slightly less than fresh compost, but more than the mature compost DOC, indicating that the mix compost did indeed represent an intermediary source of the other two composts combined.

Respiration rates from DOC treated soils were affected as expected; rates of emission from high C addition rate treatments respired approximately two times the amount of CO_2 as soils with low addition rates (Table 3.2, *P*<0.001) up to and including day 4. However, low addition rates had the highest proportion of mineralized C (Figure 3.1) for soils receiving fresh compost and mix compost. These two treatments were not significantly different from each other and mineralized, respectively, 58±13 and 46±5% of C added. Their high rate counterparts (Figure 3.1: FC-H and Mix-H) were significantly lower (*P*<0.05) in the proportion of mineralized added C, and mineralized 40 and 29% of added C, respectively. However, in absolute terms, higher C mineralization occurred in all high rate treatments, which corresponds with 10-15% higher CO_2 emission rates from mix and mature compost additions in the first 4d (Table S1: http://dx.doi.org/10.1016/j.soilbio.2014.09.004). For mature compost DOC, no different mineralization pattern between high and low rate was observed and both treatments mineralized on average 27% of C added, which was not significantly different from the mix compost at high addition rates (Figure 3.1).

3.3.2 DOC extraction and fractionation

Total DOC concentration in each treatment and the control was significantly lower on day 35 than at the start of the incubation (Table 3.2). After the first sampling (day 2), soil that had received mature compost DOC had the highest DOC concentration in soil, reaching the mean value of 49 mg kg^{-1} soil for mature compost added at the high rate, and 35 mg kg^{-1} soil for mature compost added at the low rate. Concentrations of DOC from fresh compost and
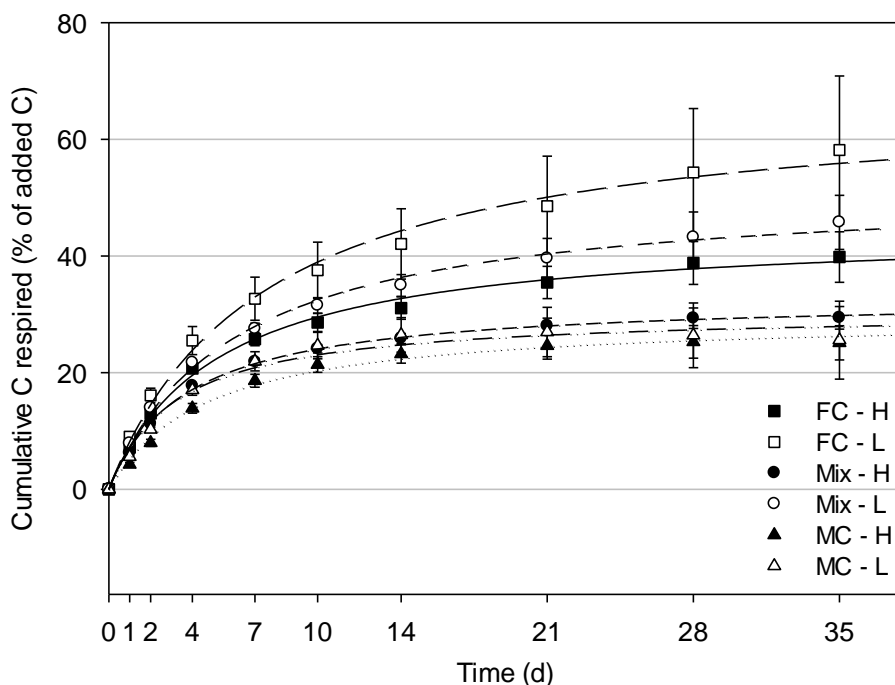
39

Figure 3.1: Cumulative rate of soil C respired as a % of C added as DOC over a 35 d incubation. Symbols represent point data calculated for fresh compost DOC high/low rate (FC-H, FC-L), mix compost DOC high/low rate (Mix-H, Mix-L), and mature compost DOC high/low rate (MC-H, MC-L) additions to soil. Different patterned lines show fitted curves: $y=(C_{max}*t)/(b+t)$, where C_{max} is maximum C mineralizable (%), t is the time in d, and b is the slope of the curve for each treatment.

mix compost-treated soils were significantly lower than mature compost on days 2 and 6, despite DOC being added at the same concentration(s) as mature compost.

Total DOC, HA, FA and Hi concentrations all had significant linear regression models with log CO_2 respiration rates ($P<0.001$, <0.001, 0.003 and <0.001, respectively) over the 35 days (Figure 3.2). Hydrophobic neutrals could not significantly explain any variation in CO_2 emission rates ($P=0.4$). The variable with the best fit to the observed data was Hi concentration ($R^2=0.81$), followed by HA, Total DOC and FA ($R^2$0.60, 0.57 and 0.29, respectively).

Over the course of the incubation, a decrease in total DOC from each soil was measured for each treatment (Table 3.2) and thus a decrease in each fraction's concentration was almost consistently exhibited. Total absolute concentrations of Hi and HA decreased the most in each treatment. Mature compost treated soils lost 18 mg DOC kg^{-1} although all HA concentrations were reduced by a factor of 3-8 from day 2-35. Fulvic acids also decreased in each soil but by less magnitude (Table 3.2). Concentrations of HoN remained relatively stable or behaved inconsistently; HoN concentrations in fresh compost and mix compost soils increased (Table 3.2). The largest loss of Hi was in mix compost soils where 10.8 mg kg^{-1} was lost while the lowest losses
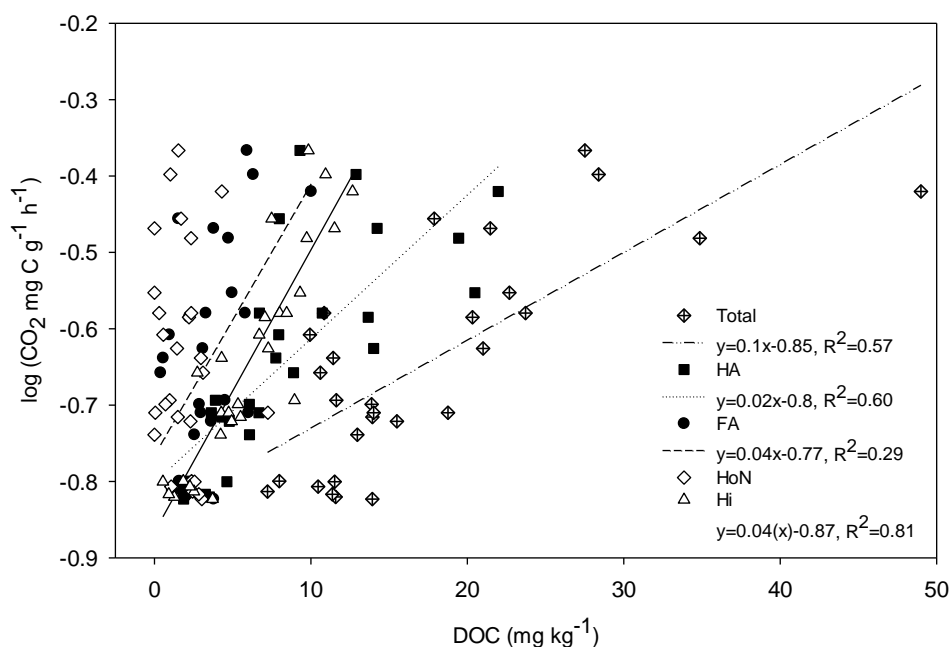

Figure 3.2: Linear regression of log CO_2 respiration rates from dissolved organic carbon (DOC) pool concentrations. Symbols represent respiration rates measured or interpolated from the same measurement time point of total DOC, humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN) and hydrophilic (Hi) pools. Regression line equations with significant (*P*<0.05) regression mean squares are presented in the legend below their corresponding variables, except for HoN (*P*>0.05).

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(7.7 mg kg^{-1}) were from fresh compost soils. While Hi decreased consistently in each soil, their depletion was almost equal to that of the HA. As a proportion of DOC however, depletion of HA was more dramatic across all treatments, including the control soil, than that of Hi.

The above trends become more apparent when considering the contribution of each pool to the total DOC (Figure 3.3). Soils with fresh compost and mix compost DOC additions had a similar pattern in quality changes over 35 d: a sharp decrease in HA proportion was observed after day 6 for high rate treatments. While low rate additions also exhibited a decrease in this pool, this was less pronounced than in high rate additions of fresh and mature compost. Furthermore, %HoN had a net increase in every treatment between day 2 and 24. To a lesser degree, %FA also increased. In high rate treatments and the control soil, %Hi was constant throughout the incubation period, although in the low rate treatments %Hi decreased over time (Figure 3.3). Remarkably, the control soil had a similar pattern as soils receiving a high rate of fresh and mature compost DOC: a significant depletion (33%) of native HA after day 6, an increase in HoN and FA proportions and a steady proportion of Hi (Figure 3.3).

3.3.3 Aromaticity of DOC

Specific UV absorption values for total extracted soil DOC did not vary significantly between DOC treatments throughout the incubation ((P>0.05) Table 3.3). Control soil DOC at day 2 was more aromatic than soils with DOC additions, but not by day 35, and this was the only treatment that exhibited a decrease in total DOC aromaticity. In contrast, HA fraction SUVA varied significantly between treatments and over time. Compost source and DOC rate of addition were significant main effects (P<0.001), but did not have a significant interaction. Soils with low rate DOC additions were more aromatic for each compost source than high rates of addition (Table 3.3). Moreover, there was a significant interaction of the compost source over time; fresh and mix compost soils increased their HA aromaticity levels over time. Conversely, the
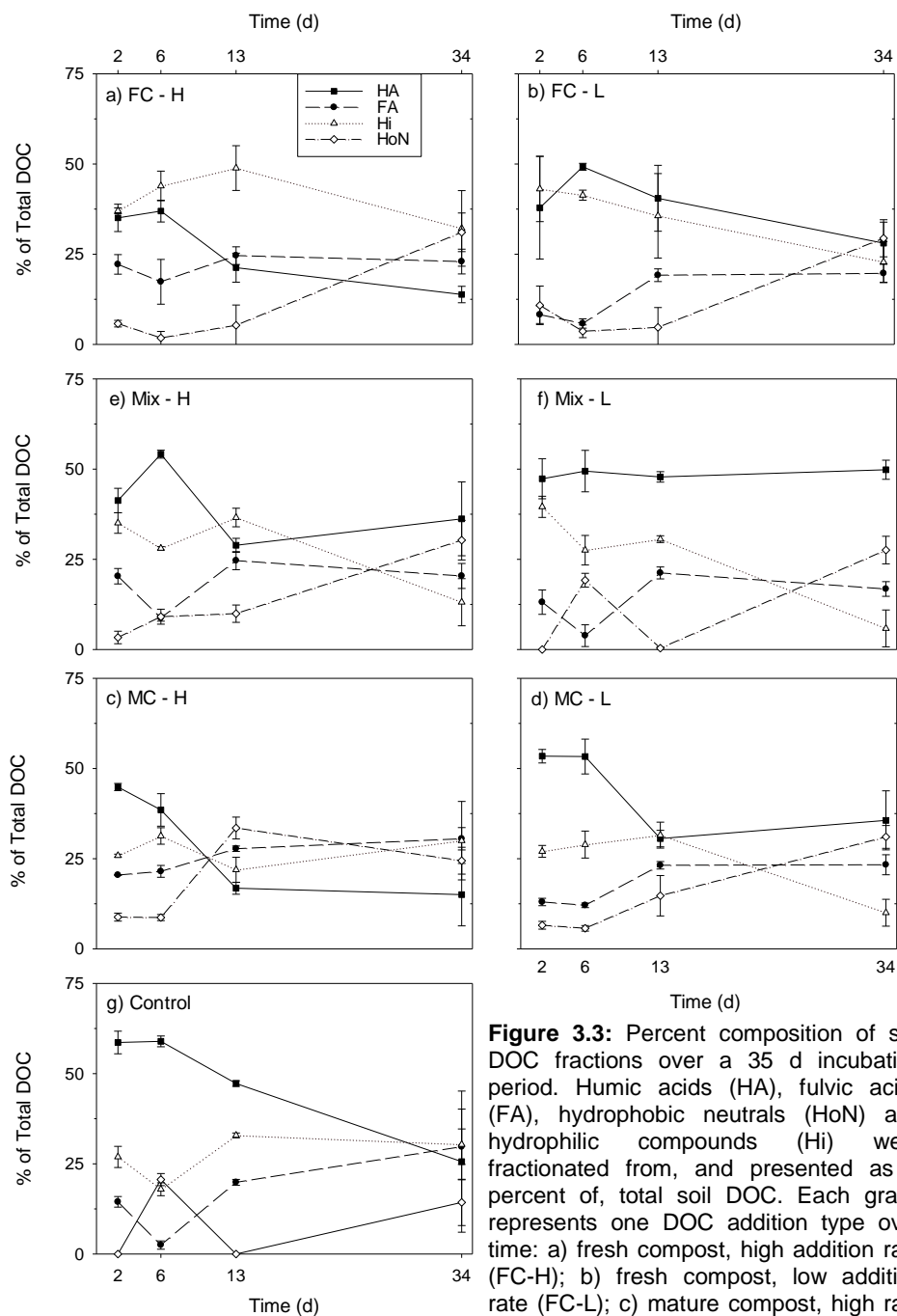
Figure 3.3: Percent composition of soil DOC fractions over a 35 d incubation period. Humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN) and hydrophilic compounds (Hi) were fractionated from, and presented as a percent of, total soil DOC. Each graph represents one DOC addition type over time: a) fresh compost, high addition rate (FC-H); b) fresh compost, low addition rate (FC-L); c) mature compost, high rate (MC-H); d) mature compost, low rate (MC-L); e) mix compost, high rate (Mix-H); f) mix compost, low rate (Mix-L); and g) Control. Bars show ±SE (n=3).

aromaticity of control soil HA fractions decreased over the same time period, while mature compost soils did not change significantly between days 2 and 35 (Table 3.3).

Table 3.3: Mean $SUVA_{254nm}$ values for dissolved organic carbon (DOC) extracted from soil receiving fresh (FC), mature (MC), or a mixture of fresh and mature (Mix) compost DOC at high (H) or low (L) addition rates, or no DOC addition (Control). Humic acid (HA) fractions and total DOC were measured at the start (day 2) and end (day 35) of the 35 d incubation. Numbers in brackets show SE (n=3).

DOC source	HA		DOC	
	Day 2 ($l\ mg^{-1}\ cm^{-1}$)	Day 34 ($l\ mg^{-1}\ cm^{-1}$)	Day 2 ($l\ mg^{-1}\ cm^{-1}$)	Day 34 ($l\ mg^{-1}\ cm^{-1}$)
FC-H	57.4 (1.9)	91.1 (10.2)	36.7 (0.2)	34.3 (3.4)
FC-L	81.9(9.1)	115.1 (11.8)	38.3 (2.2)	34.0 (2.6)
Mix-H	67.4 (0.7)	73.0 (7.9)	35.0 (2.2)	33.3 (1.7)
Mix-L	93.4 (7.1)	103.0 (8.0)	33.5 (2.7)	37.0 (2.0)
MC-H	65.6 (2.0)	66.6 (9.6)	35.0 (0.6)	33.8 (2.3)
MC-L	87.1 (4.5)	84.6 (10.4)	36.7 (1.9)	33.1 (0.7)
Control	155.3 (0.4)	106.4 (4.5)	43.0 (0.2)	34.1 (0.5)

3.4 Discussion

A pertinent hypothesis behind this experiment was that soils receiving DOC with a higher proportion of labile Hi compounds would yield higher C turnover than more hydrophobic (HA, FA, HoN) DOC additions. This was confirmed in our results, which showed higher CO_2 emission rates and thus a faster C turnover for fresh and mix compost compared to mature compost (Table 3.2). The fresh compost material had a 3-times higher extractable DOC concentration than the mature compost. Therefore, soils treated with mix compost DOC likely behaved more similarly to soils treated with fresh compost

DOC because the 50-50% mixture resulted in a DOC composition closer to fresh compost. The higher respiration rate for fresh compost DOC coincided with a faster decline in DOC and Hi concentrations, indicating that a biological process caused the decline in DOC. Specific UV absorption measurements were consistent with C mineralization dynamics and DOC fractionation, since aromaticity index SUVA (Table 3.3) for HA was lower for the treatments which respired more CO_2. Additionally, the most variation (81%) in respiration rates throughout the incubation period was explained by Hi pool concentrations as opposed to other pools or even total DOC. Both these findings support our proposition that qualification of C pools is an improvement over total DOC measurement in predicting the response of soil microbial activity levels.

Perhaps most remarkable with regards to the change in soil DOC quality measured over time was the 33% decline in the contribution of HA to total DOC in the control soil (Figure 3.3). This was unexpected due to what is known about the aromatic and recalcitrant nature of humic substances relative to the bioavailability of other DOC pools (Jandl and Sollins, 1997). To better understand the physical-chemical processes that may contribute to this, it is relevant to consider the change in SUVA characteristics of HA. All compost DOC-treated soils had a lower HA SUVA value than the control soil at day 2, indicating that the composition of treated soils' HA was more reflective of added HA, *i.e.* the dilution of native HA with less aromatic compost-derived HA reduced the aromaticity of the new conjoined pool. The depletion of humic substances in compost-DOC treated soils may be attributed to compost-derived HA being more bioavailable in nature than soil-derived HA (as evidenced by the lower HA aromaticity of soil receiving compost DOC). The bioavailability of this added HA is supported by the observation that the HA SUVA increases over time in soils amended with fresh compost DOC. This suggests that the least aromatic HA molecules are being preferentially turned over and more completely mineralized while more aromatic compounds remain in solution. The turnover of such compounds and the resulting change in their bioavailability is conceptually described by Kaiser and Kalbitz (2012). As the authors propose, and as our findings support, the turnover of freshly added

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organic matter by microbial activity results in organic matter more resistant to decomposition than its source material.

Conversely, the decrease in aromaticity of the control soil's HA fraction is indicative of a different mechanism because the depletion in size of the HA pool measured from this soil corresponds with a decrease in the pool's aromaticity. Degradation of these control soil's HA compounds, which had a higher initial SUVA than DOC treated soils, may be the result of incomplete mineralization of HA. We hypothesize that the disruption of bonds in aromatic HA molecules by partial mineralization may result in constituent molecules either remaining in the HA fraction but exhibiting less SUVA due to decreased complexity, or entering the other lower molecular weight DOC fractions. The latter explanation is supported by observations of disaggregation of HA molecules by dilution (van Zomeren and Comans, 2007), consistent with the model of Kaiser and Kalbitz (2012), and corresponds with the observation of depletion in total HA concentration. Mature compost treated soils may be in an equilibrium of these two proposed processes as the aromaticity of their HA fraction is relatively stable (unchanged SUVA) throughout the incubation.

A stabilization or increase in the concentration of FA and HoN pools was observed to correspond with a depletion of HA concentration (Table 3.2) in many treatments. Guggenberger et al. (1994) have proposed that hydrophobic compounds may be in an intermediary state of decomposition between DOC source material and Hi pools. We agree and further propose more specifically that constituents of the HA pool are resupplying the FA and/or HoN pools with DOC. Although it was not possible to directly measure the flux of materials between pools, the changes in fraction concentrations over time offer some evidence for this proposed transfer of C. Humic acids are typically represented as aromatic ring structures with a vast range of functional groups, hydrophobic, hydrogen and oxygen bonds between these rings (Sutton and Sposito, 2005). These bonds can be broken down by microbial activity, resulting in free lower molecular weight moieties either hydrophobic or hydrophilic in nature, which can then be either consumed by the soil microbial community or remain in solution as a component of another DOC subfraction, such as FA, Hi or HoN

(Guggenberger et al., 1994; Gerard H. Ros et al., 2010). The subsequent pool in which they will be found will depend on the size and nature of the compounds released from the HA: relatively long C-based molecular chains can be classified as FA or HoN, depending on the nature of their functional groups, while relatively small C-based molecules, such as sugars, proteins, carboxylic acids, and fatty acids will be found in the Hi fraction (Jandl and Sollins, 1997; Amery et al., 2009). Ros et al. (2010), although they did not account for HoN, observed the same pattern for FA in a grassland sandy soil after 35 d of incubation, suggesting that this pattern is consistent, not only for amended, but also for unamended soils. Therefore, this behaviour could be generalized for a wider range of HA and not only compost-derived ones, as further evidenced by the decline in our unamended control soil's native DOC HA.

