Bacterial diversity and denitrifier communities in arable soils

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Agricultural management is essential for achieving optimum crop production and maintaining soil quality. Soil microorganisms are responsible for nutrient cycling and are an important consideration for effective soil management. The overall goal of the present research was to better understand microbial communities in agricultural soils as they relate to soil management practices. For this, we evaluated the differential impact of two contrasting drainage practices on microbial community composition and characterized active denitrifiers from selected agricultural sites.

Field drainage is important for crop growth in arable soils. Controlled and uncontrolled tile drainage practices maintain water in the field or fully drain it, respectively. Because soil water content influences nutrient concentration, moisture, and oxygen availability, the effects of these two disparate practices on microbial community composition was compared in paired fields that had diverse land management histories. Libraries of the 16S rRNA gene were generated from DNA from 168 soil samples collected from eight fields during the 2012 growing season. Paired-end sequencing using next-generation sequencing was followed by read assembly and multivariate statistical analyses. Results showed that drainage practice exerted no measureable effect on the bacterial communities. However, bacterial communities were impacted by plant cultivar and applied fertilizer, in addition to sampled soil depth. Indicator species were only recovered for depth; plant cultivar or applied fertilizer type had no strong and specific indicator species. Among indicator species for soil depth (30-90 cm) were *Chloroflexi* (*Anaerolineae*), *Betaproteobacteria* (*Janthinobacterium*, *Herminiimonas*, *Rhodoferax*, *Polaromonas*), *Deltaproteobacteria* (*Anaeromyxobacter*, *Geobacter*), *Alphaproteobacteria* (*Novosphingobium*, *Rhodobacter*), and *Actinobacteria* (*Promicromonospora*).

Denitrification in agricultural fields transforms nitrogen applied as fertilizer, reduces crop production, and emits N_2O, which is a potent greenhouse gas. Agriculture is the highest anthropogenic source of N_2O, which underlines the importance of understanding the microbiology of denitrification for reducing greenhouse gas emissions by altered management practices. Existing denitrifier probes and primers are biased due to their development based mostly on sequence information from cultured denitrifiers. To circumvent this limitation, this study investigated active and uncultivated denitrifiers from two agricultural sites in Ottawa, Ontario. Using DNA stable-isotope probing, we enriched nucleic acids from active soil denitrifiers by exposing intact replicate soil cores to NO_3^- and $^{13}C_6$-glucose under anoxic conditions using flow-through reactors, with parallel

native substrate controls. Spectrophotometric chemistry assays and gas chromatography confirmed active NO_3^- depletion and N_2O production, respectively. Duplicate flow-through reactors were sacrificed after one and four week incubation periods to assess temporal changes due to food web dynamics. Soil DNA was extracted and processed by density gradient ultracentrifugation, followed by fractionation to separate DNA contributed by active denitrifiers (i.e., "heavy" DNA) from that of the background community (i.e., "light" DNA). Light and heavy DNA samples were analyzed by paired-end sequencing of 16S rRNA genes using next-generation sequencing. Multivariate statistics of assembled 16S rRNA genes confirmed unique taxonomic representation in heavy fractions from flow-through reactors fed $^{13}C_6$-glucose, which exceeded any site-specific or temporal shifts in putative denitrifiers. Based on high relative abundance in heavy DNA, labelled taxa affiliated with the *Betaproteobacteria* (71%; *Janthinobacterium*, *Acidovorax*, *Azoarcus*, *Dechloromonas*), *Alphaproteobacteria* (8%; *Rhizobium*), *Gammaproteobacteria* (4%; *Pseudomonas*), and *Actinobacteria* (4%; *Streptomycetaceae*). Metagenomic DNA from the original soil and recovered heavy fractions were subjected to next-generation sequencing and the results demonstrated enrichment of denitrification genes with taxonomic affiliations to *Brucella*, *Ralstonia*, and *Chromobacterium* in heavy fractions of flow-through reactors fed $^{13}C_6$-glucose. The vast majority of heavy-DNA-associated nitrite-reductase reads annotated to the copper-containing form (*nirK*), rather than the heme-containing enzyme (*nirS*). Analysis of recovered *nirK* genes demonstrated low sequence identity across common primer-binding sites used for the detection and quantification of soil denitrifiers, indicating that these active denitrifiers would not have been detected in molecular surveys of these same soils.

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%	percent
°C	degrees Celsius
µg	microgram
µM	micromolar
anammox	anaerobic ammonium oxidation
ANOVA	analysis of variance
AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
ATP	adenosine triphosphate
AXIOME	automation, extension, and integration of microbial ecology
BLAST	basic local alignment search tool
BMP	beneficial management practices
bp	base pair
Br^-	bromide
C	carbon
$C_6H_{12}O_6$	glucose
CD-HIT	cluster database at high identity with tolerance
cm	centimeter
CO_2	carbon dioxide
CsCl	cesium chloride
CTD	controlled tile drainage
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DNA-SIP	DNA stable-isotope probing
DNRA	dissimilatory nitrate reduction to ammonium
ECD	electron capture detector
EDTA	ethylenediaminetetraacetic acid
FID	flame ionization detector
FISH	fluorescent in situ hybridization
FTR	flow-through reactor
g	gram
G	gauge
GC	gas chromatography
GHG	greenhouse gas
h	hour
H^+	proton
H_2O	water
ha	hectare
HCl	hydrogen chloride
HCO_3^-	bicarbonate
He	helium
Kb	kilo base
KBr	potassium bromide
KEGG	Kyoto encyclopedia of genes and genomes
KNO_3	potassium nitrate
L	liter
LPA	linear polyacrylamide
M	molar
MEGA	molecular evolutionary genetics analysis
mg	milligram
MG-RAST	metagenomics rapid annotation using subsystem technology server
mL	milliliter
mM	millimolar
mm	millimeter
MOB	methane oxidizing bacteria
MRPP	multi-response permutation procedure
N	nitrogen
N	normal
N_2	dinitrogen gas
N_2O	nitrous oxide
nA	nanoampere
Na_2SO_3	sodium sulfite
NaCl	sodium chloride

$NaHCO_3$	sodium bicarbonate
$NaNO_2$	sodium nitrite
NaOH	sodium hydroxide
NEED	*N*-1-(naphthyl)ethylendiamine
ng	nanogram
NH_3	ammonia
NH_4^+	ammonium
NH_4Cl	ammonium chloride
NH_4NO_3	ammonium nitrate
Ni	nickel
nm	nanometer
nM	nanomolar
NMS	nonmetric multidimensional scaling
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
O_2	oxygen
OD	outer diameter
OPA	ortho-phthaldialdehyde
OTU	operational taxonomic unit
PANDAseq	paired-end assembler for DNA sequences
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
pH	power of hydrogen
pM	picomolar
PyNAST	python nearest alignment space termination tool
PPMCC	Pearson product-moment correlation coefficient
QIIME	quantitative insights into microbial ecology
qPCR	quantitative polymerase chain reaction
RDP	ribosomal database project
RNA	ribonucleic acid
s	second
SS	stainless steel
SULF	sulfanilamide
TAE	tris-acetate EDTA
TE	tris EDTA
Tg	teragram
T-RFLP	terminal restriction fragment length polymorphism
U	units of activity
UCTD	uncontrolled tile drainage
UPGMA	unweighted pair group method with arithmetic mean
v	volume
V3	16S rRNA gene variable region 3
V4	16S rRNA gene variable region 4
VCl_3	vanadium trichloride
w	weight
WFPS	water-filled pore space
WWTP	waste water treatment plant
yr	year

Chapter 1
Introduction

1.1 Soil microbial communities

1.1.1 Soil as a microbial and functional reservoir

Soil is heavily populated by microorganisms, with an estimated prokaryotic density of 10^9 cells g^{-1} in the top meter and 10^8 cells g^{-1} from 1 to 8 meters depth [1]. The majority of this microbial biomass represents uncharacterized diversity and "within the soil microbial population, there is a wealth of genetic information waiting to be discovered" [2]. Soil microorganisms provide indispensable services, including enabling crop production through agriculture, sustainability of animal productivity, and air and water quality regulation. These services depend on land management practices ensuring the maintenance of soil health, which is understood as the functioning of the underlying processes that maintain the capacity of soil to support life [3]. The microbiological component of soil is responsible for originating and consuming nutrients during biogeochemical cycling, which regenerates and maintains soil quality [4]. Organic matter degradation [5], pest control [6], and soil structure maintenance [7] are other examples of microbial life-supporting processes in soil.

1.1.2 Describing soil microbial communities

High demand of food production, extensive land management, and land-usage changes can lead to an unbalanced system that deteriorates soil. Moreover, industrialization and use of chemicals for crop production and pest management in agriculture contaminate soils and have the potential to affect the microbial communities that maintain ecosystem functioning [8]. Understanding microbial community composition is a necessary prerequisite for effective soil management to ensure that sustainable soil functionality is preserved.

Microbial communities can be described in terms of their taxonomic diversity, which encompasses measures of richness and evenness. Richness refers to the total number of species and evenness to the abundance distributions of individual species [2]. For prokaryotes, "species" are usually defined as operational taxonomic units (OTUs), which are sequences grouped by phylogenetic similarity and are used *in lieu* of the traditional species concept [9]. Measuring microbial diversity is an important

component of assessing temporal and spatial changes in community structure or function [10]. Using diversity indices as measurements of biodiversity, alongside functional assessments, is necessary to understand the importance of diversity in relation to community function. Community function has been evaluated with process level measurements that estimate microbial activity in soil. Examples of process level measurements include the dehydrogenase enzyme activity assessment of soil microbial respiration capacity [11] and the hydrolysis of fluorescein diacetate by non-specific enzymes related to microbial catabolic activity [12]. Also, metabolic fingerprints of communities can be obtained with commercial products like the Ecoplate (Biolog), which uses a suite of carbon sources to test the carbon metabolism capacity of the community [13].

Process-level measurements have been used to evaluate the community stability (i.e., resistance and resilience) as a function of taxonomic and functional diversity. For example, soil communities with reduced catabolic diversity, due to agricultural land usage, were more affected by environmental stresses (e.g., pH, salinity, heavy metals, temperature, and water disturbance) than pasture soils with a greater catabolic diversity [14]. Although the authors could not exclude the potential influence of soil physicochemical properties in the greater stability of one population over the other, they concluded that catabolic diversity was an indicator of community stability. On the other hand, inoculating differentially diverse communities into sterile soils demonstrated no correlation between diversity reduction and soil functioning based on measurements of DNA and protein activity, nitrification capacity, aerobic respiration, decomposition capacity, and resistance to heat and copper stresses [15]. However, the ability to detect altered functions at the process level depends on the monitored process [15,16] and it has been suggested that these methods do not identify changes in the community diversity due to microbial functional redundancy [2]. Nonetheless, these studies help clarify the functional role of microbial communities in soil ecosystems.

1.1.3 Factors affecting soil microbial communities

Soil microorganisms are governed by edaphic factors like salinity [17], pH [18], soil nutritional status [19], oxygen tension, and soil structure [20]. Human activities also affect soil communities and the effect of land management has been documented [19,21].

Salinity was identified as the major driver of microbial community composition based on an analysis of 202 environmental samples that included soil, sediment, and water from a wide range of

temperatures, salinity levels, pH, and nutrient content [17]. Substrate type (i.e., soil, sediment, or water) was the second driver of community composition, such that communities that originated from soil are more closely related to one another.

The effect of pH on soil microbial communities has been widely studied [22,23]. Both richness and overall community diversity (i.e., richness, evenness, and structure) are largely affected by pH [18], with higher values for both parameters at neutral pH (6-7) and lowest values at acidic pH (3-4). In fact, a survey of 98 soil samples from across North America revealed that community diversity and composition could be explained largely by pH [18]. From this investigation, pH was postulated as a good continental-scale predictor of microbial community composition, without discarding the importance of soil ecosystem type and factors at the local scale (i.e., vegetation type, soil nutritional status, moisture content).

Soil structure includes the size, shape, and arrangement of particles such as sand, silt, and clay, along with their association with organic matter [24]. The resulting aggregate formation leads to physical and chemical alterations that affect microbial communities. Moreover, a study found dominant associations of *Alphaproteobacteria* to larger soil particles (i.e., sand) and both *Holophaga* and *Acidobacterium* affiliations with smaller soil particles (i.e., silt and clay) [20]. Differential microbial distributions could result from smaller soil particles providing higher organic matter content and isolation from predators and competitors. Small soil particles possess both oxic and anoxic niches, whereas coarse particles are more exposed to oxygen, biotic interactions, and have limited nutrients.

Carbon content also plays an important role in shaping the diversity and structure of soil microbial communities. A study of 29 soil samples demonstrated that carbon-poor soils had microbial composition shifts associated with soil depth [25]. In the study, deeper soil communities were less diverse and had strongly dominant species, whereas surface communities had more OTUs and increased evenness. Conversely, communities from high-carbon content soil showed uniform diversity independent of depth, explained by carbon content and resource heterogeneity.

Land management practices have the potential to alter soil physicochemical characteristics and consequently affect the diversity and structure of microbial communities. For example, colonizing plant species, grazing, and fertilizer application influenced the rhizosphere microbial communities' structure, but no changes in diversity were detected between differently managed grasslands [19,21]. In particular, nitrogen-fixing bacteria were related to rhizosphere soil dominated by leguminous plant

species [21]. Another study revealed that the microbial community diversity and structure was similar between unfertilized and manure fertilized soil, but distinct from soil receiving sewage sludge applications [26]. The impact of land use intensity on microbial communities from grasslands has been investigated in sites with and without fertilization, mowing, and grazing [19,27]. Observations from a study analyzing the distribution of different N-cycling microorganisms showed that bacterial and denitrifier abundances were unaffected by different land use intensities. Conversely, ammonia oxidizing bacteria and archaea (AOB and AOA) were more abundant in the fertilized and mowed grasslands, but the difference was only significant for AOA. Furthermore, fertilized and frequently mowed sites showed reduced heterogeneity of soil characteristics, yet the microbial distribution of AOB and AOA was highly heterogeneous. Finally, nitrate reducers were not differentially distributed between low and high land usage intensities [27]. Therefore, factors independent from biogeochemical soil properties affected the spatial distribution of these organisms (i.e., soil structure, microclimate, and oxygen availability).

1.2 Denitrifiers

Denitrifiers are essential microbial contributors to the nitrogen cycle, releasing gaseous N-oxides and N_2 into the atmosphere. Denitrification is a major source of nitrous oxide (N_2O), a greenhouse gas (GHG) with a lifetime of 122 years [28], which has increased its concentration in the atmosphere by 18% since pre-industrialization, mainly due to human activities [29]. Denitrification in oceans is a major natural source of N_2O, contributing 20% of the 17.7 Tg N of N_2O emitted annually [29]. However, at present, N_2O released by human activities nearly equals the emissions generated by oceans and soils under natural vegetation [29]. Agriculture is the dominant anthropogenic source of N_2O; 14% of the nitrogen globally applied to soils is lost from amended agricultural fields due to denitrification [30], which contributes 16% of new annual N_2O emissions [29]. On the other hand, denitrification represents an important element of municipal wastewater treatment and bioremediation [31-34] . Therefore, it is not surprising that denitrifier communities have been explored in far ranging environments, including marine and lake sediments[31,35], soils [36,37], wastewater treatment reactors [31,32,34], surface water [31], and ground water [38].

Dissimilatory denitrification is a respiratory process by which nitrogen oxides are reduced enzymatically, coupled with oxidation of inorganic or organic molecules, driving ATP synthesis

through the proton motive force. This metabolism is widespread among facultative aerobes of the Bacteria [32,34,39]. Bacterial denitrification occurs primarily under anoxic conditions in the presence of both nitrate (NO_3^-) and organic carbon; these conditions activate nitrate transport into the cell [40] and its successive reduction to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen gas (N_2). Nonetheless, denitrification has also been observed in the presence of oxygen, in bacteria whose periplasmic nitrate reductase is able to reduce NO_3^- before it is transported into the cell [40].

1.2.1 Coding genes and enzymes involved in denitrification

The genes and enzymes involved in denitrification are well characterized [33,35,40-42] and multiple enzymes are involved in sequential nitrate reduction to dinitrogen gas (Table 1).

Table 1. Functional genes, enzymes, and reactants involved in denitrification. Modified from Verbaendert, 2011[43].

Enzyme			Gene	Substrate	Product
Name	Characteristic	Catalytic subunit			
Nitrate reductase	Membrane-bound	Nar	*narG*	NO_3^-	NO_2^-
	Periplasmic	Nap	*napA*		
Nitrite reductase	Cytochrome cd_1 periplasmic enzyme	Cd-Nir	*nirS*	NO_2^-	NO
	Copper periplasmic enzyme	Cu-Nir	*nirK*		
Nitric oxide reductase	Two-component type, membrane-bound	Nor	*norB*	NO	N_2O
	Single-component type, membrane-bound	qNor	*qnorB*		
	Membrane bound	qCuANOR	unknown		
Nitrous oxide reductase	Periplasmic in Gram-negative bacteria	Nos	*nosZ*	N_2O	N_2

Nitrate reductase is found not only in denitrifiers, but in all nitrate reducing organisms [37], including *Escherichia coli*, which makes the corresponding gene less useful for dissimilatory denitrifier community assessments. Also, both the periplasmic and membrane bound versions of the enzyme might be found in the same organism and expressed in different environmental conditions [44]. The phylogeny of the *narG* gene, which encodes the catalytic subunit of the membrane-bound nitrate reductase, approximates that of the 16S rRNA gene, although the same observation did not hold true for *napA*-based phylogenies [33].

Nitrite reductase produces the first gaseous compound of denitrification, making it a crucial enzyme for the process, catalyzing the key denitrification reaction [33]. For this reason, the genes encoding the catalytic subunit of the two existing versions of this enzyme have been studied widely and represent key genes for monitoring denitrifier communities. Nitrite reductase distributions are limited to denitrifying bacteria, both *nirS* and *nirK* have not yet been reported as occurring in the same strain [45]. Both *nirS* and *nirK* phylogenies are not consistent with 16S rRNA gene phylogeny [33].

The nitric oxide reductase gene (*qnorB*) is not exclusive to dissimilatory denitrifying bacteria [35], due to a possible NO detoxification role in other microorganisms [40,46]. Two enzyme variants have been known for decades and a third type was recently discovered for which the coding gene remains unknown [33]. Both *norB* and *qnorB* are mutually exclusive [35] and not well related to the 16S rRNA gene phylogeny of denitrifiers [33].

The *nosZ* gene, encoding the nitrous oxide reductase, is not present in all denitrifiers and this absence results in truncated denitrification within some strains [34,36]. An atypical *nosZ* gene is also present in non-denitrifying bacteria that lack other denitrification genes and even within bacteria that reduce nitrate to ammonium [47], probably indicating the suitability of N_2O as electron acceptor [45] and microbial adaptation for exogenous N_2O reduction. Also, this enzyme is recognized as the most susceptible reductase involved in the process, having oxygen [48], pH [49], temperature [50], and heavy metal sensitivity [51]. Either the absence of N_2O reductase or its inhibition could potentially lead to increased N_2O production [36,40]. The gene phylogeny resembles trees generated with 16S rRNA gene [33].

Because denitrification is widely distributed among phylogenetically diverse microorganisms, partly due to horizontal gene transfer of *nir*, *nor*, and *nos* genes [33], it is not possible to study denitrifiers with a molecular approach by targeting a genetic marker such as the 16S rRNA gene.

Instead, functional genes are targeted for characterizing denitrifier communities [32,33,40,52]. As a result, establishing robust probes and primers for these markers are important research goals for qualitative and quantitative monitoring of denitrifier communities.

Existing primers for PCR amplification of denitrifier functional genes are biased because they were designed based on limited genomic information from cultured strains. For example, Heylen and coworkers examined 277 denitrifying isolates from activated sludge but only ~50% of the isolates produced an amplicon for *nirS* or *nirK* using previously published primers [53]. Another recent effort to improve existing primers for the amplification of *nirK* from *Rhodanobacter* spp. involved the design of a genus-specific primer set [38], demonstrating that the design of universal primer sets will be challenging and certainly not feasible solely by using available gene sequences from cultivated strains. To date, commonly used primers for *nirK* amplification include nirK876 and nirK1040 [54], cd3aF and R3cd are preferred for *nirS* amplification [34,55,56], and nosZ2F and nosZ2R are employed for *nosZ* amplification [36]. However, some functional genes might not be as informative as others when evaluating denitrifier responses to environmental stimulus. For example, soil community structure changes were observed using *nirK* and *nirS* with DGGE fingerprinting, but not with *nosZ* [57]. Similarly, gene abundances did not capture the environmental impact on denitrifiers when using *nosZ* as a marker. The *nirK* gene was the most valuable tool in this research because it seemed to be more sensitive to external factors, followed by *nirS* [57].

One pitfall of analyzing DNA extracted directly from an environmental sample is that the detected genes might not be expressed or may belong to organisms that are not active, yet still contribute DNA [41]. Cultivation approaches bias toward community members that are readily grown, which may not represent those involved in a particular environment or process *in situ* [58]. A high proportion of microorganisms are recalcitrant to cultivation, mostly due to the large number of parameters that remain unknown in selecting culture media and conditions for microbial growth [52]. Evidence suggests that the study of denitrifier communities is best addressed by combining both traditional microbiology (i.e., cultivation and isolation of denitrifiers) [52] and molecular methods, encompassing genomic and metagenomic data (e.g., PCR, qPCR, DGGE, FISH, and gene sequencing) [38]. In this way, researchers retrieve genomic sequences that represent the diversity of the functional genes and best enable the development of appropriate tools for studying the composition and diversity of microorganisms in the environment.

1.2.2 Denitrification in soil and influencing factors

Among the environments in which denitrification has been studied, soil is notable because it harbors such a diverse pool of microorganisms [59], for which the majority are uncultured and uncharacterized. Soil originates two thirds of atmospheric N_2O [60] and agricultural soils are the largest source of N_2O because of fertilizer N application through mineral fertilizers, manure, and recycled crop residues [30,36,60]. Factors affecting soil denitrification are oxygen tension, carbon and nitrogen properties and availability, pH, temperature, and other microbial populations through food web dynamics. To what extent each factor is responsible for observed denitrification rates and for indirectly altering other factors of importance for the process is not well known.

Because agriculture is of high economic and social importance, many studies have assessed agricultural management practices (e.g., fertilization, tillage, irrigation, harvest) and their effects on microbially mediated processes of the N cycle. As summarized in the subsequent sections, understanding factors influencing soil denitrification is important for ensuring that soil management practices result in lower N_2O emissions, reduced fertilizer applications, and increased agriculture biomass yields.

1.2.2.1 Nitrogen effect

Nitrogen inputs affect soil bacterial communities by changing the relative abundance of specific bacterial groups [61]. The type of fertilizer employed also impacts the microbial communities found in agricultural soils, as has been shown for denitrifiers enriched in the presence of urea [56]. Organic fertilizers can lead to increased nitrogen turnover that also benefits crop yields [56,62], for example by increasing endophytic nitrifiers [62]. N inputs during fertilization are followed by denitrification and N_2O emission increases because the molecule is not fully reduced in the presence of NO_3^-, which is a more energetically favorable electron acceptor [63]. This effect can be increased by organic fertilizers that contribute organic C [64] and higher water content to soil [63]. Commonly, N_2O emissions increase in fields fertilized with higher N content materials and emissions increase if the N applied is released rapidly, as is the case with inorganic fertilizers [65].

1.2.2.2 Carbon effect

Denitrifiers are mostly heterotrophic microorganisms; as a result, C availability is an important factor influencing soil denitrification. Carbon is a direct controller of microbial communities in soils [25], but carbon can also affect the communities indirectly. For example, soil carbon promotes soil respiration and microbial activity, generating O_2-limited microsites that increase denitrification [66]. Denitrification activity is also affected by carbon concentration. Henry and collaborators demonstrated that C limitation can increase N_2O emissions. They also demonstrated that the type of soil C affects the composition of the denitrifier communities, but not denitrifier abundance [67]. However, the detected effects were not attributed to the C:N ratio; the authors concluded that C is not a major factor controlling these parameters of denitrifier populations.

1.2.2.3 Oxygen effect

Denitrification is considered an anaerobic process [68] because the denitrification pathway is active in anoxic environments in the presence of NO_3^- or NO_2^- [33]. Oxygen availability is an important regulator of the transcription of genes involved in the denitrification pathway [69]. In addition, oxygen decreases denitrification rates significantly because it is a more energetically favorable electron acceptor than NO_3^- [70]. Despite this, some strains denitrify under oxic conditions [44], where oxygen availability may lead to incomplete denitrification because the N_2O reductase is oxygen sensitive [40]. Soil oxygen content is affected by water content and texture [63]. Soil N_2O emissions were highest when water-filled pore space (WFPS) increased, yet it is important to note that ammonia oxidizers likely contributed three quarters of total evolved N_2O [49]. Given the importance of WFPS on denitrifier-mediated N_2O emissions, rain is an important element modifying WFPS in agricultural fields, which can lead to dominant denitrifier N_2O emissions at WFPS over 80% [65].

1.2.2.4 Plant effect

An effect of N input is seen in plant primary production, where increased N input reduces plant diversity in grasslands and increases plant biomass in monoculture plots [61]. Evidence of plant influence on rhizosphere microbial communities has been documented and may relate directly to plant-life stage (e.g., exudate composition) or soil conditions modified by plant-life stage demands

(e.g., water demand) [56]. Plant organic C contributions play a role in denitrification [63]. These contributions may exceed the effects of plant community composition, according to the observation that higher N inputs lead to higher C availability in the soil, promoting copiotrophs over oligotrophs, as evidenced by *Bacteroidetes* increases and *Acidobacteria* reductions [61]. However, the plant-species dependence hypothesis has been formulated for denitrifiers and other communities of importance for the N cycle [71]. Plant influences on microbial communities might be dependent on microbial taxonomy or function; denitrifiers might nonetheless be influenced by plant presence [72]. The rhizosphere possesses an increased abundance of denitrifying enzymes over the bulk soil [73], probably due to C supply and O_2 content, which may have important consequences for N_2O production potential.

1.2.2.5 pH and fertilizer effect

Soil pH is linked to the amounts and types of fertilizer applied. For example, reduced soil pH is associated with increasing ammonium nitrate inputs, decreasing from 7.4 to 6.0 in a fertilization gradient of 0 to 800 kg ha^{-1} yr^{-1} and from 6.9 to 5.0 in a fertilization gradient of 0 to 267 kg ha^{-1} yr^{-1} [61]. Soil pH modifies microbial community structure [23] and influences microbial community diversity [61]. Specific taxa correlate positively or negatively with soil pH (e.g., *Acidobacteria* decrease and *Bacteroidetes* and *Actinobacteria* increase in acidic soil).