Higher rates of DOC addition corresponded with significantly more CO_2 emission, confirming that, in absolute terms, greater C mineralization occurred in soils receiving more DOC input (Table 3.2). However, we observed that not all DOC added was mineralized (Figure 3.1) and that, in relative terms, fresh and mix compost added at low rates mineralized a higher proportion of C input compared to their corresponding high rate additions (Figure 3.1). The latter observation may be a result of a priming mechanism (De Nobili et al., 2001), although this is admittedly difficult to elucidate. We assumed that the DOC added that was not mineralized was adsorbed to the soil solid phase or organic matter. The concentration and speciation of DOC in soil solution are often controlled by adsorption and desorption processes imposed by clay minerals, Al and Fe oxides, multivalent cations such as Ca^{2+}, and soil organic matter (Chantigny, 2003; Kalbitz et al., 2000). While the soil used in this experiment had only 1% clay and 3.6% organic matter content, it is relevant to consider that soils with greater proportions of either of these properties may result in less DOC being physically available for biodegradation. In particular, had our DOC solution been added to soil with a high-binding capacity, the hydrophobic fraction of DOC may bind more preferentially to mineral and OM surfaces than hydrophilics. Upon addition of DOC, any part of the solution that subsequently adsorbs to the soil solid phase becomes more protected against

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microbial action. However, when we extracted total soil DOC from the soil solid phase at the end of the 35 d incubation using an acid and base extraction, no significant differences were found between any of the treatments' total C concentrations (data not shown). This is likely a result of the solid phase having an organic C concentration several orders of magnitude higher than DOC. This is supported by other authors, who quantified DOC as a small proportion of soil total organic carbon, usually accounting for 0.05-0.40% of soil organic C (Haynes, 2005; Lundquist et al., 1999). The soil DOC proportion in this experiment ranged between 0.09% and 0.61% of SOC. We suspect the disproportion between DOC and SOC concentrations prevents us from precisely determining DOC adsorption to the soil solid phase, which may have accounted for the remaining DOC that was not mineralized over the 35 d. As these adsorption rates will vary between soils, it may be useful to replicate aspects of this experiment among different soil types. While binding capacities between soil types may influence the soil solution's DOC in terms of hydrophobic and hydrophilic profiles, the relative lability of these pools would likely remain independent of the properties of the soil mineral phase.

3.5 Conclusions

While concentrations of DOC in soils are often associated with soil microbial activity potential, our study suggests that qualifying soil DOC through subpool fractionation, particularly of the hydrophilic pool, offers more insight into the relationship between activity and DOC. A different rate of addition and range in composition of DOC fractions resulted in different soil C mineralization rates, and also affected DOC speciation and aromaticity in soil after 35 d. These findings suggest characterization of amendments prior to application on soil will provide insight into the potential that soil holds for C turnover of the DOC from those amendments. This experiment confirmed recent findings of other experiments about the biological relevance of DOC, but more importantly, it provides evidence for a relatively new view of hydrophobic

humic acid molecules as a highly reactive pool sustaining the concentrations of other bioavailable pools. Together with hydrophilic compounds, we have shown that hydrophobic humic acid pools in DOC may provide a readily available C source to the microbial community, therefore sustaining its metabolism in soil. Further research to identify the mechanism of depletion in these hydrophobic pools would be valuable for predicting how they may behave in different soils or under different land management.

Acknowledgements
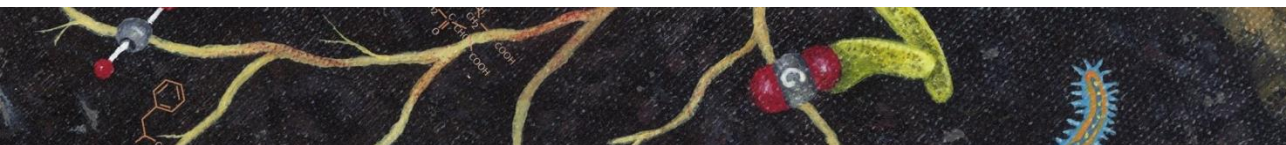
Dissolved organic carbon characteristics
of Dutch agricultural soils:
Quality indicators and their relationship
with microbial respiration

Angela L. Straathof, M. van Agtmaal, Rob N.J. Comans, Ellis Hoffland

Abstract

Dissolved organic carbon (DOC) is a heterogeneous mixture of hydrophobic and hydrophilic C compounds, the proportions of which may change the quality of DOC and its influence on soil microbial processes. Determining DOC quality indicators may therefore be a more informative tool for predicting soil microbial respiration over total DOC alone. In this study, 46 Dutch agricultural soils under different fertilization and tillage management were characterized by DOC fractionation and specific UV absorbance (aromaticity), making this the broadest survey of multiple indicators of DOC quality for these soil types. Using backward stepwise regression, we determined how these quality indicators contributed to explaining variation in soil respiration rates. Concentrations of humic acid DOC and aromaticity of hydrophilics (Hi) were significant independent variables in a linear model ($R^2 = 0.33$), which was a more informative model than total DOC alone. Aromaticity of Hi was found to increase as the proportion of Hi decreased, suggesting reduced bioavailability of this fraction and a shift of microbial substrate preference from Hi to more hydrophobic fractions. This study documents a high variability in range between DOC fractions and aromaticity, even from soils with similar absolute DOC concentrations. Our results demonstrate the potential for DOC quality to have added value as an activity indicator relative to total DOC, and to reveal mechanistic relationships between DOC as a substrate and microbial processes in agricultural soils.

4.1 Introduction

Soils are a significant global reservoir of terrestrial carbon (C). Soil C, however, especially in agricultural soils, is susceptible to loss brought on by changes in land use and management practices (Chantigny, 2003; Janzen, 2006). A particularly transient phase of C in soils is the pool of dissolved organic carbon (DOC), which, as an intermediary phase between organic and inorganic C, is an important pool for various soil processes (Kalbitz et al., 2000). These processes include nutrient mineralization (Boyer and Groffman, 1996; Magill and Aber, 2000) and mobility (Qualls et al., 1991), and soil microbial activity (Brooks et al., 1999; Jandl and Sollins, 1997). The relationships between DOC and these processes are well-established, despite DOC contributing a relatively small proportion to total agricultural soil C (0.05-0.4%) (Haynes, 2005). However, with regards to soil microbial activity, there is still a large amount of variation in rates that cannot be explained by absolute concentrations of DOC (Neff and Asner, 2001).

Variation in microbial activity rates relative to soil DOC may be due to the heterogeneous nature of the DOC solution, the constituents of which range from low molecular weight, aliphatic organic- and amino-acids, to more aromatic fulvic and humic acids (Amery et al., 2009; Stevenson, 1994). The range in the proportion of those DOC compounds which may be bioavailable has also been reported to be quite large: from 14-88% in forest soils (Kalbitz et al., 2003a; Qualls and Haines, 1992) and 17-46% in agricultural soils (Embacher et al., 2007; Marschner and Kalbitz, 2003). Characterization of these more bioavailable DOC compounds may therefore provide insight into microbial activity variation that cannot be explained by total DOC.

Agricultural soils' DOC concentrations and properties are often quantified for comparison with forest or "natural" ecosystems (Chantigny, 2003; McDowell et al., 2006; Zsolnay, 1996) but less frequently for comparison with other agricultural soils, and even less-so in connection with biological properties. In an extensive review of dissolved organic matter (DOM) in terrestrial systems, Chantigny (2003) identified the need to determine the

significance of DOM composition for soil biological functions, especially composition under different agricultural management practices. Methods of determining soil DOC composition include measurement of proportions of hydrophobic and hydrophilic compounds (Jandl and Sollins, 1997; R.G. Qualls et al., 1991; Van Zomeren and Comans, 2007) and/or measuring the aromaticity of DOC (Amery et al., 2008; Kalbitz et al., 2003a&b; Straathof et al., 2014).

Since little appears to be known about the variability among agricultural soils' DOC characteristics, this study primarily aimed to investigate the potential range that exists in different soil types, specifically of the quality indicators DOC fractions and DOC (fractions') aromaticity. Furthermore, we wanted to determine how these quality indicators may be influenced by management practices at the field-scale; thus, a second aim of this study was to determine relationships between tillage or fertilization practices and DOC quality. Having characterized DOC and investigated the influence of management practices, our third and ultimate aim in this study was to determine relationships that may exist between DOC quality and microbial activity, relative to other soil properties known to influence microbial activity. We hypothesized that those indicators of DOC quality (fractions and/or aromaticity) would be more informative of microbial activity levels from soils than total DOC alone, and that this in turn may eventually lead to useful management-practice advice for managing DOC quality that may optimize mineralization driven by microbial activity.

4.2 Materials and Methods

4.2.1 Soil sample collection and conditioning

Forty-six arable fields from across the Netherlands (Figure 4.1) were sampled in February-March 2013 (after frost recession but before crop emergence). This time of year is also when many farmers submit soil samples for nutrient status determination. Forty-two out of the 46 farmers surveyed

Figure 4.1: Locations in The Netherlands of 46 field sites where soil samples were obtained.

provided information about the previous season's tillage regime and fertilization (including organic matter (OM)) application. In this survey, we did not sample fields that had had tillage, OM application, or crop emergence already in 2013. A 3 kg soil sample representative of each field was made by taking about 60 soil cores along a criss-crossing W-pattern from an area of about 2 ha. Sampling depth was from the soil surface to 20 cm. These cores were all pooled and manually homogenized on-site into one bulk sample, which was kept at 4°C during transportation to the laboratory.

Soils were processed directly upon arrival to the laboratory. Each bulk sample was separated into two equal parts: one for analysis of properties by BLGG laboratories (Wageningen, The Netherlands) and one for analysis of a different set of properties by the Chemical-Biological Soil Laboratory of Wageningen UR. Samples at BLGG were oven-dried (40°C), ground, and used to determine OM, total C, total N and $CaCO_3$ by near-infrared analysis (Malley et al., 1999). The other half of each sample was kept at field moist conditions and sieved to 4 mm to homogenize samples and remove large, non-soil particles. Soil moisture was determined from the mass differential measured by drying a subsample at 105°C for 24 h, and soil water-holding capacity (WHC) was determined by bringing a field-moist subsample to saturation. The remainder of the soil sample was then separated into three equal-sized samples in plastic bags. These three samples per field were then pre-incubated for 3 days at 9°C (mean ambient air temperature in The Netherlands in February-March) and 60% WHC, which was achieved by adding ultra-pure water (UPW) to samples below 60% WHC, or air-drying samples above 60% WHC. Sample equilibration during this period was for two intended purposes: 1) to minimize the effects of variable temperature and moisture conditions occurring at different fields over the four weeks sampling period, and 2) to allow soils time to approach chemical equilibrium, which may have been disrupted during sampling and handling. After separation and equilibration, the measurement of each soil property was performed in triplicate and the average of the three samples was used as the input datum.

4.2.2 DOC fractionation and aromaticity measurements

Extractions of DOC were made using a 1:2 soil to UPW solution ratio, which was equilibrated for 1 h via horizontal shaking at 220 rpm. The suspension was then centrifuged for 20 min at 3000 g and ultra-centrifuged for 10 min at 11700 g, after which the supernatant was syringe-filtered through a 0.45 μm cellulose nitrate membrane that was pre-rinsed with UPW to prevent

contamination of DOC originating from the membrane. pH was measured on this filtered sample. The filtered DOC solution was then fractionated according to the rapid-batch procedure developed by Van Zomeren and Comans (2007). This fractionation method is based on operational definitions determined by the International Humic Substances Society (IHSS) and adapted from methods described by Aiken et al. (1985). The result is the separation of four fractions of DOC: humic acids (HA), fulvic acids (FA), hydrophobic neutrals (HoN), and hydrophilic compounds (Hi), the concentrations of which are either measured directly (HA, FA, Hi) or calculated by differences (HoN) before and after isolation.

The starting total DOC extraction was acidified to pH 1 with 6 M HCl, which precipitates HA out of the starting solution. This acidified solution was then centrifuged (10 min at 3000 g), separating the HA precipitate from the supernatant (containing FA+HoN+Hi), which was poured-off. The supernatant DOC concentration was measured on a San++ 6 channel Segmented Flow Analyser (SFA) (Skalar, The Netherlands). The HA pellet was resuspended in 0.1 M KOH and DOC content in this suspension was measured on a Shimadzu total organic carbon 5050A analyser (Shimadzu Corporation, Kyoto, Japan). The supernatant from the centrifugation step was then equilibrated with the non-ionic, macroporous resin DAX-8 (Sigma-Aldrich) for 1 h at 220 rpm horizontal shaking at a 1:5 resin to solution ratio. This equilibration with DAX-8 pulled hydrophobic FA and HoN compounds out of solution by binding them to the surface of the resin. The compounds that remained in solution after separation from the resin were Hi and their DOC concentration was measured on the SFA. Finally, the resin adsorbing the FA and HoN fractions was equilibrated in 0.1 M KOH for 1 h at 220 rpm horizontal shaking at a 1:5 resin to solution ratio, re-dissolving the FA pool. This step was repeated until the DOC concentration of solution after equilibration was not different from the blank-resin (i.e. all FA was desorbed). The DOC concentration of desorbed FA was also measured on the SFA. The concentration of the HoN pool was not measured directly (as it remains bound to the resin even under alkaline conditions) but calculated as the difference between the concentration of the FA+HoN+Hi sample and the FA

and Hi concentrations. All DOC-fractionated soils in this study were between 90-120% mass balances, relative to the original total DOC concentration.

Aromaticity of the total DOC, HA, FA and Hi fractions was determined by specific ultraviolet absorbance (SUVA) of the respective solutions at 254 nm (Weishaar et al., 2003). Each sample had 1.5 ml solution analyzed on a spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham MA, USA) with a 1 cm path length. Blank samples for total DOC, HA, FA and Hi were UPW, 0.1 M KOH, 0.1 M KOH after equilibration with blank resin, and 0.1 M HCl after equilibration with blank resin, respectively. Specific UV absorbance (SUVA, 1 g^{-1} cm^{-1}) per sample was calculated as UV absorbance normalized by sample DOC concentration per cm path length (Amery et al., 2008). As the HoN fraction remains adsorbed to the DAX-8 resin, no aromaticity data is obtainable for this fraction.

4.2.3 Microbial respiration and biomass determination

Basal respiration values for each soil were determined from CO_2 emission rates of incubated soils. The dry weight-equivalent (DWE) of 100 g fresh soil was weighed into a 335 ml glass bottle and incubated in the dark at 20°C, in triplicate. Soil moisture in each bottle was maintained at starting levels (60% WHC) by adding UPW as needed, at least once per week. After the start of incubation (T0), emissions were measured nine days after T0 (T1), three weeks after T1 (T2), and 11 weeks after T2 (T3). At the start of a measurement, each glass bottle was flushed with N_2 gas, and then sealed with a rubber septum for 4 h (T1), 6 h (T2), or 12 h (T3). Accumulated CO_2 concentrations in the bottle's headspace were then measured through the septum using an INNOVA 1412 Photoacoustic field gas-monitor (LumaSense Technologies, Ballerup Denmark) at the end of the sealed period. Cumulative CO_2 emissions for the 14 weeks of incubation were calculated by linear interpolation of T1, T2 and T3, which produced a better fit than exponential interpolation. In downstream

analysis of respiration rates, cumulative CO_2 values were used after log-transformation.

Microbial biomass N was determined using the chloroform fumigation-extraction method (Brookes et al., 1985) on 20 g DWE fresh soil. Before and after 24 h fumigation with chloroform, a soil subsample was equilibrated for 1 h in a 1:10 DWE soil to solution ration in 0.5 M K_2SO_4 using horizontal shaking at 220 rpm. The equilibrated solution was centrifuged for 10 min at 3000 g and the supernatant was filtered through a 0.45 μm cellulose nitrate membrane. Total soluble N in the filtrate was measured on a San^{++} 6 channel segmented flow analyser (Skalar, The Netherlands). The microbial biomass N fraction of total soluble N released during fumigation was calculated using the conversion rate of 0.54 (Brookes et al., 1985).

4.2.4 Data analysis

Statistical analysis on data from this survey was conducted in SAS 9.3 (SAS Institute). Analysis was performed on the average of three measurements from 46 soils, except where management practice was an effect because then only 42 soils were included (due to limited information from the farmer survey responses). One-way analysis of variance was performed using the SAS *proc mix* statement with tillage and fertilization practice as class variables. Limitations in degrees of freedom did not permit exploration of management-practice interactions. Management-practice effects on DOC and DOC fractions were tested using Tukey's adjusted least squared means comparisons, where the significance limit was set to $P<0.05$. Correlations between DOC parameters were also tested using Pearson's product moment correlation coefficient (r). Linear regression analysis was performed using the *proc reg* statement to investigate relationships between DOC quality indicators and other soil parameters. Non-normally distributed (according to the Shapiro-Wilk test statistic) response variable data were log-transformed before regression, which also resulted in their meeting requirements of homogeneity of variance

($P<0.001$). Residuals of input variables were independently distributed and covariance was tested using Spearman rank correlation. No significant linear regression models were determined from *proc reg* between SUVA data and DOC fractions, but visualization of the Hi SUVA data suggested a nonlinear relationship. Therefore, curve-fitting and subsequent graphing of the SUVA data was performed in Sigmaplot 11.0 (Systat Software Inc.) using inverse first-order nonlinear regression.

For regression with microbial respiration as the response variable, analysis was performed using *proc reg* backward selection stepwise statements where slstay=0.05 (upper cut-off level of significance for keeping model parameters) and the C_p statistic (Mallows, 1973) was within two units of the number of equation variables (including the y-intercept). Six models were generated from different sets of input variables: 1) general soil properties that are known from literature to influence soil microbial respiration (OM, total DOC, pH, microbial biomass, $CaCO_3$ and C:N), 2) General abiotic soil properties (general soil properties except for microbial biomass), 3) DOC fractions (HA, FA, HoN, Hi), 4) DOC aromaticity (SUVA of HA, FA, Hi and total DOC), 5) DOC quality indicators to test for interactions between fractions and aromaticity (DOC fractions data with DOC aromaticity data), and 6) all measured soil properties. Only significant ($P <0.05$) models had coefficients determined for their independent variables.

4.3 Results

4.3.1 DOC properties: fractions and aromaticity

Among the 46 soils measured in this experiment, DOC ranged broadly with regards to total concentration extracted, but also DOC quality as indicated by both fraction profiles (Figure 4.2) and aromaticity measurements (Figure 4.3). Total DOC ranged from 11.3 - 292.3 mg kg^{-1} (Figure 4.2), with a median concentration of 35.5 mg kg^{-1}. The presence of four peat soils (Figure 4.2) in

this survey resulted in non-normal distribution of this dataset and a high standard deviation of the mean (56.6 ±61.25 mg kg^{-1}). Concentrations of DOC fractions also ranged broadly, although hydrophobic compounds (HA + FA + HoN) were consistently cumulatively higher in concentration than Hi. Humic acids made up ≥50% of soil DOC in 24 (more than half) of the soils. Hydrophobic compounds made up at least 60% of total DOC in every sample, and up to about 95% of total DOC in some samples (i.e. Hi compounds only made up 5-40% of total DOC) (Figure 4.2). Concentrations of each fraction were positively correlated with total DOC concentration, but the proportion of each fraction had no strong correlation with total DOC concentration (HA r = 0.11; FA r = 0.13; HoN r = -0.11; Hi r = -0.21).
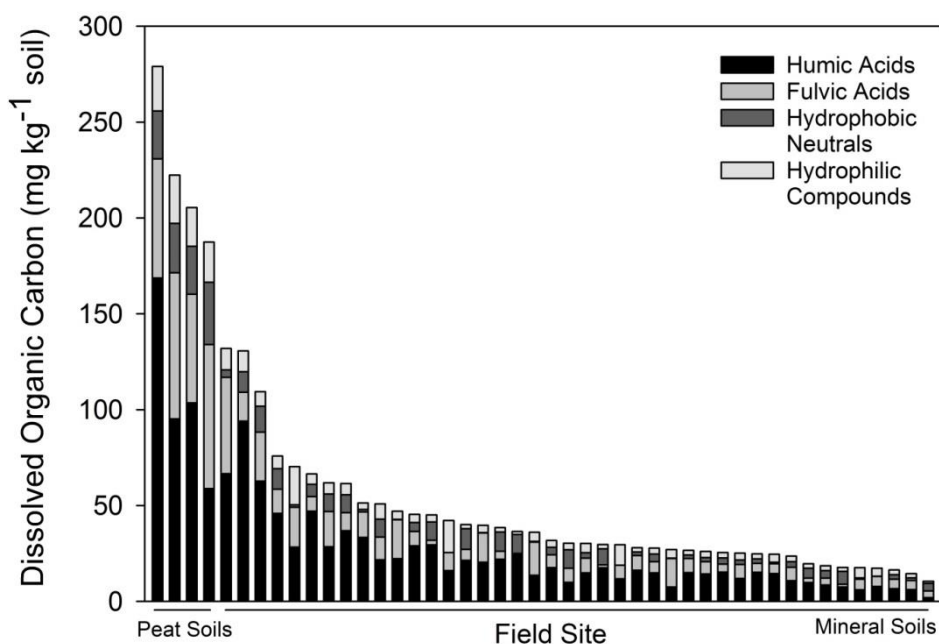

Figure 4.2: Dissolved organic carbon (DOC) profiles of 46 soils after fractionation. The sum of the four DOC fractions is equal to the total DOC (n=3) measured from each site. Four peat soils and 42 mineral soils are indicated on the x-axis.
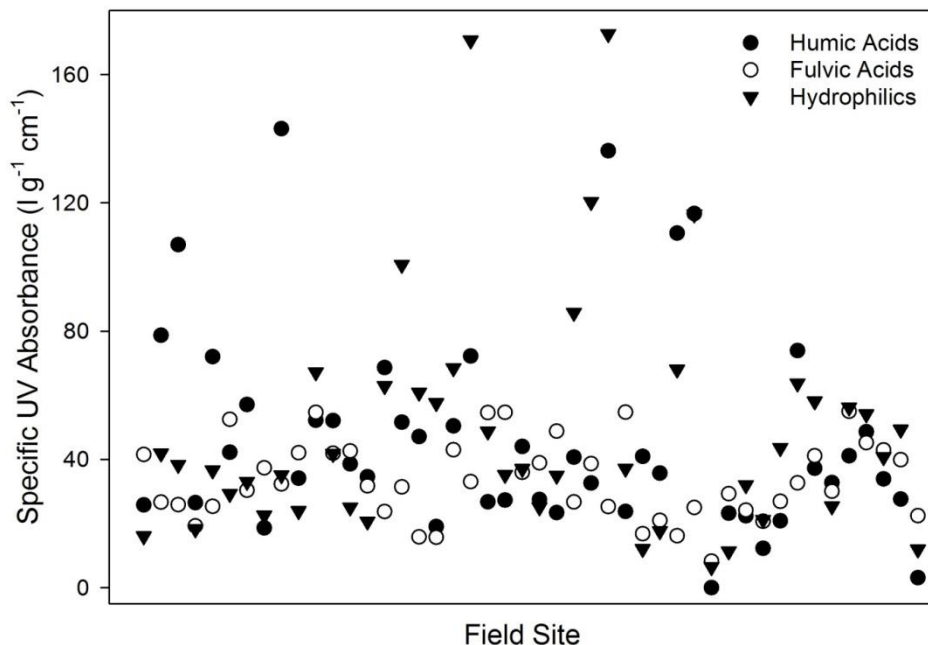
Figure 4.3: Specific UV absorbance at 254 nm of total dissolved organic carbon (DOC) and three DOC fractions (n=3). Data at the same x-axis values belong to the same soil sample from that field site.

Even greater variability was seen among sites' soil aromaticity values (SUVA). Figure 4.3 shows the diversity of aromaticity profiles of the 46 soils in this experiment. No significant (P <0.05) linear relationship could be found between DOC fractions (proportions) and SUVA concentrations. The DOC fractions that were most aromatic in any sample were about equally split between HA (in 16 fields), FA (14) and Hi (16), i.e. no fraction was consistently the most aromatic (Figure 4.3). While total DOC SUVA was significantly positively associated ($R^2 = 0.8$) with HA SUVA, this was deemed the result of the collinearity between total DOC and HA absolute concentrations ($R^2 = 0.9$). However, initial data visualization of Hi SUVA properties prompted nonlinear regression of this value as a function of the proportion of Hi in the total DOC solution (Figure 4.4). The significant (P <0.05) relationship determined between

these two properties had an R^2 of 0.58, which was higher than any linear relationships (which were not significant).
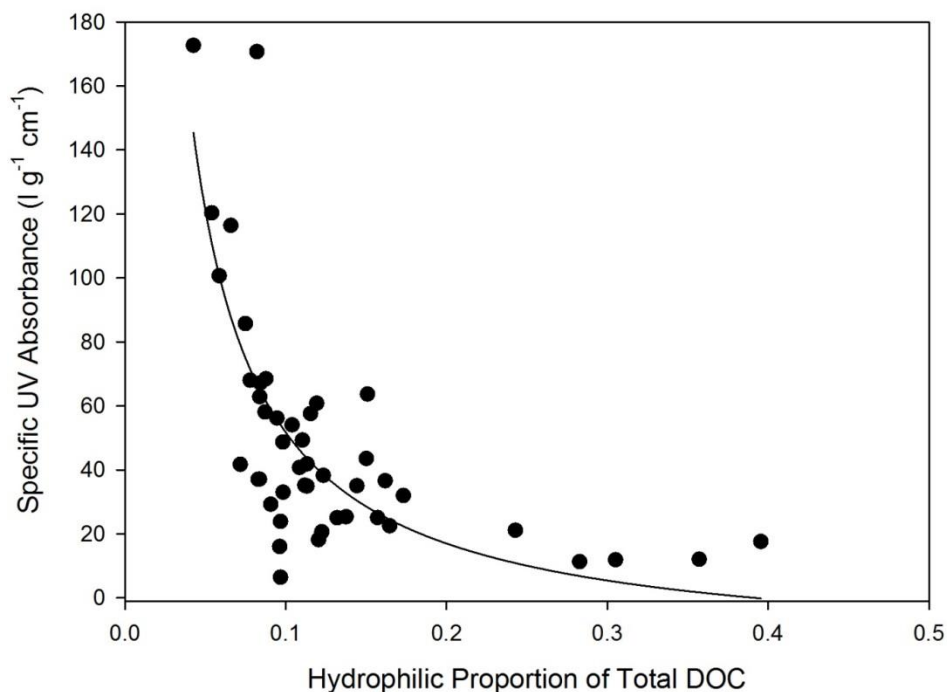

Figure 4.4: The hydrophilic (Hi) dissolved organic carbon (DOC) fraction as a proportion of total DOC relative to the specific UV absorbance at 254 nm of the Hi fraction of 46 soils. Plotted is the inverse first-order polynomial equation (P <0.05) with intercept (P =0.06) and independent variable coefficient (P <0.001).

4.3.2 Influence of field management practices

Tillage practice (conventional, conservation, or no till) and fertilizer source (artificial, liquid manure, solid manure or compost) did not significantly (P >0.05) affect OM content, pH, total DOC, DOC quality indicators, or microbial biomass, according to the results of one-way ANOVAs. Means were calculated for each tillage practice and fertilizer source for informative purposes (Table 4.1). Analysing total DOC and DOC fractions normalized by soil OM

Table 4.1: Least square multiple means comparison per management practice of soil total dissolved organic carbon (DOC) and four DOC fractions from 42 arable Dutch fields. Values are least squared means after Tukey's adjustment and values in brackets are the standard error of the mean.

Management practice	n	Total DOC		Humic Acids		Fulvic Acids		Hydrophobic neutrals		Hydrophilics	
		mg kg^{-1}	mg kg^{-1} OM^{-1}	mg kg^{-1}	mg kg^{-1} OM^{-1}	mg kg^{-1}	mg kg^{-1} OM^{-1}	mg kg^{-1}	mg kg^{-1} OM^{-1}	mg kg^{-1}	mg kg^{-1} OM^{-1}
Artificial fertilizer	13	25.7 (8.20)	5.7 (0.79)	14.5 (4.35)	3.2 (0.53)	6.1 (2.65)	1.1 (0.18)	2.7 (1.03)	0.7 (0.16)	2.7 (0.85)	0.8 (0.21)
Liquid manure	17	34.3 (7.30)	4.6 (0.70)	16.2 (3.87)	2.1* (0.47)	9.4 (2.18)	1.2 (0.16)	3.5 (0.92)	0.5 (0.14)	4.1 (0.76)	0.7 (0.19)
Solid manure	4	40.0 (14.76)	8.5 (1.41)	27.2 (7.83)	5.7* (0.96)	5.2 (4.41)	0.9 (0.33)	5.0 (1.86)	1.3 (0.30)	3.0 (1.53)	0.6 (0.39)
Compost	8	25.1 (10.38)	6.8 (1.00)	13.4 (5.51)	3.9 (0.58)	6.7 (3.10)	1.6 (0.23)	2.9 (1.31)	0.68 (0.20)	2.8 (1.08)	0.8 (0.27)
Conventional till	22	22.1 (6.76)	6.4 (0.65)	12.7* (3.58)	3.9 (0.44)	4.8 (2.02)	1.2 (0.15)	2.9 (0.85)	0.8 (0.13)	2.4 (0.70)	0.7 (0.17)
Conservation till	9	46.7 (10.43)	6.8 (1.00)	29.0* (5.53)	4.1 (0.68)	10.0 (3.11)	1.2 (0.23)	5.4 (0.31)	0.8 (0.20)	4.0 (1.08)	0.6 (0.27)
No till	11	22.0 (8.88)	6.00 (0.85)	11.8 (4.71)	3.2 (0.58)	5.7 (2.65)	1.3 (0.20)	2.2 (1.12)	0.7 (0.17)	3.1 (0.92)	1.0 (0.23)

* means within that column are significantly different from one another ($P < 0.05$)

content resulted in different trends in the means per management practice (Table 4.1), although this did not result in significant differences between these effects. Humic acids showed significant differences as a result of tillage or fertilizer source: significantly higher HA/OM was associated with solid manure application and higher concentrations of HA relative to conventional till was associated with conservation till (Table 4.1). Conservation till fields also had significantly higher cumulative CO_2 respiration rates than conventionally tilled fields (8.7 µ C g^{-1} d^{-1} and 4.1 µ C g^{-1} d^{-1}, respectively, as determined after log-transformation).

4.3.3 Relationships between DOC and microbial respiration

Results of linear regression analysis obtained via backward-step procedures identified the most influential parameters explaining variation in (log-transformed) cumulative soil respiration rates (Table 4.2). Six models with various input parameters were tested, and one linear model was made using total DOC for comparison to the performance of models including DOC quality indicators in place of total DOC.

The independent variable that explained the most variation in soil respiration was microbial biomass ($R^2 = 0.77$). Any model that included microbial biomass (General Soil Properties and All Measured Soil Properties) selected this parameter from the backward procedure as the only variable in the model (Table 4.2). When microbial biomass was removed as an input parameter, to consider only abiotic soil properties, the coefficient of determination decreased to 0.28, in which case OM became the only independent variable included.

When considering only the DOC quality indicators measured on these soils, significant ($P < 0.05$) models could also result in explaining respiration. The second-best model ($R^2 = 0.33$) resulted from DOC quality indicators as input parameters (the four DOC fraction absolute concentrations and four DOC aromaticity measurements (Table 4.2)). In this model the independent variables included were HA concentration and Hi SUVA.

Table 4.2: Regression equations determined from backward-step analysis, in which microbial respiration (log 14 week cumulative CO_2 emission) was the dependent variable (y) and the input parameters varied. Models are linear and significant (P <0.05). Total dissolved organic carbon (DOC) was included as a stand-alone model for comparison to DOC fractions.

Model name (tested input variables)	R^2	Standard error of estimate	Independent variable(s) in linear model
General soil properties (OM[a], total DOC, pH, microbial biomass, C:N, $CaCO_3$)	0.77	0.0435	Microbial biomass
General abiotic soil properties (OM, total DOC, pH, C:N, $CaCO_3$)	0.28	0.0775	OM
DOC fractions (Humic acids, fulvic acids, hydrophobic neutrals, hydrophilic compounds)	0.24	0.0793	Fulvic acids
DOC aromaticity (SUVA[b] of Humic acids, fulvic acids, hydrophobic neutrals, hydrophilic compounds, total DOC)	0.16	0.0844	Fulvic acid SUVA and hydrophilic SUVA
DOC quality indicators (all input variables of "DOC fractions" and "DOC aromaticity" models)	0.33	0.0753	Humic acids and hydrophilic SUVA
All measured soil properties (all input variables of "General soil properties" and "DOC quality indicators" models)	0.77	0.0435	Microbial biomass
Total DOC (total DOC)	0.25	0.0790	Total DOC

[a]Organic matter content
[b]Specific UV absorption at 245 nm

4.4 Discussion

The range of DOC fractions as a proportion of total DOC measured in this study indicates this characteristic behaves independently of total DOC concentration. The concentrations of the four DOC fractions measured in this study agree with previous research indicating humic acids constitute approximately 50% of soil DOC (Kaiser et al., 1996), and that 10-40% of soil DOC is made up of hydrophilic compounds (Guggenberger et al., 1994), although this is lower than reported in other studies (Cook and Allan, 1992).

Management practices had little to no effect on either the concentrations of total DOC, or the composition of its profile with regards to fractions or aromaticity (Table 4.1), which may be the result of measurable effects already having subsided before our sampling period. Still, the lack of significance was surprising considering the effects that OM inputs and tillage are known to have on soil OM content (Rasmussen and Collins, 1991), the quality of which is often reflected in DOC quality (Chantigny, 2003; De Troyer et al., 2011; Kalbitz et al., 2000). Although, as it has been suggested that DOC is more dynamic than other soil C pools in terms of C fluxing through it (Kalbitz et al., 2000), DOC may only be sensitive to tillage and OM input in the short-term. Concentrations of DOC have been previously reported to rapidly return to background levels after OM addition (Chantigny, 2003). This rapid degradation of added OM also agrees with recent findings linking DOC additions to soil: in an incubation study DOC added from various OM sources was significant in effects on respiration and DOC fraction characteristics only up until 6 days after addition (Straathof et al., 2014). Therefore, as our study did not include soils that had had a recent (<4 months) application of OM, these effects may have already subsided in the 46 soils of this survey. More research into the short-term effects of OM additions on DOC fractions in different soil types may therefore be valuable for determining potential microbial responses and turnover rates.

The low proportion of Hi compounds (Figure 4.2) and their subsequently high aromaticity values (Figure 4.3) may also be a result of the sampling time of this study and the exclusion of soils with crop emergence or

recent OM application. Root exudates from growing plants (i.e. arable crops) are often low-weight molecular C compounds (Van Hees et al., 2005; Strobel, 2001) that are measured in the hydrophilic fraction of DOC (Straathof, unpublished). As total DOC has been found to increase throughout the growing season in arable fields (Campbell et al., 1999; Embacher et al., 2007), we propose a large proportion of reported increases may be the result of crop-root-exudate Hi contributions. The low proportion and high aromaticity of Hi fractions measured in this experiment (Figure 4.4) suggest the latter is a response to low-Hi DOC. Kaiser and Kalbitz (2012) have previously proposed a conceptual model of soil C recalcitrance, in which C at depth is microbially-derived and thus more recalcitrant than plant-derived C, which is more prevalent in the rhizosphere. Although we did not measure along a depth gradient, the lack of recent Hi C input in our soils (either from root exudation or OM application) may result in a relative deprivation of the soil microbial community of Hi C. The remaining compounds of the reduced Hi pool may therefore be more aromatic because they are microbially-derived by-products of decomposition (Kalbitz et al., 2003b), and/or because they are remnant compounds from preferential turnover of more bioavailable (i.e. less aromatic) C that had previously been present in the soil solution. The high aromaticity of this residual Hi may therefore shift substrate preference of the microbial community from low-molecular weight Hi compounds to more hydrophobic compounds (Kalbitz et al., 2003b). This shift may have implications for why hydrophobic compounds (HA and FA) were significant independent model variables (as opposed to Hi) for soil respiration (Table 4.2) (despite their previously reported low-bioavailability (Jandl and Sollins, 1997)), and supports recent observations of relatively bioavailable HA (Straathof et al., 2014).

The most important variable for explaining microbial respiration measured in this study was the amount of microbial biomass (Table 4.2). On account of our hypothesis that soil chemical parameters are also valuable respiration indicators, we compared our DOC-quality models to this microbial-biomass model: When considering only DOC fractions as model input variables to microbial respiration, FA performed just as well as total DOC as an

independent variable in the linear model (Table 4.2), which may indicate that FA is an important fraction for microbial activity *in situ*. The transient nature of FA may contribute to its biological relevance, as it has been proposed as an intermediary phase between Hi and more hydrophobic compounds (Guggenberger et al., 1994; Ros et al., 2010). Fulvic acid aromaticity was also generally lower than the HA or Hi fractions (Figure 4.3), which may contribute to a relatively more bioavailable FA pool. Nonetheless, it is interesting to note the lack of significance in the DOC fraction model of either HA (which constituted the largest proportion of total DOC among the soils) or the presumably more bioavailable Hi. Although neither DOC fraction concentration nor aromaticity models increased the coefficient of variation relative to the total DOC model, considering all DOC quality indicators together in one model did (Table 4.2). It was this latter model in which HA and Hi properties became significant independent variables, indicating an interaction between the (non-colinear) variables HA concentration and Hi aromaticity. The depletion of Hi compounds has also recently been linked to concurrent depletion of HA (Straathof et al., 2014) and Kalbitz et al. (2003b) describe how, in the absence of carbohydrates as a substrate, microorganisms rely on hydrophobic compounds. The apparent feedback between these two operationally distinct DOC fractions is obviously also relevant for physiological responses of the soil microbial community and the biological relevance of HA DOC may be underestimated. In general, we found that the relationships between DOC quality indicators and microbial respiration were complex but significant, supporting our hypothesis that these parameters are not unimportant to consider as components of soil microbial activity.

4.5 Conclusions

This study innovatively links a variety of DOC properties to agricultural management and microbial activity in a comprehensive survey of soil types. The range in DOC fraction-prevalence and aromaticity in these soils showed

high variability of these quality indicators, independent of total DOC concentrations. Total DOC was not statistically more relevant for microbial respiration rates than fulvic acid DOC concentrations. The added value of determining DOC quality characteristics also includes a greater amount of variation in soil respiration rates explained by humic acid (HA) concentrations and hydrophilic (Hi) aromaticity. Hi aromaticity was also found to increase as the proportion of Hi decreased, supporting previous conceptual models which suggested increased recalcitrance of low-molecular weight compounds as their concentrations decrease. This shift in Hi bioavailability has apparent implications for the bioavailability of the HA fraction and the interaction between these two DOC characteristics for microbial substrate preference should be mechanistically explored in future research. We recommend DOC characterization as a soil-chemical-based method of determining potential soil microbial respiration and as a tool to further identify mechanistic relationships between DOC properties and turnover in soils. Specific follow-up research should also include shifts in these DOC characteristics in different soils in response to occurrences of management practices more recent than were measured here.

Acknowledgements
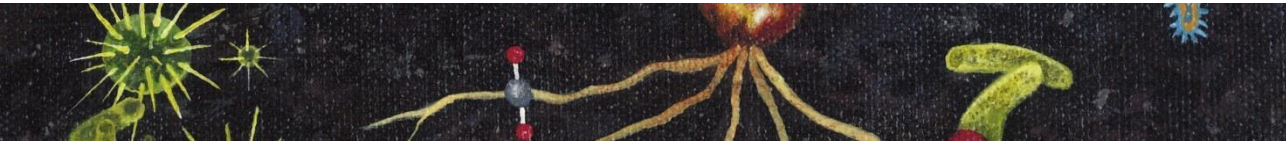
Soil parameters linked to volatile-mediated suppression of plant pathogens

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Abstract

There is increasing evidence that volatile organic compounds have a role in suppressing soil-borne plant pathogens but it is thus far unknown which edaphic properties are indicative of a soil's capacity to produce pathogen-suppressing volatiles. We measured the growth-suppressive effects of volatiles emitted from a broad range of agricultural soils on the agronomically important pathogens *Rhizoctonia solani, Fusarium oxysporum* and *Pythium intermedium. In vitro* growth suppression caused by exposure to soil volatiles was linked to edaphic properties, microbial community composition and field history using a multivariate statistical approach. Our results show volatile-mediated suppression of mycelial development for all pathogens; however, the range of effects and the significant edaphic variables differ per pathogen. Suppression of *R. solani* by volatiles was positively correlated with organic matter content, microbial biomass and amount of litter saprophytes but negatively correlated with pH, Shannon diversity and amount of *Acidobacteria.* Suppression of *F. oxysporum* and *P. intermedium*, however, was more affected by field history. *P. intermedium* suppression was also negatively correlated with soil sulphur content. Regression modelling of the three pathogens' overall suppression rate identified microbial activity, dissolved organic carbon (substrate availability) and crop history as the most influential variables. This study identified the overall and pathogen-specific drivers of growth-suppressive volatiles of soil-borne pathogens, which may be valuable components of a soil's potential for natural disease suppression.

5.1 Introduction

Soil-borne plant pathogens cause crop loss world-wide and there is a need for enhancing natural control mechanisms as a component of sustainable agriculture. In soils, one natural phenomenon that decreases disease manifestation of such pathogens is called general disease suppression (GDS), which is proposed to be related to substrate-driven microbial activity (Hoitink and Boehm, 2003). The edaphic properties that determine a soil's capacity for GDS, however, remain largely unidentified (van Bruggen and Semenov, 2000), but an indicator of this capacity would be valuable for moving towards prediction of natural suppression and risk of disease outbreak. One aspect of soil that is positively correlated with GDS is pathogen suppression (Termorshuizen and Jeger, 2008), which occurs when a pathogen's germination and/or hyphal extension is restricted by the soil microbial community through either resource competition or the production of antifungal compounds (Watson and Ford, 1972). The latter may be in the form of volatile organic compounds (VOCs), which have recently been proposed as important agents in pathogen suppression (as an outcome of fungistasis) and, thus, may contribute to GDS (Garbeva et al., 2011). The diffusive nature of VOCs facilitates their permeation of the soil matrix, resulting in a greater effective range relative to other suppressive compounds or organisms.

The effect of exposure to volatiles on growth of several phylogenetically different, agronomically important soil-borne plant pathogens has been tested, including *Rhizoctonia solani., Fusarium spp.* (Garbeva et al., 2014a; Kai et al., 2009)*, and Pythium spp.* (Chaurasia et al., 2005; Garbeva et al., 2014a). The growth of these pathogens is reported to be inhibited by VOCs released from various bacteria and fungi (Weisskopf and Bailly, 2013), including soil-dwelling *Bacillus spp., Burkholderia spp., Pseudomonas spp., Serratia spp.,* and *Stenotrophomonas spp.* (Fiddaman and Rossall, 1994; Kai et al., 2007; Pandey et al., 1997). Growth stimulation, however, has also been reported, including observations of volatiles that are suppressive against some pathogenic species but promote the growth of others (Wheatley, 2002). Most

volatile-pathogen interaction studies have been performed with bacterial isolates (Campos et al., 2010) on artificial media, outside the indigenous environment of both the volatile-producers and the pathogens. This limits the conclusions that can be made about volatiles emitted by the collective soil community, or volatiles produced as a result of microbe-microbe or edaphic-microbial interactions in the soil.