N-fertilizer type influences soil pH [72], as seen in soils fertilized with sewage sludge (pH 4.7) or ammonium sulfate (pH 4.0), in comparison to the unfertilized control plot (pH 5.6), those receiving calcium nitrate (pH 6.3), or manure (pH 6). Also associated with lower pH was a decrease in nitrate reducers, denitrifiers, and AOA abundance (AOB were not influenced); the greatest reduction was in the ammonium sulfate plots. A complementary study showed that the denitrifier composition was notably different in the acidic pH plots [64]. However, denitrifier potential activity and community composition were not related.

Soil pH is affected by urea addition, decreasing from 4.6 to 4.1 when urea was added as sole fertilizer source and from 5.5 when manure is the fertilizer to 5.1 when a mixture of manure/urea was used [56]. In such plots, denitrifiers increased as a result of N supplementation. This increase was more pronounced when urea was added to the fertilizer mixture than when manure or straw were the sole fertilizers. Denitrification activity responses to pH are different when evaluated shortly after pH

alteration and after long-term establishment [49]. The assumption that denitrification decreases as pH decreases [63] was recently tested by acidifying soil from its original pH of 7.4 to pH 5.6 (WFPS 50%) and 6 (WFPS 65%). The result was lower N_2O emissions in both cases and denitrification dominating within the acidified soil sample with a WFPS of 65%. The same study demonstrated superior denitrification activity from soil maintained at pH 4.5 (82% of N_2O emissions) than from soil maintained at pH 7.0 since 1961 (Craibstone, Scotland). One possibility is that acid-resistant denitrifiers become selected in acidic soils, facilitating denitrification at low pH, with N_2O reduction inhibited by low pH [49]. For basification of long-term low pH soil from 4.5 to 7, the study showed a shift from denitrification as the main source of N_2O (82% of N_2O produced) to ammonia oxidation (87% of N_2O produced). Similarly, basification of soil from original pH 7.4 to pH 8.1 at two WFPS (50% and 65%) showed that ammonia oxidation contributed more N_2O than denitrification, which was related to an increased amount of ammonia (NH_3) at higher pH and possible mitigation of pH inhibition of denitrifier N_2O reduction [49].

1.2.2.6 Denitrifier community effect

Soil denitrifiers have a direct impact on nitrogen biogeochemistry and N_2O fluxes. Whether this influence is due to community structure, abundance, or diversity is not yet clear. Some evidence indicates that denitrifier abundance is the most direct controller [71,72,74], whereas another study showed that community structure is an important element [75]. Conversely, some authors found no relationship between denitrifier activity and community abundance [76] nor structure [64,71,72]. As expected, the microbial component of N cycling is convoluted and more information is required to better understand the factors that affect soil denitrifier activity and community composition.

1.2.2.7 Agricultural management effect

As described above, factors that influence denitrification are carbon availability, NO_3^- concentration, and O_2 tension. In agricultural soils, these parameters are modified by anthropogenic soil alterations and also by climate events. Factors that alter soil O_2 content are rainfall events and irrigation that change soil WFPS [63], reducing or eliminating oxygen in soil. Soil carbon content is influenced by organic fertilizers [64], plant exudates [56], and plant cultivar [65]. Soil nitrogen content is modified by fertilizer type and application rate [61]. Soil structure is important because it

influences O_2 tension and soil drainage [77]. Also, soil mechanical operations might increase soil erosion and reduce organic matter content [78]. Structure also suffers from grazing livestock in fields [79], whereas tillage modifies soil structure and might promote N_2O emissions [65].

1.2.3 Alternative soil processes contributing N_2O and N_2

Nitrogen cycling is an intricate network of reactions executed by diverse functional populations that may share NO_X pools and niches. These groups of microorganisms are thought to become active in different environmental conditions and may compete for substrates, cross-feed metabolites, transform chemicals, and differentially contribute to the pool of N cycle substrates. Co-denitrification, ammonia oxidation, anaerobic ammonium oxidation (anammox), and nitrate ammonification, also called dissimilatory nitrate reduction to ammonium (DNRA), are capable of transforming nitrogen in soil and together can contribute N_2O and N_2.

Co-denitrification, suggested by some authors to be referred to as denitrification, implies the use of N atoms from substrates other than those involved in the denitrification pathway (e.g., azide, salicylhydroxamic acid, amino acids). Such atoms would react with NO to form N_2O [80], consequently increasing produced N_2O and N_2. To date, few reports address co-denitrification separately and understanding of co-substrate preference, utilization mechanisms, and importance is limited. Some evidence from an experiment using temperate grassland soil found that co-denitrification might exceed denitrification by accounting for 92% of N_2 produced; the study also highlighted the fungal importance in N_2O production via co-denitrification [81].

DNRA might be a source of N_2O in soil. Although poorly studied, DNRA was recently shown to correlate with pH, sand content, NO_2^- concentration, low redox potential, and low NO_3^- content ($C:NO_3^-$ ratio and bulk density), and to negatively correlate with C and organic N content in soil [82], which contrasts with the hypothesis that C abundance correlates with DNRA activity [83]. DNRA capacity in temperate arable soils was demonstrated [82], but the activity seemed to be limited to a narrow set of conditions, which might be found in the rhizosphere. Therefore, the authors suggested it is unlikely that DNRA contributes significantly to the N budget in temperate arable soil. In contrast to these findings, a pristine forest was found to have DNRA outcompete denitrification for NO_3^- reduction without significant N_2O production [84].

Anammox produces N_2 from NO, using ammonia as electron donor. The process involves hydrazine as an intermediate and an anammoxosome organelle is essential for the process. Anammox activity is mostly found in marine environments [85,86] and estimates suggest that 50% of global N_2 derives from anammox activity [87]. Anammox has been detected in freshwater [88] and different soil types, including agricultural soils where anammox activity is not thought to be substantial in comparison to denitrification [89]. Anammox bacteria have an important role in wastewater treatment where implementation of this process over nitrification/denitrification can reduce treatment costs by diminishing aeration requirements for nitrification and by avoiding C supplementation for treatment of effluents that have minimal organic carbon for denitrification [87].

Ammonia oxidation by nitrifying AOB contributes NO_2^-, but also produces N_2O as byproduct of chemical decomposition of the hydroxylamine intermediate. Furthermore, AOB can denitrify, which is referred to as nitrifier denitrification. This process was first observed in *Nitrosomonas* spp. [90] and later verified for all betaproteobacterial AOB [91]. Heterotrophic niftrification has been observed in soil microbial isolates. Notably, N_2O production under oxic conditions for *Alcaligenes faecalis* goes beyond the observed activity of *Nitrosomonas*, suggesting a possible important role of heterotrophic nitrification in N_2O production [92]. However, direct evidence of such a process in soil is lacking.

Categorizing bacterial groups as devoted to a certain metabolic process can be misleading when evaluating interactions among microorganisms and the overall role of such species in the environment. Illustrating how flexible the boundaries of microbial metabolism can be, methanotrophs can denitrify [93] and the possibility exists that methane-oxidizing bacteria (MOB) produce N_2O in soils by ammonia oxidation, resulting from ammonia competition for the methane monooxygenase enzyme active site [94] due to homology between ammonium monoxygenase and methane monooxygenase [95].

At the activity level, denitrification can be distinguished from anammox in aquatic environments using isotope-labelled substrate incubations (i.e., $^{15}NO_3^-$ and $^{14}NH_4^+$ incubations), where anammox produces $^{29}N_2$, whereas denitrification yields $^{30}N_2$. In soils, co-denitrification would generate $^{29}N_2$, making that isotope the contribution of anammox and co-denitrification [89]. Denitrification activity comprises both bacterial and fungal activity, which can be attributed to the source microorganism through incubations in the presence of antimicrobial compounds (e.g., streptomycin for bacterial activity inhibition and cycloheximide for fungal activity inhibition) to estimate the relative contribution of each organism [81]. Recent work based on isotopologue values for N_2O production by

denitrifying bacteria and fungi might advance denitrification attribution to the source microorganism [96]. DNRA estimations remain a challenge and although experimental approaches have been described, they only suggest the presence of the process and do not estimate rate contributions [82]. At the genomic level, N_2O production by ammonia oxidizers is estimated using the *amoA* gene. N_2 emissions from anaerobic ammonia oxidizers are estimated with anammox-associated *Planctomycetales* 16S rRNA genes [88]. In turn, denitrifiers are linked to N_2O emissions with *nirK*, *nirS*, and *nosZ* gene detection and quantification [34,74,76].

1.3 Methods in microbial ecology

Microbial ecology and taxonomy use 16S rRNA genes to explore, classify, and identify microbial species [97]. Fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) can visually differentiate and enumerate bacterial species in a sample [98,99]. Fingerprinting methods allow the comparison of multiple samples over time or in response to treatments or disturbances.

Recently, technological advances made microbial diversity characterizations possible with Illumina, Ion Torrent, and Roche's 454 sequencing platforms. Different methodologies for massively parallel sequencing of taxonomic gene markers are available and constantly improved, allowing characterization of microbial communities of many disparate environments. A pioneering study compared different soil ecosystems with Illumina 16S rRNA gene sequencing and found high species richness, but poor phylum richness in agricultural soils, whereas forest soil had higher phylum richness [100]. Also, they reported *Bacteroidetes*, *Betaproteobacteria*, and *Alphaproteobacteria* as the most abundant lineages in the forest and arable soils studied. This new era of "big data" acquisition and data analysis promises to continue advancing our understanding of microbial communities' composition and dynamics.

Microbial communities can also be studied through the analysis of metagenomic libraries. The soil metagenome refers to the entire collection of microbial genomes from a particular soil [101]. Once the DNA is extracted from a soil sample, it can be cloned into a vector and transformed into an appropriate host. A metagenomic library comprises the obtained clones and can be screened for sequences that associate to a function or for the metabolic activity itself [102].

One goal in microbial ecology is associating microbial identity and function [59]. One methodological approach for linking function to particular microbial taxa is by combining fluorescent in situ hybridization (FISH) and microautoradiography. FISH uses fluorescent rRNA gene-binding probes to identify microorganisms, whereas microautoradiography detects radiolabeled substrate uptake in the same microbial cells [103].

A molecular approach for targeting nucleic acids of active and potentially uncultivated microorganisms is DNA stable-isotope probing (DNA-SIP) [104]. This method allows for a specific functional group of organisms to be enriched while incorporating a stable-isotope labeled substrate into their DNA. Subsequent density-gradient ultracentrifugation of the DNA and molecular analyses can identify the active microorganisms [105], regardless of their ability to be isolated in pure culture. The combination of DNA-SIP and metagenomics is valuable in the discovery of novel enzymes that can be employed for industrial benefit [106]. Likewise, DNA-SIP has revealed novel versions of functional genes involved in known metabolic pathways [58]. Now, exploration of microbial diversity and metabolism from genomic material of the active population obtained through DNA-SIP is facilitated by the advent of high-throughput sequencing [107].

1.4 Research description

Agricultural practices influence soil microbial communities and denitrifier populations [57,61,65]. Microbial community structure assessments have been conducted previously with fingerprinting methods [64], gene abundance counts [74], and recently by sequencing [61]. This information can then be linked with activity assays to estimate microbial potential for different processes [72,108]. Available data are in many cases qualitative or semi-quantitative in terms of microbial composition and quantitative in terms of microbial processes rates. This study addressed the role of agricultural practices as factors that shape soil bacterial communities and denitrifier populations and potential activities. Also, this study explored denitrifying gene sequences from active uncultured members of soil communities, which will improve current database coverage for future denitrification research.

1.4.1 Research overview

Across Canada, the Watershed Evaluation of Beneficial Management Practices program studies the impact of agricultural beneficial management practices (BMPs) at the watershed level. One of the study sites identified as WEBs (derived from Watershed Evaluation of Beneficial Management Practices), is located within the South Nation Watershed in Ottawa, Ontario where the effect of controlled (CTD) versus uncontrolled (UCTD) tile drainage is evaluated.

UCTD consists of perforated belowground pipes (i.e., "tiles") through which groundwater drains from the fields. Using this drainage method may negatively impact water quality once the drainage enters receiving waters. The alternative is implementation of CTD that enables regulation of the field water table depth during crop growth. CTD increases crop yield and reduces nutrient loading of surface water by prolonging the residence time of the drained water in the field [109,110]. Although the use of CTD is now an approved BMP, it is possible for this practice to promote anoxic conditions that increase GHG emissions (i.e., N_2O). Therefore, characterization of the microbial community and denitrifier populations in fields under controlled and uncontrolled drainage management will assist in defining the possible role of this BMP in the release of NO and N_2O.

Fertilizer type might affect the microbial community in CTD and UCTD fields. This can be explored by studying WEBs fields, which receive either mineral fertilizers or a mixture of mineral and organic fertilizers. Also, a second site in the South Nation watershed operating under CTD and manure fertilization, known as the Winchester site, was included. Its characterization will aid in recognizing the effect of agricultural practices on the microbial component of soil.

An important goal of microbial community studies is to identify the active microorganisms in a given situation. For this research, active denitrifiers are a target of interest due to the importance of the guild for agriculture and the environment. DNA-SIP was used on selected samples to enrich the active community under nitrate-reducing conditions for phylogenetic analysis. The experimental setup allowed denitrification-rate calculation. Finally, metagenomic sequencing of the active denitrifier DNA allowed functional-gene recovery and exploration.

1.4.2 Objectives and hypotheses

This research aimed to explore the effect that two different drainage practices (i.e., CTD and UCTD), employed for agriculture, have on microbial diversity. The hypothesis is that the implemented drainage practice modifies moisture content and oxygen availability in soil, thus potentially altering the community composition. This research also aimed to characterize active denitrifiers from differentially managed soils, amended with either manure or mineral nitrogen fertilizers and operated under CTD or UCTD systems. The hypothesis is that using culture-independent techniques will lead to recovery of novel denitrifiers that are distinct from cultured isolates. Another aim was to recover and explore nitrite reductase sequences by combining DNA-SIP and metagenomics. The hypothesis is that gene sequences recovered from active microorganisms without the prerequisite of cultivation will identify denitrifier genes that differ from those of known cultivated representatives.

Chapter 2
Materials and methods

My thesis research involved the characterization of total bacterial communities and denitrifying organisms from two distinct agricultural sites with varying drainage management practices and fertilizer application regimes. Bacterial community profiling with next-generation sequencing involved collecting soil samples during the 2012 growing season. Additional soil cores were collected in October 2012 for denitrifying community characterization with DNA-SIP and metagenomics.

2.1 Microbial community characterization

2.1.1 Sampling sites

Two study sites were used for this research, both located in Ottawa, Ontario (Table 2). First, the WEBs site (named after the Watershed Evaluation of Beneficial Management Practices study) is divided into fields operated under contrasting management practices. The site was amended with either a mineral-based or a mixture of mineral- and manure-based nitrogen fertilizers. These fields were operated under CTD or UCTD systems, which maintain water in the field or fully drain it, respectively. Second, the Winchester site is divided into fields operated under manure fertilization and CTD.

Table 2. Study sites characteristics and implemented management practices.

Site	Coordinates	Fields	Drainage mode[1]	Fertilizer	Crop
WEBs	45°16’ N, 75°10’ W	1	UCTD	Mineral-based	Soybean
		2	CTD	Mineral-based	Soybean
	45°15’ N, 75°11’ W	11 and 12	CTD	Mineral- and manure-based	Forage
		13 and 14	UCTD	Mineral- and manure-based	Forage
Winchester	45°3’ N, 75°20’ W	1 to 6	CTD	Manure	Silage corn

[1]CTD is controlled tile drainage, UCTD is uncontrolled tile drainage.

WEBs fields 1 and 2 were amended only with mineral fertilizer (urea) over the past ten years. Corn and soybean were grown on these fields, exclusively. On May 16, 2012 the fields were planted with Soybean (Dekalb 26-10RY). Soybean emerged on May 23, 2012 and herbicide (Roundup) was applied between May 23 and 28, 2012. Harvest occurred on September 24, 2012. Drainage for field 2 was controlled; water was kept in the field from May 17, 2012 to November 20, 2012.

WEBs fields 11 to 14 received both mineral fertilizer (urea) and manure (dairy lagoon and soiled bedding). These fields have grown soybean (2005), corn (2006-2010), and forage (2011-2012). During 2012, no fertilizer, pesticide, or herbicide was applied and minor reseeding was done on damaged patches. The fields grew alfalfa and were harvested on June 14, July 13, August 21, and October 25, 2012. Drainage for fields 11 and 12 was controlled; water was kept in the fields from May 18, 2012 to November 19, 2012.

Winchester's fields 1 to 6 receive manure and grow corn. On May 31, 2012 they received manure. Corn was planted on June 6, 2012 and emerged on June 14, 2012. Herbicide (Roundup) was applied on June 28, 2012. Corn was harvested on October 29, 2012. Drainage for all fields was controlled; water was kept in the fields starting May 31, 2012 and only released from fields 1-3 on November 30, 2012.

During the growing season of 2012, soil samples from WEBs and Winchester were collected for DNA-based microbial community characterization at multiple time points. Soil samples from different depths (0-30, 30-60, and 60-90 cm for WEBs and 0-15, 15-30, and 30-60 cm for Winchester) and different locations with varying proximity to tiles (above tile and between tiles for WEBs and between tiles for Winchester) were collected and stored (unsieved) at -20°C until DNA extraction (Appendix A).

2.1.2 DNA extraction

DNA was extracted from 0.25 to 0.30 g of soil using the PowerSoil DNA Isolation Kit (MO BIO), which uses chemical and mechanical cellular lysis and removes humic acids, proteins, and other contaminants through precipitation and washing steps. Mechanical lysis was done for 45 seconds at 5 m s^{-1} in a bead beater (FastPrep 24, MP Biomedicals). Purified DNA was stored at -20°C in EDTA-free 10 mM Tris solution provided by the manufacturer until use.

DNA was subject to electrophoresis in 1% (w/v) agarose gels (BioShop) in TAE buffer, gels contained ethidium bromide (1 µg mL^{-1}, Calbiochem) for nucleic acid visualization. For reference, a 1 Kb Plus DNA Ladder (Invitrogen) was included as a marker. Gel images were acquired with an AlphaImager HP (Alpha Innotech). In addition, DNA concentration and quality was evaluated using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.1.3 Next-generation sequencing

2.1.3.1 16S rRNA gene library construction

A fragment of approximately 465 bp from the 16S rRNA gene was amplified by PCR from 168 soil DNA samples (WEBs and Winchester sites) using a modified version of a previously published protocol [111]. Modification included migration from V3-region amplification to V3/V4-region amplification for obtaining two variable regions of the 16S rRNA gene. Primers employed for the PCR were 341F (5'-CCTACGGGAGGCAGCAG) and 806R (5'-GGACTACHVGGGTATCTAAT) [98,112] with adaptors for Illumina next-generation sequencing. Each sample was PCR amplified in triplicate. Each PCR mixture contained 1.5 µL of bovine serum albumin (10 mg mL^{-1}, Sigma-Aldrich), 2.5 µL of ThermoPol reaction buffer (10X, New England BioLabs), 0.05 µL dNTPs (100 nM, New England BioLabs), 0.05 µL forward primer (100 µM, Integrated DNA Technologies), 0.5 µL reverse-indexed primer (10 µM, Integrated DNA Technologies), 0.125 µL *Taq* DNA polymerase (5000 U mL^{-1}, New England BioLabs), 1 µL soil DNA template (2-20 ng μL^{-1}), and nuclease free H_2O (Thermo Scientific) to 25 µL. The reaction was done in a T100 Thermal Cycler (Bio-Rad). The program had an initial denaturation step of 30 seconds at 95°C, 30 cycles of 15 seconds at 95°C, 30 seconds at 50°C, and 30 seconds at 68°C, followed by a final extension step of 5 seconds at 68°C. Controls included non-template and positive reactions.

Individual PCR products were subjected to electrophoresis to verify amplification, specificity, and size. For each soil DNA sample, three independent PCR amplifications were combined and then gel quantified using the band analysis tool in AlphaView Software (Alpha Innotech). Purified and spectrophotometrically quantified V3/V4 amplicons served as quantification standards. Equal amounts of all soil PCR-products were combined in one sample. The mix was electrophoresed in a 1% agarose gel and visualized by UV transillumination. The 16S rRNA gene amplicon band was

excised and then purified with the Wizard SV Gel and PCR Clean-up System (Promega). The obtained 16S rRNA V3/V4 amplicon mixture was eluted in Buffer EB (Qiagen).

Library quantification was done with spectrophotometry, gel quantification, and qPCR. Primers employed for the qPCR were 341F (5'-CCTACGGGAGGCAGCAG) and 518R (5'-ATTACCGCGGCTGCTGG) [98]. Sample mix contained 1.2 µL of bovine serum albumin (10 mg mL^{-1}, Sigma-Aldrich), 10 µL of SsoAdvanced SYBR Green Supermix (2X, Bio-Rad), 0.04 µL forward primer (100 µM, Invitrogen), 0.04 µL reverse-indexed primer (100 µM, Invitrogen), 1 µL DNA library, and nuclease-free H_2O (Thermo Scientific) to 20 µL. The reaction was done in a C1000 Thermal Cycler with a CFX96 optical module (Bio-Rad). The program had an initial denaturation step of 30 seconds at 95°C, 35 cycles of 5 seconds at 95°C, and 20 seconds at 50°C, finally melt curve analysis from 65°C to 95°C with increments of 0.5°C held for 2 seconds. Purified and spectrophotometrically quantified V3/V4 amplicons served as a quantification standard.

2.1.3.2 Illumina sequencing

Template was prepared for sequencing according to manufacturer guidelines using the MiSeq Reagent Kit v2 (500 cycles, Illumina). Briefly, template was denatured with NaOH and diluted to 12.5-17 pM, then mixed with denatured Illumina PhiX control at the same molarity in a 19:1 ratio. Template was loaded into a MiSeq v2 Reagent Tray (Illumina) and sequencing proceeded using the MiSeq System (Illumina).

During the Illumina run, clusters of monoclonal V3/V4 sequences in the library were generated and then sequenced to generate V3/V4 paired-end reads of 250 bp, including an additional index read linking each sequence to the sample of origin. Image analysis, base calling, quality score calculation, and demultiplexing (read sorting by index) were done using MiSeq Control Software (version 2.3.0.3).

2.1.4 Bioinformatic analysis

Obtained 16S rRNA gene sequences were processed using QIIME [113] and AXIOME [114] for taxonomic exploration of the communities as described below. PANDAseq was used for paired-end read assembly [115]. Sequences were clustered with CD-HIT using a 97% similarity threshold to

yield OTUs [116]. OTUs were assigned to bacterial taxonomy using the RDP classifier [117] based on the Greengenes database [118]. Chimeric sequences were filtered with UCHIME [119].

AXIOME generated principal coordinate analysis (PCoA) [120] and nonmetric multidimensional scaling (NMS) ordinations [121]. The former identifies patterns in the data and summarizes them in the ordination using synthetic variables or axes that express the percentage of the variance captured from the original dataset, the latter finds patterns based on a ranked distance matrix. Both ordinations were done using Bray-Curtis distances [122], which measures the quotient of the shared abundances and the total abundance, therefore representing the difference between communities. Multi-response permutation procedure (MRPP) analysis tested individual community membership to a predefined group and tested group segregation [123]. MRPP was controlled by AXIOME as well as indicator species analysis [124], which identified members of the community that are associated with a particular group according to group fidelity and specificity. Indicator value and median sequence abundance cutoff to limit indicator species were 0.7 and 100, respectively, with $p < 0.05$. Indicator species analysis for crop and fertilizer type applied was performed on data from WEBs fields 1 and 2, WEBs fields 11-14, and Winchester fields together. Indicator species analysis for drainage practice was performed on data from WEBs fields 1 and 2 together and WEBs fields 11-14 together. Depth indicator species analysis was performed independently on WEBs field 1, WEBs field 2, WEBs fields 11-12, WEBs fields 13-14, and Winchester fields together. All AXIOME-mediated analyses were based on a rarefied OTU table. Representative OTU sequences were aligned with PyNAST [125] and the alignment used to build a phylogeny using FastTree [126] to compute UniFrac distances [127] (the evolutionary divergence exclusive to each community) used for QIIME-generated PCoA ordination plots. Pearson product-moment correlation coefficient (PPMCC) and pairwise Wilcoxon signed rank test comparisons were used for statistical analysis of taxa behavior in R [128].

2.2 Denitrifying community characterization

2.2.1 Sampling sites

Fields 1 (45°16’18.578” N, 75°10’6.743” W) and 2 (45°16’19.485” N, 75°10’3.483” W) from WEBs site and the boundary between fields 3 and 4 (45°3’42.221” N, 75°20’31.976” W) from Winchester site were sampled on October 22, 2012, from 15 to 20 cm depth and from between tile locations. For detailed site and field characteristics, refer to section 2.1.1.

2.2.2 DNA-SIP

2.2.2.1 Denitrifying incubation in flow-through reactors

DNA-SIP was conducted in flow-through reactors (FTRs), allowing enrichment of the active community under nitrate-reducing conditions and denitrification rate calculation. Soil samples were incubated using FTRs [129], which consist of an air-tight cell containing an undisturbed soil core (Figure 1). The soil sample contained in a Plexiglass ring (2 cm height, 4.7 cm diameter) was covered by a polypropylene-membrane filter (0.2 µm pore size, 50 mm diameter, Pall Life Sciences) and a fiberglass filter (extra-thick, 1 µm pore size, 47 mm diameter, Pall Life Sciences) at the upper and lower ends. O-rings (50 mm diameter) were placed on top of the fiberglass filters and the reactors then closed with Plexiglass lids at the upper and lower ends held in place with screws. The bottom lid had an input channel used to supply a feed solution to the soil core and the top lid had an output channel used to recover the flow through. Carved rings on the lids promoted uniform flow of the feed solution through the sample.

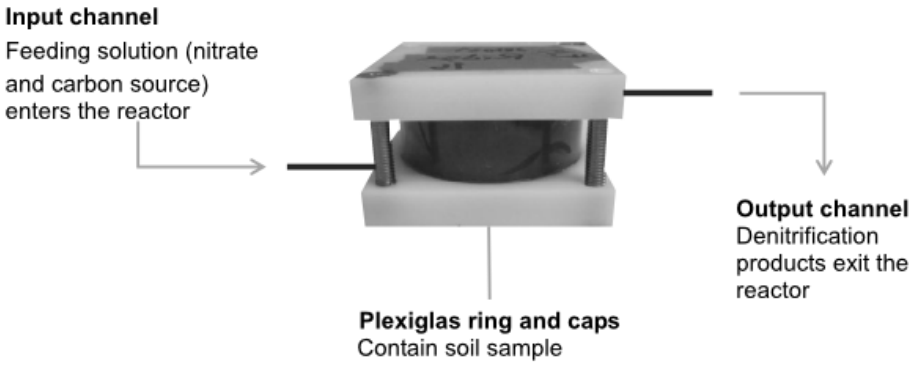

Figure 1. Flow-through reactor functional scheme.

Feeding solution was kept at room temperature in amber glass bottles and was purged continuously with humidified and filtered argon gas (Praxair) to ensure the absence of O_2. The solution was conducted through Viton tubing lines (0.89 mm internal diameter, opaque black, Ismatec and Masterflex) at a flow rate controlled by an 8-channel peristaltic pump (Minipuls 3, Gilson).

The DNA-SIP incubation in FTRs was conducted at room temperature, protected from light, and consisted of two experimental stages. First, there was a starvation period of 22 days in which the reactors were fed with a sterile, O_2-free solution of KNO_3 (1 mM), KBr (0.5 mM), and NaCl (8 mM)

at a flow rate of 1 mL h^{-1}. This was followed by a denitrification period in which the reactors were fed with a sterile, O_2- free solution of KNO_3 (1 mM), KBr (0.5 mM), NaCl (8 mM), and either $^{12}C_6$-glucose or $^{13}C_6$-glucose (0.31 mM) at a flow rate of 1 mL h^{-1}. Bromide was used as a nonreactive tracer.

Eight FTRs were included for each of three agricultural sites studied (refer to section 2.2.1); all were subjected to the starvation period after which four FTRs were fed with $^{12}C_6$-glucose solution and four FTRs with $^{13}C_6$-glucose solution, constituting four pairs of FTRs per agricultural site. Two randomly chosen pairs were sacrificed after 8 days of denitrification (total incubation time 30 days) and the remaining two pairs were sacrificed after 27 days of denitrification (total incubation time 49 days). For the FTRs with longer incubation times, the feeding flow rate was increased from 1 to 2 mL h^{-1} at denitrification day 18 (total incubation-time day 40). After the incubation, the soil was recovered and stored at -20°C until DNA extraction.

2.2.2.2 Denitrifying incubation monitoring

Nitrogen metabolites were monitored periodically during the incubation using spectrophotometric methods and gas chromatographic techniques to verify that denitrification activity was induced when required and that it was the dominant respiratory process occurring in the FTRs. Both input and output solutions were monitored for NO_3^-, NO_2^-, pH, and Br^-; the output solution was also measured for CO_2, N_2O, and NH_3/NH_4^+. Gas sampling for CO_2 and N_2O analysis was done in air-tight vials.

2.2.2.2.1 Solution chemistry

Flow-throw samples were collected every other day and stored at -20°C until analysis. Nitrate was quantified as total NO_x following its reduction to NO_2^- according to an established protocol [130]. Briefly, 100 µL standards and samples were mixed in triplicate in a 96-well plate (clear, flat bottom, Greiner), with the addition of 100 µL saturated VCl_3 solution (Sigma-Aldrich; 800 mg in 100 mL of 1 M HCl) and 100 µL Griess reagent (SULF, Sigma-Aldrich, 2% w/v in 5% HCl, and NEED, Sigma-Aldrich, 0.1% w/v in water, which were prepared separately and premixed in equal volumes immediately before use). The plate was incubated for 30 minutes at 37°C and then the absorbance read at 550 nm using a plate reader (FilterMaxF5 Multi-Mode Microplate Reader, Molecular

Devices). Nitrate concentration in the sample was determined as the remainder of NO_x minus NO_2^-. Soft Max Pro 6.2.2 (Molecular Devices) was used to analyze sample data. Standards ranged from 2.5 to 350 µM KNO_3 (ACS, Bio Basic Inc.).

Nitrite was quantified according to a previously published method [130]. The procedure was similar as for NO_3^- quantification except for the use of the saturated VCl_3 solution step, which was omitted. Soft Max Pro 6.2.2 (Molecular Devices) was used to analyze sample data. Standards ranged from 2.5 to 350 µM $NaNO_2$ (reagent grade, Bio Shop).

Ammonia and ammonium were quantified according to a previously published method [131]. Briefly, in a 96-well plate (dark, flat bottom, Greiner), 100 µL standards and samples in triplicate were mixed with 200 µL of ortho-phthaldialdehyde (OPA) solution. OPA solution was prepared at least 24 hours before use by combining 500 mL of 30 g L^{-1} borate buffer (20 Mule Team), 2.5 mL of 8 g L^{-1} Na_2SO_3 (98% pure; Sigma-Aldrich), and 25 mL of 40 g L^{-1} OPA in ethanol (97% pure; Sigma) and stored at room temperature in a dark glass bottle. The plate was incubated for four hours at room temperature in the dark and then the fluorescence read with excitation at 360 nm and emission at 465 nm using a plate reader (FilterMaxF5 Multi-Mode Microplate Reader, Molecular Devices). Soft Max Pro 6.2.2 (Molecular Devices) was used to analyze sample data. Standards ranged from 0.5 to 75 µM NH_4Cl (ACS; BDH).

pH was measured twice per week with Mettler Toledo's In Lab Expert Pro pH electrode. Bromide was measured every other day with Thermo Scientific Orion bromide electrode.

2.2.2.2.2 Total dissolved inorganic carbon as carbon dioxide

Two mL of standard or sample collected in an air-tight vial were sucked into a disposable syringe (10 mL capacity with 23G needle) containing 1 mL of HCl (6 N) for acidification. The needle was then replaced by a rubber stopper (5 x 11 mm for 8-9 OD mm tubing, VWR) and 4 mL of He gas (Ultra High Purity, Praxair) were injected into the syringe through the rubber stopper. Syringe contents were mixed and 30 minutes were allowed for headspace equilibration. One mL of headspace was drawn into a disposable syringe (1 mL capacity with 25G needle) and 500 µL were injected into a gas chromatograph (GC-2014, Shimadzu Scientific Instruments) equipped with a Porapak Q 80-100 column (6' x 1/8'' x 0.085'' SS, Alltech), a methanizer, and a FID. The retention time for CO_2 was 1.3 minutes with the following GC settings: injector temperature at 100°C, oven temperature at 80°C,

methanizer at 380°C, FID at 250°C, He as carrier gas (Ultra High Purity, Praxair) flowing at 20 mL min^{-1}, and supply of compressed air (Ultra Zero, Praxair) and hydrogen (Ultra High Purity, Praxair). Samples were analyzed immediately after collection twice a week. Peak area was integrated with GC Solution software (version 2.31.00, Shimadzu Corporation). Standards were analyzed in duplicate and ranged from 0.25 to 6 mM of $NaHCO_3$ in water (ACS, Bio Basic Inc.). The methodology was developed based on reported methods [132,133].

2.2.2.2.3 Nitrous oxide

One mL of sample was collected in an air-tight vial purged with He and allowed to equilibrate with the headspace for one hour. Four hundred µL of head space were taken into a disposable syringe (1 mL capacity with 25G needle) and injected into a gas chromatograph (GC-2014, Shimadzu Scientific Instruments) equipped with a Porapak Q 80-100 column (6' x 1/8'' x 0.085'' SS, Alltech) and an ECD. The retention time for N_2O was 1.2-1.3 minutes with the following GC settings: injector temperature at 120°C, oven temperature at 100°C, ^{63}Ni ECD at 325°C with 1.50 nA current, and P5 carrier gas (4.97% methane in argon, Certified Standard, Praxair) flowing at 25 mL min^{-1}. Samples were analyzed twice a week immediately after equilibration. Peak area was integrated with GC Solution software (version 2.31.00, Shimadzu Corporation). Nitrous oxide (Extendapak, Praxair) was used as reference for peak identification. This methodology was developed based on reported methods [134-136].

2.2.2.3 Denitrification potential

For each incubated soil and feeding flow rate, the NO_3^--N reduction rate was calculated once stable concentrations of NO_3^- were obtained. The formula used is [129]:

$$R = \frac{(C_0 - C_{OUT})Q}{V}$$

Where C_0 is the NO_3^--N concentration in the inflow, C_{OUT} is the NO_3^--N measured in the outflow, Q is the solution feeding rate, and V is 27.7 cm^3, the reactor volume. A two-way analysis of variance (ANOVA: two-factor with replication) was done using the Data Analysis Tools in Excel (Microsoft).

2.2.2.4 DNA ultracentrifugation and gradient fractionation

Extracted DNA was prepared for ultracentrifugation and the resulting gradient fractionated according to a previous protocol [105]. Briefly, DNA was mixed with CsCl solution and ultracentrifuged to form a density gradient in which the DNA was separated by density. The gradient was then divided into 12 fractions with fractions 3-5 containing "heavy" DNA. Refractive index of each fraction was measured with a digital hand-held refractometer (AR200, Reichert). The DNA was recovered using 4 µL of linear polyacrylamide (LPA; co-precipitant pink, Bioline), washed, dried, and suspended in TE buffer. The DNA was stored at 4°C when processed or at -20°C for long-term storage.

2.2.2.5 DNA-fraction characterization

Fingerprint patterns of PCR amplicons of the 16S rRNA gene V3 region were generated for each DNA fraction using DGGE as described by Muyzer et al. 1993 [98,137]. Briefly, PCR sample mix was similar to the one described in section 2.1.3.1, except 0.1 µL *Taq* DNA polymerase was used. Primers 341f-GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG) and 518r (5'-ATTACCGCGGCTGCT GG) were employed. The program had an initial denaturation step of 5 minutes at 95°C, 35 cycles of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C, followed by a final extension step of 7 minutes at 72°C. PCR amplicons were run in 10% polyacrylamide (acrylamide/bisacrylamide 37.5:1 solution 40% w/v, Bio Basic Canada Inc.) gels with a denaturing gradient from 30% to 70% (100% denaturant is 7 M urea; Bio Basic Inc. and 40% redistilled formamide; Invitrogen) for 840 minutes at 85 V in a DGGE-2001-110 system (CBS Scientific). Five µL of each PCR were loaded into each gel well alongside a custom ladder used as reference for normalization. Gels were post stained for 1.5 hours with SYBR Green I (Invitrogen) in TAE buffer and imaged with a Molecular Imager (Pharos FX Plus, Bio-Rad) equipped with an external laser and Quantity One 4.6.9 image acquisition software. Gel images were normalized and clustered with GelCompar II version 6.6 (Applied Maths) using Pearson correlations of densitometric curves and UPGMA clustering.

2.2.3 Heavy- and light- DNA fractions DGGE-band sequencing

Predominant bands identified from the DGGE fingerprints were excised from the gel. Each band-containing gel fragment was suspended in 50 µL of TE buffer and kept at 4ºC for 12 hours. The suspension was used as PCR template for re-amplification and amplicon purity was confirmed with DGGE as in section 2.2.2.5. PCR product was purified with the Wizard SV Gel and PCR Clean-up System and sequenced with Sanger technology at the TCAG DNA Sequencing Facility of the Centre for Applied Genomics (Toronto, ON). Sequences were then assigned microbial identity using the ribosomal database project (RDP) classifier [117] and BLAST [138].

2.2.4 Heavy- and light-DNA fraction next-generation sequencing and analysis

Eighteen samples corresponding to nine composite-heavy and nine composite-light DNA fractions were prepared from the density gradient DNA fractions described in section 2.2.2.4 as follows. A heavy DNA sample corresponds to a mixture of fractions 4 and 5, whereas a light DNA sample corresponds to a mixture of fractions 10 and 11 of DNA extracted from reactors fed with $^{13}C_6$-glucose. For the reactors sacrificed after eight days of denitrifying enrichment, a total of six heavy and six light DNA samples were obtained (two duplicate reactors per agricultural site times three sites), whereas a total of three heavy and three light DNA samples were obtained for the reactors sacrificed after 27 days of enrichment (two duplicate reactors per agricultural site pooled into a single sample times three sites). The 18 samples were prepared for Illumina sequencing as mentioned in section 2.1.3. Finally, obtained 16S rRNA gene sequences were analyzed as mentioned in section 2.1.4. For indicator species analysis, the indicator value and sequence abundance cutoff values to limit indicator species were 0.8 and 2000, respectively, with $p < 0.05$.

2.2.5 Bulk soil DNA and heavy DNA metagenomic sequencing and analysis

Four samples were selected for nitrite reductase-coding DNA analysis and sequence retrieval comparison between denitrifier-enriched and unenriched soil. Samples included: a) DNA from bulk soil from WEBs field 2 (45°16’19.485” N, 75°10’3.483” W) sampled on October 22, 2012, b) DNA from bulk soil from Winchester’s fields 3 and 4 boundary (45°3’42.221” N, 75°20’31.976” W) sampled on October 22, 2012, c) DNA-SIP heavy DNA from WEBs field 2 (fractions 4, 5, and 6;

reactor E2-1), d) DNA-SIP heavy DNA from Winchester's fields 3 and 4 boundary (fractions 4, 5, and 6; reactor I-2).

2.2.5.1 Metagenomic-DNA library preparation and sequencing

DNA from all samples was sent to the DNA Services Facility of the University of Illinois at Chicago for shotgun metagenomic sequencing. Briefly, the Nextera XT DNA Sample Preparation Kit (Illumina) was employed to independently fragment and tag the DNA of each sample using transposons. The fragmented DNA was then subjected to PCR to attach sample-association indices and sequencing adapters. The samples were then combined to produce a library that was paired-end sequenced in an Illumina MiSeq. Image analysis, base calling, quality score calculation, and demultiplexing (read sorting by index) were done using MiSeq Control Software (version 2.3.0.3).

2.2.5.2 Bioinformatic analysis

Reads were uploaded to MG-RAST [139] for sequence annotation. The metagenomes were automatically assigned the following identifiers: 4543546.3 (WEBs field 2 bulk soil read1), 4543547.3 (WEBs field 2 bulk soil read 2), 4543549.3 (Winchester's fields 3 and 4 boundary bulk soil read 1), 4543550.3 (Winchester's fields 3 and 4 boundary bulk soil read 2), 4543544.3 (WEBs field 2 heavy DNA read 1), 4543545.3 (WEBs field 2 heavy DNA read 2), 4542721.3 (Winchester's fields 3 and 4 boundary heavy DNA read 1), and 4543548.3 (Winchester's fields 3 and 4 boundary heavy DNA read 2). Each metagenome was processed with the online analysis tools provided by MG-RAST as follows. All functional annotations were obtained by comparing the sequences against the GenBank database with maximum E-value cutoff of 0.00001, 60% minimum identity cutoff, and minimum alignment length cutoff of 15 amino acids. Next, the search term "nitrite reductase" was used to filter the obtained hits. Further hit selection was manually conducted to discard sequences that did not correspond to dissimilatory nitrite reductases. The obtained annotations were exported to the MG-RAST workbench for download. The sequences were catalogued into *nirS* or *nirK* based on their header description. The classified sequences were respectively aligned to *Proteobacteria nirS* and *nirK* sequences from the KEGG database [140] using MAFFT 7 [141]. The only modification to the default settings for multiple alignments was to allow adjustment of nucleotide sequence direction. The alignments were exported into MEGA 6 [142] for analysis and primer region identification.

Chapter 3
Results and discussion

3.1 Microbial community characterization

3.1.1 Agricultural soil DNA next-generation sequencing

Arable soils are managed with the main purpose of sustainably increasing crop yields. Food demand increases with economic development and population growth. Furthermore, climate change presents risks for crop production with altered climate trends that affect growing season length, water availability, and temperature [143,144]. Farming, on the other hand, represents a risk to the environment associated with increased GHG emissions, groundwater contamination, and a decline of soil fertility and growing capacity due to poor management and excessive land use [29,145]. As a result, research into agriculture practices can help establish best management practices to help ensure food security while reducing environmental impact.

This research evaluated the effect that two drainage practices had on the microbial communities in two agricultural sites in Ottawa, Ontario. The selected fields from the WEBs site were fields 1 and 2 with implemented UCTD and CTD, respectively, fields 11 and 12 with CTD, and fields 13 and 14 with UCTD. Finally, Winchester field boundaries 2/3 and 4/5 with CTD were studied. Sampling was done in 2012 and included different soil depths and position with respect to drainage pipes. Moisture was expected to increase with depth. That said, above-tile locations were expected to be associated with lower moisture than the between tile locations, because the tiles collect and drain water primarily from above their physical location.

Sequencing of amplified 16S rRNA gene sequences from DNA extracts obtained from all soil samples collected from the WEBs and Winchester sites resulted in 11,450,780 paired-end reads. Each sequenced sample contributed an average of 68,159 sequences. The largest number of sequences contributed for a sample was 156,580 and the smallest was 16,695. After assembly of paired-end reads, clustering, and chimera checking, the number of remaining sequences was reduced to 10,622,116 (minimum 15,687, maximum 149,322). For analyses requiring an equal sequence contribution from each sample (i.e., Bray-Curtis PCoA, UniFrac PCoA ordinations, and MRPP), all samples were rarefied to 15,687 sequences. For identifying indicator species, the sample data were

partitioned into relevant sample sets and within-sample series searches involved rarefying to the number of sequences contributed by the lowest sample in each series.

3.1.1.1 Identifying factors influencing agricultural soil microbial communities

The field community composition was evaluated with next-generation sequencing to elucidate the effect that the drainage practice imposed had on soil microbial communities. Sequences corresponding to the V3/V4 region of the 16S rRNA gene were obtained from 168 samples from the WEBs and Winchester sites (Appendix A).

The microbial communities in each of the soil samples were analyzed with multivariate statistics. The main factors that visually influenced the ordination of the soil communities were plant cultivar and fertilizer application (Figure 2A). In addition, soil depth was strongly associated with separation of soil bacterial communities (Figure 2B). Together, these factors were more clearly responsible for distinguishing sample data than the sampled field location (Figure 2C) or drainage practice (Figure 2D). PCoA using the UniFrac metric showed similar trends (data not shown). Depth was one of the most important factors influencing soil bacterial community composition (Figure 2B). Indeed, the MRPP values showed support for significant depth-specific grouping (T=-41.1; A=0.10, p=0.001). Similarly, previous reports identified depth as an important factor determining microbial community composition, even suggesting that its impact might be equivalent to that of ecosystem origin [146]. The documented changes have been linked to modified soil properties along the depth gradients [147].

Although agricultural soil bacterial communities were most similar when originating from soils sharing common management practices and field histories (e.g., Figure 2A, 2C), communities from soybean and corn cover were distinct from communities beneath alfalfa (Figure 2A). These groupings were supported by MRPP values (p=0.001), with a high negative value for between-group separation (T=-75.9), although with relatively low within-group similarity (A=0.13) presumably due to the variability introduced by soil depth. Previous research has identified plant type as a major driver of community composition, mainly due to the environment created by root exudates in the rhizosphere [148]. Although soybean and alfalfa communities could be predicted to be similar to one another, because both are leguminous plants, examining field histories provides a potential explanation for this lack of clustering of soybean and alfalfa soil samples. The sampled soybean fields in 2012 are used

alternately to produce corn, which might cause these communities to be similar to those from corn fields. Also, the sites growing alfalfa in 2012 have had a crop rotation that included soybean and corn. Therefore, the observed ordination results may be a result of multiple factors that include fertilizer type and field history. It is important to note that the selected sites are farmed for commercial purposes, operated by their owners, which makes management of these sites difficult to control with respect to treatment and history.

Importantly, drainage practice did not appear to influence bacterial communities in the fields sampled here (Figure 2D). Furthermore, the MRPP values showed only weak statistical support for drainage practice group differences (T=-7.8; A=0.01, p=0.001). Moisture was expected to be influenced by the drainage practice, because CTD keeps water in the field and UCTD allows water to exit the field. However, we observed no trend in moisture data related to drainage practice or depth (data not shown), probably due to 2012 being a relatively dry year. The average rainfall in Ottawa between May 1 and September 30, 2012 was 74 mm, very similar to the 73 mm of 2011, in comparison to 107 mm and 82 mm in 2010 and 2013, respectively, for same time period [149].

Although the corresponding field data showed a slight increase in crop yield from CTD fields, these differences were not statistically significant [110]. Also, there is no evidence of GHG emissions increasing from CTD fields, while a considerable reduction of nitrate in CTD field groundwater is observed (unpublished report by the South Nation River Conservation Authority, 2013). Whether these observations would be replicated in a wet season remains undetermined. So while limited, these results demonstrate that CTD implementation in the fields does not modify the microbial community or GHG emissions and might nonetheless help retain N in the fields and improve plant biomass.

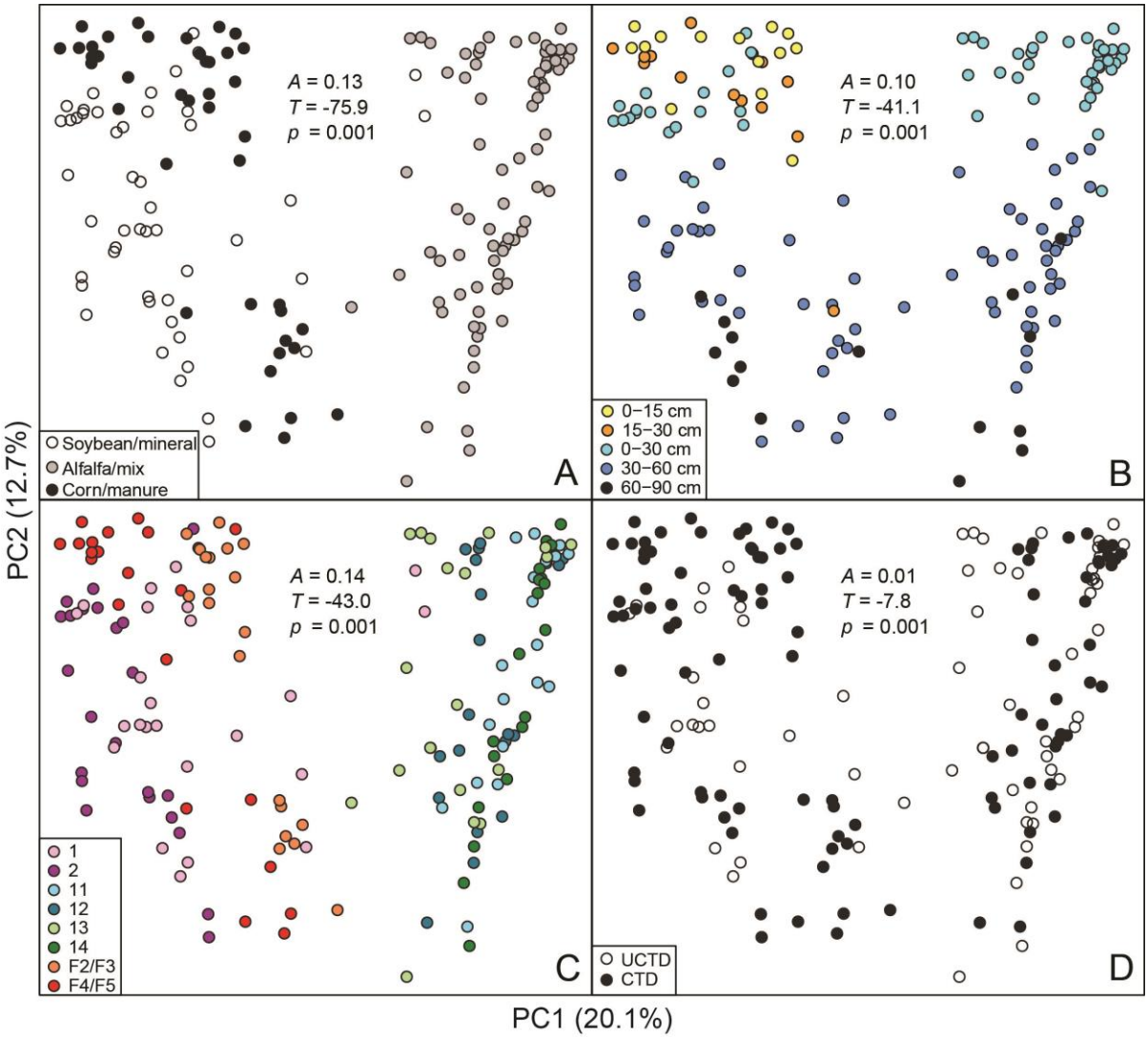

Figure 2. PCoA ordinations and inset MRPP data (based on a Bray-Curtis distance matrix) of next-generation sequencing data for agricultural soils according to crop and fertilizer type aplied (A), sampled depth (B), sampled field (C), and implemented drainage practice (D). CTD is controlled tile drainage, UCTD is uncontrolled tile drainage.

3.1.1.2 Taxa exploration

Ribosomal RNA gene sequences obtained from the next-generation sequencing effort from all samples were clustered into OTUs using a 97% similarity threshold and then classified taxonomically. Microbial phyla with sequence abundances above 10% were *Chloroflexi*,

Proteobacteria, *Actinobacteria*, and *Acidobacteria* for WEBs fields 1 and 2 (Figure 3), *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Bacteroidetes* for WEBs fields 11-14 (Figure 4), and *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* for Winchester (Figure 5). For all studied sites, the phylum *Acidobacteria* had similar average abundances in the top, middle, and deeper soil samples. For example, *Acidobacteria* abundances in WEBs fields 1 and 2 were 16.4±2.4, 16.6±2.4%, and 12.2±4.5% in soil samples from 0-30, 30-60, and 60-90 cm depth, respectively (Figure 3).

Actinobacteria relative abundances increased over the growing season and peaked around August (Figure 3, Figure 5). This was observed in all sampled depths and was particularly noticeable in deeper soil (Figure 4). For example, *Actinobacteria* 16S rRNA gene relative abundance in WEBs fields 11-14 went from 22.1±1% average in April to 40.2±6% average in November, in soils from 60-90 cm depth. In these particular fields, the phylum's overall increment in soil from 0-30 and 30-60 cm depth was less pronounced, but the trend remained visible nonetheless. A Pearson product-moment correlation coefficient (PPMCC) test showed that the abundance increment of the phylum was significant for WEBs fields 1 and 2 ($r(46)=0.69$, $p=6.6E-8$), WEBs fields 11-14 ($r(52)=0.38$, $p=0.005$), and Winchester site ($r(39)=0.46$, $p=0.002$). Also, pairwise comparisons using the Wilcoxon signed rank test showed that *Actinobacteria* abundances for the first and last month of sampling were significantly different for WEBs fields 1 and 2 ($p=4.9E-4$) and Winchester site ($p=0.031$), but not for WEBs fields 11-14 ($p=0.49$).