The effects of edaphic-microbial interactions on volatile production may be two-fold: first, the management and/or inherent properties of a soil may influence the composition of the microbial community (Ettema and Wardle, 2002; Marschner, 2003; Rousk et al., 2010), which is responsible for volatile production. Secondly, the quality and availability of substrates for microorganisms may influence the rate and profile of VOCs produced (Fiddaman and Rossall, 1994; Gray et al., 2010). Leff and Fierer (2008) measured both higher emission rates and higher diversity of VOCs emitted from litter than from mineral soils, and found organic C quality and microbial biomass were, respectively, the most influential edaphic properties of those sources. The soil environment may further confound volatile emissions, because of absorptive properties of the soil matrix; for instance, differences in recovery rates of polar, aromatic and aliphatic VOCs were significant when compounds were forced through either sand or clay soil types (Ruiz et al., 1998). Because of the myriad of influences the soil environment can have on both production and release of VOCs (Peñuelas et al., 2014), it is important to consider pathogen-suppressing VOCs as they are emitted from a variety of soils. Chuankun et al. (2004) found a widespread suppressive effect of volatiles from 146 soils on fungal spore germination. Furthermore, Campos (2010) suggested that volatile-mediated pathogen suppression is far more extensive than currently known; however, no studies have investigated volatile-mediated suppression from multiple soils for multiple plant pathogens thus far.

We conducted a large-scale survey on agricultural soils to measure a broad range of soil properties and potentially relevant parameters never before measured in the context of volatile-mediated pathogen growth. The objectives of this survey were to 1) measure the effect of volatiles emitted from

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agricultural soils on *in vitro* biomass production of the soil-borne plant pathogens *Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium intermedium*, and 2) identify the most statistically informative soil properties, microbial community structure, or field management parameters relevant for an agricultural soil's production of pathogen-suppressing volatiles. This study will therefore contribute to the development of hypothesis-driven testing of volatile-mediated pathogen suppression in soils.

5.2 Materials and Methods

5.2.1 Field selection, soil sampling and pre-treatment

A total of 50 arable fields were selected from across the Netherlands (Figure S1), covering a wide range of soil properties, e.g. texture, pH and organic matter content. Fields were sampled in February-March 2013, before the start of the growing season. Soil sampling (0-20 cm cores) was performed by taking 60 subsamples in a double W-pattern from an area of about 2 ha in each field. These subsamples were pooled and manually homogenized, resulting in a 3 kg sample per field, which was kept at 4℃ during transportation.

Upon arrival in the lab, soils were processed directly; they were split in two parts used for determining (1) chemical soil properties, (2) dissolved organic carbon (DOC) fractions, microbial biomass N, respiration, microbial community composition, and the *in vitro* suppression of pathogen growth by volatiles released by the soil. Part (1) was oven-dried (40°C), ground, and processed by BLGG (Wageningen, The Netherlands) according to standard procedures (Table 5.1). Part (2) was sieved to 4 mm. Per soil, two 1 g subsamples were taken and stored at -20°C for DNA extractions. Soil moisture was determined from the mass differential measured by drying the soil at 105°C for 24 h. Part (2) was then separated into three equal-sized samples. The three samples per field were then pre-incubated for 3 days at 9°C and 60% water-holding capacity (WHC). By equilibrating each soil under the same

Table 5.1: Edaphic parameters measured in this study. Transformation of raw data was performed only on the parameters indicated. Parameters in **bold** were included as independent variables in subsequent multivariate analysis where the *in vitro* suppression of *Rhizoctonia solani*, *Fusarium oxysporum*, and *Pythium intermedium* by soil volatiles were the dependent variables. All units indicated are per unit of soil dry weight equivalent.

Parameters	Dataset name	Method of determination	Raw data transformation	Methodological reference
Total and available N, P, **K**, C, **S**, Ca (mg kg⁻¹); subsequent **C:S and C:N ratios; Organic Matter,** sand, silt, **clay**, organic C, **CaCO₃ (%);** CEC (mmol kg⁻¹);	Soil properties	Near-infrared spectroscopy on soil dried at 40°C and ground	$\log$ (P, K, N, S, CEC); square root ($CaCO_3$)	Malley et al., 1999
pH; **P-, Na-,** Mg-, K- (mg kg⁻¹), Ca-available (kg ha⁻¹)	Soil properties	0.01 M $CaCl_2$ extraction on soil dried at 40°C and ground	$\log$ (Na)	Houba et al., 1990
Total DOC; humic acid, fulvic acid, hydrophobic neutral and **hydrophilic DOC fractions** (mg kg⁻¹)	Soil properties	Water extraction, 0.45 μm filtration, and subsequent rapid-batch fractionation	$\log$ (total DOC); square root (hydrophilics)	Thurman and Malcolm, 1981; Van Zomeren and Comans, 2007
Aromaticity of total DOC and of humic acid, fulvic acid, and hydrophilic DOC fractions (1 mg⁻¹ cm⁻¹)	Soil properties	Specific UV absorption at 254nm	$\log$	Weishaar et al., 2003; Amery et al., 2008
CO_2 emission (μg g⁻¹ h⁻¹) after 1, 3, and 14 weeks incubation and **cumulative CO_2** (μg g⁻¹)	Soil properties	Headspace concentration measurements using photoacoustic gas monitor	$\log$	Straathof et al., 2014 (modified)
Microbial biomass N (mg kg⁻¹)	Soil properties	Chloroform fumigation extraction	$\log$	Brookes et al., 1985; Joergensen and Mueller, 1996
Bacterial community analysis	Microbial community	454 pyrosequencing	Hellinger	Legendre and Gallagher, 2001
Fungal community and functional group analysis	Microbial community	454 pyrosequencing	Hellinger	Legendre and Gallagher, 2001
Oomycete community analysis	Microbial community	454 pyrosequencing	Hellinger	Legendre and Gallagher, 2001
Tillage practice, organic matter application and source, artificial fertilizer application, crops in rotation, cover crop	Field history	Survey with farmers	Dummy-coded: presence/absence	
Suppression of hyphal biomass relative to soil-free control: R. solani, F. oxysporum, P. intermedium (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation)	N/A (dependent variables)	Petri-dish assay for measuring soil volatile effects on hyphal biomass	arcsin-square root (F. oxysporum, P. intermedium)	Garbeva et al., 2014 (modified)

conditions, the effects of variable temperature and moisture conditions between different fields over the four weeks of sampling were minimized. After separation and pre-incubation, measurements of soil properties were performed in triplicate and the averages of the three samples were used as the input data.

5.2.2 Soil properties

A brief summary of all measured soil properties and the respective methodological references can be found in Table 5.1. For the measurement of soil dissolved organic carbon (DOC), field-moist soil was suspended in a 1:2 (w:w) extraction, where one part fresh soil (mass of dry-weight equivalent (DWE) was suspended in two parts ultra-pure water (UPW). Samples were equilibrated for 1 h on a horizontal shaker, centrifuged 20 min at 3000 g, and ultra-centrifuged 10 min at 11700 g. The supernatant was filtered through a pre-rinsed 0.45 μm cellulose nitrate membrane and a subsample of the filtrate was analysed for total DOC with a TOC-5050A analyzer (Shimadzu Corporation, Kyoto, Japan). The remaining filtrate was fractionated into four DOC fractions (Table 5.1) based on their hydrophobicity (Thurman and Malcolm, 1981) using a batch fractionation procedure (Van Zomeren and Comans, 2007). The aromaticity of Total DOC, humic acids, fulvic acids and the hydrophilic fraction were also measured by each solution's absorption of UV light at 254 nm (Genesys 10S UVeVIS, Thermo Fisher Scientific Inc., Waltham MA, USA), normalized by sample DOC concentration per cm path length.

Basal respiration rates were determined by CO_2 emission from incubated soils. The DWE of 100 g fresh soil was weighed into a 335 ml glass bottle and incubated in the dark at 20°C. Soil moisture in each bottle was maintained at starting levels (60% WHC) by adding UPW as needed, at least once per week. After the start of incubation (T0), emissions were measured nine days after T0 (T1), three weeks after T1 (T2), and 11 weeks after T2 (T3). At the start of a measurement period, each glass bottle was flushed with N_2 gas, and then sealed with a rubber septum for 4 h (T1), 6 h (T2), or 12 h (T3). Accumulated CO_2 concentrations in the bottle's headspace were then measured

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through the septum using an INNOVA 1412 Photoacoustic field gas-monitor (LumaSense Technologies, Ballerup Denmark). Cumulative CO_2 emissions for the 14 weeks of incubation (T1-T3) were calculated by linear interpolation of T1, T2 and T3, which produced a better fit than exponential interpolation.

Microbial biomass N was determined using the chloroform fumigation-extraction method (Brookes et al., 1985) on 20 g DWE fresh soil. Before and after a 24 h fumigation, a soil subsample was equilibrated for 1 h in 80 ml 0.5 M K_2SO_4, and the solution was filtered through a pre-rinsed 0.45 µm cellulose nitrate membrane. Total soluble N in the filtrate was measured on a San^{++} 6 channel segmented flow analyser (Skalar, The Netherlands).

5.2.3 Community analysis

Microbial community composition was assessed using 454 pyrosequencing. Two DNA extractions per soil were performed using Mobio 96-well Powersoil® extraction kit according to the manual. Amplicons for barcoded pyrosequencing (10 bp unique barcode per sample) of bacterial 16S ribosomal DNA fragments, and fungal and oomycetal ITS regions were generated using PCR reactions (primers, sequencing adapters and PCR conditions are listed in Table S1). PCR product quality was examined on a 1% agarose gel and subsequently purified using gel electrophoresis, followed by gel extraction (QIAGEN Inc., Valencia, CA). Concentrations of amplified DNA were measured by the Qubit® 2.0 Fluorometer (Life Technologies) and samples were pooled equimolar. Sequencing was performed by Macrogen (Macrogen Inc., South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA).

The obtained 454 sequences were filtered and analyzed using Mothur version 1.32.1. Briefly, primer and barcode information was identified in sequences allowing 0 errors. 16S sequences were trimmed based on a Phred score of 30, or 400 bases. Chimeras were identified by Uchime (Edgar et al., 2011). Sequences were aligned to the Silva reference alignment (Pruesse et al., 2007) followed by a classification (Wang et al., 2007). A distance matrix was

calculated (distance cutoff 0.10), and clustered using average neighbour clustering. Operational taxonomic units (OTUs) were determined at 97% similarity. A representative sequence was taken for each OTU and blasted against the NCBI database. An indication of taxonomy was based on the first five blast hits. ITS sequences were similarly filtered and analyzed using a minimum sequence length of 180 and a maximum length of 400 bases. ITS sequences (after chimera removal) were aligned and clustered using cd-hit-est (Li and Godzik, 2006) version 4.5.4 (parameters: word size 9, compare both strands, cluster sequences into most similar clusters instead of first cluster). The same workflow was used for 18S sequences, but a minimum length of 200 bases and a maximum length of 450 bases was applied.

rDNA sequences obtained from the two DNA extractions of each soil were pooled and rarefied to the minimum number of reads. The bacterial sequences were grouped on phylum level. Phyla were included in further analysis if a phylum contained over 0.05% of the total reads. The OTUs of the fungal dataset that could be assigned at the species level were each classified into one of eight potential functional groups (pathogenic, arbuscular mycorrhizal, coprophilic, endophytic, hyperparasitic (i.e. parasitizing on fungi), nematophagous, saprophytic on wood, saprophytic on litter), based on literature screening. For 40-50% of the total reads, a potential function could be indicated. The oomycete sequencing data were also classified into functional groups. However, in contrast to the other two datasets, the data were only pooled and not rarefied, due to the apparent absence or low number of reads in several soils. Shannon diversity index was calculated from the fungal, bacterial and combined dataset.

5.2.4 Field management survey

For each of the 50 soils, an interview with the farmers was conducted to document management practices, including questions on tillage, fertilization, previous crop and crop rotation. Of the 50 fields sampled, 46 fields had successfully completed management surveys; four fields were not included in

subsequent analysis where field management variables were input. Results were grouped according to different management practices: different tillage practices were categorized into three groups, (1) conventional tillage, (2) reduced tillage, or (3) no tillage; fertilizer application was categorized into four groups, (1) artificial fertilizer, (2) liquid manure, (3) solid manure, or (4) compost; cover crop was grouped based on presence or absence of a cover crop during the previous field season.

5.2.5 Volatile Assay

An experimental set-up was designed to determine the growth response of three different soil-borne plant pathogens to volatile organic compounds (VOC) released from soils. The three pathogens selected were the basidiomycete *Rhizoctonia solani* AG2-2-IIIB (strain 02-337, Sugarbeet Research Institute (IRS), isolated from *Beta vulgaris*), the ascomycete *Fusarium oxysporum* f. sp. *tulipae* (strain TuA, Applied Plant Research (PPO) Wageningen University and Research Centre, PPO Lisse isolated from *Tulipa* bulbs) and the oomycete *Pythium intermedium* (strain P52, PPO, Wageningen University and Research Centre, isolated from *Narcissus* bulbs). The experiment was designed to ensure enough airspace between pathogen and soil so the exposure to volatiles produced by the soils was enabled without physical contact between the pathogens and soil, an assay modified from Garbeva et al., 2014 (Figure S2). For each pathogen, 20 g DWE soil (60% WHC) was spread evenly on the bottom of a 90 mm Petri dish and incubated for 1 week at 10°C before the start of the experiment. For each Petri dish, a 4 mm layer of Water Yeast Agar (WYA; 20 g agar, 1 g KH_2PO_4, 0.1g $(NH_4)_2SO_4$, 0.1 g yeast extract (Difco) L^{-1}, pH 6.5) was poured into the lid. Agar plugs of 6 mm diameter Potato Dextrose Agar (PDA; 19.5 g L^{-1} (Oxoid)) colonized by *R. solani, F. oxysporum* or *P. intermedium*, incubated 5-10 days at 20°C, taken from the growing front, were transferred to WYA plates and incubated at 10°C. After 48 h, a WYA agar disc (Ø 6 mm) containing the pathogen mycelium was placed in the center of the (agar-filled) lid. The lid was then carefully placed on top of the

bottom (soil-containing) compartment and sealed using Parafilm (Figure S2). Plates were incubated for 10 days at 10°C. Petri dishes without soil were used as controls to measure the development of mycelium under conditions without soil-released volatiles. The assay was performed with six replicates. Mycelial biomass determination was done according to the method of Garbeva et al. (2014b), with some modifications. Briefly, pathogen mycelia were harvested by melting and dissolving the colonized agar from the lids of the Petri dishes in a glass beaker with water in a microwave oven (c. 100°C), followed by sieving the mycelium with a tea strainer and three washing steps with water (c. 90°C) to remove agar residues. For measurements of dry biomass weight, mycelia were frozen at -20°C and freeze-dried for 24 h.

5.3 Data Analysis

5.3.1 Statistics

All statistical analyses were performed in R (3.0.0) with the R packages vegan, packfor, ade4, leaps, car, and ape. For the soil properties data, normality and homogeneity of variances were examined using the Shapiro-Wilk test and Levene's test, respectively. Variables that did not meet these assumptions were log-transformed or square root-transformed (Table 5.1). To study the community composition of the fungal and oomycete community, the OTUs from the sequencing that could be assigned to species were grouped by function. The bacterial community was grouped by taxa. The sequencing datasets were Hellinger-transformed to minimize the effects of large abundances and zero values in the community dataset (Legendre and Gallagher, 2001). The field management data were dummy-coded because the data were categorically either absent or present. Pathogen suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to the soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation). The *F. oxysporum* and *P. intermedium* suppression datasets

were arcsin-square-root-transformed and three growth-promoting outlier soils were removed from the *P. intermedium* dataset to meet the basic assumptions of normality and homoscedasticity. These soils prevented normal distribution of suppression rate data and were excluded from subsequent analysis. They were not outliers with regards to any other measured parameters.

5.3.2 Selection of parameters for regression analysis

Covariation between all measured soil properties was first examined using a principal component analysis (PCA). The resulting plots of the first two PCs (Figure S3A and B) were used to make a selection of the most relevant parameters (Table 5.1), to avoid covariability and reduce the number of input parameters for downstream regression analyses. The decision-making process for selecting soil properties included: 1) if two parameters' PCA vectors had overlapping length and direction, the most biologically relevant of the two was selected (*e.g.* available nutrients from $CaCl_2$ extraction were chosen over less soluble bound elements (Houba et al., 1990)), 2) if two parameters have an inverse relationship, the most biologically relevant of the two was selected (*e.g.* DOC proportion of humic acids' vector is divergent from the proportion of hydrophilic compounds, but the latter has been found to be more closely related to rates of microbial respiration (Straathof et al., 2014)), and 3) parameters previously identified in the literature as being associated with disease suppression in agricultural soils (*e.g.* microbial respiration and biomass (Janvier et al., 2007)). For microbial community and field history datasets, all parameters were used as input.

5.3.3 Multiple linear regression analysis

For the soil property and microbial community datasets, a multiple linear regression analysis was performed, followed by permutation tests to determine the contribution and significance of the selected parameters in the

suppression rates of each pathogen by volatiles (the dependent variable). To create a regression model, this was followed by a forward selection procedure with double stopping criterion, adjusted R² and $\alpha<0.05$ (Blanchet et al., 2008). Correlations between significant ($P<0.05$) parameters and suppression rates were tested with the Pearson correlation coefficient (*r*). A similar approach was taken for the field history dataset, but using multiple logistic regression models (due to the binary nature of this dataset) and forward selection to create a parsimonious model (Blanchet et al., 2008).

5.3.4 Multivariate regression analysis

Preliminary observations of pathogen growth response to volatiles revealed that no one soil or group of soils was highly suppressive to all three pathogens. Furthermore, a lack of common significant variables among the three pathogens resulting from the univariate multiple linear regression prompted analysis of multivariate regression. Redundancy analysis (RDA) was performed for each dataset to test the contribution and significance of the selected soil property, microbial community, and field history parameters on suppression by volatiles of all three pathogens in combination. Within the RDA plot, an ordination value was generated for the overall pathogen suppression rates. This three-way ordination value became the dependent variable in subsequent regression; a permutation test and then forward selection procedure with double stopping criterion, adjusted R² and $\alpha<0.05$ (Blanchet et al., 2008) was performed to create parsimonious models and identify the most relevant parameters. To assess the contribution of each variable in the model, partial R^2 values were calculated. Furthermore, for each model, each significant ($P<0.05$) variable was removed one-by-one to assess its respective contribution to the significance level of the original model and coefficient of variation value. The subsequent parameters selected in the forward selection procedure to replace removed variables were also considered in terms of their contribution to explaining variation in pathogen-suppression. All of the parameters found to be significant in the multiple linear regression for each pathogen, and the

multivariate regression within the RDA (all models run) were combined into one dataset for a final regression analyses on all significant parameters only.

5.4 Results and Discussion

In part due to the geographical dispersion of the 50 arable fields sampled across The Netherlands (Figure S1), a wide variety of soil properties and management strategies was obtained. This in turn resulted in a broad range of soil properties measured: soils contained from 1-45% clay and 8-97% sand. Organic matter (OM) content ranged from 1.3-41% (mean 6.4%), although mineral soil (excluding five peat soils) mean OM content was 4.1%, which agrees with the 4.3% mean OM content of Dutch mineral arable soils found by Reijneveld et al. (2009). The mean pH of all 50 soils was 6.4, again similar to values previously reported (6.6 (Reijneveld et al., 2009)), indicating our selection of soils is representative of arable soils in The Netherlands. Other soil properties varied broadly as well and not necessarily collinearly (Figure S3), and 17 were ultimately selected as input variables into the regression analysis (Table 5.1). Tillage regimes, manure applications, and the use of a cover crop varied among the selected soils as a result of the spread in edaphic properties and the variety of crops. Corn was the most frequently grown crop at sampled sites (n=24), followed by wheat (n=19). Furthermore, the crop rotation on the sampled fields often included flower bulbs, sugar beet, potato and/or onion.

The soils also varied in their compositions of indigenous bacteria, fungi and oomycetes. The 16S sequencing resulted in a minimum of 14789 bacterial reads per field, from 16 phyla. Most reads were assigned to *Proteobacteria* (19-41% of the reads per field) and *Acidobacteria* (13-34% of the reads per field). More rare phyla included *Tenericutes* and *Spirochaetes,* which each had maximum 42 reads per sampled site. Most fungi inhabiting these soils (minimum 2745 reads per field) were classified within the functional group litter saprophytes, yielding in total 23-90% of the reads per field, whereas most

of the oomycetes were from the family Pythiales (>99%) in the genera *Phytophthora* or *Pythium*.