Proteobacteria was the most abundant phylum in the studied soils throughout the season, independent of sampled depth or field. In all sites the average abundance was in the range of 34-42%, with standard deviations from 2.1 to 12.7 (Figure 3, Figure 4, and Figure 5). *Chloroflexi* were abundant in WEBs fields 1 and 2 and Winchester (Figure 3 and Figure 5). Importantly, deeper soil samples showed the greatest abundance of the phylum across the entire season. For example, *Chloroflexi* average abundance in Winchester was 3.6±0.8 and 3.8±1.1% in soils from 0-30 and 30-60 cm depth, respectively, but 13.4±4.2% in soil from 60-90 cm depth (Figure 5). *Chloroflexi*-abundance pairwise comparisons using the Wilcoxon signed rank test for WEBs fields 1 and 2 showed that the phylum abundance in soil from 0-30 cm depth was significantly different from abundance in soil from 30-60 and 60-90 cm depth ($p=1.7E-5$ and $p=0.031$, respectively), but abundances in soil from 30-60 and 60-90 cm depth were not significantly different ($p=0.148$). For Winchester, *Chloroflexi* abundances in soil from 0-15 and 15-30 cm depth were not significantly different ($p=0.615$), but

Chloroflexi abundance in soil from 30-60 cm depth was significantly different from abundance in soil from 0-15 and 15-30 cm depth (p=0.002 for both cases). Finally, *Bacteroidetes* was only above 10% abundance in WEBs fields 11-14 (Figure 4).

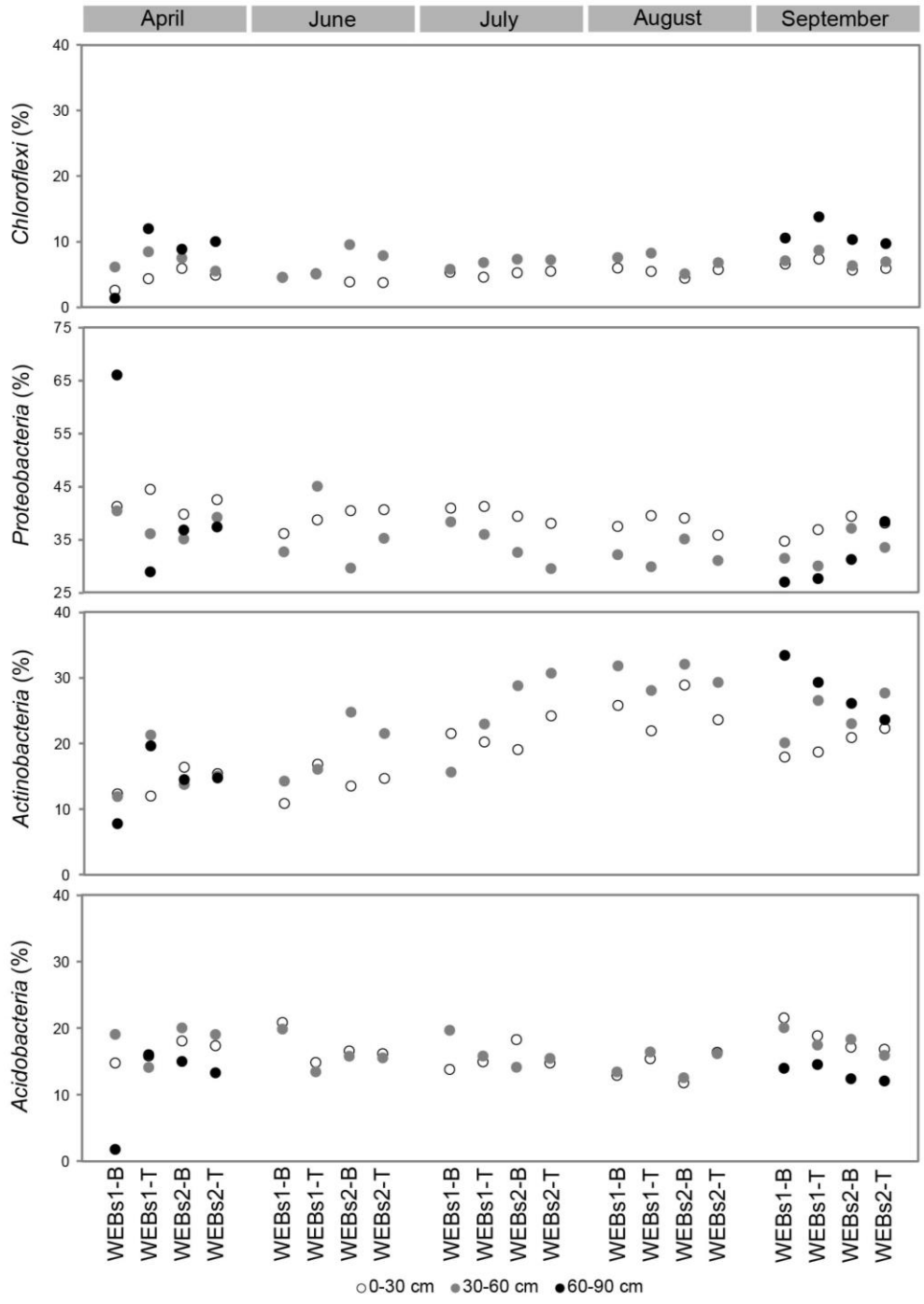

Figure 3. *Choroflexi, Proteobacteria, Actinobacteria,* and *Acidobacteria* relative abundance for soils from WEBs fields 1 and 2 for between tile (B) and above tile (T) sampling locations for sampled months of 2012 at different depths (0-30 cm in clear circles, 30-60 cm in grey circles, and 60-90 cm in black circles).

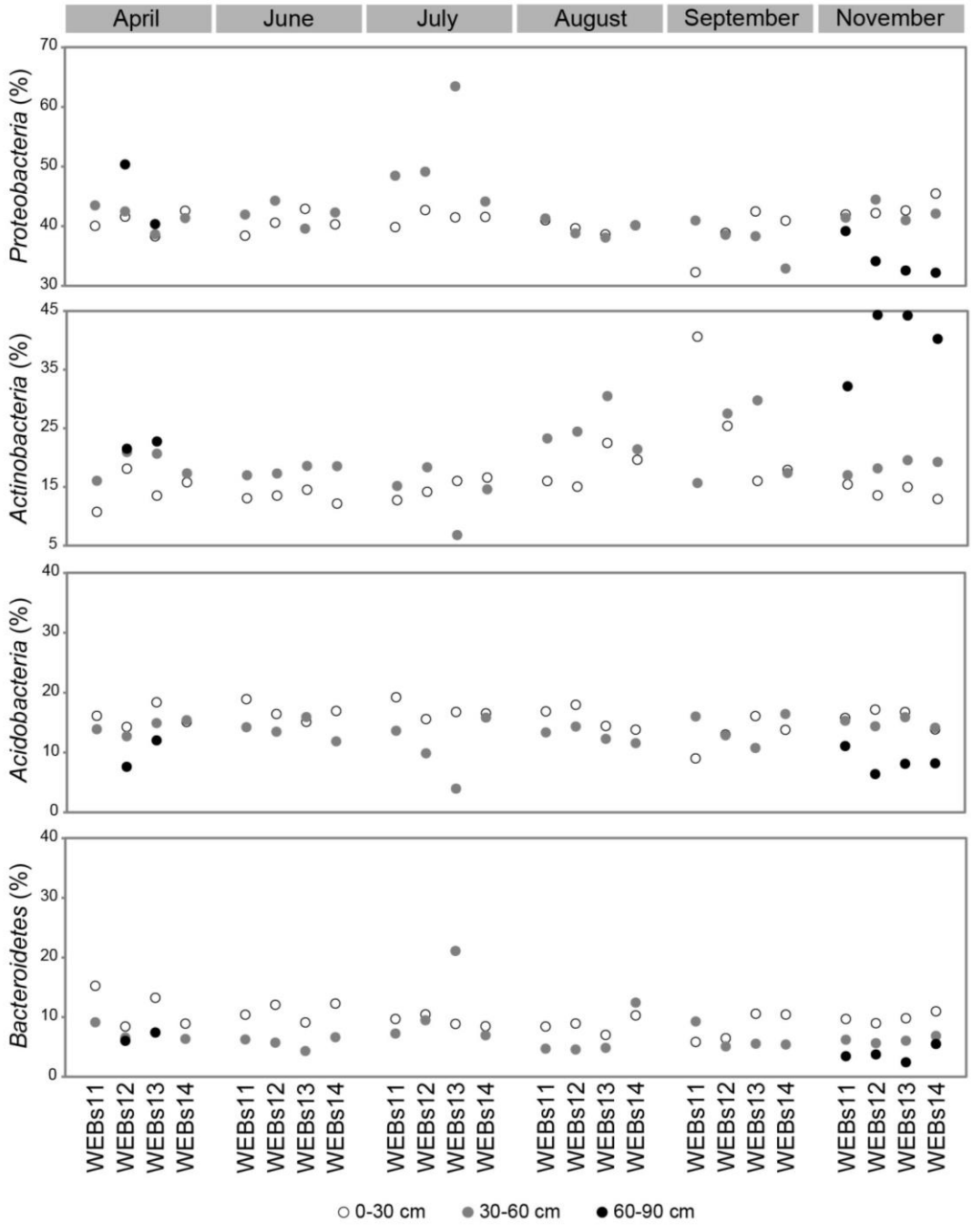

Figure 4. ***Proteobacteria, Actinobacteria, Acidobacteria,*** **and** ***Bacteroidetes*** **relative abundance for soils from WEBs fields 11-14 for sampled months of 2012 at different depths (0-30 cm in clear circles, 30-60 cm in grey circles, and 60-90 cm in black circles).**

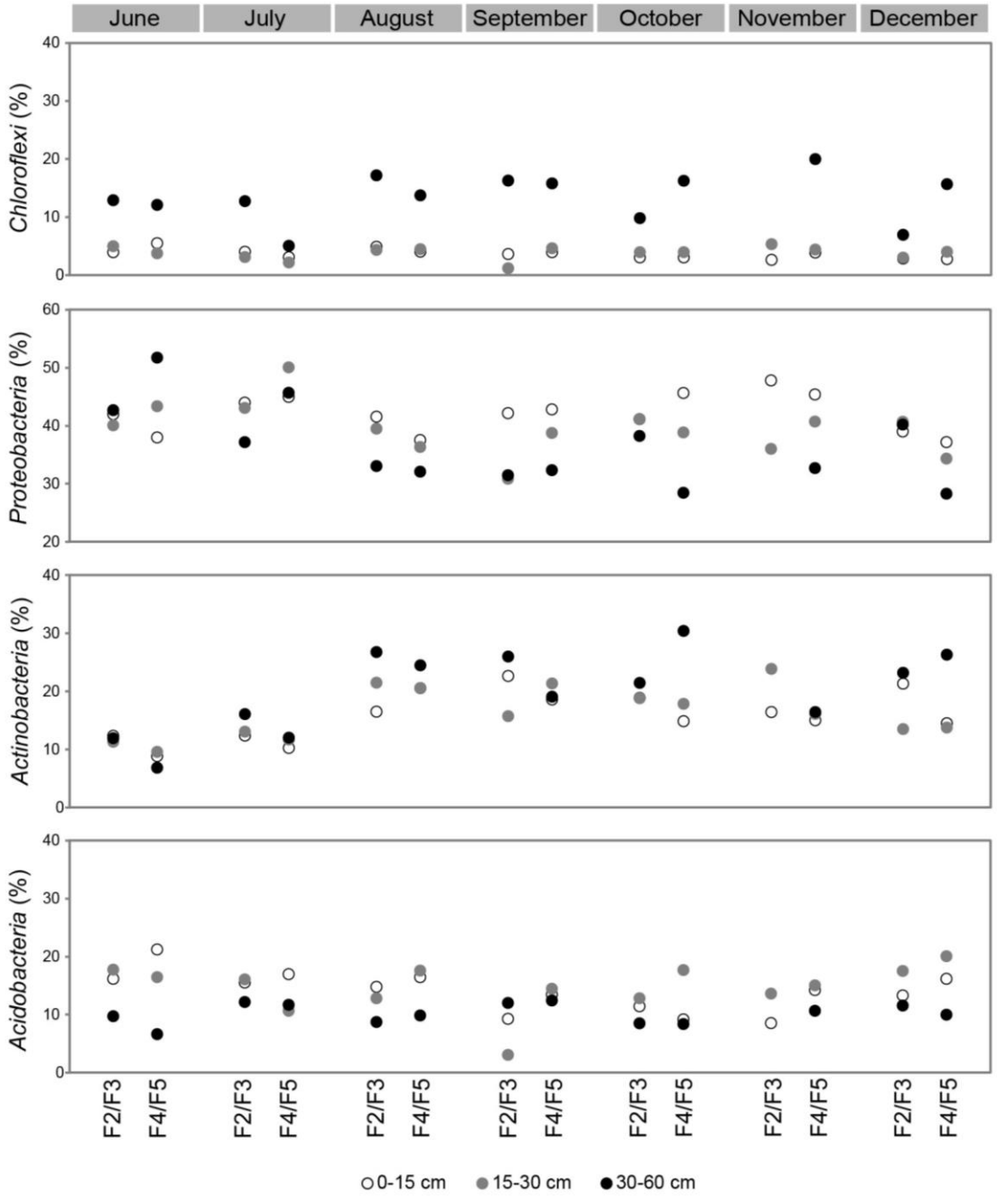

Figure 5. ***Choroflexi, Proteobacteria, Actinobacteria,*** **and** ***Acidobacteria*** **relative abundance for soils from Winchester fields 2/3 and 4/5 boundaries for sampled months of 2012 at different depths (0-15 cm in clear circles, 15-30 cm in grey circles, and 30-60 cm in black circles).**

The importance of *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* for soil bacteria community structure has been noted previously [22,61,150]. Several studies have detected abundance changes for these phyla in response to pH, nitrogen, and carbon. A study of 88 soils from North and South America, for example, showed that *Acidobacteria*, *Actinobacteria*, and *Bacteroidetes* varied in response to pH. Soil samples with pH below 5 had increased *Acidobacteria* abundance and lower *Actinobacteria* and *Bacteroidetes*, which increased in soils above pH 6 [22]. Another study showed opposite findings with respect to pH when characterizing the community from soils amended with different NH_4NO_3 inputs; researchers observed trends that included *Acidobacteria* decrease at low pH and *Actinobacteria* and *Bacteroidetes* increase at low pH. The response was related to the enhanced plant productivity and consequent increase in soil C, attested to by increases in *Bacteriodetes* and *Betaproteobacteria* (copiotrophs) and decreases in *Acidobacteria* (oligotrophs) in highly fertilized soil [61]. The pH of Winchester soil increases with depth, being 6.2, 6.5, and 7.4 for soil sampled at depth 0-15, 15-30, and 30-60, respectively (measured in the laboratory for September samples). These pH values might be in the range that does not affect the communities (Figure 5). Also, the data show consistent proportions of *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* across sites during the sampled months, despite the difference of crops, fertilizer type applied, drainage practice imposed, and overall field histories. One possibility is that differential management in these fields does not alter edaphic factors to the point where an altered structure would be evident at the phylum level, despite the fact that the communities from these sites differ from each other (Figure 2).

A seasonal study characterizing a wheat field recognized community structure variation during the sampling year [151]. In that study, the July community was different from communities profiled in January, May, and September. Also, the communities obtained from soils sampled in May and September were similar. The authors interpreted the recovery of similar communities in these months as a result of high nutrient availability caused by fertilization and residues left after harvest, respectively. A different study found that microbial communities were more active in July; DNA and RNA fingerprints for that time point showed that the communities were different. Soil moisture and air temperature were associated to these changes [147]. In the current data set, *Actinobacteria* increase could be attributed to nutrient load associated to the crop. For WEBs sites 1 and 2 and Winchester, the emergence of the crop coincided with cumulative increasing abundance of the phylum. Also, WEBs fields 11-14 showed the least noticeable *Actinobacteria* increase, which might relate to multiple harvests from these sites. In agreement with this hypothesis, *Actinobacteria* was

reported to increase in response to glucose and sucrose [152] and to decrease in a harvested site in comparison to an unmanaged site [153]. Studies have shown that improved soils (i.e., soils managed to increase nutrient content) have a different microbial community structure than those from unmanaged sites [154]. However, the reasons for this observation vary and are not fully established. My data comes from managed and improved sites and captured seasonal variations for *Actinobacteria* and a characteristic depth distribution profile for *Chloroflexi*. Further work and soil characterization needs to be done to elucidate the factors contributing to these observations in the selected fields.

3.1.1.3 Indicator species analysis

Indicator species analysis was used to recognize the bacterial OTUs ("species") that were associated with particular crop and fertilizer groups (soybean/mineral, alfalfa/mix, and corn/manure), drainage practice groups (CTD and UCTD), and depth groups (0-15, 15-30, 0-30, 30-60, and 60-90). No significant indicators were found for crop, fertilizer, or drainage groups. For depth, very few indicator species were associated with surface soil, in contrast to many indicators identified for deeper soil samples across all sites (Figure 6-Figure 10).

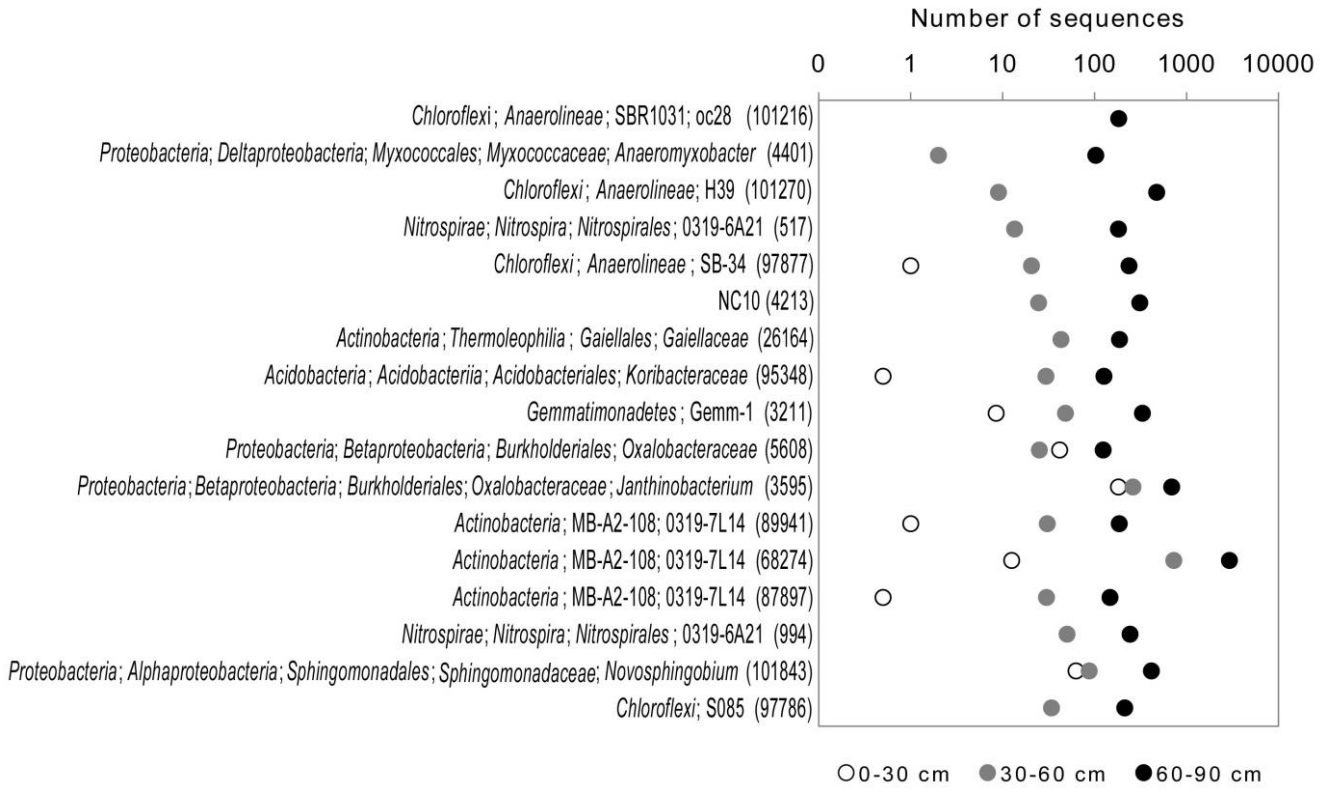

Figure 6. Indicator species associated with soil from 60-90 cm depth for WEBS field 1 with indicator value ≥ 0.7, sequence abundance median ≥ 100, and $p \leq 0.05$. The corresponding OTU number is shown in brackets. Additionally, OTU abundance median for soil from 0-30 cm depth (clear circles), 30-60 cm depth (grey circles), and 60-90 cm depth (black circles) are shown.

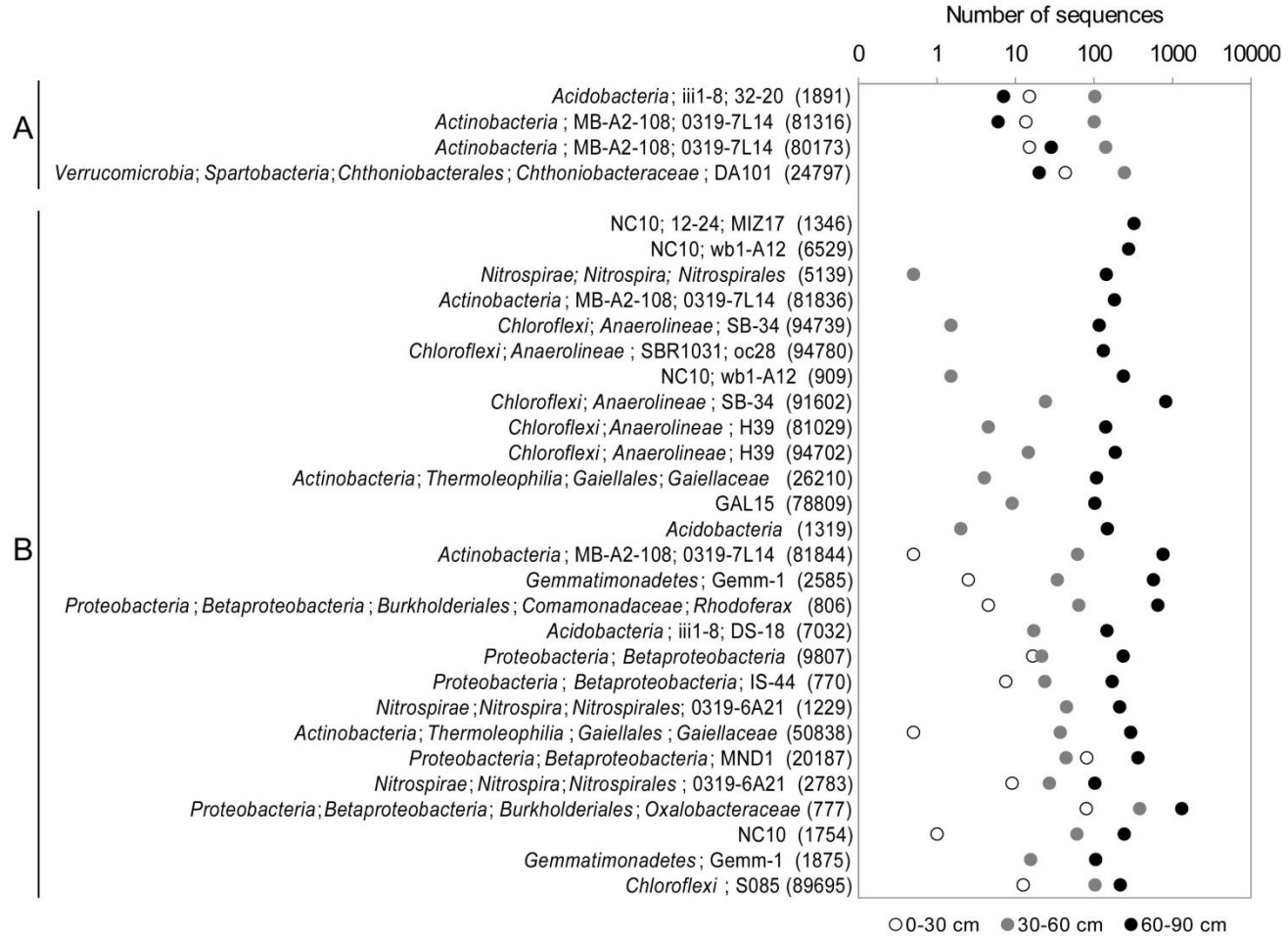

Figure 7. Indicator species associated with soil from 30-60 cm depth (A) and 60-90 cm depth (B) for WEBS field 2 with indicator value ≥ 0.7, sequence abundance median ≥ 100, and $p \leq 0.05$. The corresponding OTU number is shown in brackets. Additionally, OTU abundance median for soil from 0-30 cm depth (clear circles), 30-60 cm depth (grey circles), and 60-90 cm depth (black circles) are shown.

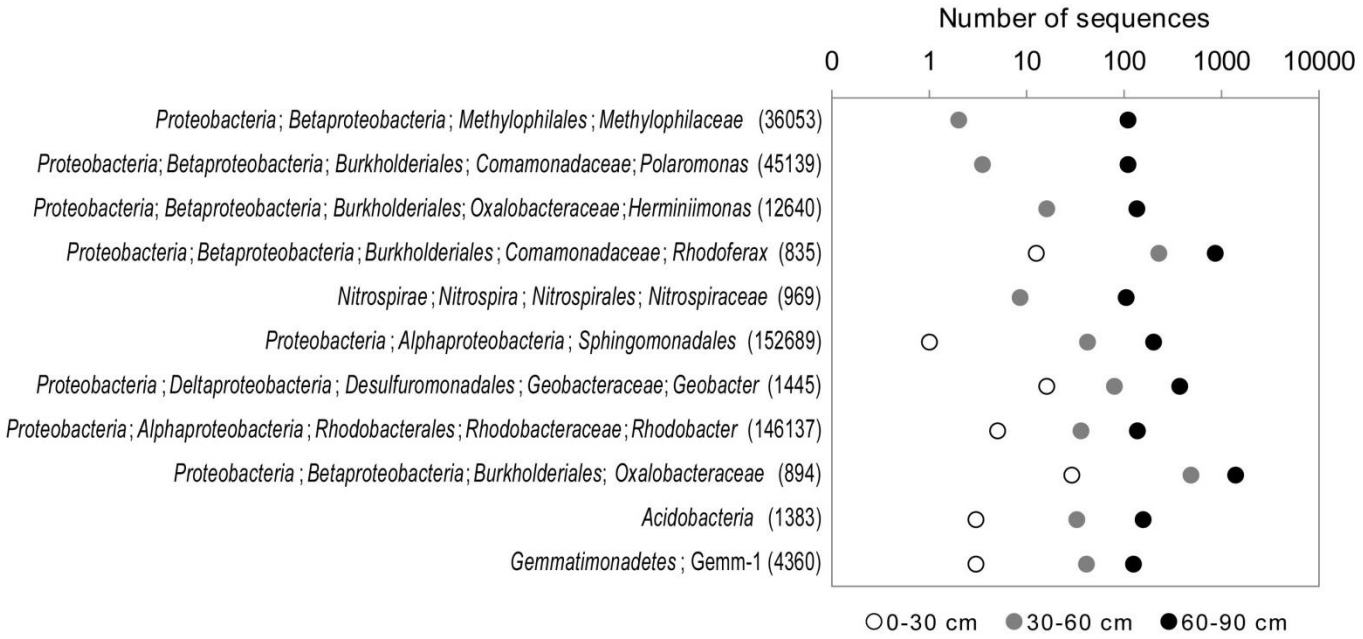

Figure 8. Indicator species associated with soil from 60-90 cm depth for WEBS fields 11 and 12 with indicator value ≥ 0.7, sequence abundance median ≥ 100, and $p \leq 0.05$. The corresponding OTU number is shown in brackets. Additionally, OTU abundance median for soil from 0-30 cm depth (clear circles), 30-60 cm depth (grey circles), and 60-90 cm depth (black circles) are shown.