Variability among soils was measured in *in vitro* pathogen growth suppression by soil VOCs (Figure 5.1) and was determined to be a general soil phenomenon with pathogen-specific outcomes (Table 5.2). Volatile-mediated effects differed among the tested soil pathogens (Table 5.2). However, relating these overall pathogen response effects to measured parameters identified microbial activity, previous crop type, and multiple C- and S-related parameters as the most statistically influential (Table 5.3). Each of *R. solani*, *F. oxysporum*, and *P. intermedium* exhibited a range of responses from suppression to promotion of hyphal biomass production, but 91% of pathogen-soil combinations resulted in at least some suppression relative to the soil-free control. This is similar to results showing fungal growth can be either inhibited (up to 60%) or stimulated (up to 35%) by the volatiles of bacterial isolates (Mackie and Wheatley, 1999; Wheatley, 2002). When comparing responses to soil volatiles between pathogens (Figure 5.1), patterns failed to emerge; *i.e.* volatiles from soils that were promoting the growth of *F. oxysporum* were not necessarily promoting growth of *R. solani* (Figure 5.1A) or *P. intermedium* (Figure 5.1B), and *vice versa*.

The observation of pathogen-specific response effects (Figure 5.1) to volatiles emitted from the 50 soils in our study agrees with previous research which found species-specific responses to volatiles from various bacterial and fungal isolates under laboratory conditions (Bruce et al., 2000; Garbeva et al., 2014a; Mackie and Wheatley, 1999). Pathogen-specific response variability may result from volatile compounds affecting different sites of action on the pathogen, or from differences between each pathogen's ability to detoxify the VOCs (Kai et al., 2009). Our results support previous study that reported both taxon- and genus-specific sensitivity between pathogens; generally oomycetes tend to be very sensitive to volatiles while *Fusarium spp.* were reported to have relative tolerance towards them (Hunziker et al., 2015; Weisskopf and Bailly, 2013). These observed differences in tolerance to volatile exposure may also be related to the biology and morphology of each pathogen. Hunziker et al. (2015)
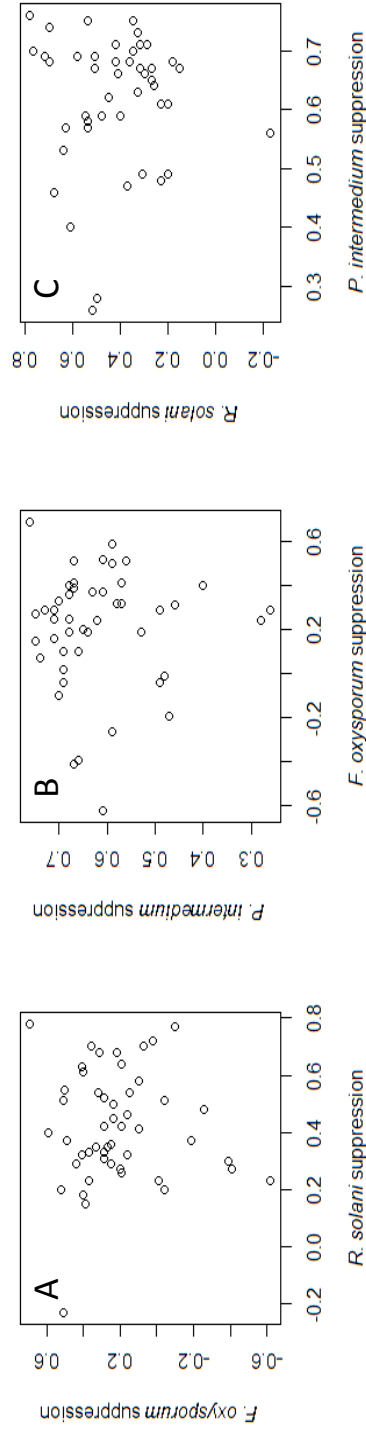
Figure 5.1: *In vitro* suppression rates of *Rhizoctonia solani*, *Fusarium oxysporum*, and *Pythium intermedium* by volatiles from 50 arable soils, plotted against each other pathogen. Pathogen suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation). Axes scaling differs between A, B, and C as comparisons are for between pathogens, not between plots.

Table 5.2: Multiple linear regression models determined from soil properties, microbial community, and field history parameters measured from 50 soils; the dependent variable was *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* or *Pythium intermedium* by soil volatiles. Significant model parameters (SMP) presented explain significant (*P*<0.05) amounts of variation in volatile suppression rates. Parameters are derived from a reduced regression model after a forward selection procedure, which was only run on significant (*P*<0.05) models, and not non-significant (ns) models.

Pathogen	Dataset	Model type	R^2	SMP with respective partial R^2 (in brackets)
R. solani	Soil properties	linear	0.51	organic matter (0.40), pH (0.27), microbial biomass N (0.26)
	Microbial community	linear	0.44	Shannon diversity index (0.28), litter saprophytes (0.17), *Acidobacteria* (0.02)
	Field history	logistic	0.18	bulbs (0.10), potato (0.08)
F. oxysporum	Soil properties	linear	ns	
	Microbial community	linear	ns	
	Field history	logistic	0.08	reduced tillage (0.08)
P. intermedium	Soil properties	linear	0.16	S-total (0.18), microbial biomass N (0.08)
	Microbial community	linear	ns	
	Field history	logistic	0.39	solid manure (0.24), bulbs (0.09), corn (0.08)

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Table 5.3: Multivariate regression models determined from soil properties, microbial community, and/or field history parameters measured from 50 soils; the dependent variable was overall *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* by soil volatiles, combined. Significant model parameters (SMP) presented explain significant ($P<0.05$) amounts of variation in volatile suppression rates. SMP are derived from a reduced regression model after a forward selection procedure, which was only run on significant ($P<0.05$) models, and not non-significant (ns) models. The first model listed for each dataset is the best model determined. For comparison, SMP were removed, the model rerun, and the alternative models listed below the best model.

Dataset	Removed variable	R^2	*P*	Significant model parameters with respective partial R^2 (in brackets)
Soil properties		0.24	<0.001	DOC[a] (0.08), CO_2[b] (0.08), C:S (0.05)
	DOC	0.21	0.029	CO_2 (0.08), S-total (0.09), OM[c] (0.07)
	C:S	0.19	0.016	DOC (0.08), CO_2 (0.08), C:N (0.02)
	CO_2	0.20	0.049	DOC (0.08), C:S (0.05), Na (0.03)
Microbial community		ns	ns	
Field History[d]		0.18	0.004	corn (0.09), potato (0.07), solid manure (0.07)
	corn	0.16	0.045	potato (0.07), solid manure (0.07), liquid manure (0.02)
	potato	0.14	0.070	corn (0.09), solid manure (0.07)
	solid manure	0.14	0.025	potato (0.07), corn (0.09)
All significant parameters from soil properties + microbial community + field history		0.27	<0.001	CO_2 (0.09), corn (0.05), DOC (0.07),
	CO_2	0.13	0.002	DOC (0.07), corn (0.05)
	corn	0.22	<0.001	CO_2 (0.09), DOC (0.07), C:S (0.04)
	DOC	0.25	<0.001	CO_2 (0.09), corn (0.05), S-total (0.07)

[a]DOC=dissolved organic carbon

[b]Cumulative microbial respiration after 14 weeks incubation

[c]Organic matter content (%)

[d]Indicated field crop or management practice was either present or absent in previous field seasons

have suggested that cell wall differences between pathogens may contribute to the permeability and, consequently, the inhibitory effect of volatiles. Relatively VOC-sensitive oomycete (*P. intermedium*) cell walls contain cellulose whereas fungal (*R. solani* and *F. oxysporum*) hyphae are built with a chitin matrix. Strong differences in lysis have been observed between *Rhizoctonia* and *Fusarium* after contact with non-volatile anti-microbial metabolites, which Potgieter and Alexander (1966) attributed to differences in cell wall structure and composition between the two fungi.

5.4.1 Suppression of *R. solani* by volatiles

Of the three pathogens, *R. solani* was most consistently suppressed by volatiles, with only one soil emitting volatiles that promoted *R. solani* growth (Figure 5.1A and C) by about 20% more than the soil-free control. The best multiple linear regression model determined for *R. solani* explained a proportion of 0.51 variation (Table 5.2), and was produced from the dataset of soil properties. While OM content and microbial biomass N were positively correlated with *R. solani* suppression rates (Figure 5.2A and 2C, respectively), pH was negatively correlated with growth suppression by volatiles (Figure 5.2B). A moderate correlation ($r=0.51$) was found between OM content and microbial biomass N, and OM content and pH ($r=-0.52$), but no relationship was found between microbial biomass N and pH. The decomposition of complex OM constituents may result in the release of VOCs (Isidorov and Jdanova, 2002) as intermediary compounds of decomposition processes (Dickschat et al., 2005; Gray et al., 2010). With regards to pH, while it may act as a direct determinant of a volatile's partitioning between the solution and gas phase, the potential effects of this property so tightly link soil chemical and microbiological feedback that it is difficult to disentangle whether its effect is direct or interactive.

When considering the microbial community dataset, another significant model included the Shannon diversity index value, the fungal functional group litter saprophytes, and the taxonomic group *Acidobacteria* as significant model

parameters (Table 5.2). *Acidobacteria* were present in each of the 50 soils, had about 1800-5000 reads after rarefication, and were negatively correlated with volatiles suppressing *R. solani* (Figure 5.2F). Abundance of *Acidobacteria* has been shown to positively correlate to VOC-based pathogen suppression in other experiments (Van Agtmaal, unpublished). As the Shannon diversity index of the total microbial community increased, *R. solani* suppression decreased (Figure 5.2D). On the contrary, an increase in suppression was measured as litter saprophyte read numbers increased (Figure 5.2E).

With regards to the historical field management practices at each site, a lower (0.18) amount of variation could be accounted for by that model, although this was still significant ($P<0.05$) (Table 5.2). Field sites that included bulbs and/or potato in their crop rotation were most relevant in this model (Table 5.2) and appeared to slightly decrease *R. solani* suppression by volatiles (Figures5.2G and H). Plant species are known to differentially alter soil microbial community composition (Berg and Smalla, 2009). This effect also applies to crops grown in long-term agricultural soils (Maul and Drinkwater, 2009) which may offer an explanation for this observation as the composition of the microbial community can influence the volatile profiles emitted.

Figure 5.2 (next page): Relationships between *in vitro* suppression of *Rhizoctonia solani* by soil volatiles and properties of 50 arable soils: A) Organic matter (% (log-scaled)), B) pH, C) microbial biomass N (mg kg^{-1} (log-scaled)), D) Shannon diversity index, E) *Acidobacteria* (Total OTU reads), F) Litter saprophytes (Total OTU reads), G) Potatoes in the crop rotation, and H) Bulbs in the crop rotation. Properties A-H were significant ($P<0.05$) model parameters determined by forward-step regression where suppression of *R. solani* by soil volatiles was the dependent variable. Suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation).
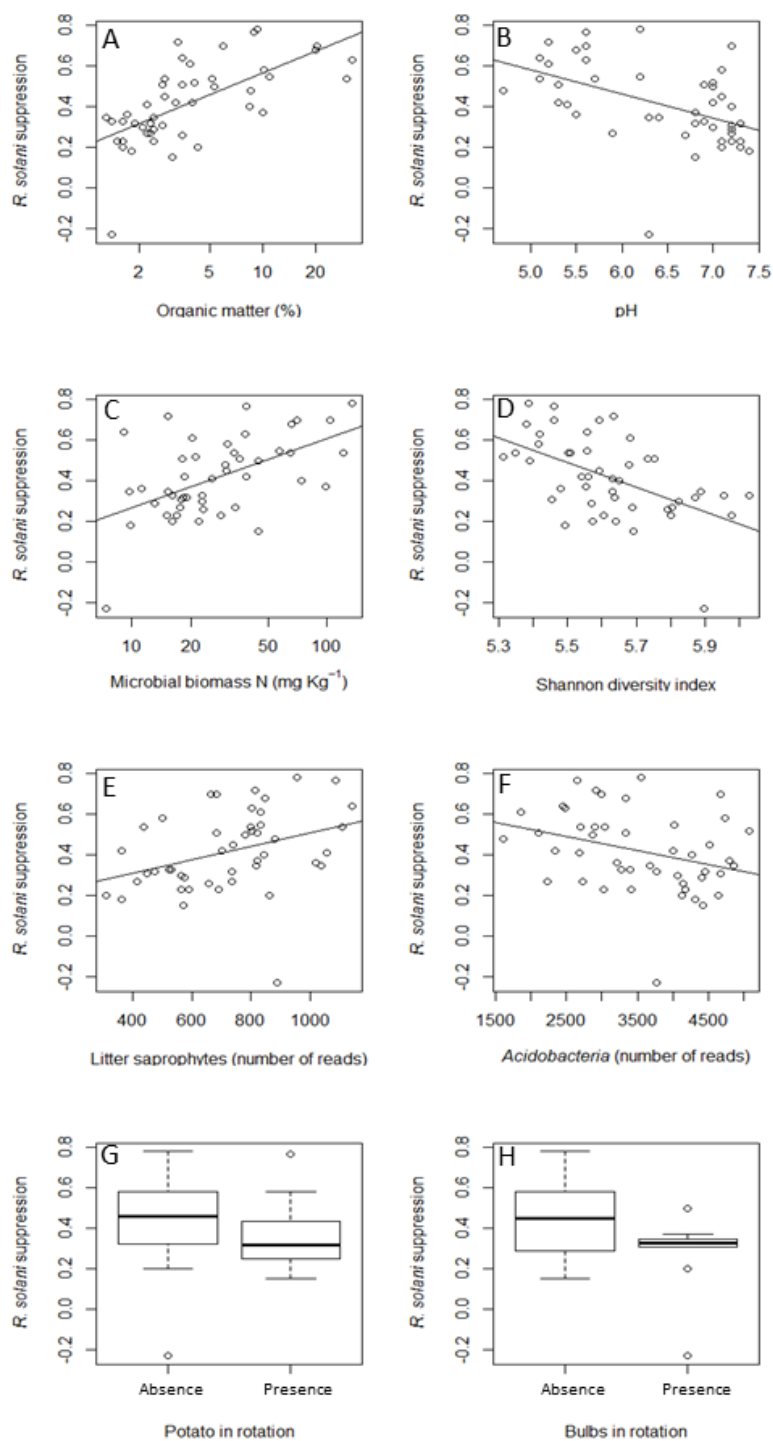
5.4.2 Suppression of *F. oxysporum* by volatiles

Volatiles from the soils measured in this experiment were least effective in suppressing *F. oxysporum in vitro*. The only parameter from all three measured datasets which significantly contributed to a model explaining variation in suppression of *F. oxysporum* ($R^2 = 0.08$) was the practice of reduced tillage (Table 5.2). Field sites using reduced tillage (n=9) had slightly higher suppression levels of *F. oxysporum* than sites using conventional or no-till management (Figure 5.3). Although most soils were suppressive to some degree, the mean rate of suppression for *F. oxysporum* relative to the control (0.2) was much lower than for *R. solani* or *P. intermedium* (0.7 and 0.6, respectively). There were also nine soils from which volatiles promoted biomass production of this pathogen (Figure 5.1A and 1B), although the remainder were suppressive to at least some degree (<0.7).
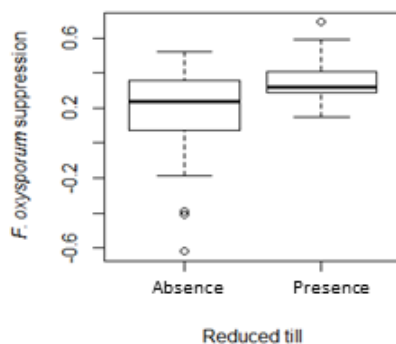

Figure 5.3: Relationship between *in vitro* suppression of *Fusarium oxysporum* by soil volatiles and the practice of reduced tillage in 50 arable soils. Reduced tillage was a significant (*P*<0.05) model parameter determined by forward-step regression where suppression of *F. oxysporum* by soil volatiles was the dependent variable. Suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression, <0 = stimulation).

Different *Fusarium* species have been reported to be more tolerant to VOCs, e.g. *F. solani* was resistant to bacterial VOCs (Kai et al., 2007) and *F. oxysporum* has been shown to have only limited sensitivity to volatiles produced by antagonistic strains (Hunziker et al., 2015; Weisskopf and Bailly, 2013). The relative tolerance of *Fusarium* to microbial volatiles and specifically bacterial volatiles may be one of the underlying reasons for the lack of edaphic variables corresponding with *F. oxysporum* volatile-mediated suppression. This may, however, be strain-specific or dependent on the volatile profile the fungus is exposed to, as strong inhibitory responses of *F. oxysporum* upon volatile exposure have also been found (Garbeva et al., 2014a).

5.4.3 Suppression of *P. intermedium* by volatiles

Several oomycetes (*Pythium spp.* and *Phytophthora spp.*) have consistently been shown to be highly sensitive to microbial volatiles (Van Agtmaal, unpublished; Hunziker et al., 2015). From the soil properties dataset, two significant parameters contributed to a model predictive of suppression with a R^2 of 0.16: S-total and microbial biomass N (Table 5.2). When correlated individually against suppression, relationships were slightly negative (Figure 5.4A and 4B, respectively), although variability between sites was high for both parameters. Sulphur-based volatile compounds like dimethyl disulphate (DMDS) and dimethyl trisulphate (DMTS) have been shown to be produced by soil microbes (Kai et al., 2007), e.g. the major compound emitted (94%) by an *Achromobacter* isolate was DMDS (Minerdi et al., 2011). Sulphur-containing compounds have been related to reduced *Pythium* infections in cucumber, both after direct addition of DMDS to soil or after incorporation of S-rich *Allium* crop residues (Arnault et al., 2013). As production of S-containing VOCs from bacterial isolates seems dependent on nutrient availability (including S) in the growth medium (Garbeva et al., 2014a), this would imply that S-availability, quality, or interactions with other soil properties may also influence emission of S-containing VOCs. The nature of this relationship, however, may be too complex to elucidate via our statistical approach.
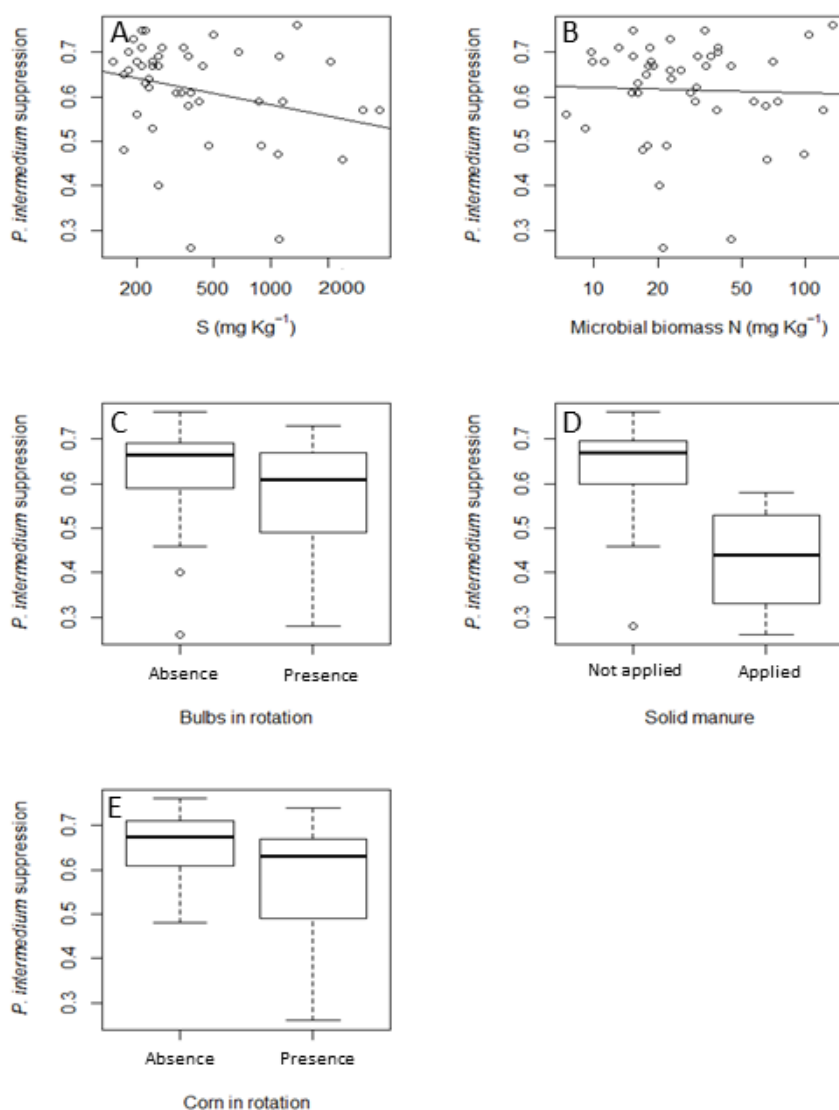
Figure 5.4: Relationships between *in vitro* suppression of *Pythium intermedium* by soil volatiles and properties of 50 arable soils: A) Total sulphur (mg kg^{-1} (log-scaled)), B) Microbial biomass N (mg kg^{-1} (log-scaled)), C) Bulbs in the crop rotation, D) Application of solid manure, and E) Corn in the crop rotation. Properties A-E were significant ($P<0.05$) model parameters determined by forward-step regression where suppression of *P. intermedium* by soil volatiles was the dependent variable. Suppression by volatiles was converted to the proportion of reduction of mycelial biomass in comparison to a soil-free control (control = 0 (no suppression), 1 = maximum suppression).

A model with a better fit ($R^2 = 0.39$) to the *P. intermedium* suppression rates was determined from the field history data. The presence of bulbs in the crop rotation (Figure 5.4C), the presence of corn in the crop rotation (Figure 5.4E), and the application of solid manure (Figure 5.4D) were all negatively correlated with the suppression rates of *P. intermedium*. The latter, however, was the most explanatory parameter in terms of its partial R^2 value (Table 5.2).

5.4.4 Multivariate analysis of overall pathogen suppression by volatiles

Multivariate analysis of the overall pathogen response to volatiles was prompted by the observation that there was no one soil or group of soils producing volatiles highly suppressive to all three pathogens (Figure 5.1). Furthermore, a lack of common significant variables among the three pathogens resulting from the univariate multiple linear regression (Table 5.2) warranted a multivariate approach to determine whether the combined response rate would have significant model parameters. Therefore, the parameters of all three datasets were combined using the overall response rate's ordination of the three pathogens as one dependent variable (Table 5.3). The ordination of each point on the RDA plot (Figure S4A, B and C) was more spread out compared to clustering that had been seen in PCA plots (Figure S3A and B), suggesting that while soils shared similar properties, this did not translate to commonalities in overall suppression of the pathogens.

Significant models were obtained from the soil properties and field history datasets, but not from the microbial community dataset (Table 5.3). Parameters in the most explanatory model of soil properties ($R^2 = 0.24$) were different from those identified in the univariate multiple linear regression. Instead, DOC, cumulative CO_2 production, and C:S ratio were the most significant of the 17 soil properties included. Removing each of these significant parameters did not drastically reduce the model's coefficient of variation, but it did indicate relationships between parameters beyond collinearity. Parameters removed from regression analysis are not necessarily

replaced by parameters with the most similar RDA vectors (Figure S4A). For example, removing CO_2 as a model input parameter results in Na as a new significant parameter (Table 5.3).

Dissolved organic carbon is a known substrate for soil microorganisms (Haynes, 2005). Substrate quality impacts VOC composition, as resource variations have been shown to change the type, amount and suppressiveness of volatiles (Ezra and Strobel, 2003; Gray et al., 2010; Wheatley et al., 1997). Furthermore, resource availability and quality of DOC is reflected in microbial activity rates (CO_2) (Straathof et al., 2014; Chapter 4 (this thesis)), which have been found to positively correlate to VOC production rates and microbial biomass in soil and litter samples (Leff and Fierer, 2008). Any combination of these effects may thus result in the significance of DOC for the overall pathogens suppression measured.

The best field history model for overall pathogen suppression yielded an R^2 of 0.18 in which the presence of corn and/or potato in the crop rotation, along with the application (or lack thereof) of solid manure were significant independent variables (Table 5.3). About 40% of fields had had corn and about 30% had had potatoes in their rotations in the last five years. Conversely, only five fields had received applications of solid manure, and are relative outliers in their RDA ordination (Figure S4B). When solid manure was removed from the model, potato and corn still explained significant amounts of variation in the volatile-mediated suppression of the three pathogens combined (Table 5.3).

By reducing the number of input parameters into the multivariate regression models (dataset "All significant parameters") for overall suppression, the highest coefficient of variation was achieved ($R^2 = 0.27$ (Table 5.3)). In this case, cumulative CO_2 production, DOC and corn were the significant model parameters. Removal of CO_2 resulted in the highest decrease of variation explained: 14% lower than when it is included in the model, and it was not replaced by any other variables (*i.e.* DOC and corn remained the only two significant parameters (Table 5.3)). The importance of this parameter further supports the notion that overall pathogen suppression by volatiles is driven by the consortium of soil microorganisms. Furthermore, these results suggest that

microbial activity is more relevant to volatile production than the absence or presence of particular microbial species, which were not significant model parameters contributing to overall suppression variation.

5.5 Conclusions and Future Directions

We have presented here the first multi-soil survey of the effects of volatiles emitted from soils on *in vitro* biomass production of three different pathogens. The edaphic parameters we have identified as being significant in their effect on the combination of these pathogens are also properties that have been directly linked to microbial metabolic activity, either as a substrate source (DOC) or an activity indicator (CO_2). While this link has been previously postulated, our statistical confirmation of the relevance of these parameters should provide an impetus for future hypothesis-testing. The focus of this future experimental work is recommended to 1) more mechanistically explore the role of microbial substrate, including DOC, and its influence on VOC production/quality, and then 2) determine management practices which effect substrate-driven microbial activity and thus, may enhance VOC-mediated pathogen suppression *in situ*. Previously it has been shown that VOCs from soil positively correlate to reduced disease incidence *in situ* (Van Agtmaal et al., unpublished) which supports the potential for VOC-mediated suppression in agricultural fields. This implies VOC-mediated pathogen suppression could be an important component of general disease suppression in agricultural soil, and should be considered as a natural control mechanism for reducing crop-loss and moving towards sustainable agriculture.

Acknowledgements

Please see supplementary information for Chapter 5 in the following pages.

Table S1: Primers, adapters, PCR conditions and mastermix used for the 454 amplicon libraries of bacterial 16S, and fungal and oomycetal ITS rDNA fragment sequencing.

|	Bacteria 16S	Fungal ITS	Oomycete ITS	References
Primer F	577F (5'-AYTGGGYDT AAAGNG-3')	ITS86F (5'-GTGAAT CATCGAATCTTTGAA-3')	OOMUP18Sc (5'-TGCGGA AGGATCATTACCACAC-3')	577F/926R: Rosenzweig et al. (2012)
Primer R	926R (5'-CCGTCAATT CMTTTRAGT-3')	ITS4 (5'-TCCTCCGCT TATTGATATGC-3')	ITS2-OOM (5'-GCAGCG TTCTTCATCGAATGT -3')	
sequencing adapter F	(5'-CCATCTCATCCCTGC GTGTCTCCGACTCAG-3')	(5'-CCATCTCATCCCTGC GTGTCTCCGACTCAG-3')	(5'-CATCTCATCCCTGC GTGTCTCCGACTCAG-3')	ITS86F: White et al. (1990)
sequencing adapter R	(5'-CCTATCCCCTGTGTG CCTTGGCAGTCTCAG-3')	(5'-CCTATCCCCTGTGTG CCTTGGCAGTCTCAG-3')	(5'-CTATCCCCTGTGTG CCTTGGCAGTCTCAG-3')	ITS4: Turenne et al. (1999)
PCR conditions	2 minutes 94°C, 30 cycles 94°C 45s, 59°C 45s, 72°C 60s, final annealing: 10 minutes 72°C	2 minutes 94°C, 30 cycles 94°C 45s, 59°C 45s, 72°C 60s, final annealing: 10 minutes 72°C	2 minutes 94°C, 30 cycles 94°C 45s, 57°C 45s, 72°C 60s, final annealing: 10 minutes 72°C	OOMUP18Sc, ITS2-OOM: Lievens et al. (2004); Lievens and Thomma, (2005)
PCR mastermix	25 µl, 0.15 mM each DNTP, 0.5 µM of each primer, 1 unit Titanium taq DNA polymerase, 1x Titanium taq PCR buffer (Clontech Laboratories, Palo alto, USA)	25 µl, 0.15 mM each DNTP, 0.5 µM of each primer, 1 unit Titanium taq DNA polymerase, 1x Titanium taq PCR buffer (Clontech Laboratories, Palo alto, USA)	25 µl, 0.15 mM each DNTP, 0.5 µM of each primer, 1 unit Titanium taq DNA polymerase, 1x Titanium taq PCR buffer (Clontech Laboratories, Palo alto, USA)	
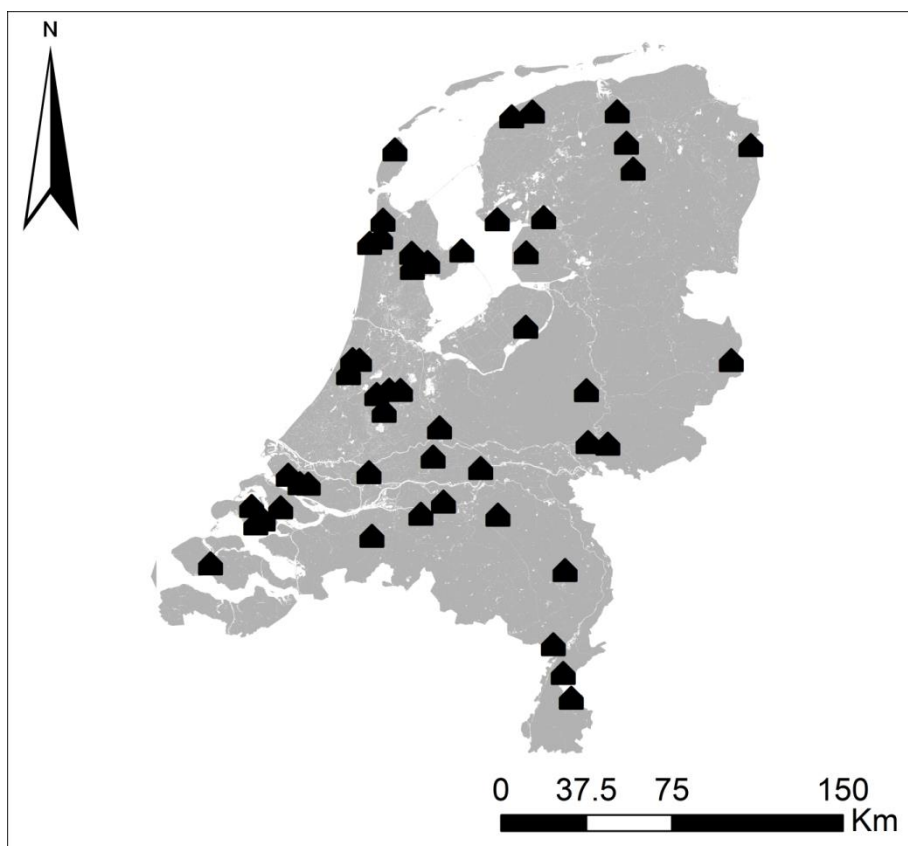
Figure S1: Locations in The Netherlands of 50 arable agricultural fields sampled.
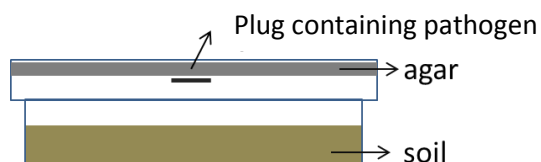

Figure S2: Illustration of the set-up used to determine the effect of soil-released volatiles on pathogen biomass. The bottom Petri-dish compartment contains fresh soil. The inner side of the lid compartment contains water yeast agar with a plug directly in the centre containing pathogen mycelium as inoculum. Para-film seals the lid to the bottom compartment.
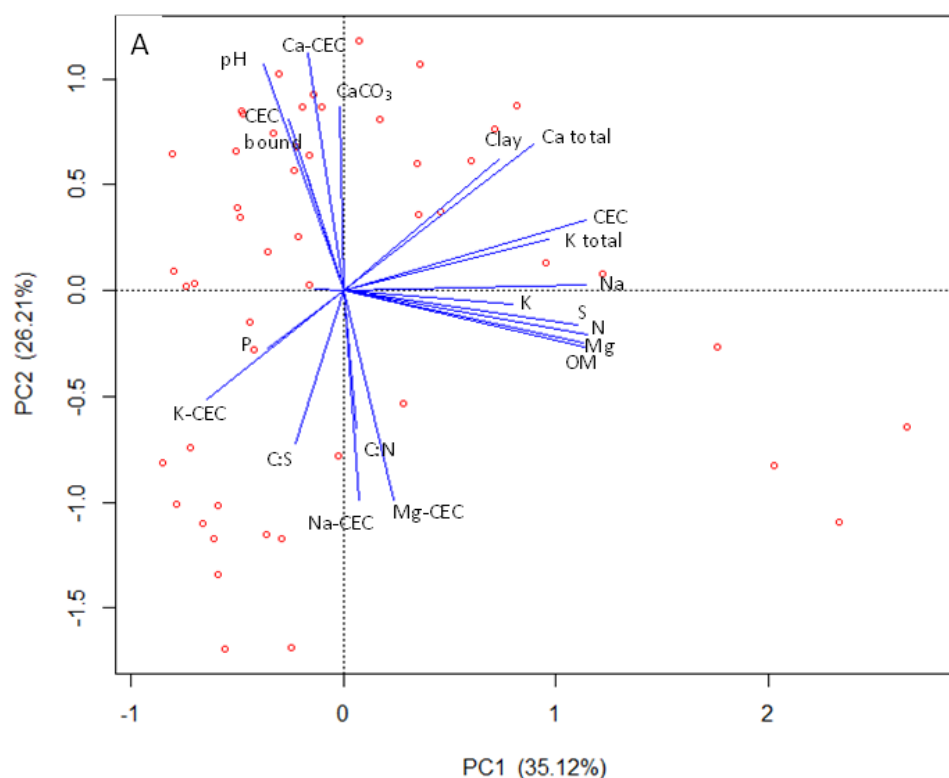
Figure S3A: Principal component analysis of 50 soils' soil properties measured using routine near-infrared based or CaCl$_2$ extractions (OM=organic matter; CEC=cation exchange capacity).
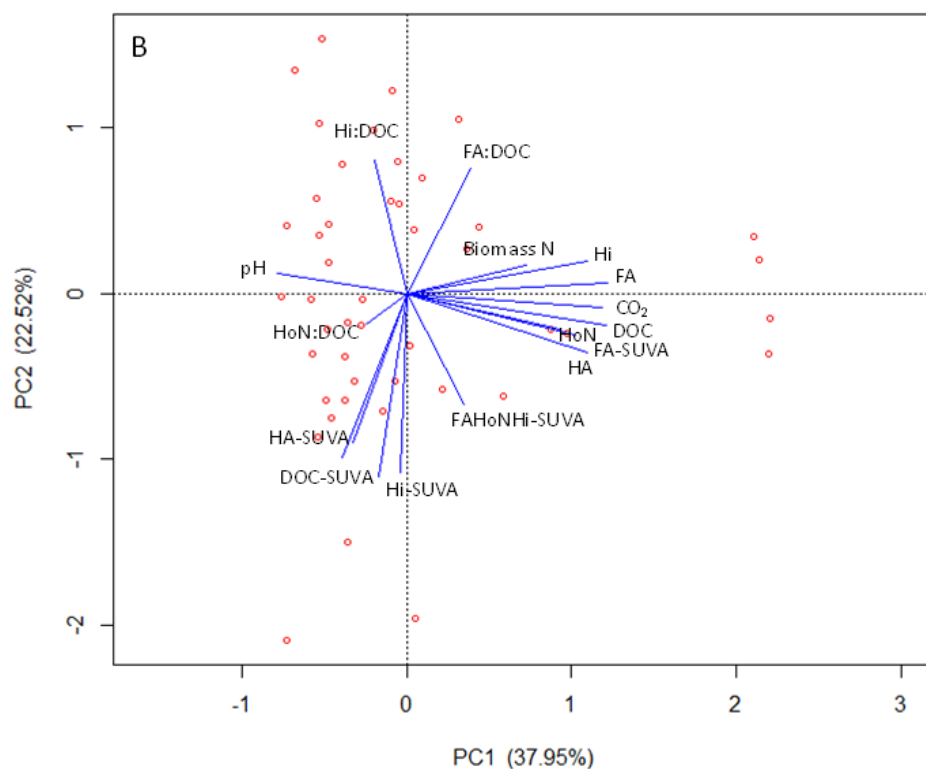
Figure S3B: Principal component analysis of 50 soils' soil properties measured using ultra-pure water extractions and dissolved organic carbon (DOC) fractionation (HA=humic acids; FA=fulvic acids; HoN=hydrophobic neutrals; Hi=hydrophilic compounds; SUVA=specific ultra violet absorption at 245 nm).

Figure S4A: Redundancy analysis (RDA) plot of overall *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* (combined) by soil volatiles from 50 arable soils in relation to soil properties (DOC=dissolved organic carbon).

Figure S4B: Redundancy analysis (RDA) plot of overall *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* (combined) by soil volatiles from 50 arable soils in relation to field management practices.
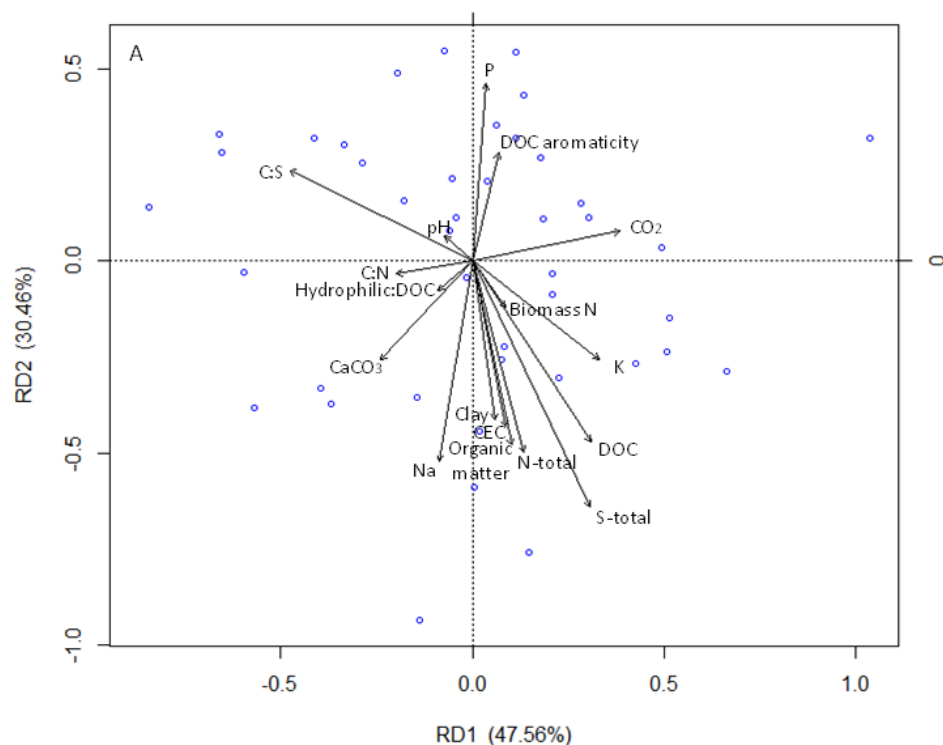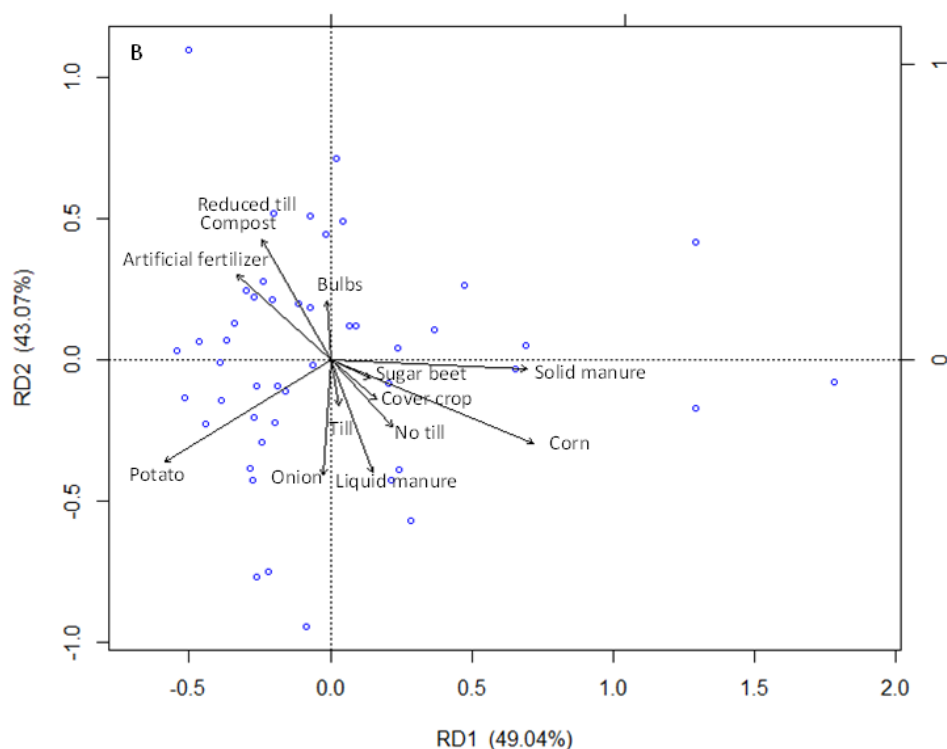
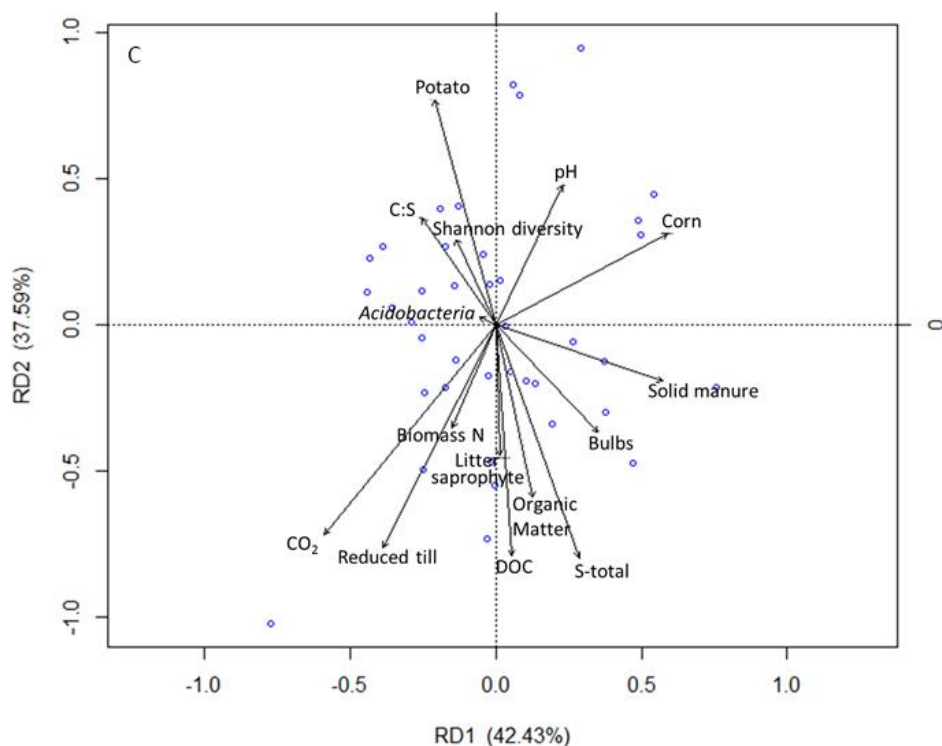**Figure S4C**: Redundancy analysis (RDA) plot of overall *in vitro* suppression of *Rhizoctonia solani, Fusarium oxysporum,* and *Pythium intermedium* (combined) by soil volatiles from 50 arable soils in relation to all significant (*P*<0.05) regression model parameters from soil properties, microbial community and field history datasets.