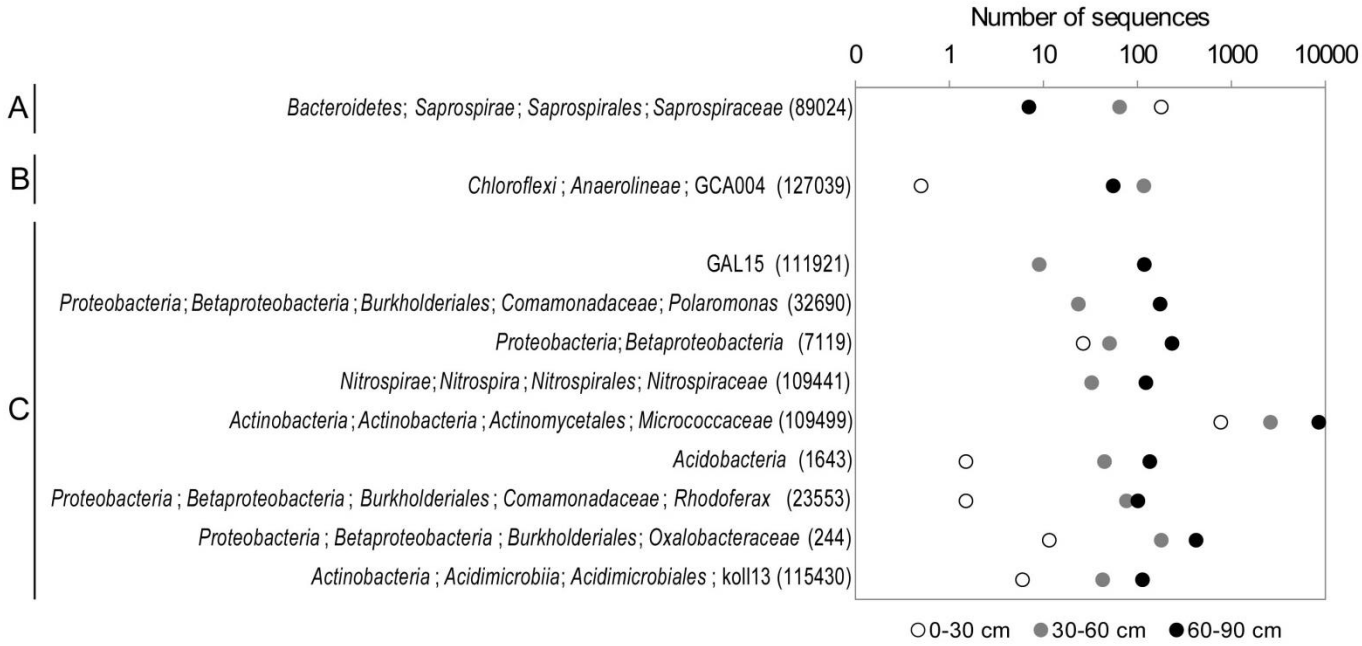

Figure 9. Indicator species associated with soil from 0-30 cm depth (A), 30-60 cm depth (B), and 60-90 cm depth (C) for WEBS fields 13 and 14 with indicator value ≥ 0.7, sequence abundance median ≥ 100, and $p \leq 0.05$. The corresponding OTU number is shown in brackets. Additionally, OTU abundance median for soil from 0-30 cm depth (clear circles), 30-60 cm depth (grey circles), and 60-90 cm depth (black circles) are shown.

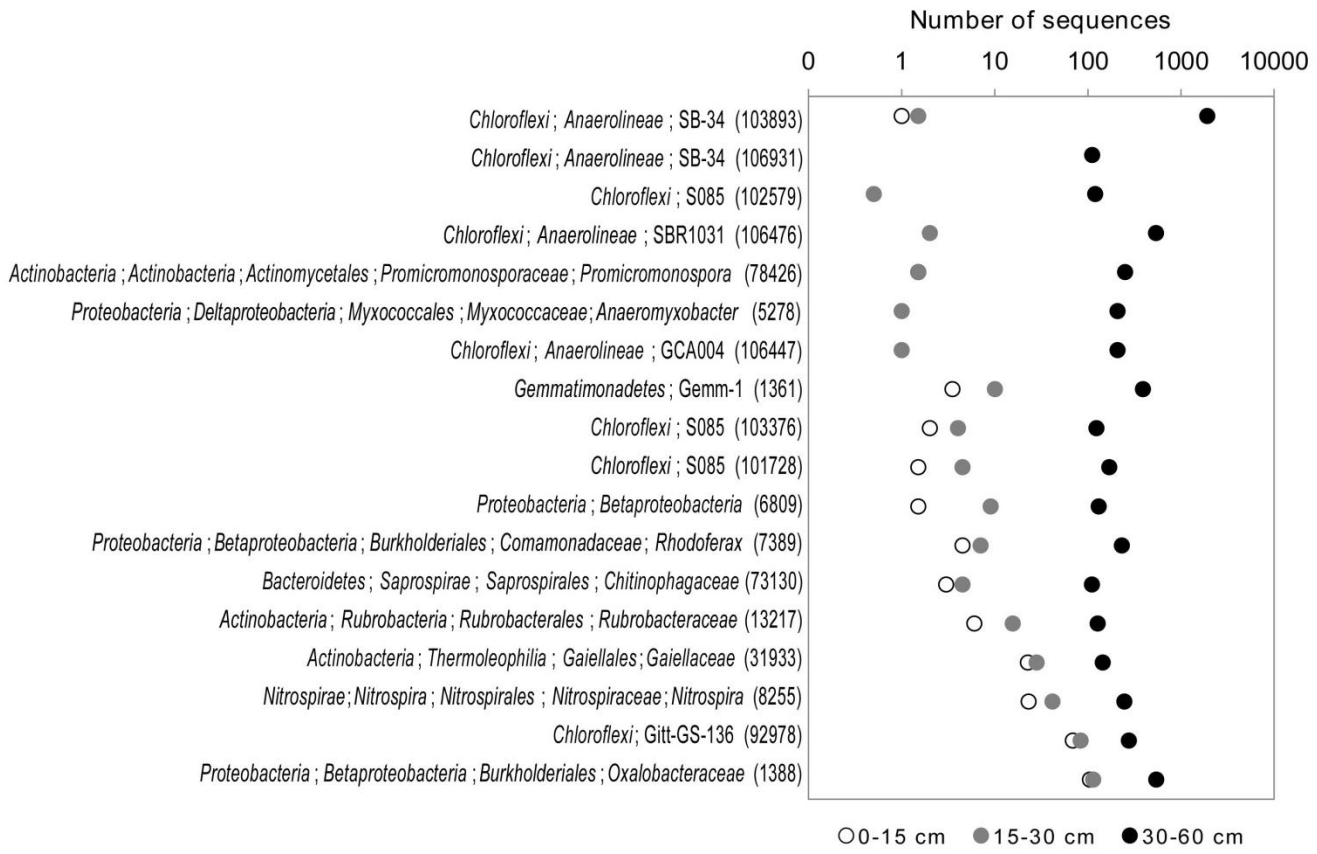

Figure 10. Indicator species associated with soil from 30-60 cm depth for Winchester with indicator value ≥ 0.7, sequence abundance median ≥ 100, and $p \leq 0.05$. The corresponding OTU number is shown in brackets. Additionally, OTU abundance median for soil from 0-15 cm depth (clear circles), 15-30 cm depth (grey circles), and 30-60 cm depth (black circles) are shown.

Alphaproteobacteria have been associated with C-rich environments like the rhizosphere [155]. Accordingly, relatively few indicators in my research belonged to this class in deeper soils (Figure 6 and Figure 8). *Geobacter* from the *Deltaproteobacteria* class was recovered both from an Fe(III) enrichment and untreated saturated C horizon [155], which highlights their ability to thrive in oxygen-limited conditions, like those that might be found in deeper soil layers (Figure 8). Likewise, the same study found spore-forming microorganisms like *Anaeromyxobacter* in horizons with lower pH, lower nutrients, and changing moisture conditions. This genus was also an indicator of the WEBs and Winchester deeper soil samples (Figure 6 and Figure 10). *Betaproteobacteria*, despite being favored by C-rich environments [146] like those found in the rhizosphere, were indicators for deeper soil in the present study (Figure 6-Figure 10). Notably, some of the detected indicators are capable of using electron acceptors different from oxygen for respiration. For example, identified members of the *Oxalobacteraceae* (*Janthinobacterium*, *Herminiimonas*) and *Comamonadaceae* (*Polaromonas*) (Figure 6, Figure 8, and Figure 9), are capable of denitrification [45,156,157].

Other phylum-level distributions have also been observed previously in response to depth. The phylum *Actinobacteria* was previously found to increase in abundance with deeper soil horizons [153]. Members of the *Actinomycetales* order, for example, have sporulation capacity that favors survival in nutrient-content variable environments [158]. The phylum was represented among the indicators by families *Gaiellaceae*, *Micrococcaceae*, *Promicromonosporaceae* (*Promicromonospora*), and *Rubrobacteraceae* in soil samples from 30-90 cm depth (Figure 6, Figure 7, Figure 9, and Figure 10). The phylum *Gemmationadetes* has been found to increase in intermediate soil horizons, the distribution was attributed to pH, with alkaline pH favoring the phylum [153]. Accordingly, class Gemm-1 was identified as an indicator in the sites from 30-90 cm depth (Figure 6-Figure 8 and Figure 10). The phylum *Bacteroidetes* has been observed to decrease with soil depth [146] and are recognized copiotrophs [159], which might explain why the data show this phylum associated with surface soil (Figure 9). The phylum *Verrucomicrobia* was more abundant in soils from 10-50 cm depth [146], consistent with my findings (data not shown) and was identified in one site as an indicator from 30-60 cm depth (Figure 7). However, the ecology of *Verrucomicrobia* is largely unexplored.

The phylum *Chloroflexi* featured as an important indicator for soil from 30-90 cm depth (Figure 6, Figure 7, Figure 9, and Figure 10). *Chloroflexi* are commonly found in soils [153,155] and recent work showed that *Chloroflexi* representatives require up to 12 week-incubation periods to grow on media [160]. Because deeper soil has lower diversity [146] and evidence suggests that deeper soil

conditions might reduce the chance of observing dominant species [147], it could be hypothesized that microorganisms that have low growth rates and enzymes suited to low-nutrient content environments might benefit from the reduced competition of this niche. *Acidobacteria* have been suggested to withstand soil moisture variation and to be adapted to low nutrient environments by means of high substrate affinity enzymes [161], which would explain why the phylum appears as indicator for deep soil in the present study (Figure 6-Figure 9). Similarly, a *Nitrospira* genome analysis in conjunction with experimental data have shown adaptation to low-nutrient environments [162] and this microorganism was an indicator for deeper soil (Figure 6-Figure 10).

3.2 Denitrifying community characterization

Cultivation-independent and isotope-based incubations, followed by metagenomics, were used to identify the complement of denitrifying bacteria from intact agricultural soil cores fed $^{13}C_6$-glucose, using flow-through reactors (FTRs) under water-saturated conditions. The studied sites have been amended historically with either manure- or mineral-based fertilizers and operated under CTD or UCTD systems. Two incubation time points were employed to evaluate denitrifier temporal shifts. Also, nitrate reducing rates were calculated. Finally, nitrite reductase enzymes recovered from denitrifier-enriched DNA were analyzed to evaluate the likelihood to recover these genes in a denitrifier molecular survey of these soils.

3.2.1 Denitrifying flow-through reactors behavior

Eight FTRs per agricultural field were prepared for DNA-SIP incubation to enrich nucleic acids from the denitrifying population without the requirement of cultivation. The FTR microcosms helped preserve the *in situ* spatial distribution of the soil microbial communities during the study. Commonly, soil denitrification rate evaluations [49,67] and DNA-SIP studies [58,163,164] employ homogenized and sieved soil, which can influence the observed rates and alter microbial substrate uptake.

The DNA-SIP incubation had two stages. First, a 21-day starvation period with NO_3^- addition was imposed to promote the uptake of readily available carbon sources in the soil. Second, a denitrification period was triggered by $^{12}C_6$-glucose or $^{13}C_6$-glucose supply to four replicate reactors

along with NO_3^-, constituting of four pairs of denitrifying reactors per agricultural field studied, to be sacrificed at two different time points. During the incubation, both the feeding solution and outflow were monitored periodically to evaluate the community metabolic response to the imposed incubation conditions.

During the starvation period, the community was expected to reach a steady state with minimum denitrification activity, verified by quantitative recovery of fed NO_3^- and a low recovery of CO_2 (Figure 11). In this stage, nitrite was below the detection limit and N_2O showed low levels that declined over time (data not shown). These observations suggested that the community had used the available carbon for reduction of provided NO_3^- and thereafter metabolic activities were at a minimum given the absence of electron donors for respiration and energy production. The second part of the incubation was initiated once a steady state was achieved.

After glucose was supplied, denitrification was induced and became the dominant respiratory process occurring in the FTRs, as seen by NO_3^- reduction and CO_2 production (Figure 11). At that point of the incubation, anoxic conditions, electron acceptor (NO_3^-), and electron donor (glucose) were present, meeting the requisites for denitrification [69]. Nitrite and N_2O were also detected as products of the denitrification process. Nitrite levels were low during the denitrification stage of the incubation ranging from below the detection limit (i.e., 10 µM) to 537 µM. Observed values implied nitrite was being used actively by nitrite reductase in sequential denitrification reactions for generation of chemical energy.

Precautions were taken to prevent other carbon assimilation pathways from occurring in the FTRs. All feeding solutions were kept anoxic and the soil cores were protected from light. Among alternate metabolic pathways, fermentation was of concern because it occurs in oxygen-limited environments [165]. The pH of the non-buffered solution ranged from 6 to 7.5 showing no acidification of the flow through, suggesting that minimal fermentation occurred. Expected pH values attributed to acetic or butyric acids evolved from glucose fermentation, according to a batch experiment, would be around pH 5 [165]. Alternatively, to prevent fermentation, some researchers prefer to use succinate for DNA-SIP studies, representing a non-fermentable substrate [164], although we did not do this here. Also, ammonia and ammonium were quantified as ammonium to corroborate that N did not deviate from denitrification due to DNRA, which has been associated with both C-rich [83] and C-deficient [82] environments. As expected, ammonia and ammonium were present in concentrations below 20 µM with an average of 7 µM during the length of the incubation, indicating that ammonium was not being

actively produced. Nitric oxide consumption through anammox was not expected in the studied soil, because previous molecular surveys were unable to detect anammox bacteria in the same field sites (Moore and Neufeld unpublished work, 2011).

During the denitrification stage of the incubation, N_2O was detected in all reactor outflows (data not shown), indicating that incomplete denitrification was occurring. This might have been because a constant supply of NO_3^- was maintained, NO_3^- is more thermodynamically favorable as an electron acceptor than N_2O [166]. This may also have been because of enzyme kinetics, if initial reactions of denitrification yielded more product than N_2O reductase could transform [167]. Alternatively, the denitrifier community itself might have been dominated by populations of denitrifiers that do not possess the nitrous oxide reductase gene [34]. However, once the flow rate was increased from 1 to 2 mL h^{-1}, N_2O was no longer detected in the outflow. This indicated that the supply of more organic carbon was necessary for N_2O reduction, implying that C was limiting when the flow was kept at 1 mL h^{-1}. Similarly, previous studies have shown that C content can be sufficient to support nitrate reduction and still be limited for denitrification enzyme activity [67]. The N_2O detection method was qualitative and not quantitative, so the described N_2O behavior during the incubation is based on qualitative observations.

Carbon dioxide was quantified and represented dissolved inorganic carbon, which is expected as a product of the metabolism of the substrate provided as electron donor. The following balanced reaction dictates the stoichiometry of the reaction:

$$5C_6H_{12}O_6 + 24NO_3^- \rightarrow 6H^+ + 30HCO_3^- + 12N_2 + 12H_2O$$

According to the reaction and considering that the input solution had 0.31 mM glucose, the generated CO_2 could have a maximum of 1.86 mM. The average value for CO_2 observed during the denitrification stage of the incubation was 0.81 mM, with a minimum and maximum of 0.32 mM and 1.17 mM, respectively (Figure 11). Mass balances for C and N were not calculated because all carbon and nitrogen species were not quantified. Also, a portion of the C and N must have been consumed during microbial proliferation for cellular component synthesis. However, FTR behavior denotes that denitrification was the main process during the second stage of the incubation.

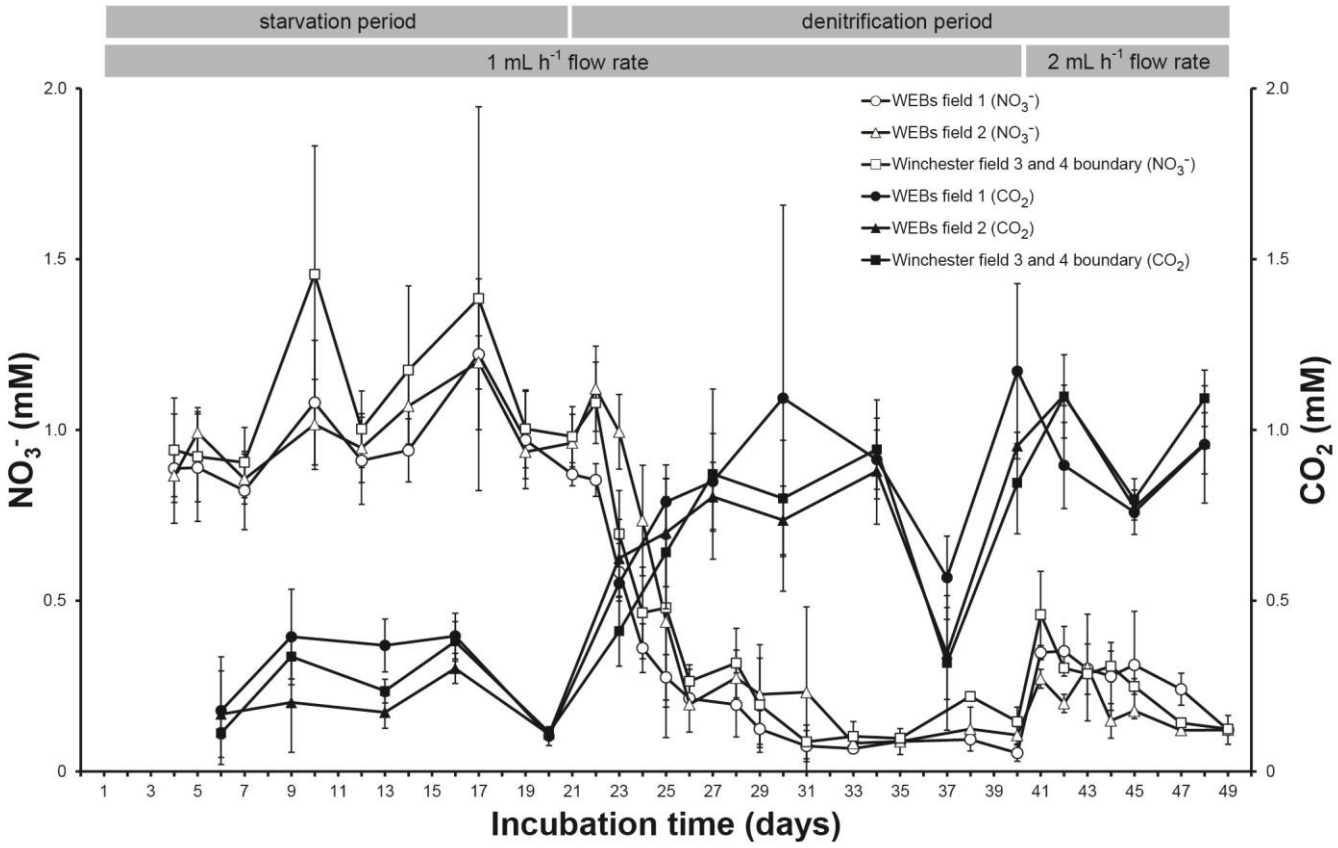

Figure 11. NO_3^- consumption and CO_2 production during the FTR DNA-SIP incubation. Both the starvation and denitrification periods (i.e., 22 and 27 days, respectively) of the incubation are shown. During the starvation stage NO_3^- was supplied to the reactors and during the denitrification stage both NO_3^- and $^{13}C_6$-glucose were supplied to the reactors. At two incubation time points (i.e., days 30 and 49) two randomly chosen FTRs were sacrificed from each studied field (i.e., WEBs field 1, WEBs field 2, and Winchester fields 3 and 4 boundary). For the FTRs with longer incubation times, the feeding flow rate was increased from 1 to 2 mL h^{-1} at incubation day 40. Error bars represent the standard deviation for replicate FTRs.

The glucose concentration in the feed solution was 1.5 times the required C to reduce the supplied N. The first incubation time point (i.e., 8 days after the denitrification stage had started) was reached when 5 to 9 µmoles of ^{13}C had been consumed per cm^3 of incubated soil, because previous reports suggested a consumption range of 5-500 µmoles of ^{13}C per gram of soil for successful labeling [105]. The soil retrieved at the second time point (i.e., 27 days after the denitrification stage had started) metabolized from 31.5 to 35 µmoles of ^{13}C per cm^3 of incubated soil. The incubation time was kept to the minimum necessary to avoid cross-feeding and consequent labeling of non-denitrifying bacteria.

3.2.2 Community denitrifying capacity

The denitrification potential for the three field soils incubated under denitrifying conditions was calculated at the two imposed feeding flow rates as the nitrate reduction rate during stabilized conditions, using the equation presented in section 2.2.2.3 (Table 3).

Table 3. Denitrification potential of agricultural soils during the FTR incubations at two imposed feeding flow rates during stabilized conditions.

Flow rate (mL h^{-1})	Nitrate reduction rate (N·nmol cm^{-3} h^{-1}) [1]		
	WEBs (1)[2]	WEBs (2)[2]	Winchester (3 and 4 boundary)[2]
1	35.91±1.57	34.17±1.00	35.66±2.57
2	66.14±4.55	65.86±3.91	71.49±4.11

[1] Mean ± SD, n=4

[2] Site (field)

Site and field of origin of the studied soils (i.e., soil characteristics and management practice histories) might affect observed nitrate reduction rates, but that effect might differ according to the feeding solution flow rate imposed to the FTRs during the incubation. A two-way analysis of variance (two-way ANOVA) tested the nitrate reduction capacity of the soils under imposed 1 and 2 mL h^{-1} feeding solution flow rates. The soils did not show significantly different nitrate reduction rates, despite proceeding from different sites and fields and having diverse management histories ($F(2,18)$ =

2.57, $p = 0.105$). However, the soils showed significantly different nitrate reduction rates when challenged with different feeding flow rates ($F(1,18) = 607.52$, $p = 2.54E\text{-}15$). The interaction of site and field of origin of the studied soils and feeding flow rate had no significant effect on the observed nitrate reduction rates ($F(2,18) = 1.61$, $p = 0.230$).

WEBs fields 1 and 2 have disparate drainage modes that might change moisture content in the CTD field (WEBs field 2). However, recorded moisture data from the study sites revealed no trend associated with drainage practices or depth (data not shown), which could be attributed to a particularly dry year. Drainage practice impacts on the community, if present, should persist to consider moisture a long term modifier of the denitrifier community in these fields, referred to as a distal control [42], but the rates were similar (Table 3). Because evidence of drainage influences on active denitrifiers is lacking, the possibility exists that the management practice has no impact on that part of the community. An alternative possibility is that the community responded to the imposed laboratory conditions, indicating that there is a common seed of denitrifiers that readily responds to events favoring denitrification in the same manner.

The Winchester site differed from WEBs fields 1 and 2 in that it had received manure as a fertilizing agent for the past ten years, instead of commercial fertilizers. Another difference between the fields was crop rotation (see section 2.1.1). The denitrification rates obtained for Winchester were similar to those obtained for the WEBs fields (Table 3), implying that despite the diverse background of the soils, the potential for denitrification was equivalent. Reports suggest that the fertilizer applied to agricultural fields affects the microbial communities, particularly when accompanied by a pH shift [72]. Importantly, a pH change does not always affects microbial community structure [61]. In addition, the denitrifier community composition does not always correlate with potential denitrification activity [64].

For the studied WEBs fields 1 and 2 and Winchester site, no differences in the denitrification potential were found, despite contrasting drainage practices and fertilization regimes applied to the fields. However, increasing the rate of N and C supplementation caused denitrification rates to increase significantly (Table 3). This is an important observation and has major implications in scenarios of fertilizer application, root exudate secretion, and organic matter degradation, as reported by previous research [60,73].

3.2.3 Heavy and light DNA fingerprints

3.2.3.1 Clustering DGGE fingerprints

Soil DNA from the FTRs was extracted and processed by density gradient ultracentrifugation, followed by fractionation to separate DNA contributed by active denitrifiers (i.e., heavy DNA) from that of the background community (i.e., light DNA). DGGE of the amplified 16S rRNA gene was used to generate fingerprints of the microbial communities concentrated in the heavy and light DNA fractions. Fingerprints from the six reactors fed with $^{13}C_6$-glucose showed that heavy DNA corresponded to fraction 5 of the gradient from each reactor and it grouped separately from the light DNA corresponding to fraction 11 (Figure 12). Such grouping is expected as a consequence of the directed DNA labeling and confirms sufficient label incorporation during the SIP incubation. Interestingly, three main bands (Figure 12, bands 1-3) were common to heavy DNA in all the reactors, despite them being derived from distinct agricultural field sites.