Chapter 6

General Discussion

Angela L. Straathof

6.1 Introduction

Dissolved organic carbon (DOC) has long been presumed to be a vital substrate for soil microorganisms (Haynes, 2005; Kalbitz et al., 2000). This thesis aimed to investigate the importance of DOC properties for various processes of the soil microbial community. These microbial processes included turnover of DOC fractions, both basal and substrate-induced microbial respiration rates, and production of pathogen-suppressing volatile organic compounds (VOCs), and were all measured as functions of the total soil microbial community. The soil microbial community is known to act collectively to suppress the proliferation of soil-borne plant pathogens, in a process known as general disease suppression (GDS). By determining the role of DOC in microbial processes linked to GDS, this thesis contributes knowledge that may aid in identifying of an indicator of a soil's GDS capacity. In this final chapter, I synthesize and contextualize the major findings of my experimental chapters (Chapters 2-5) with respect to the six thesis objectives described in Chapter 1. I then summarize some recommendations for future research opportunities, especially for considering DOC quality in the ongoing search for an indicator of GDS.

6.2 New insights into dissolved organic carbon

Before DOC and its inherent properties could be linked to soil microbial processes, this thesis had to consider the potential range of these properties. Therefore, I first had to assess the suitability of a rapid-batch fractionation procedure (Van Zomeren and Comans, 2007) for determining DOC characteristics among soils and organic amendments. The principles of this fractionation procedure have been previously applied to determine the bioavailability of some DOC fractions (Jandl and Sollins, 1997; Qualls and Haines, 1992) but this thesis relies on this procedure as the primary method of DOC characterization in all experimental chapters. The improvements of this

rapid-batch method over previous approaches to DOC fractionation (Aiken et al., 1985) make it appropriate to be used in experiments with a high-frequency of DOC sampling (Chapter 3) as well as with large sample sizes of organic matter (OM) and soil types (Chapters 2, and 4 and 5, respectively). I found the rapid-batch DOC fractionation procedure to be suitable for all of these applications; it is sensitive and sufficiently replicable to detect changes in DOC characteristics over time, and appropriate to conduct in combination with other measurements of DOC characteristics (e.g. aromaticity (Amery et al., 2008) (Chapters 3 and 4)). Application of this DOC fractionation method therefore allowed me to consider DOC fractions as they might vary in field soils, in soil after OM application, and as they might influence microbial processes.

6.2.1 Fractions of dissolved organic carbon and the influence of organic amendments

One of the difficulties in developing the experimental designs of this thesis was considering the wide range in DOC concentrations that has been reported in literature for both soils (Cook and Allan, 1992; Van Hees et al., 2005; Zsolnay, 1996) and organic amendments (Termorshuizen et al., 2006; Wei et al., 2014). Each experimental chapter confirmed these previously reported ranges in total DOC concentrations, and further, showed high variability in the DOC fraction profiles that was independent of total DOC concentration (Chapters 2 and 4). Fractions of DOC are known to vary in their different physical and chemical traits relative to one another (Amery et al., 2009). The range I initially observed proportions of hydrophilic vs hydrophobic fractions (Chapters 2 and 4) supported the hypothesis that previously reported discrepancies in DOC concentration and microbial processes (Neff and Asner, 2001) may be attributed to differences in DOC quality.

One of the earliest observations I made in this thesis was that the DOC fractions of organic amendments range widely (55-90% hydrophobic compounds) and independently of total DOC concentration. Concentrations of DOC fractions are driven by the conditions under which they are processed

(Straathof and Comans, 2015 (Chapter 2)). Amendments are often selected on the basis of nutrient status or OM content (Hargreaves et al., 2008), not on the basis of DOC content or quality. They are also often applied to inhibit pathogen outbreaks and support GDS (Termorshuizen et al., 2006), but there is currently no recommended physical or chemical properties for selecting a disease-suppressive amendment (Bonanomi et al., 2010). In Chapter 2, I propose that the proportions of DOC fractions measured may be valuable to consider when selecting a soil organic amendment, depending on the purposes of application (e.g. improving soil nutrient status and/or promoting GDS and/or influencing DOC quality). This led to the development of my second thesis objective: to measure the influence of amendment DOC on soil DOC.

In Chapter 3, I demonstrated that amendment DOC quality shifts soil DOC quality, but only in the short-term ($\leq$ 6 d) (Straathof et al., 2014). However, no significant effects were found when determining the influence of organic amendment type on DOC fractions in 42 agricultural soils (Chapter 4). Organic amendments had been applied to these soils more than 4 months before sampling. As such, the effects of amendment DOC on soil DOC in Chapters 3 and 4 appear time-dependent. Therefore, I am led to conclude, in agreement with Chantigny (2003), that the effects of DOC added to soil are immediate and significant, but short-lived. The legacy of these effects, however, may impact plant production throughout the growing season if they coincide with seedling emergence (Scheuerell et al., 2005). Therefore, it may be especially valuable to characterize the DOC quality of organic amendments applied close to the time of crop emergence, if producers want an indicator of how soil DOC properties (and the microbial processes linked to those properties) may be influenced. More experimentation is needed, however, to confirm the relevance of any influence on microbial processes for GDS and plant productivity.

6.2.2 Dissolved organic carbon as a substrate for soil microbial respiration

Two objectives of this thesis (Chapter 1) specifically aimed to determine how DOC quality indicators influence rates of soil microbial respiration. Chapters 3, 4, and 5 all present evidence that DOC quality is important for microbial activity. In Chapter 3, I found that by comparing basal respiration rates to respiration rates induced by substrates with different DOC fraction profiles, I could interpret the relative biological importance of the added fractions (Straathof et al., 2014). Applications of amendment DOC with higher proportions of hydrophilic (Hi) DOC fractions resulted in the highest substrate-induced respiration (SIR) rates. Concentrations of the Hi fraction also contributed the most to variation in respiration rates from all soil treatments in that experiment. This supported previous findings of high bioavailability of this fraction (De Troyer et al., 2011; Jandl and Sollins, 1997) but was not confirmed by the results of Chapter 4 when DOC fractions were model-input parameters for respiration rates among 46 soils. In that experiment, fulvic acid (FA) accounted for as much variation in respiration rates as the linear regression model that correlated total DOC and respiration rates (Chapter 4), suggesting that fulvic acids are also a biologically relevant fraction.

In order to explain the influence of isolated DOC fractions on microbial respiration rates more mechanistically, a follow-up experiment to Chapter 3 was conducted, which is not included in this thesis. Total DOC, or humic acid (HA), FA, or Hi fractions from the end of the rapid-batch fractionation method were added to soil and incubated 6 h in a SIR assay (Campbell et al., 2003). Significant SIR rates from the HA fraction were measured, but later deemed an artefact of the 0.1 M KOH solution used to re-suspend HA (Van Zomeren and Comans, 2007) and the 0.01 M HCl used the moderate the pH of that solution. Blank 0.1 M KOH + 0.01 M HCl, and 0.1 M KOH + 0.01 M HCl + 30 ppm glucose also released significantly higher SIR rates than additions of water, total DOC, FA or Hi to the soil. This is one methodological limitation of the DOC fractionation procedure, and of only measuring short-term SIR.

Background-solution effects of HA fractions may be minimized when SIR is measured in a longer-term incubations (i.e. several weeks), but this would not account for any shifts in the soil microbial community, which may adapt differently depending on added salt concentrations (Rousk et al., 2010). Measuring the effects of isolated fractions on microbial respiration therefore remains an important avenue of experimentation which may help explain the statistical significance of Hi and FA fractions identified in Chapters 3 and 4. Measuring turnover of an isolated HA fraction may also lead to mechanistic explanations for the observed depletion of HA concentration in Chapter 4. However, alternative methods of fraction-isolation should first be considered: these may include dialysis of fraction solutions to reduce the ionic strength (Canellas et al., 2010), or equilibration of the solutions with a cation-exchange resin (Schmidt et al., 2007).

6.2.3 Considering the relationship between hydrophilic and hydrophobic dissolved organic carbon

In all experiments where DOC quality was considered in the context of microbial activity, a link was found between the fractions of Hi and hydrophobic HA. The observation of HA depletion in Chapter 3 suggests that this fraction was either consumed or degraded by the soil microbial community. This seemed to correspond with relatively stable concentrations of the Hi fraction. Therefore, although I could not determine this experimentally, I proposed that HA constituents were fuelling microbial activity upon entering less hydrophobic pools. This possible explanation also offers an explanation for observations regarding FA: FA proportions in Chapter 3 remained stable while other fractions were preferentially depleted. The FA fraction was also as relevant as total DOC for statistically explaining variation in basal respiration rates in Chapter 4.

It has been recently proposed that HA molecules may act as carrier molecules of more labile functional moieties (Sutton and Sposito, 2005). These adsorbed compounds may desorb from HA molecules and thus be more

susceptible to microbial decomposition. Molecules desorbed from HA may then enter less hydrophobic pools (FA or HoN), as I postulate in Chapter 3, in a fragmentation pathway similar to that proposed by Leenheer and Croué (2003). In Chapter 4, I provide further support for this possible explanation by describing a statistically significant model with aromaticity of the Hi fraction and concentration of the HA fraction accounting for microbial activity variation. This is conceptually presented in Figure 6.1 which illustrates how shifts in microbial substrate consumption potentially occur under changing properties of the Hi fraction. This shift in substrate utilization offers a possible explanation for the observed depletion of HA even when a comparably-sized Hi fraction was in the soil solution (Chapter 3). It also potentially accounts for why aromaticity measurements alone did not explain high amounts of variation in microbial activity rates in Chapter 4. The nature of this feedback between hydrophobic and hydrophilic fractions would be interesting to illuminate in future research, because it may have implications for other microbial functions, as it would plausibly result in a shift of the microbial community as substrates of the native community are consumed.

It may be useful to reconsider fractions of DOC as a continuum of hydrophobicity, rather than in terms of aromaticity or bioavailability. Aromaticity does not appear to have a strong relationship with biodegradability (Chapter 4) and bioavailability is a somewhat subjective term because it depends on the composition of the microbial community. Reliance of the microbial community on low molecular weight (LMW) C (Boddy et al., 2007; Van Hees et al., 2005) may be determined by properties of other C compounds in solution, or by properties of LMW C other than their molecular weight. This thesis, in particular Chapters 3 and 4, also provides evidence for previous suggestions that FA and HoN fractions contain compounds that are intermediary between HA and Hi (Guggenberger et al., 1994). The measurement of C fluxes through these fractions in both directions may also provide further insight into how and why HA compounds supplement the Hi fraction.
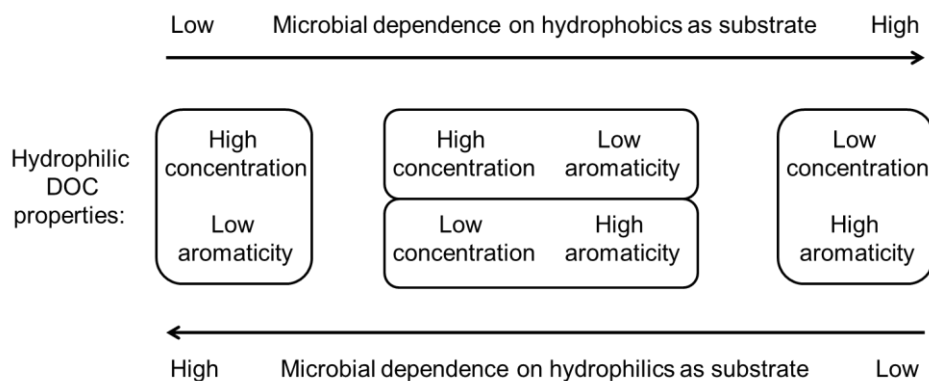
Figure 6.1: Conceptual diagram of dissolved organic carbon (DOC) hydrophilic fraction properties and the consumption of that fraction by the soil microbial community. Arrows indicate increasing or decreasing consumption rates; as microbial dependence on hydrophobic fractions for substrate increases, dependence on hydrophilic fractions decreases and vice versa. Properties may be "high" or "low" relative to other hydrophilics, and/or relative to hydrophobics.

6.2.4 A call to reconsider humic acids

This thesis demonstrated the biological relevance of the soluble HA fraction in Chapters 3 and 4. Although HA is typically described as recalcitrant (Kalbitz et al., 2000) and relatively non-biodegradable (Haynes, 2005) fraction, some studies have shown HA biodegradation (Boyer and Groffman, 1996) or short-term depletion (Ros et al., 2010) in soils. These studies, however, failed to provide a mechanistic explanation for this phenomenon. I propose here that the use of HA as a C substrate for soil microorganisms is linked to the prevalence and aromaticity of other DOC fractions (Figure 6.1). However, this remains to be established experimentally. Therefore, I recommend that future research on the biodegradability of HA aim to determine how these molecules decompose in the presence or absence of Hi compounds with different properties. The biggest limitation to exploring the mechanism underlying HA depletion may be a lack of means to trace products of HA decomposition through DOC solutions and/or into the microbial food web.

6.2.5 Dissolved organic carbon as a substrate for volatile production

Another soil microbial process measured in the context of DOC quality was the production of pathogen-suppressing volatiles by the soil microbial community (Chapter 5). This was done specifically to address my fifth thesis objective: to determine whether DOC quality effects on microbial activity rates (Chapters 3 and 4) were also reflected in suppression of soil-borne plant pathogenic biomass.

Volatiles have previously been identified as capable of suppressing pathogens *in vitro* (Weisskopf and Bailly, 2013; Garbeva et al., 2014; Van Agtmaal, unpublished (Figure 6.2)) and pathogen-suppression is a factor potentially contributing to GDS (Termorshuizen and Jeger, 2008). The results of Chapter 4 contribute to the growing body of evidence that VOCs may play an important role of suppressing soil-borne plant pathogens *in situ*. Hypothesis 1, which suggested that GDS is determined by soil microbial activity and volatile production, has been explored in more detail in the thesis *Suppression of Soil-Borne Plant Pathogens* (Van Agtmaal, 2015). Experiments conducted in *Suppression of Soil-Borne Plant Pathogens* explored in more detail whether soils producing pathogen-suppressing volatiles also had lower incidences of diseased plants. The results of a combined volatile-assay and bioassay measuring the inhibition of *P. intermedium* are presented in Figure 6.2. Two soils inoculated with *P. intermedium* produced hyacinth bulb biomass not significantly different from the uninoculated control soils (Figure 6.2a; untreated soils and peat-amended soils). These same two soils also produced VOCs that significantly reduced the biomass of *P. intermedium* grown on agar exposed to these volatiles (Figure 6.2c). This observation of soils producing pathogen-suppressing volatiles *and* inhibiting disease manifestation (Figure 6.2) suggests that VOCs are potentially important components of GDS. The statistical relationship described in Chapter 4 between DOC and overall pathogen-suppression by volatiles therefore supports the hypothesis that DOC may play a role in disease suppression, via volatile production, although a direct link remains hypothetical.
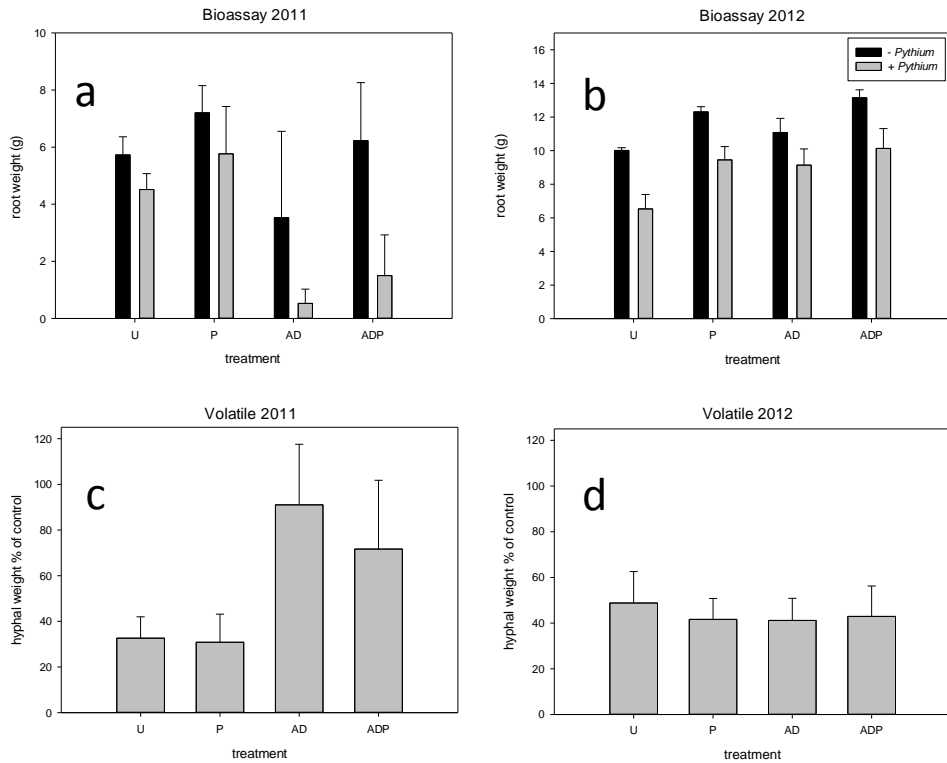
Figure 6.2: Root biomass of Hyacinth bulbs after a bioassay with *Pythium intermedium* inoculation, and hyphal biomass production of *P. intermedium* exposed to soil volatiles *in situ*. Top row shows average root biomass of Hyacinth bulbs grown in differently managed soils (U = untreated, P = peat addition, AD = anaerobic disinfestation; soil treatments applied in 2011 only) with and without *P. intermedium* addition in 2011 (a) and 2012 (b). Bottom row shows average hyphal weight of *P. intermedium* that had been exposed to volatiles produced from differently managed soils in 2011 (c) and 2012 (d). *P. intermedium* biomass is presented as a percentage of the biomass produced in a soil-free control. Error bars represent standard deviation.

This figure (unpublished) is courtesy of M. van Agtmaal, modified from the thesis *Suppression of Soil-Borne Plant Pathogens* (2015).

Each of the three hypotheses put forward in the project *Predicting Disease Suppression of Agricultural Soils* (Chapter 1) has been tested in this thesis. Properties that have been identified as potential indicators of a soil's capacity for GDS include microbial biomass, microbial activity, and pathogen-suppression (Bonanomi et al., 2010; Janvier et al., 2007; Termorshuizen and Jeger, 2008). This thesis has statistically linked microbial activity to DOC quality, and production of pathogen-suppressing volatiles to DOC concentration. Therefore, I hypothesize that, with further research, mechanistic links can still be identified between DOC quality and GDS.

6.3 On Future Research Considerations

This thesis has sequentially linked organic amendment DOC quality to soil DOC quality, and soil DOC quality to microbial activity and volatile-induced pathogen suppression *in vitro*. To close the gap that remains in application of this knowledge, it would be useful to investigate such effects *in situ*. Long-term field experiments should be used to validate the results of this thesis under more agronomically-relevant conditions. These long-term experiments would be most useful in combination with bioassays for investigating the qualities of organic amendments that best promote GDS in agricultural soils.

Many results of this thesis indicate a statistical relationship between DOC properties and soil microbial processes. In particular, statistical evidence for the role of DOC in volatile production, and the role of volatile production in disease suppression, lends support to the hypothesis that DOC may yet prove significant in identifying an indicator GDS. These statistics are useful in establishing that DOC characteristics and processes are linked, and in inspiring hypotheses for causal mechanisms. However, these causal mechanisms must be identified via future experiments before DOC quality can be used as a tool to manipulate these processes, or before management recommendations to promote GDS can be made.

Furthermore, based on the results of this thesis's experimental chapters, I make the following recommendations and considerations for the field of DOC research:

1) The biological relevance of hydrophobic DOC fractions, especially HA, is currently undervalued. In order to understand the decomposition of these compounds, and the role of recalcitrance in organic-C bioavailability, future experiments should quantify HA DOC concentrations and determine how these change over time in parallel with microbial activity measurements.

2) Stable-isotope labelling of compounds in DOC solutions via ^{13}C and stable-isotope probing of the soil microbial community would allow for tracing of substrate fluxes and uptake. This is necessary to confirm the flow of compounds between hydrophobic and hydrophilic fractions, and to determine the consumption rates of these compounds by microorganisms. This technique would provide a means of mechanistically determining microbial substrate consumption, however:

3) ^{13}C labelling of aromatic compounds such as HA presents challenges because of their degree of degradation. Perhaps for tracing these compounds, natural abundance of isotopes may be a more appropriate approach.

4) Manipulation of soil DOC quality (e.g. via organic amendment application) is recommended in combination with measuring soil microbial community shifts. This would confirm if a shift in microbial consumption from hydrophilics to hydrophobics (or vice versa (Figure 6.1)) is a result of community composition shifts, or substrate-use shifts, or both.

5) A statistical relationship has been identified between DOC, microbial respiration, and production of pathogen-suppressing volatiles. However, it remains unknown if this effect is the result of an absolute increase in volatile production, or an influence on the types of volatiles produced. Therefore, identifying volatile profiles and/or volatile compounds produced when different qualities of DOC are used as substrate would determine the specific mechanism of this effect.

6) Experiments in the form of bioassays and field-trials must be conducted with labelled ^{13}C compounds in order to discover the fundamental

link between organic amendment management, soil DOC properties, and *in situ* GDS of soil-borne plant pathogens.

Conclusions

This thesis has demonstrated the importance of not only total DOC concentration, but also the quality of DOC fractions for various microbial processes, including respiration, and production of pathogen-suppressing volatiles. I have demonstrated the biological relevance of DOC fractions, particularly humic acids, which were previously dismissed as too recalcitrant to be considered bioavailable. This thesis, therefore, provides support for the value of DOC fractionation in supplement to measurements of total DOC concentration. Whether or not an experimental design makes use of DOC fractionation (or other methods of DOC qualification) will always depend on the experimental objectives. However, I strongly encourage researchers to consider the value of soil DOC quality in the context of their research questions. The results of this thesis also contribute evidence to support the hypothesis that DOC and organic amendment quality are relevant for identifying an indicator of GDS of soil-borne plant pathogens.

I am confident in speculating that the next frontier of soil science lies in being able to mechanistically link the quality of DOC, and soil organic matter in general, to the performance of soil organisms, including microbial processes beyond those investigated in this thesis. The implications of thesis extend beyond soil science and advance our fundamental understanding of microbial C-cycling.

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Summary

An important form of C in agricultural soils is organic C, which is presumed to be relatively bioavailable in dissolved form (DOC). Soil microorganisms consume DOC to fuel various metabolic processes, and the rate of consumption, or the nature of these processes, may be influenced by the quality of DOC available. Since DOC is a heterogeneous mixture of compounds varying in hydrophobicity and aromaticity, some fractions of DOC are potentially more relevant for microbial processes than others. These processes may include microbial activity (which decomposes organic C and releases CO_2) and production of volatile organic compounds (VOCs). Both of these processes have been linked in previous research to general disease suppression (GDS), which is an important natural aspect of soils that protects crops from soil-borne plant pathogen disease infestation. It therefore stands to reason that DOC quality, via an influence on microbial function, may contribute to identifying an indicator of GDS, which would be very valuable for crop producers.

This thesis was motivated by a lack of scientific evidence to identify the DOC properties that are most important for soil microbial processes. Chapter 1 provides an overview of previous research framed in support of the hypotheses that organic matter quality may influence DOC quality, and that DOC quality may in turn influence soil microbial activities. By linking properties of DOC to indicators of microbial processes in both controlled experiments and statistically explorative studies, this thesis aimed to provide evidence for the role of DOC quality in these processes, and support the hypothesis that DOC is an important component of GDS in agricultural soils. This was done in a series of four experiments presented as four thesis chapters.

Chapter 2's objective was to determine how DOC quality differed among organic amendments and if these differences were the result of the processing conditions of those amendments. Eleven composts were processed under various methods, and were also made of many different input materials. This experiment used DOC fractionation to determine concentrations and proportions of hydrophobic (humic acid (HA), fulvic acid (FA), hydrophobic

neutral (HoN)) and hydrophilic (Hi) compounds in each compost. Duration and temperature of composting were the most important aspects of processing: these treatments negatively corresponded with Hi, suggesting preferential turnover of this fraction during the thermophilic composting phase. The results of this experiment led to the development of the hypothesis that the ranges measured in amendment DOC quality may subsequently affect soil DOC quality and microbial activity rates when added to soil.

To test this hypothesis, an experiment was designed in which DOC extracted from composts was added to soils at the same concentration, but with different ratios of hydrophobic:hydrophilic compounds. This experiment is presented in Chapter 3. High-Hi treated soils had the highest respiration rates, but only up to 6 days after DOC addition. Linear-regression modelling identified the Hi fraction as having the highest coefficient explaining variation in respiration rates. An important peripheral observation reported in this chapter is the depletion of the HA fraction. Because HA is generally considered an aromatic, recalcitrant fraction, the evidence of this fraction being decomposed (by as much as one-third) over a period of 35 d suggests that HA is more biologically relevant than previously assumed.

In the experimental work preceding Chapter 4, this thesis characterizes the DOC of only one soil type. Therefore, to determine how DOC properties may differ among soil types, and may be influenced by field-management practices, 50 agricultural soils were characterized. Concentrations of soil DOC fractions, (fractions') aromaticity and basal respiration were measured and statistically analysed. Neither tillage nor organic matter applications significantly influenced DOC properties; the fact that these management treatments had been applied the previous growing season suggests their effects are short-lived. This observation agrees with the results of Chapter 3 (<6 d of significant treatment effects of compost DOC). Another result of Chapter 3 supported by Chapter 4's soil survey is the potential biological relevance of the HA fraction. This fraction, along with the aromaticity of Hi, accounted for 33% of the variation measured in basal respiration rates. Soils with lower proportions of Hi had the highest aromaticity of Hi, but not necessarily lower respiration

rates. This observation led to the hypothesis that as Hi substrates decrease in bioavailability, substrate utilization of the more hydrophobic fractions increases.

While microbial activity rates in Chapters 3 and 4 could be linked to DOC quality, microbial activity is just one potential aspect of GDS in soils. Another aspect is the microbial community's potential to suppress pathogens via VOCs, which has been previously reported in the literature. Therefore, the objective of Chapter 5 was to determine if volatiles from the 50 soils sampled were able to suppress pathogens *in vitro*, and if so, to statistically explore the relationship between suppression and other soil properties, including DOC. The measurements of pathogen-suppression by volatiles were done in combination with DOC fraction and aromaticity measurements, 454-pyrosequencing of the total soil microbial community, and extensive analysis of soil chemical properties. The results of Chapter 5 are that different soil properties account for variation among different pathogens. However, these properties were generally indicators of collective microbial function, as opposed to specific groups of organisms. When overall pathogen suppression was modelled, activity rates and total DOC concentrations were the significant soil properties contributing to variation.

The statistical significance of activity and DOC in relation with pathogen suppression are important for supporting and developing hypotheses for links with GDS, but causal relationships can only be identified through experiments investigating mechanisms of GDS. These are the types of recommendations made for future research in Chapter 6, along with the use of isotopic labelling techniques to trace soil organic C decomposition through DOC fractions and into the microbial community. Chapter 6 emphasises the novelty and value of this thesis' results in supporting the importance of DOC quality for microbial function, particularly the undervalued role of hydrophobic DOC. The potential for characteristics of DOC to be identified as GDS indicators is still possible, and this thesis concludes by encouraging future research into this relationship.

Samenvatting

Organische koolstof (C) is de meest voorkomende vorm van C in landbouwgronden. Een deel ervan is opgelost in het bodemvocht (DOC, *dissolved organic C*). Algemeen wordt aangenomen dat micro-organismen in de bodem deze opgeloste fractie goed kunnen omzetten. De manier waarop en de snelheid waarmee DOC wordt omgezet, hangen waarschijnlijk af van de samenstelling van DOC. DOC is namelijk een mengsel van verbindingen die variëren in hydrofobie en aromaticiteit en daarmee waarschijnlijk in afbreekbaarheid door microorganismen. Bij de afbraak kunnen naast CO_2 ook vluchtige organische verbindingen (*volatile organic compounds*; VOCs) worden geproduceerd. Zowel CO_2, als de productie van VOCs zijn in eerder onderzoek gerelateerd aan algemene ziektewerendheid van de bodems (*general disease suppression*; GDS). GDS is een belangrijke natuurlijke bodemeigenschap, die aangeeft in welke mate gewassen beschermd zijn tegen bodempathogenen. Via haar invloed op het microbieel functioneren van de bodem zou de samenstelling van DOC een waardevolle indicator voor GDS kunnen zijn.

Aanleiding voor het onderzoek in dit proefschrift, was het gebrek aan kennis over de deeigenschappen van DOC die belangrijk zijn voor bodemmicrobiologische processen. Hoofdstuk 1 geeft een overzicht van de bestaande kennis over de relatie tussen organische stof in de bodem en DOC. Dit overzicht ondersteunt de hypothese dat de kwaliteit van DOC is gerelateerd aan de kwaliteit van organische stof en de activiteit van micro-organismen in de bodem. De overige vier hoofdstukken in dit proefschrift beschrijven het onderzoek van deze hypothese in van vier experimenten. Een deel van deze hoofstukken beschrijft experimenten die zijn uitgevoerd onder gecontroleerde omstandigheden.Een ander deel beschrijft statistische verkenningen van gegevens over grondmonsters uit akkers van verschillende boerenbedrijven.

Het doel van hoofdstuk 2 was om vast te stellen of organische meststoffen, met name composten, verschillen in kwaliteit van DOC. en of deze verschillen te maken hebben met de omstandigheden waaronder de composten zijn geproduceerd. Elf verschillende composten werden onderzocht die werden

geproduceerd uit verschillende uitgangsmaterialen. Behalve de concentratie is ook de samenstelling van DOC in de composten bepaald door deze te fractioneren in hydrofobe zuren (humuszuren en fulvozuren), neutrale verbindingen en hydrofiele verbindingen. De samenstelling van DOC bleek in belangrijke mate bepaald door de duur van en temperatuur tijdens de compostering. Hoe langer de compostering en hoe hoger de temperatuur, hoe kleiner de fractie hydrofiele verbindingen. Dit duidt op een selectieve omzetting van deze verbindingen tijdens de thermofiele fase van de compostering. Deze variatie in DOC tussen composten gaf aanleiding te veronderstellen dat verschillende composten na toediening uiteenlopende effecten op de DOC van de bodem zouden kunnen hebben.

Om deze veronderstelling te toetsen, werd een experiment opgezet waarin DOC uit composten in een vaste concentratie, maar variërende verhouding hydrofobe/hydrofiele verbindingen, werd gemengd met een grondmonster. Dit experiment is beschreven in hoofdstuk 3. Behandelingen met hogere concentraties hydrofiele verbindingen hadden de eerste zes dagen na toediening de hoogste respiratiesnelheid. Een belangrijke observatie was bovendien dat de humuszuurfractie afnam na toediening aan de grond, terwijl algemeen wordt aangenomen dat deze fractie slecht afbreekbaar is vanwege haar aromaticiteit. De waarneming dat maar liefst een derde van de humuszuurfractie werd afgebroken binnen 35 dagen, wat suggereert dat humuszuur biologisch belangrijker is dan tot nu toe werd gedacht.

In hoofdstuk 4 staat beschreven hoe DOC uit 50 verschillende grondmonsters van akkerbouwbedrijven werd geanalyseerd. Het doel was om vast te stellen of de samenstelling van DOC varieert tussen bodemtypen en of deze afhangt van het type bodembeheer. De concentratie en aromaticiteit van verschillende componenten van DOC, alsmede de bodemrespiratie werden gemeten en de resultaten werden statistisch geanalyseerd. Noch grondbewerking, noch toediening van organische stof, bleken variatie in DOC eigenschappen te verklaren, ondanks dat deze in het vorige groeiseizoen waren toegediend. Dit suggereert dat , zelfs als deze beheersmaatregelen al effect hebben gehad, dan ditvan korte duur geweest is. Dit is in overeenstemming met

de resultaten van hoofdstuk 3, waarin blijkt dat de toediening van compost na 6 dagen ook geen effect meer heeft op bodemrespiratie. Een ander resultaat uit hoofdstuk 3 werd ook bevestigd: humuszuur blijkt inderdaad mogelijk een biologisch relevante component van DOC te zijn. Een derde van de variatie in bodemrespiratie bleek te verklaren te zijn door de grootte van de humuszuurfractie en de aromaticiteit van de hydrofiele fractie,. In grondmonsters met een kleinere hydrofiele fractie bleek deze aromatischer te zijn. Echter, de bodemrespiratie in deze monsters was niet *per se* lager. Deze genoemde statistische verbanden leidden tot de hypothese dat de hydrofobe componenten in toenemende mate biologisch worden afgebroken naarmate de beschikbaarheid van de hydrofiele substraten afneemt.

In de hoofdstukken 3 en 4 is weliswaar aangetoond dat er een relatie bestaat tussen DOC en metabolische activiteit van de microbiële gemeenschap . Echter, microbiële activiteit is slechts één mogelijk aspect van GDS. Een ander aspect is het vermogen om pathogenen te onderdrukken d.m.v. VOCs. Het doel van hoofdstuk 5 was om vast te stellen of VOCs die werden geproduceerd door de 50 grondmonsters van akkerbouwbedrijven, *in vitro* pathogene schimmels konden onderdrukken en aan of deze statistisch gerelateerd kon worden aan bepaaldebodemeigenschappen. De onderdrukking van pathogenen door VOCs werd gemeten in combinatie met analyse van de samenstelling van DOC alsmede een groot aantal andere bodemchemische parameters en een analyse van de samenstelling van de bodemmicrobiële gemeenschap middels 454-*pyrosequencing*. Voor elk van de drie pathogene schimmels verklaarde een andere combinatie van bodemeigenschappen de variatie in onderdrukking. Deze bodemeigenschappen waren indicatoren voor algemene microbiële activiteit. Aanwezigheid van bepaalde microbiële groepen correleerde echter niet met onderdrukking van de pathogenen. Wanneer de onderdrukking tegen alle drie de geteste pathogenen werd samengenomen, bleken de microbiële activiteit van de en de totale DOC concentratie significante verklarende factoren voor onderdrukking van bodempathogenen *in vitro*.

Deze laatste bevinding inspireert tot het formuleren van nieuwe hypotheses over de relatie tussen microbiële activiteit en DOC enerzijds en GDS

anderzijds, waarbij causale verbanden uiteraard alleen kunnen worden vastgesteld door de mechanismen van GDS te onderzoeken. Hiervoor worden aanbevelingen gedaan in hoofdstuk 6. Ook zou de rol van de verschillende fracties van DOC als intermediair in de afbraak van organisch C in de bodem onderzocht kunnen worden met behulp van koolstofisotopen. Hoofdstuk 6 benadrukt bovendien de tot nu toe ondergewaardeerde rol van de hydrofobe fracties van DOC als substraat voor microbiële activiteit in de bodem. Het hoofdstuk besluit met een aanmoediging tot nader onderzoek naar DOC als indicator voor GDS.

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In addition to my supervisors in Soil Quality, I had a fantastic team of collaborators on our STW project. Prof. Dr Wietse de Boer of the Netherlands Institute of Ecology (NIOO) always had an important microbial-ecological perspective to contribute, and really promoted an inquisitive atmosphere in our meetings, with lots of fundamental questions, which I think was very valuable to the project. To complement (counter?) this perspective, was Dr Aad

Termorshuizen of BLGG AgroXpertus (later SoilCares Research). Aad had an incredible knack for bringing any far-fetching and fundamental research questions back to the task at hand: how can we help *farmers*? What does this mean *in the field*? What has this to do with *GDS*? Together, Rob, Ellis, Wietse, and Aad made a great leadership team, with important scientific contributions to this thesis, and to the realization of project milestones. The input from our STW Users Committee members was invaluable: very special thanks to Gera van Os, Jan van Aartrijk, Michiel Rutgers, Bram Hanse, STW program officer Henry van der Valk, and project management assistant Quirine Ruis. The experience of working on an STW project was especially rewarding because of the consistent and helpful feedback I received from my keen and insightful Users Committee. Further expertise was provided to my PhD thesis by Mark van Iersel of Van Iersel Compost, Albert Dortmans of Orgaworld, Joeke Postma of Plant Research International, and Gerard Korthals of the Centre for Soil Ecology. Each of their contributions is very much appreciated.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)
- Dissolved organic carbon cycling in agricultural soils (2011)

Writing of project proposal
- Predicting disease suppressiveness of agricultural soils (2011)

Post-graduate courses (7.5 ECTS)
- Molecular advances in ecology; PE&RC (2011)
- Soil, biodiversity and life; PE&RC (2012)
- Meta-analysis; PE&RC (2012)
- Dynamics of organic matter in soil; University of Copenhagen (2013)

Laboratory training and working visits (2 ECTS)
- Dissolved organic carbon fractionation method; ECN (Energy Research Centre of the Netherlands) (2011)

Invited review of (unpublished) journal manuscript (2 ECTS)
- Water: bacterial transport, organic waste (2014)
- Applied Microbiology and Biotechnology: microbial diversity, organic waste (2015)

Competence strengthening / skills courses (3.1 ECTS)
- Competence assessment; WGS (2011)
- Reviewing a scientific paper; WGS (2011)
- Techniques for writing and presenting a scientific paper; WGSD (2014)
- Career orientation; WGS (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)
- PE&RC PhD Introductory weekend retreat (2011)
- PE&RC Last phase weekend retreat (2014)
- WGS PhD Workshop carousel (2014)

Discussion groups / local seminars / other scientific meetings (5.4 ECTS)
- NIOO-wide seminars and microbial ecology seminars (2011-2014)

- KNPV Working group Soilborne plant pathogens and soil microbiology (2012-2014)
- Centre for soil ecology on tour meetings (2013-2014)
- Netherlands Annual Ecology Meeting (2014)
- Workshop: tips and tricks for successful grant application (NIOO) (2014)
- Workshop: bacterial volatiles (NIOO) (2014)

International symposia, workshops and conferences (8.5 ECTS)
- Ecology of Soil Microorganisms; poster presentation; Prague, Czech Republic (2011)
- Wageningen Soil Conference; poster presentation; Wageningen, the Netherlands (2011)
- Biological Control of Fungal and Bacterial Plant Pathogens-Meeting of the International Organization for Biological and Integrated Control (IOBC); poster presentation; Reims, France (2012)
- European Geoscience Union General Assembly; oral presentation; Vienna, Austria (2013)

Lecturing / supervision of practicals / tutorials (2.1 ECTS)
- The carbon dilemma (2012-2013)
- Soil-plant interactions (2013-2014)

Supervision of MSc students
- R. Chincarini: biological relevance of operationally defined dissolved organic carbon fractions
- L. Bin: dissolved organic carbon dynamics in the rhizosphere

Curriculum Vitae

Angela Lynsey Straathof was born on the 29th of November, 1985, in Arnprior, Ontario, Canada. Growing up on her family's dairy farm fostered Angela's interest in both agricultural and environmental sciences. In September 2004, she began her bachelor's degree in Environment and Resource Studies, Honours Co-op (minor Physical Geography), at the University of Waterloo. Angela went on to complete a Master's degree in Land Resource Science at the University of Guelph, from 2008-2010. During this time she also served as Chair of the School of Environmental Sciences Graduate Student Association. Her MSc thesis, *The effect of mycorrhizal inoculation on trace gas flux of N_2O and bacterial communities of different soil types,* developed her passion for the topic of soil biochemical cycling, and the relevance of the soil microbial community. In January 2011, Angela moved to The Netherlands, and joined the Soil Quality group of Wageningen UR to complete her PhD. Her PhD research was conducted under the project *Predicting Disease Suppressiveness of Agricultural Soils*, funded by STW. In addition to completion of this thesis, Angela collaborated on peer-reviewed publications with colleagues at the Netherlands Institute of Ecology, Institut de Physique du Globe de Paris, and Wageningen UR's departments of Soil Chemistry and Soil Biology. Angela is currently based at the University of Manchester, England, where in May 2015 she began a post-doctoral research position in the Soil and Ecosystems Ecology group. Angela really, really loves soil.