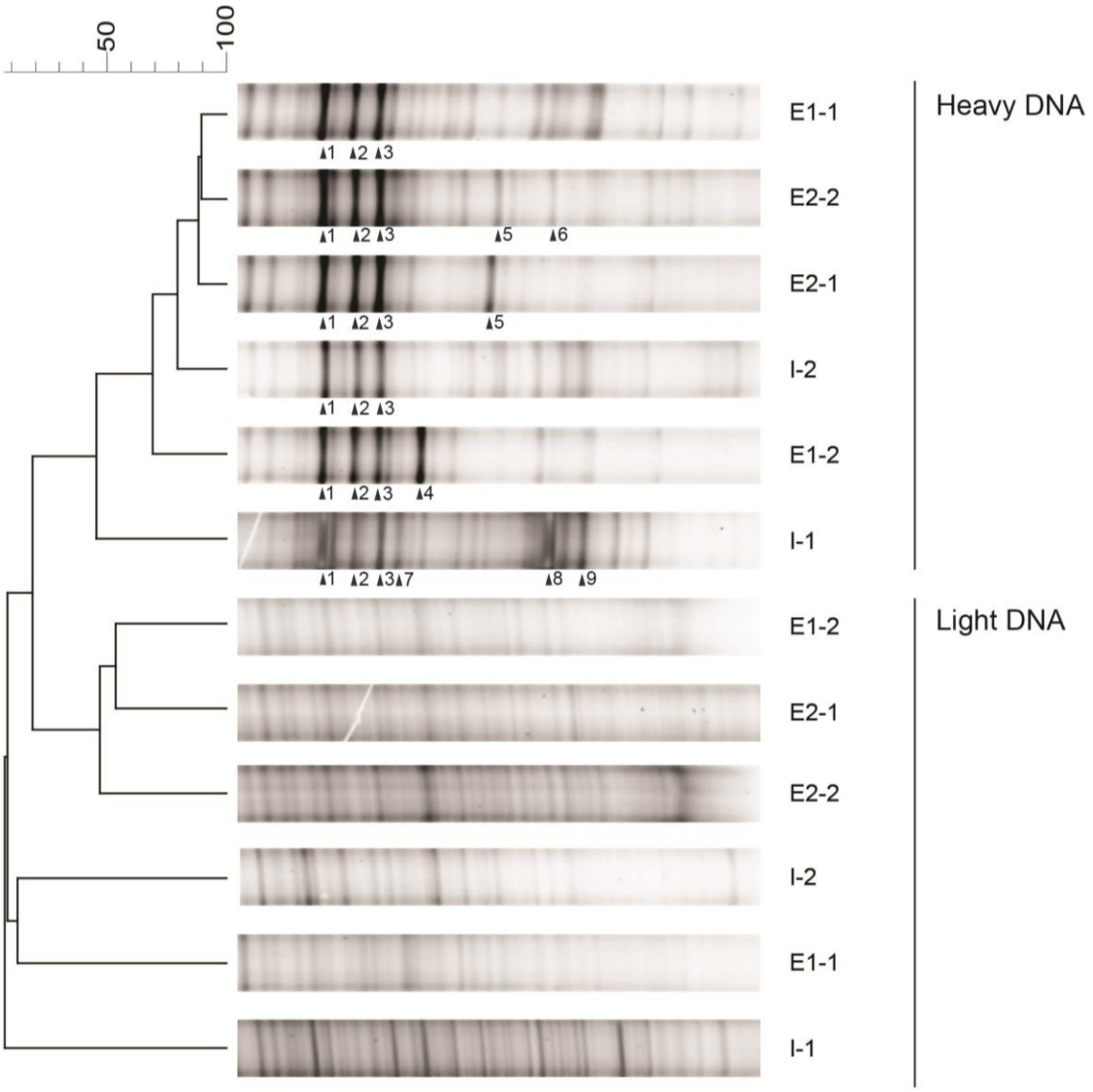

Figure 12. An UPGMA dendogram of the 16S rRNA gene DGGE fingerprints of heavy and light DNA-SIP fractions from FTRs sacrificed after 30 days of incubation (i.e., 8 days of denitrification activity after glucose addition) with numbered picked bands (triangles). Two replicate FTRs from WEBs field 1 (E1-1 and E1-2), WEBs field 2 (E2-1 and E2-2), and Winchester fields 3 and 4 boundary (I-1 and I-2) are shown.

3.2.3.2 Identified taxa by DGGE-band sequencing

Dominant putative denitrifier DGGE bands (numbered in Figure 12) were excised, re-amplified, and sequenced. Taxonomic assignment using the RDP classifier [117] assigned all but one sequence to the *Betaproteobacteria* class (Table 4); the exception was band 7, which was assigned to *Flavobacteria*. Sequenced bands classified to the *Burkholderiales* order, included members of the *Oxalobacteraceae* family (Figure 12, bands 1-3), a band from the *Comamonadaceae* family (Figure 12, band 4), and a band from the *Burkholderiaceae* family (Figure 12, band 5). The order *Rhodocyclales* was represented by the *Rhodocyclaceae* family (Figure 12, band 6). Finally, a member of the order *Flavobacteriales*, family *Flavobacteriaceae* (Figure 12, band 7) and two members of the order *Neisseriales*, family *Neisseriaceae* (Figure 12, bands 8 and 9) were retrieved. For comparison, the sequences were submitted to BLAST [138] for taxa attribution with the GenBank database [168] (Table 4). Agreement between databases was not consistent at the genus level, but family-level affiliations were consistent for all band sequences.

Table 4. Identity of abundant DNA-SIP denitrifiers obtained through DGGE-band sequencing.

Band	Family	Genus	
		RDP (% similarity)	GenBank (% similarity, E value)
1	*Oxalobacteraceae*	*Oxalicibacterium* (33)	*Janthinobacterium* (96, 7E-48)
2	*Oxalobacteraceae*	*Herminiimonas* (39)	*Massilia* (99, 1E-53)
3	*Oxalobacteraceae*	*Massilia* (36)	*Massilia* (99, 4E-53)
4	*Comamonadaceae*	*Acidovorax* (97)	*Acidovorax* (100, 2E-55)
5	*Burkholderiaceae*	*Pandoraea* (28)	*Ralstonia* (96, 4E-39)
6	*Rhodocyclaceae*	*Azonexus* (28)	*Dechloromonas*, *Azonexus*, *Azoarcus*, *Thauera* (98, 1E-29)
7	*Flavobacteriaceae*	*Meridianimaribacter* (15)	NA
8	*Neisseriaceae*	*Pseudogulbenkiania* (97)	*Chromobacterium* (100, 2E-54)
9	*Neisseriaceae*	*Pseudogulbenkiania* (51)	*Chromobacterium* (93, 3E-47)

NA, not applicable because the sequence was not successfully classified.

Acidovorax sp. is a denitrifier that has been found in waste water treatment plant (WWTP) systems [169], sludge [52], and drinking water denitrification reactors, including *Acidovorax facilis* [170]. *Ralstonia* and *Thauera*, that inhabit soil, have denitrifiers among their member species [45], such as *R. basilensis* M91-3 [171], *T. aromatica*, and *T. mechernichensis* [52]. *Azonexus* has both denitrifying and non-denitrifying species, including *A. fungiphilus* LMG 19789^T, which has no known denitrifying capacity and *A. caeni*, (isolated from WWTP sludge), which can reduce nitrate to N_2. A close relative of *Azonexus* is *Dechloromonas agitata* CKB^T [172], a species with denitrifying capacity [52]. *Azoarcus* has many denitrifier representatives isolated from aquatic and soil environments, including strains of *A. tolulyticus*, *A. toluclasticus*, and *A. toluvorans* [173]. Bulk soil *Azoarcus* species have been suggested to have an anaerobic metabolism and to use nitrate as electron acceptor [174].

Janthinobacterium sp. was affiliated with the heavy DNA bands. Strain A1-13, which was isolated from arable soil, was reported as able to reduce nitrate with high N_2O contributions [156]. Another band affiliated with *Pseudogulbenkiania* sp. The strain NH8B, which was isolated from an agricultural soil, reduces N_2O, which might implicate this genus as a N_2O emission mitigating agent [175]. Interestingly, *P. subflava* BP-5^T is not able to reduce nitrite to N_2, whereas *Chromobacterium subtsugae* PRAA4-1^T, one of its closest relatives, has this capacity [176] and within this genus *C. violaceum* is able to reduce nitrate and nitrite [177].

Both *Massilia* spp. [164] and *Meridianimaribacter flavus* [178] were affiliated with the DGGE band sequences and were found previously to be unable to reduce nitrate. Researchers were unable to detect a nitrite reductase gene in *Massilia* strains although the authors speculated primer mismatches prevented amplification; the nitrous oxide reductase gene was detected and its activity confirmed using SIP incubations under N_2O reducing conditions with succinate as electron donor [164]. *Meridianimaribacter* is a relatively new genus with little published information. *Herminiimonas* sp. SP-B was isolated from cold marine sediment and identified as psychrophilic denitrifier, using a mixture of acetate, propionate, and butyrate as electron donors to reduce nitrate [157]. This genus has five known species, of which three have shown nitrate reduction [179].

Pandoraea has been also isolated from soil, but is more commonly known as an opportunistic pathogen [180]. The presence of a nitrate reductase coding gene involved in DNRA is known for *Pandoraea* sp. RB-44 and *P. pnomenusa*, *narZ* genes X636_23310 and U875_11400, respectively, annotated in the KEGG database [140]. *Oxalicibacterium* has never been found to grow anaerobically

by reducing nitrate or nitrite, although *Herminiimonas, Janthinobacterium*, and *Massilia* species are among its close relatives [181,182].

3.2.4 Heavy and light DNA next-generation sequencing

Sequences corresponding to the V3/V4 region of the 16S rRNA were obtained from 18 samples as follows: 6 heavy-DNA samples from soil incubated under denitrification conditions for 8 days, 3 heavy-DNA samples from soil incubated under denitrification conditions for 27 days, 6 light-DNA samples from soil incubated under denitrification conditions for 8 days, and 3 light-DNA samples from soil incubated under denitrification conditions for 27 days.

Sequencing 16S rRNA gene amplicons from the heavy and light fractions from the FTR resulted in 568,443 paired-end sequences. Each sequenced sample contributed an average of 31,580 paired-end sequences (minimum 12,545 and maximum 79,018). After assembly of paired-end sequences, clustering by similarity, and chimera check, the remaining number of assembled sequences was 558,857 (minimum 12,486 and maximum 77,789). For beta-diversity analysis requiring equal sequence contributions from each sample (i.e., Bray-Curtis PCoA, UniFrac PCoA, NMS, MRPP, and indicator species), these were rarefied to 12,486 sequences.

3.2.4.1 Clustering next-generation sequencing community data

UniFrac-based PCoA ordination revealed relevant taxa associated with the samples (Figure 13A) and showed that heavy DNA separated from light DNA (Figure 13B). In addition, samples that were incubated the longest grouped more closely together (Figure 13C). In comparison, a PCoA ordination based on a Bray-Curtis distance matrix showed heavy and light DNA separation, but no incubation-length effect (data not shown). Taxa associated with the heavy DNA were *Rhizobiales*, *Comamonadaceae*, *Oxalobacteraceae*, *Neisseriaceae*, *Azoarcus*, *Acidovorax*, and *Janthinobacterium* (Figure 13A), which overlapped with DGGE results (Table 4).

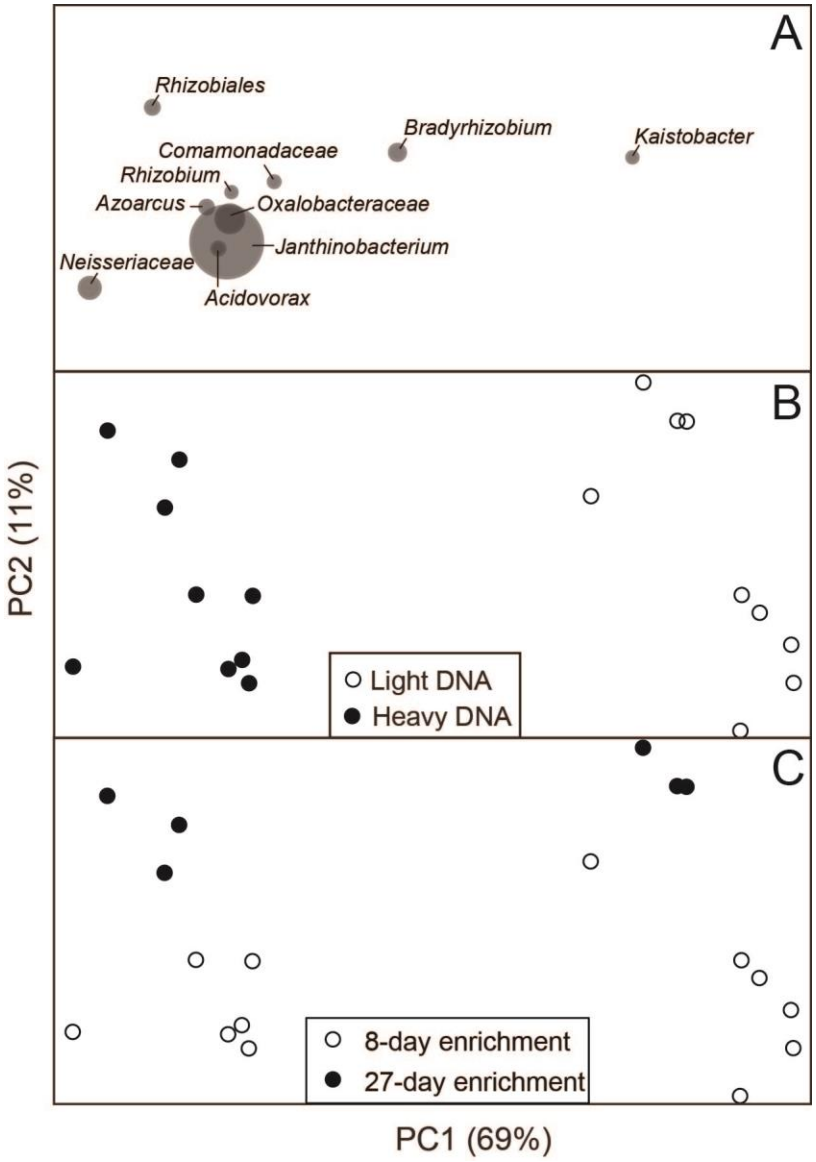

Figure 13. Weighted-UniFrac PCoA ordination of next-generation sequencing data of heavy and light DNA-SIP fractions from FTRs with associated taxa (A), colored by DNA-type (B) and incubation time point under denitrifying conditions (C).

Alternative ordination methods confirmed separation of heavy and light DNA samples (Figure 14A) and also did not indicate changes in community composition with increased incubation time (Figure 14B). A MRPP test supported heavy and light DNA difference (p=0.001) with a high negative value for between-group separation (T=-10.2) and high within-group similarity (A=0.22),

which can also be seen with the spread of the samples on the ordination. MRPP values showed no support for incubation-time differences (T=-0.3, A=0.01, and p=0.24).

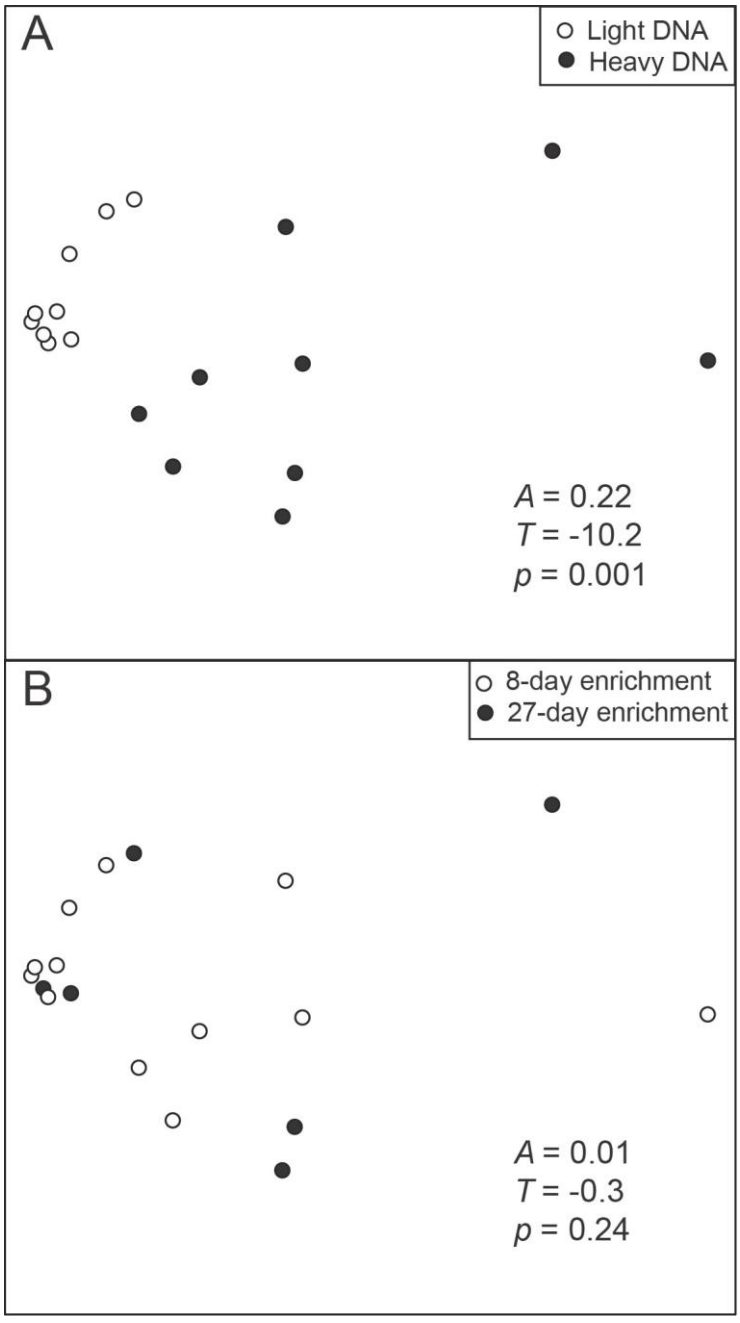

Figure 14. NMS ordination and inset MRPP data (based on a Bray-Curtis distance matrix) of next-generation sequencing data of heavy and light DNA-SIP fractions from FTRs colored by DNA-type (A) and incubation time point under denitrifying conditions (B).

NMS and PCoA ordinations possess different characteristics that shape their suitability for identifying patterns in ecological data. Generally, NMS is recommended due to its freedom in pattern search because it is not limited to identifying linear relationships. PCoA is an ordination method that is being used increasingly because of its amenability to any distance matrix [183]. For the Illumina sequencing data, both methods identified the grouping of samples according to light or heavy DNA, which is due to the differential taxonomic representation of denitrifiers in the heavy DNA. Also, results imply similarity of the active communities enriched from three fields from two agricultural sites irrespective of their soil of origin and corresponding site management practices.

3.2.4.2 Taxa exploration and indicator species

Comparison of the taxonomic profiles from heavy and light DNA showed distinct patterns for heavy and light DNA (Figure 15A and Table 5). The heavy DNA was dominated by the *Betaproteobacteria* class (Figure 15B) of the *Oxalobacteraceae* and *Comamonadaceae* families (Figure 15C). Abundant heavy DNA genera included *Janthinobacterium*, *Acidovorax*, *Ralstonia*, and *Azoarcus* (Figure 15D). Several *Alphaproteobacteria* were also represented in the heavy DNA, in addition to a group of the *Actinobacteria* phylum (Table 5). Similarly, previous characterization of 199 cultivated denitrifiers classified the majority of isolates as *Betaproteobacteria* (50.4%), followed by *Alphaproteobacteria* (36.8%), *Gammaproteobacteria* (5.6%), and *Epsilonproteobacteria* (2%) [52].

Indicator species analysis of the DNA-SIP fractions revealed species or taxa associated with the denitrifiers, indicating microorganisms capable of reducing nitrate in the established conditions (Table 6). The identified indicators were highly similar to taxa associated with the heavy DNA itself (Table 5). *Janthinobacterium lividum* was a prominent indicator species with high sequence abundance, despite being undetected by most denitrification studies.

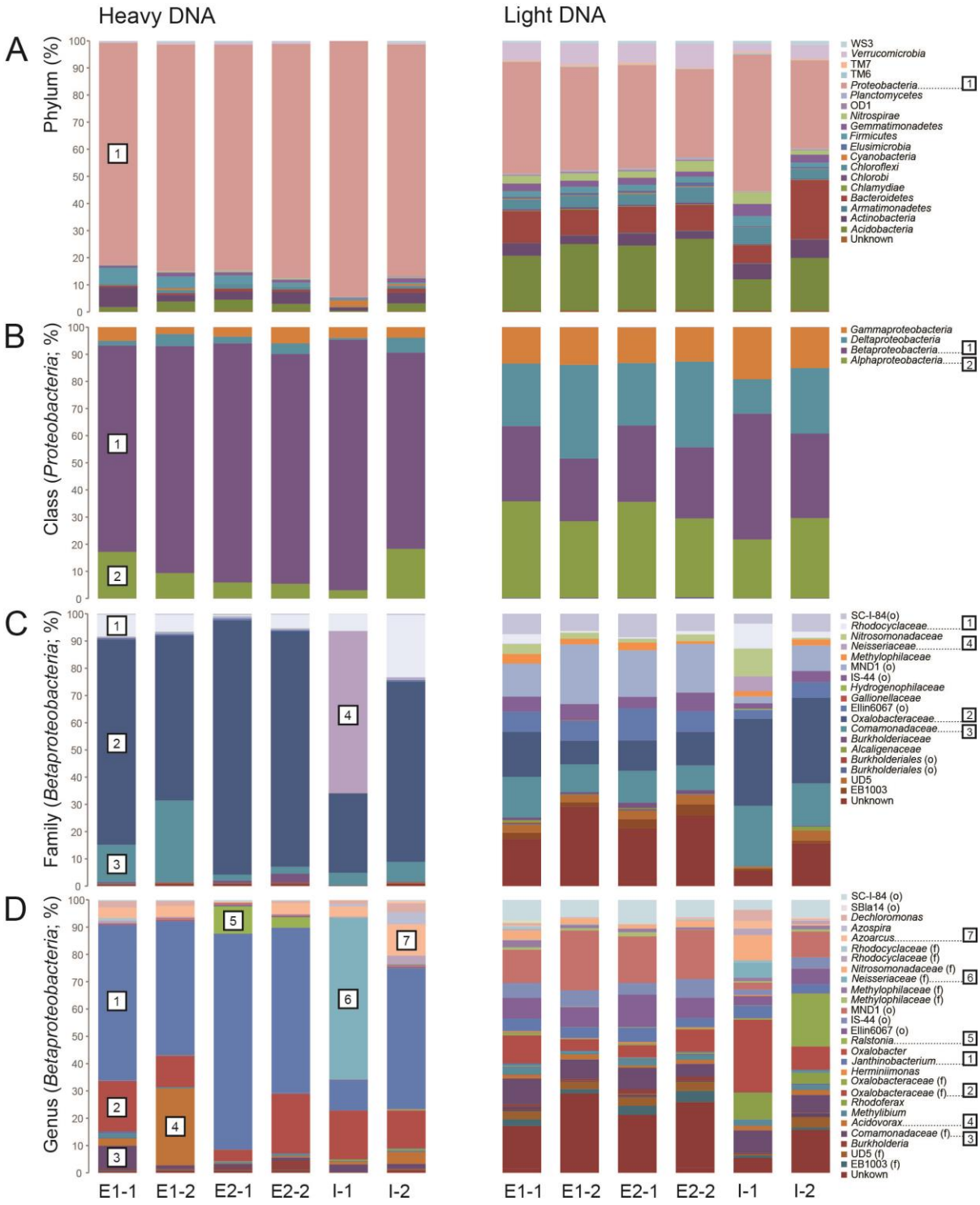

Figure 15. Taxa associated with DNA-SIP fractions after 8 days of denitrification at the phylum (A), class (B), family (C), and genus (D) levels. Two replicate FTRs from WEBs field 1 (E1-1 and E1-2), WEBs field 2 (E2-1 and E2-2), and Winchester fields 3 and 4 boundary (I-1 and I-2) are shown. Boxed numbers point to taxa in the figure legends.

Table 5. Relative abundance of *Actinobacteria* and *Proteobacteria* taxa above 0.5% associated with heavy DNA and light DNA in brackets for different incubation time points under denitrifying conditions obtained from next-generation sequencing.

Taxonomic position (phylum, class, order, family, and genus)	16S rRNA gene sequence abundance (%)							
	8 days [1]			**27 days** [2]			**8 days** [3]	**27 days** [4]
	WEBs1	WEBs2	Winchester	WEBs1	WEBs2	Winchester	**Average**	**Average**
Actinobacteria	4.8 (3.8)	3.7 (3.6)	2.5 (6.2)	5.6 (9.3)	3.5 (10.5)	6.0 (12.7)	**3.7 (4.5)**	**5.1 (10.8)**
Actinobacteria	3.9 (1.5)	2.7 (1.3)	2.0 (3.6)	4.7 (5.2)	2.8 (3.9)	5.6 (5.3)	**2.8 (2.2)**	**4.4 (4.8)**
Actinomycetales								
Streptomycetaceae	2.6 (0.1)	0.6 (0.1)	0.6 (0.4)	1.4 (0.1)	0.3 (0.2)	3.4 (0.3)	**1.3 (0.2)**	**1.7 (0.2)**
Proteobacteria	82.7 (39.7)	84.7 (35.3)	90.0 (41.4)	87.2 (44.5)	85.3 (42.8)	90.6 (40.4)	**85.8 (38.8)**	**87.7 (42.6)**
Alphaproteobacteria	10.9 (12.7)	4.7 (11.4)	9.2 (10.2)	42.6 (21.6)	28.4 (19.3)	22.4 (11.0)	**8.3 (11.4)**	**31.1 (17.3)**
Rhizobiales								
Other	0.2 (0.1)	0.2 (0.1)	0.3 (0.1)	11.6 (0.2)	13.0 (0.2)	8.8 (0.2)	**0.2 (0.1)**	**11.1 (0.2)**
Bradyrhizobiaceae	1.8 (1.7)	1.1 (1.5)	0.6 (1.4)	7.7 (3.1)	6.4 (2.7)	2.4 (1.1)	**1.1 (1.5)**	**5.5 (2.3)**
Bradyrhizobium	1.6 (1.7)	0.7 (1.4)	0.5 (1.2)	7.0 (3.0)	5.5 (2.5)	2.3 (1.0)	**0.9 (1.4)**	**4.9 (2.2)**
Phyllobacteriaceae	0.7 (0.2)	0.4 (0.1)	0.5 (0.4)	3.8 (0.5)	1.7 (0.3)	0.7 (0.5)	**0.5 (0.3)**	**2.1 (0.4)**
Other	0.2 (0.1)	0.1 (0.0)	0.3 (0.2)	1.7 (0.2)	0.9 (0.1)	0.5 (0.2)	**0.2 (0.1)**	**1.0 (0.1)**

Mesorhizobium	0.4 (0.1)	0.3 (0.1)	0.1 (0.2)	2.2 (0.2)	0.8 (0.2)	0.1 (0.3)	**0.3 (0.1)**	**1.0 (0.2)**
Rhizobiaceae	5.7 (0.2)	0.5 (0.1)	4.1 (0.5)	14.1 (0.6)	4.3 (0.3)	6.9 (0.3)	**3.4 (0.3)**	**8.4 (0.4)**
Other	2.7 (0.0)	0.0 (0.0)	1.5 (0.2)	0.3 (0.0)	0.1 (0.0)	4.7 (0.1)	**1.4 (0.1)**	**1.7 (0.0)**
Agrobacterium	0.4 (0.1)	0.1 (0.0)	0.8 (0.1)	10.7 (0.2)	1.6 (0.1)	1.8 (0.2)	**0.4 (0.1)**	**4.7 (0.2)**
Rhizobium	2.6 (0.1)	0.4 (0.1)	1.8 (0.2)	3.1 (0.3)	2.6 (0.2)	0.4 (0.1)	**1.6 (0.1)**	**2.1 (0.2)**
Rhodospirillales								
Rhodospirillaceae	0.7 (1.8)	0.5 (1.6)	0.9 (1.1)	0.8 (1.8)	1.0 (1.6)	0.5 (0.7)	**0.7 (1.5)**	**0.8 (1.4)**
Azospirillum	0.0 (0.0)	0.0 (0.0)	0.7 (0.0)	0.6 (0.0)	0.6 (0.0)	0.4 (0.0)	**0.2 (0.0)**	**0.5 (0.0)**
Betaproteobacteria	66.0 (10.1)	73.2 (9.6)	74.5 (16.7)	42.3 (8.8)	53.4 (11.3)	60.0 (15.7)	**71.2 (12.1)**	**51.9 (11.9)**
Burkholderiales								
Comamonadaceae	14.7 (1.3)	1.8 (1.0)	4.3 (3.4)	10.1 (1.5)	5.8 (1.4)	3.3 (2.2)	**6.9 (1.9)**	**6.4 (1.7)**
Other	3.0 (0.8)	0.9 (0.6)	1.8 (1.3)	3.4 (0.9)	3.1 (0.9)	2.4 (0.6)	**1.9 (0.9)**	**3.0 (0.8)**
Acidovorax	10.7 (0.2)	0.3 (0.1)	1.8 (0.3)	6.1 (0.2)	1.7 (0.1)	0.2 (0.3)	**4.3 (0.2)**	**2.7 (0.2)**
Oxalobacteraceae	44.6 (1.3)	65.8 (1.1)	33.1 (5.3)	21.3 (1.8)	31.5 (1.8)	33.0 (3.2)	**47.9 (2.6)**	**28.6 (2.2)**
Other	9.6 (0.8)	9.5 (0.6)	12.1 (3.5)	7.0 (0.7)	5.8 (0.6)	10.1 (2.1)	**10.4 (1.6)**	**7.6 (1.1)**
Janthinobacterium	34.7 (0.4)	51.1 (0.4)	20.8 (0.7)	10.4 (0.8)	21.6 (1.0)	22.8 (0.9)	**35.6 (0.5)**	**18.3 (0.9)**
Ralstonia	0.0 (0.0)	5.1 (0.0)	0.0 (0.0)	1.6 (0.0)	3.4 (0.0)	0.0 (0.0)	**1.7 (0.0)**	**1.7 (0.0)**
Neisseriales								
Neisseriaceae	0.0 (0.0)	0.0 (0.0)	26.0 (0.6)	0.0 (0.0)	0.0 (0.0)	2.1 (0.0)	**8.7 (0.2)**	**0.7 (0.0)**
Other	0.0 (0.0)	0.0 (0.0)	25.9 (0.6)	0.0 (0.0)	0.0 (0.0)	1.8 (0.0)	**8.6 (0.2)**	**0.6 (0.0)**
Rhodocyclales								
Rhodocyclaceae	4.7 (0.2)	2.0 (0.1)	9.9 (1.2)	9.3 (0.1)	13.1 (0.1)	20.6 (0.5)	**5.5 (0.5)**	**14.3 (0.3)**
Azoarcus	2.6 (0.0)	1.6 (0.0)	5.2 (0.3)	0.7 (0.0)	2.2 (0.0)	6.8 (0.2)	**3.1 (0.1)**	**3.2 (0.1)**

Azospira	0.0 (0.0)	0.0 (0.0)	1.7 (0.0)	1.0 (0.0)	4.3 (0.0)	0.0 (0.0)	**0.6 (0.0)**	**1.8 (0.0)**
Dechloromonas	1.4 (0.1)	0.3 (0.0)	1.6 (0.5)	7.2 (0.0)	5.4 (0.0)	3.4 (0.1)	**1.1 (0.2)**	**5.3 (0.0)**
Rhodocyclus	0.0 (0.0)	0.0 (0.0)	0.3 (0.0)	0.0 (0.0)	0.0 (0.0)	9.3 (0.1)	**0.1 (0.0)**	**3.1 (0.0)**
Gammaproteobacteria	3.1 (5.4)	4.0 (4.6)	3.5 (7.3)	0.9 (6.9)	1.3 (5.1)	6.9 (5.3)	**3.6 (5.7)**	**3.1 (5.7)**
Pseudomonadales								
Pseudomonadaceae	2.0 (0.3)	2.8 (0.2)	1.9 (2.7)	0.2 (1.4)	0.6 (0.2)	1.0 (1.1)	**2.2 (1.1)**	**0.6 (0.9)**
Pseudomonas	2.0 (0.3)	2.8 (0.2)	1.9 (2.7)	0.2 (1.4)	0.6 (0.2)	1.0 (1.1)	**2.2 (1.1)**	**0.6 (0.9)**
Xanthomonadales								
Xanthomonadaceae	0.3 (0.8)	0.4 (0.6)	1.2 (1.8)	0.3 (1.1)	0.4 (1.5)	5.8 (1.5)	**0.7 (1.1)**	**2.2 (1.4)**
Lysobacter	0.0 (0.0)	0.1 (0.0)	0.9 (0.3)	0.0 (0.1)	0.0 (0.1)	5.5 (0.5)	**0.4 (0.1)**	**1.8 (0.2)**
Deltaproteobacteria	2.6 (11.3)	2.7 (9.5)	2.7 (7.1)	1.3 (7.1)	2.2 (6.9)	1.3 (8.4)	**2.6 (9.3)**	**1.6 (7.5)**

All percentages were obtained for each taxon at the corresponding hierarchical level, which added to the partiality of this list, causes the addition of values to not yield 100%.

"Other" refers to sequences that were classified to the immediate higher taxonomical rank, but were not classified at the current level.

Order level was included with hierarchical orientation purposes and deliberately lack abundances.

[1] Average of two independently sequenced samples from two reactors.

[2] Value obtained from sequencing a composite sample from two independent reactors.

[3] Average of six independently sequenced samples from six reactors from three sites.

[4] Average of three independently sequenced composite samples from six reactors from three sites.

Table 6. Indicator species associated with denitrifier DNA obtained from DNA-SIP (indicator value ≥ 0.8, sequence abundance ≥ 2000, and $p \leq 0.05$).

OTU	Consensus lineage	Sequence count	Indicator value
20368	*Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*	8703	1.00
348	*Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Oxalobacteraceae*; *Janthinobacterium*; *lividum*	3500	0.98
202	*Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Oxalobacteraceae*; *Janthinobacterium*	67170	0.98
361	*Proteobacteria*; *Betaproteobacteria*; *Rhodocyclales*; *Rhodocyclaceae*; *Azoarcus*	6647	0.97
20701	*Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Rhizobiaceae*	3286	0.96
20567	*Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Rhizobiaceae*; *Agrobacterium*	4580	0.95
20576	*Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Rhizobiaceae*; *Rhizobium*	4503	0.92
17646	*Actinobacteria*; *Actinobacteria*; *Actinomycetales*; *Streptomycetaceae*	3753	0.88
331	*Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Oxalobacteraceae*	21974	0.88
20556	*Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*	2433	0.88
1515	*Proteobacteria*; *Betaproteobacteria*; *Rhodocyclales*; *Rhodocyclaceae*; *Dechloromonas*	3930	0.87
1005	*Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Comamonadaceae*; *Acidovorax*	2352	0.84

Oxalobacteraceae was the most abundant family in the active fractions, regardless of incubation length (Table 5), with *Janthinobacterium* being the most abundant genus among the observed denitrifiers at both incubation lengths. *Janthinobacterium* is not widely covered in the literature and might be an important denitrifier according to these results, especially given its presence as a depth-dependent indicator species in WEBs site (Figure 6). Furthermore, it could be of environmental concern given its high N_2O emission capacity [156].

Incubation length helped us identify *Agrobacterium* as an active denitrifier, although it was observable after the short incubation as well. Moreover, this genus was more predominant in WEBs site field 1, with 10.7% abundance. An unclassified *Rhizobiales* family ("other" in Table 5) became notably abundant after 27 days of enrichment (average of 11.1%), whereas it was poorly represented after 8 days of denitrifying activity (average of 0.2%). This was observed for other *Alphaproteobacteria* as well. Other examples are the *Rhizobiaceae* family and *Bradyrhizobium*. Similarly, *Dechloromonas*, a *Betaproteobacteria*, was an active genus after 8 days of incubation, but it greatly increased in abundance with incubation time, going from an average abundance of 1.1 to 5.3%. Conversely, *Azoarcus*, *Rhizobium*, and the *Streptomycetaceae* family had similar abundances across sites and incubation lengths, whereas *Acidovorax* was very abundant in one of two reactors from the WEBs site field 1 (reactor E1-2, Figure 15D) and was active in samples from all sites.

Azospirillum, *Ralstonia*, and *Azospira* were active only in a few FTRs after the short incubation and appeared in the active fraction after the long incubation, which might be due to sample heterogeneity. The growth of *R. solanacearum,* for example, has been shown to be reduced by 8 mM nitrite concentrations at pH 7 [184]. The reactors showed higher nitrite concentrations than this, which might explain the delayed appearance of the microorganism. *Azospirillum* was an important genus recovered from rice paddy soil with a denitrifier-targeted single-cell isolation effort, which resulted in 20 isolates out of 37 from this lineage [185]. Also, *Azospirillum lipoferum* and *B. japonicum* stimulated each other's capacity for denitrification, where the presence of the former enhanced the growth of the latter [186], which might explain why in the present study both genera appeared after the longer incubation under denitrifying conditions.

Several unclassified taxa from the *Neisseriaceae* family were only observed in the Winchester site's active fraction with important abundance (25.9%; "other" in Table 5).

Two activated sludge studies support the denitrifier taxa mentioned above by retrieving either denitrifier isolates or 16S rRNA gene sequences from active denitrifiers using DNA-SIP. In the first

study, 10% of the isolates were *Acidovorax*, 5.5% *Pseudomonas*, 4% *Dechloromonas*, 3% *Rhizobium*, 1% *Azospira*, and 0.5% *Neisseraceae* [52]. Similarly, from 201 clones obtained from denitrifier DNA identified with labeled acetate, 14% of the clones were related to *Acidovorax*, 4% to *Dechloromonas*, and 0.5% to *Pseudomonas*. From 137 clones obtained from denitrifier DNA enriched with labeled methanol incubations, 3% of clones were associated to *Ralstonia* [187]. Notably, genera like *Ochrobactrum*, *Paracoccus*, *Comamonas*, *Thauera*, *Rhodobacter*, and *Arcobacter* were not found in our SIP heavy DNA despite being abundant denitrifiers in these reviewed studies. Finally, a denitrifier cultivation study using soil planted with corn associated 73% of isolates to *Agrobacterium tumefaciens* and 12% to *Streptomyces cinnabarinus*, where the end products of denitrification for these isolates are N_2O and N_2, respectively [188]. Overall, the SIP-identified active denitrifiers agree with those reported in the literature. However, this research shows evidence of their activity under the employed incubation conditions and captured the effect of incubation length. Furthermore, the reported active denitrifier abundance data might be closer to phenomena observed in agricultural fields because a cultivation-independent method was used.

3.2.5 Bulk-soil DNA and heavy DNA metagenomic sequencing

3.2.5.1 Comparison of the metagenome of bulk vs. denitrifier-enriched soil DNA

Denitrifier-enriched DNA and the original soil DNA samples were sequenced directly in order to access the metagenomic information in the heavy DNA. As described in previous sections, the denitrifying incubation resulted in a taxonomic shift within the heavy DNA (Figure 15), which confirmed denitrifier enrichment. After metagenomic sequencing of the selected samples, the expectation was to observe a higher proportion of denitrifier DNA (e.g., nitrite reductase genes) in heavy DNA samples compared to corresponding libraries from the original soil samples. However, this was not the case. Very similar gene abundances of *nirK* and *nirS* were observed, in relation to the total number of 16S rRNA genes recovered for each sample (Table 7). This similar proportion of nitrite reductase genes concealed distinct taxonomic affiliations in the bulk soil and heavy DNA (Figure 16).

Table 7. Nir sequences annotated by MG-RAST by comparison to GenBank sequences (E-value ≤ 0.00001, identity ≥ 60%, and minimum alignment length of 15 amino acids) from original-soil and denitrifier-enriched DNA for WEBs and Winchester after metagenomic paired-end next-generation sequencing.

Library	Sequence abundance		
	nirK[1]	*nirS*[1]	16S rRNA
WEBs bulk soil read 1	43 (0.89)	3 (0.06)	4,810
WEBs bulk soil read 2	74 (2.32)	2 (0.06)	3,191
WEBs heavy DNA read 1	572 (1.65)	25 (0.07)	34,638
WEBs heavy DNA read 2	243 (1.64)	11 (0.07)	14,832
Winchester bulk soil read 1	180 (2.25)	4 (0.05)	8,011
Winchester bulk soil read 2	109 (2.29)	2 (0.04)	4,769
Winchester heavy DNA read 1	51 (0.91)	22 (0.39)	5,628
Winchester heavy DNA read 2	43 (1.18)	12 (0.33)	3,636

[1] Number of gene sequences identified and % relative to 16S rRNA gene in brackets.

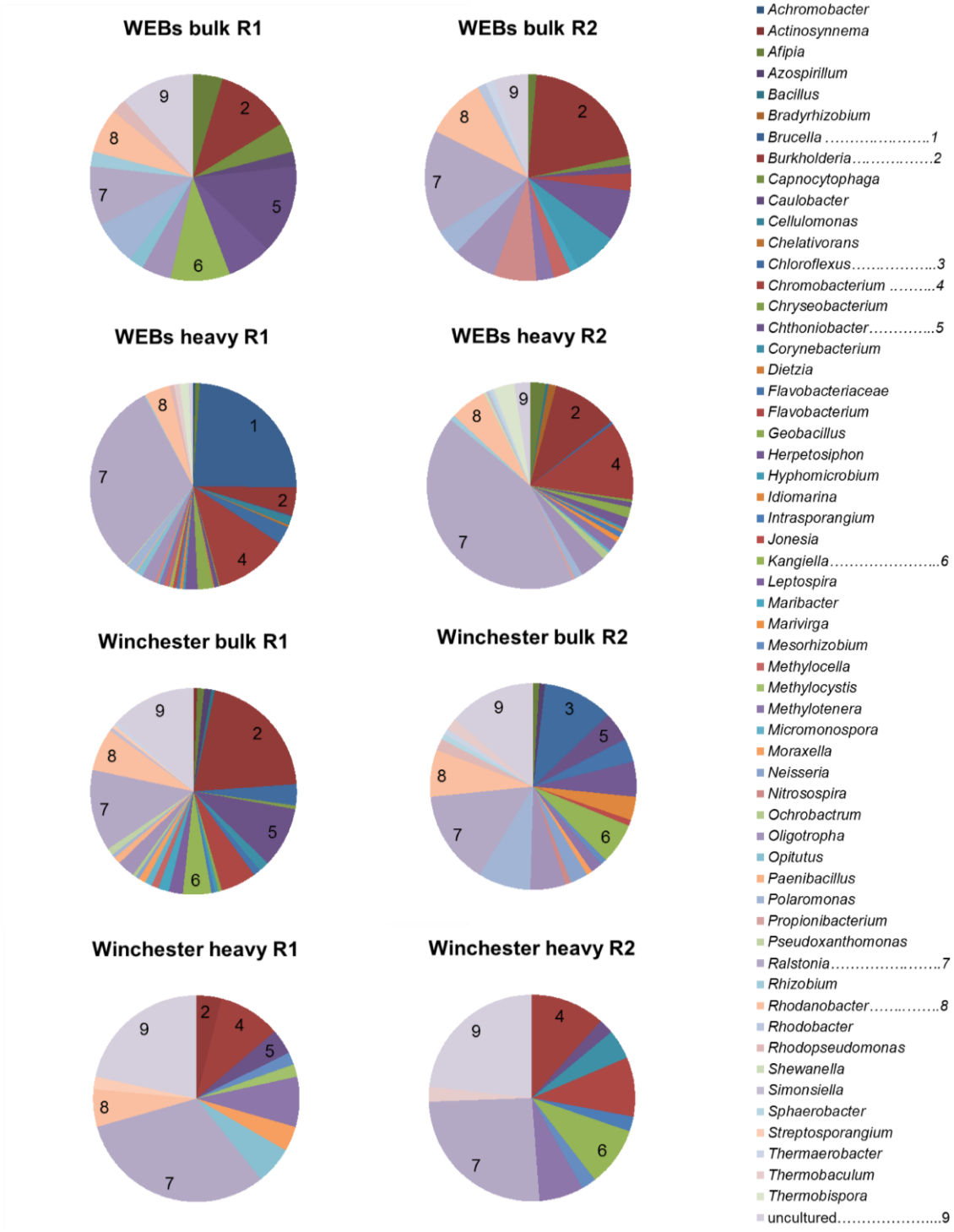

Figure 16. Taxonomic affiliation of identified *nirK* genes for WEBs and Winchester bulk soil and heavy DNA read 1 (R1) and read 2 (R2) metagenomic libraries. Numbers point to taxonomic affiliation in the figure legend.

3.2.5.2 Identification of nitrite reductase genes

The metagenomic data indicated that *nirK*-containing denitrifiers were dominant (Table 7). This observation is consistent given recent findings on Nir-type niche selection. A soil study found that abundance for both Nir genes was partly determined by soil structure: *nirS* community structure responded to soil nitrate, clay, and pH, whereas *nirK* was naturally influenced by copper; *nirK* genotype was responsive to management practices implemented in the studied sites [189]. The *nirS*/*nirK* ratio in soil has also been observed to respond to cattle influence and to correlate with pH, nitrate, and moisture [75]. The results of this study cannot be directly compared to available literature, because Nir gene recovery is commonly done with prior amplification using primers that might selectively and partially recover these genes [38]. This study shows evidence of environmental selection of Nir genes and *nirK* in the WEBs and Winchester fields. *Brucella*, *Ralstonia*, and *Chromobacterium* related *nirK* sequences were abundant in heavy DNA compared to bulk soil DNA (Figure 16).

3.2.5.3 Similarity of *nirK* priming sites and targeting primers

Initially, curated proteobacterial *nirS* and *nirK* sequences from the KEGG database were aligned to evaluate their primer-binding sites (data not shown). Notably, *nirS* genes from this database are more conserved across the primer-binding sites than *nirK* genes. Conversely, previous reports state that *nirS* primer design could be more challenging [32]. The *nirK* genes recovered from WEBs metagenomic sequencing were aligned to genes of *Proteobacteria* isolates and then the primer-binding sites of the obtained sequences were compared to different primers (i.e., forward primers nirK1F [31], F1aCu [32], and nirK876 [54] and reverse primers nirK5R [31], R3Cu [32], and nirK1040 [54]) (Figure 17). The number of matches and mismatches for curated *nirK* sequences with respect to the same primers were determined (Table 8).

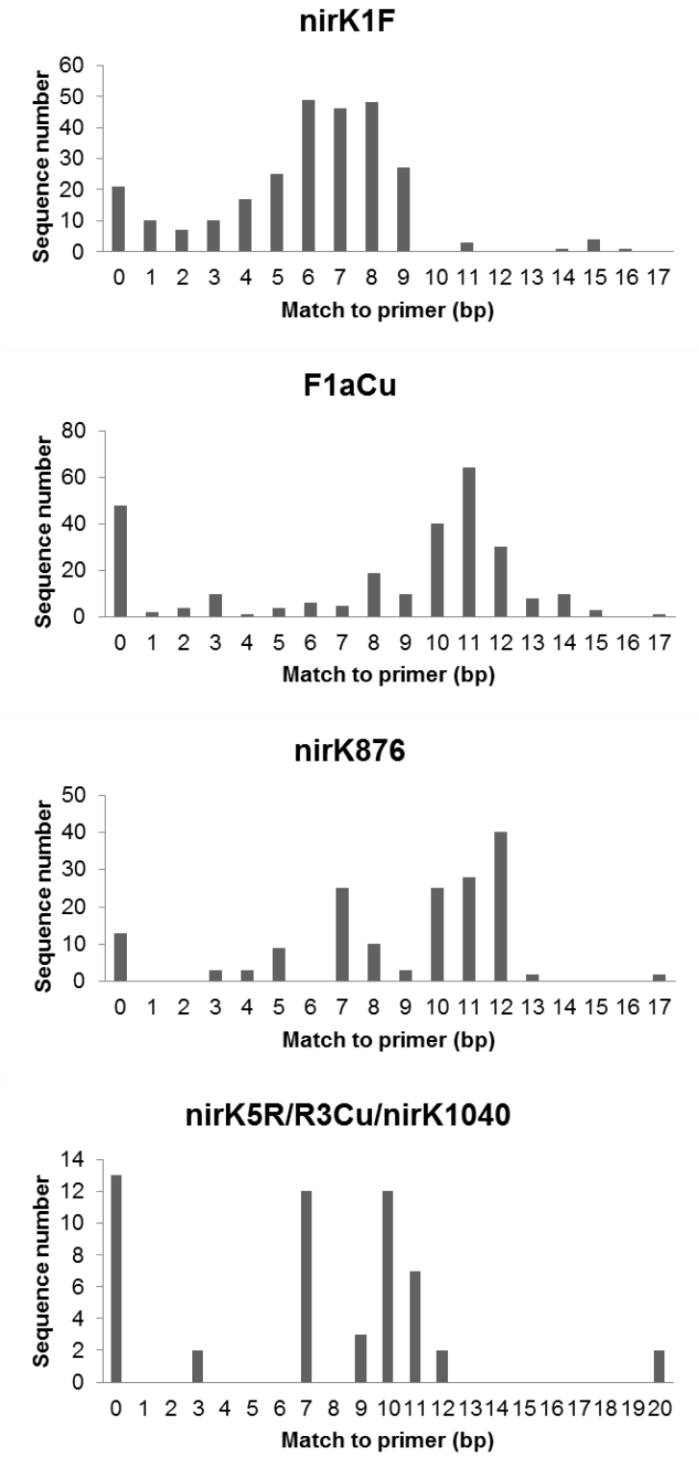

Figure 17. Evaluation of match between published *nirK* primers (i.e., nirK1F, F1aCu, nirK876, nirK5R, R3Cu, and nirK1040) and the primer-binding region for identified *nirK* partial genes from WEBs heavy DNA read 1 metagenomic library.

Table 8. Gene primer-binding region evaluation for selected curated *nirK* sequences from the KEGG database.

Microorganism	Sequence	Primer	Match/Mismatch
Burkholderia pseudomallei 1710b	BURPS1710b_A0477	nirK1F	(11/6)
		F1aCu	(12/5)
		nirK876	(11/6)
		nirK5R/R3Cu/nirK1040	(9/11)
Burkholderia pseudomallei 1710b	BURPS1710b_A0520	nirK1F	(6/11)
		F1aCu	(11/6)
		nirK876	(11/6)
		nirK5R/R3Cu/nirK1040	(9/11)
Chromobacterium violaceum	CV_2007	nirK1F	(8/9)
		F1aCu	(12/5)
		nirK876	(12/5)
		nirK5R/R3Cu/nirK1040	(12/8)
Oligotropha carboxidovorans OM5 (Mississippi)	OCAR_5468	nirK1F	(7/10)
		F1aCu	(10/7)
		nirK876	(12/5)
		nirK5R/R3Cu/nirK1040	(12/8)
Oligotropha carboxidovorans OM5 (Mississippi)	OCAR_7249	nirK1F	(10/7)
		F1aCu	(12/5)
		nirK876	(12/5)
		nirK5R/R3Cu/nirK1040	(6/14)
Pseudomonas denitrificans	H681_24715	nirK1F	(15/2)
		F1aCu	(17/0)
		nirK876	(17/0)
		nirK5R/R3Cu/nirK1040	(20/0)
Ralstonia pickettii 12D	Rpic12D_4128	nirK1F	(7/10)
		F1aCu	(12/5)
		nirK876	(12/5)
		nirK5R/R3Cu/nirK1040	(10/10)
Ralstonia pickettii 12J	Rpic_4015	nirK1F	(7/10)
		F1aCu	(12/5)
		nirK876	(12/5)
		nirK5R/R3Cu/nirK1040	(10/10)
Ralstonia solanacearum FQY_4	F504_4973	nirK1F	(7/10)
		F1aCu	(12/5)
		nirK876	(12/5)
		nirK5R/R3Cu/nirK1040	(10/10)

For partial sequences retrieved from this research, primer-binding regions showed few perfect matches to tested *nirK* primers (Figure 17). For example, identified *nirK* sequences that were similar to that of *Chromobacterium violaceum* showed lower sequence similarity to primers than observed for curated sequence from the same species. The same was observed for *Burkholderia pseudomallei* related sequences. Among *nirK* gene sequences retrieved from the KEGG database, both high and low primer-binding region similarities were observed (Table 8). Sequences with low similarity to primers belong to *Actinobacillus* sp., *Azoarcus* sp., *Azozpirillum* sp., *Burkholderia* sp., *Chromobacterium violaceum*, *Kangiella koreensis*, and *Ralstonia solanacearum*. On the contrary, curated sequences of *Pseudomonas denitrificans*, *Rhodopseudomonas* sp., *Brucella* sp., *Rhizobium etli*, *Sinorhizobium* sp., and *Achromobacter xylosoxidans* had primer-binding sites that showed high similarity to amplification primers.

An important observation among denitrifiers is that some might possess more than one copy of the nitrite reductase gene; *Afipia* sp. 1NLS2 illustrates this scenario with three *nirK* copies that are dissimilar; and *Oligotropha carboxidovorans* strain Om5 has two non-identical gene copies [45]. The possibility exists that the data captured different gene versions from the same active denitrifier, which could cause some differences in matches and mismatches for sequences related to the same strain (data not shown).

A comparison of the taxa associated with retrieved *nirK* sequences and the bacterial taxonomic assignment based on 16S rRNA gene found in the heavy DNA (Figure 16, Figure 18, and Figure 19), reveals some disagreement. Previously researchers tried to pair nitrite reductase gene and 16S rRNA gene phylogenies and realized that the functional gene is not useful for taxonomic inference, probably due to horizontal gene transfer that complicates taxonomy attribution based on functional gene sequences [33]. However, denitrifier diversity and function can be studied simultaneously with DNA-SIP and a handful of studies are available using specialized reactor biomass [190], activated sludge [187,191,192], and soil [58,175]. The present research is the first to recover functional genes and go beyond denitrifier-community taxonomic evaluation by using metagenomic sequencing of SIP-derived heavy DNA. My results (Figure 17) support previous findings indicating that available primers are likely to miss a fraction of the denitrifiers and this study proved that fraction to be potentially important for the denitrification process.

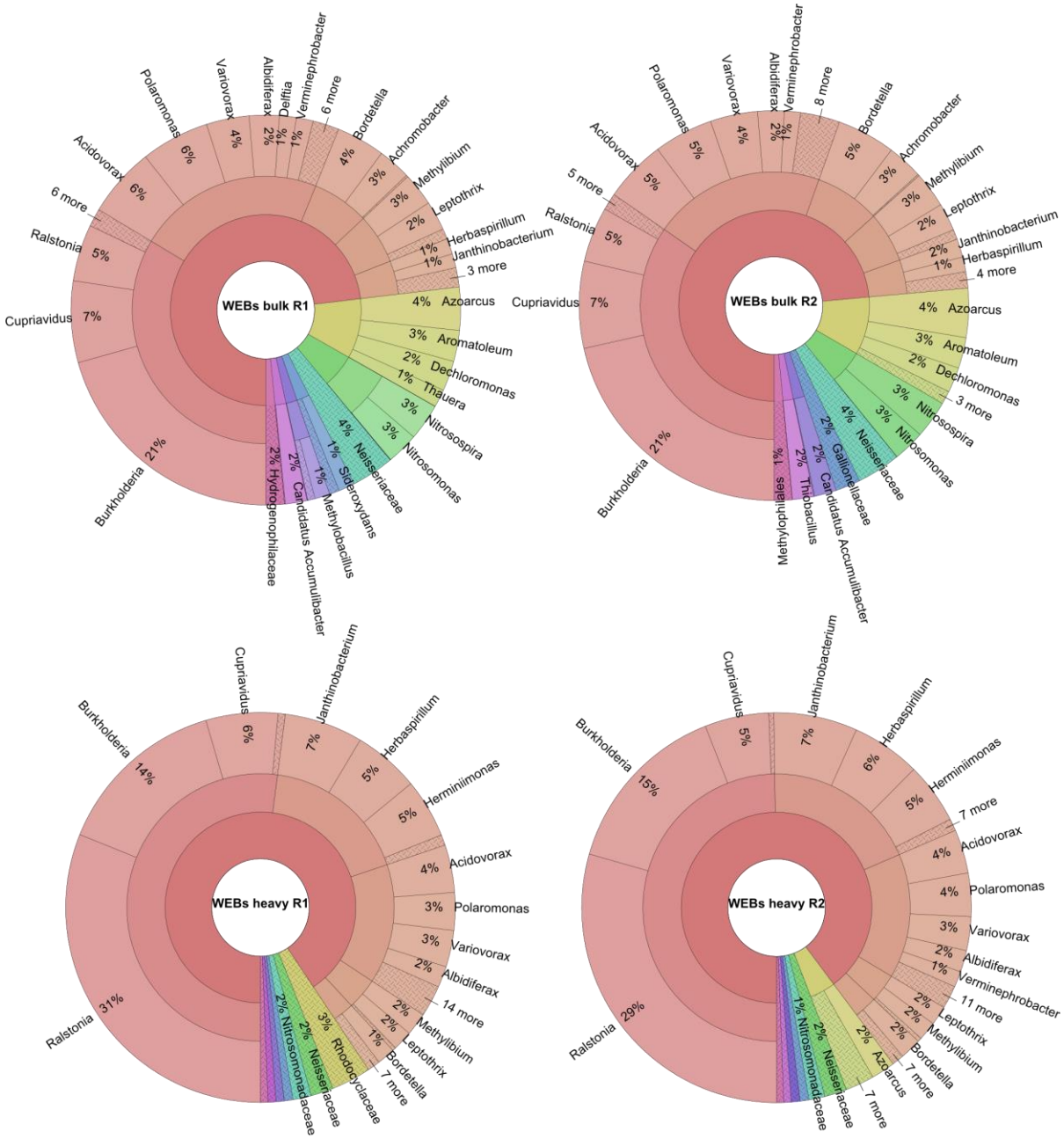

Figure 18. Taxa associated to WEBs bulk soil and heavy DNA read 1 (R1) and read 2 (R2) metagenomic libraries based on the 16S rRNA gene for the *Betaproteobacteria*.

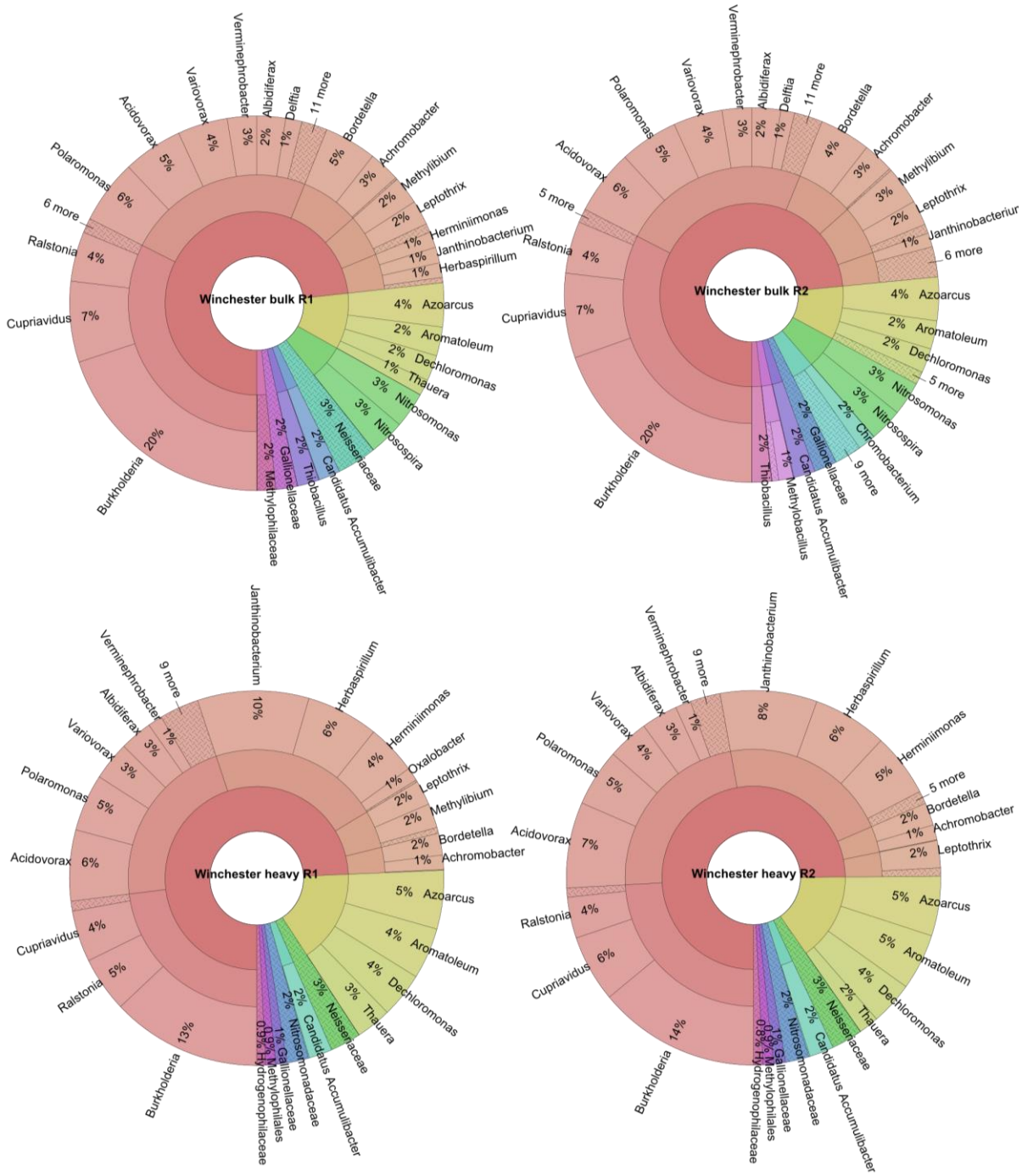

Figure 19. Taxa associated to Winchester bulk soil and heavy DNA read 1 (R1) and read 2 (R2) metagenomic libraries based on the 16S rRNA gene for the *Betaproteobacteria*.

Chapter 4
Conclusions and future research

This research was done as part of a major project called "Grow more and emit less", aiming to evaluate the impact of agricultural management in GHG emissions. Agricultural practices have the potential to affect the soil microbial community structure and diversity [19,21] by modifying pH [18], soil nutrient content [19], oxygen, and soil structure characteristics [20]. However, particular sites must be characterized because observations might vary from one site to another given the number of interacting variables (i.e., soil edaphic factors and microbial communities, climate, crop, and management practices).

The first objective of this research consisted of characterizing the microbial community from soils with contrasting drainage management to identify community responses to the imposed practice. It was hypothesized that the community would be influenced by the drainage practice established in the sites and moisture and oxygen variations were expected in response to drainage practice. However, results revealed that the selection of CTD or UCTD for field management did not influence the site's microbial community (Figure 2D). The community data collected across the farming season of 2012 showed that cultivar type and fertilizer type applied explained most of the between-communities variation (Figure 2A), followed by soil depth (Figure 2B). These factors have been acknowledged as major modifiers of microbial composition in soil [146,148].

The studied sites are owned and operated by farmers according to market demands. Therefore, the fields' management has several constraints, one of which is the implemented drainage system and the type of fertilizer employed (i.e., manure, commercial, or a mixture). On the other hand, crop rotation is flexible and all the characterized fields have grown similar crops in recent years. As a result, and also due to lack of soil characterization, our analysis was not able to determine which factors caused the observed ordination grouping (Figure 2). However, I identified that the drainage management used in these fields did not impact soil microbial communities in the dry 2012 growing season (average rainfall in Ottawa between May 1 and September 30, 2012 of 74 mm). Along with field data collected by other parties as part of this integrated and collaborative project, my research suggests that CTD may be employed in fields without enhancing GHG emissions. Nonetheless, further work is necessary to better characterize the factors influencing the microbial communities, which led to the observed bacterial community structure in these soils. Such work includes detailed soil

characterization and monitoring the communities in upcoming years to confirm that the drainage practice would not affect the community under different weather scenarios, since 2012 was a year with little precipitation.

My study revealed that *Actinobacteria* relative abundance increased during the growing season for WEBs fields 1 and 2 ($r(46)=0.69$, $p=6.6E-8$; Figure 3), WEBs fields 11-14 ($r(52)=0.38$, $p=0.005$; Figure 4), and Winchester site ($r(39)=0.46$, $p=0.002$; Figure 5), possibly reflecting nutrient accumulation in the field as the crop grows. *Actinobacteria* have been shown to increase with carbon source supplementation [152] and decrease with harvesting [153]. Also, *Chloroflexi* abundance significantly increased within deeper soil samples ($p=1.7E-5$; Figure 3 and $p=0.002$; Figure 5). *Chloroflexi* are likely able to withstand deeper soil conditions, despite having low growth rates due to reduced competition associated with lower microbial diversity in deeper soil. Other obtained indicator species for deeper soil, like *Acidobacteria* and *Nitrospira*, are equipped with high substrate affinity enzymes and other adaptations for nutrient-limited niches [161,162]. However, assigning functional significance to these depth-associated OTUs is difficult. Some phyla are poorly characterized and few representatives are available due to their resistance to cultivation. Currently, genomic sequencing serves the purpose of predicting potential functional roles for members of these phyla.

The second objective of this research involved the study of denitrification in the farming sites employed to detect active denitrifiers and retrieve their genomic information. Denitrification has been known for more than one hundred years and was initially discovered by Gayon and Dupetit (1883) in sewage [193]. Since then, microbiological methods used for denitrification research have included microbial cultivation and phenotypic characterization through biochemical tests that, alongside enzymatic characterization, established baseline data for subsequent denitrification studies. Formerly employed methods biased denitrifier representatives in databases to those that were readily grown on media. Currently, the advent of genome sequencing helps understand and fill gaps on microbial physiology, allowing the reevaluation of species and strains that before could not be affiliated with denitrification activity. Furthermore, Shapleigh and Philippot acknowledged the difficulty in assigning denitrification function to microorganisms due to the limitations of evaluation methods employed (i.e., biochemical, molecular, or genome based) [45,68].

Among the best studied denitrifiers are *Paracoccus denitrificans,* which belongs to the *Alphaproteobacteria,* and *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* from the *Gammaproteobacteria*. Other genera with high numbers of denitrifier representatives are *Brucella*,

Burkholderia, *Ralstonia*, *Thauera*, and *Shewanella*. Many of these microorganisms were used as models to develop primers to identify and quantify denitrifiers in the environment. The best primers to amplify Nir sequences, to my knowledge, are nirK876 and nirK1040 for *nirK* designed with degeneracies to be as universal as possible [54]. Both cd3aF and R3cd are recognized as being primers of choice for the heme-containing nitrite reductase [34,55,56]. Despite being designed for universal gene amplification, it is known these primers fail to recognize denitrification potential in some species [38,53].

To circumvent the issues of culture bias and primer-dependent detection of denitrifiers, DNA-SIP was chosen in this project to detect relevant and potentially active denitrifiers in the study fields (Figure 15). Interestingly, a common group of denitrifiers was found in the studied sites (e.g., *Janthinobacterium*, *Acidovorax*, *Azoarcus*, *Dechloromonas*, *Rhizobium*, and *Pseudomonas*), despite the diverse history of the fields, which could indicate that under denitrifying conditions, a core microbial consortium thrives regardless of soil origin and "background" community. However, the community behavior in the field during the growing season and especially in denitrification-favoring events like fertilizer application and rain should not be deduced from these experiments without *in situ* testing to corroborate the identities of naturally denitrifying microorganisms in the field.

An important contribution of my research is the identification of an active core of denitrifiers that may be highly important for affecting plant growth and soil biogeochemical activity. Furthermore, a major contribution of this study is the recovery of putative *nirK* sequences obtained without the prerequisite of cultivation and without relying on primers for detection. Our sequences showed low similarity across regions used for primer binding (Figure 17), which suggests that molecular surveys of these sites would have failed to identify potentially active denitrifiers. Also, active denitrifiers from the studied sites carried *nirK* genes rather than *nirS* genes (Table 7). Denitrifier Nir-type distribution in the environment has been studied; both enzymes are functionally equivalent. Initially, *nirS* genes were reported to be more abundant than *nirK* [194], suggesting that one Nir type could be more widely distributed than the other. Later, evidence suggested that the environment could differentially affect *nirS*- and *nirK*-carrying microorganisms. For example, the presence of plants in a plot changed the *nirS*/*nirK* ratio [72]. Recently, community phylogeny studies were used to make inferences about the possible underlying causes of differential distribution of Nir genes in different environments. A study found that the global Nir-type distribution was related to environment salinity for aquatic

denitrifier communities[195]. The authors also postulated that different Nir-type harboring denitrifiers in soil respond to different environmental factors, such as nitrogen species.

Future work to assess functional denitrifier enrichment could involve RNA extraction for RNA-SIP, cDNA synthesis, and shotgun sequencing. This approach would be potentially more sensitive to Nir gene enrichment after labelled substrate incubation. Also, assembly of the obtained Nir sequences could be attempted to yield complete Nir genes for analysis and primer design. Heavy DNA analysis was only done for Nir genes, but other functional genes (i.e., *nosZ*) should also be explored to complement my results. In addition, evaluating multiple match length and confidence thresholds for Nir gene annotation would be an important next step to confirm that annotations associated with ^{13}C-DNA sequences (Table 7, Figure 16, and Figure 17) were genuine nitrite reductase homologues.

In sum, this work assessed agricultural management impacts on soil microbial communities and employed FTRs for the first time for DNA-SIP denitrifier functional gene recovery. The employed methodologies facilitated the study of the total bacterial community in response to agricultural management practices and the characterization of a fraction of the community responsible for GHG emission, demonstrating the value of novel molecular techniques for environmental microbiology studies.

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Appendix A

List of soil samples

Soil samples used for WEBs and Winchester bacterial diversity analyses.

Site	Field	Sampled date	Depth (cm)	Position
WEBs	1	16-Apr-12	0-30	B
WEBs	1	16-Apr-12	30-60	B
WEBs	1	16-Apr-12	60-90	B
WEBs	1	18-Apr-12	0-30	T
WEBs	1	18-Apr-12	30-60	T
WEBs	1	18-Apr-12	60-90	T
WEBs	2	16-Apr-12	0-30	B
WEBs	2	16-Apr-12	30-60	B
WEBs	2	16-Apr-12	60-90	B
WEBs	2	18-Apr-12	0-30	T
WEBs	2	18-Apr-12	30-60	T
WEBs	2	18-Apr-12	60-90	T
WEBs	1	19-Jun-12	0-30	B
WEBs	1	19-Jun-12	30-60	B
WEBs	1	19-Jun-12	0-30	T
WEBs	1	19-Jun-12	30-60	T
WEBs	2	19-Jun-12	0-30	B
WEBs	2	19-Jun-12	30-60	B
WEBs	2	19-Jun-12	0-30	T
WEBs	2	19-Jun-12	30-60	T
WEBs	1	25-Jul-12	0-30	B
WEBs	1	25-Jul-12	30-60	B
WEBs	1	25-Jul-12	0-30	T
WEBs	1	25-Jul-12	30-60	T
WEBs	2	25-Jul-12	0-30	B
WEBs	2	25-Jul-12	30-60	B
WEBs	2	25-Jul-12	0-30	T
WEBs	2	25-Jul-12	30-60	T
WEBs	1	22-Aug-12	0-30	B
WEBs	1	22-Aug-12	30-60	B
WEBs	1	22-Aug-12	0-30	T
WEBs	1	22-Aug-12	30-60	T
WEBs	2	22-Aug-12	0-30	B

WEBs	2	22-Aug-12	30-60	B
WEBs	2	22-Aug-12	0-30	T
WEBs	2	22-Aug-12	30-60	T
WEBs	1	11-Sep-12	0-30	B
WEBs	1	11-Sep-12	30-60	B
WEBs	1	11-Sep-12	60-90	B
WEBs	1	11-Sep-12	0-30	T
WEBs	1	11-Sep-12	30-60	T
WEBs	1	11-Sep-12	60-90	T
WEBs	2	11-Sep-12	0-30	B
WEBs	2	11-Sep-12	30-60	B
WEBs	2	11-Sep-12	60-90	B
WEBs	2	11-Sep-12	0-30	T
WEBs	2	11-Sep-12	30-60	T
WEBs	2	11-Sep-12	60-90	T
WEBs	11	16-Apr-12	0-30	B
WEBs	11	16-Apr-12	30-60	B
WEBs	11	16-Apr-12	60-90	B**
WEBs	11	20-Apr-12	0-30	T
WEBs	11	20-Apr-12	30-60	T
WEBs	11	20-Apr-12	60-90	T**
WEBs	12	16-Apr-12	0-30	B
WEBs	12	16-Apr-12	30-60	B
WEBs	12	16-Apr-12	60-90	B
WEBs	12	20-Apr-12	0-30	T
WEBs	12	20-Apr-12	30-60	T
WEBs	12	20-Apr-12	60-90	T
WEBs	13	16-Apr-12	0-30	B
WEBs	13	16-Apr-12	30-60	B
WEBs	13	16-Apr-12	60-90	B
WEBs	13	20-Apr-12	0-30	T
WEBs	13	20-Apr-12	30-60	T
WEBs	13	20-Apr-12	60-90	T**
WEBs	14	16-Apr-12	0-30	B
WEBs	14	16-Apr-12	30-60	B
WEBs	14	16-Apr-12	60-90	B**
WEBs	14	20-Apr-12	0-30	T
WEBs	14	20-Apr-12	30-60	T
WEBs	14	20-Apr-12	60-90	T**
WEBs	11	22-Jun-12	0-30	B

WEBs	11	22-Jun-12	30-60	B
WEBs	11	22-Jun-12	0-30	T
WEBs	11	22-Jun-12	30-60	T
WEBs	12	22-Jun-12	0-30	B
WEBs	12	22-Jun-12	30-60	B
WEBs	12	22-Jun-12	0-30	T
WEBs	12	22-Jun-12	30-60	T
WEBs	13	22-Jun-12	0-30	B
WEBs	13	22-Jun-12	30-60	B
WEBs	13	22-Jun-12	0-30	T
WEBs	13	22-Jun-12	30-60	T
WEBs	14	22-Jun-12	0-30	B
WEBs	14	22-Jun-12	30-60	B
WEBs	14	22-Jun-12	0-30	T
WEBs	14	22-Jun-12	30-60	T
WEBs	11	10-Jul-12	0-30	NA
WEBs	11	10-Jul-12	30-60	NA
WEBs	12	10-Jul-12	0-30	NA
WEBs	12	10-Jul-12	30-60	NA
WEBs	13	10-Jul-12	0-30	NA
WEBs	13	10-Jul-12	30-60	NA
WEBs	14	10-Jul-12	0-30	NA
WEBs	14	10-Jul-12	30-60	NA
WEBs	11	25-Jul-12	0-30	NA
WEBs	11	25-Jul-12	30-60	NA
WEBs	12	25-Jul-12	0-30	NA
WEBs	12	25-Jul-12	30-60	NA
WEBs	13	25-Jul-12	0-30	NA
WEBs	13	25-Jul-12	30-60	NA
WEBs	14	25-Jul-12	0-30	NA
WEBs	14	25-Jul-12	30-60	NA
WEBs	11	22-Aug-12	0-30	NA
WEBs	11	22-Aug-12	30-60	NA
WEBs	12	22-Aug-12	0-30	NA
WEBs	12	22-Aug-12	30-60	NA
WEBs	13	22-Aug-12	0-30	NA
WEBs	13	22-Aug-12	30-60	NA
WEBs	14	22-Aug-12	0-30	NA
WEBs	14	22-Aug-12	30-60	NA
WEBs	11	20-Sep-12	0-30	NA

WEBs	11	20-Sep-12	30-60	NA
WEBs	12	20-Sep-12	0-30	NA
WEBs	12	20-Sep-12	30-60	NA
WEBs	13	20-Sep-12	0-30	NA
WEBs	13	20-Sep-12	30-60	NA
WEBs	14	20-Sep-12	0-30	NA
WEBs	14	20-Sep-12	30-60	NA
WEBs	11	7-Nov-12	0-30	NA
WEBs	11	7-Nov-12	30-60	NA
WEBs	11	7-Nov-12	60-90	NA
WEBs	12	7-Nov-12	0-30	NA
WEBs	12	7-Nov-12	30-60	NA
WEBs	12	7-Nov-12	60-90	NA
WEBs	13	7-Nov-12	0-30	NA
WEBs	13	7-Nov-12	30-60	NA
WEBs	13	7-Nov-12	60-90	NA
WEBs	14	7-Nov-12	0-30	NA
WEBs	14	7-Nov-12	30-60	NA
WEBs	14	7-Nov-12	60-90	NA
Winchester	F2/F3	25-Jun-12	0-15	B
Winchester	F2/F3	25-Jun-12	15-30	B
Winchester	F2/F3	25-Jun-12	30-60	B
Winchester	F4/F5	25-Jun-12	0-15	B
Winchester	F4/F5	25-Jun-12	15-30	B
Winchester	F4/F5	25-Jun-12	30-60	B
Winchester	F2/F3	25-Jul-12	0-15	B
Winchester	F2/F3	25-Jul-12	15-30	B
Winchester	F2/F3	25-Jul-12	30-60	B
Winchester	F4/F5	25-Jul-12	0-15	B
Winchester	F4/F5	25-Jul-12	15-30	B
Winchester	F4/F5	25-Jul-12	30-60	B
Winchester	F2/F3	20-Aug-12	0-15	B
Winchester	F2/F3	20-Aug-12	15-30	B
Winchester	F2/F3	20-Aug-12	30-60	B
Winchester	F4/F5	20-Aug-12	0-15	B
Winchester	F4/F5	20-Aug-12	15-30	B
Winchester	F4/F5	20-Aug-12	30-60	B
Winchester	F2/F3	17-Sep-12	0-15	B
Winchester	F2/F3	17-Sep-12	15-30	B
Winchester	F2/F3	17-Sep-12	30-60	B

Winchester	F4/F5	17-Sep-12	0-15	B
Winchester	F4/F5	17-Sep-12	15-30	B
Winchester	F4/F5	17-Sep-12	30-60	B
Winchester	F2/F3	22-Oct-12	0-15	B
Winchester	F2/F3	22-Oct-12	15-30	B
Winchester	F2/F3	22-Oct-12	30-60	B
Winchester	F4/F5	22-Oct-12	0-15	B
Winchester	F4/F5	22-Oct-12	15-30	B
Winchester	F4/F5	22-Oct-12	30-60	B
Winchester	F2/F3	20-Nov-12	0-15	B
Winchester	F2/F3	20-Nov-12	15-30	B
Winchester	F2/F3	20-Nov-12	30-60	B**
Winchester	F4/F5	20-Nov-12	0-15	B
Winchester	F4/F5	20-Nov-12	15-30	B
Winchester	F4/F5	20-Nov-12	30-60	B
Winchester	F2/F3	7-Dec-12	0-15	B
Winchester	F2/F3	7-Dec-12	15-30	B
Winchester	F2/F3	7-Dec-12	30-60	B
Winchester	F4/F5	7-Dec-12	0-15	B
Winchester	F4/F5	7-Dec-12	15-30	B
Winchester	F4/F5	7-Dec-12	30-60	B

B, between tile samples
T, above tile samples
NA, not applicable
** These samples were excluded due to DNA extraction and amplification problems.