Denitrification, Dissimilatory Nitrate Reduction, and Methanogenesis in the Gut of Earthworms (Oligochaeta): Assessment of Greenhouse Gases and Genetic Markers

Dissertation

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Peter Stefan Depkat-Jakob

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Acting dean:	Prof. Dr. Rhett Kempe
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Doctoral Committee:

Prof. PhD H. Drake	1st reviewer
Prof. Dr. O. Meyer	2nd reviewer
Prof. Dr. G. Gebauer	Chairman
Prof. Dr. H. Feldhaar	
Prof. Dr. G. Rambold	

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EQUATIONS

ABBREVIATIONS, CHEMICAL FORMULAS, AND ENZYMES

AIX-plates	agar plates for cloning containing ampicillin, IPTG, and X-Gal
anoxic tube	butyl rubber stopped aluminium crimp sealed glass tube (24 ml)
*Ban*I	endonuclease originally derived from *Bacillus anaerinolyticus*
BES	2-bromoethane sulfonate
BLAST	basic local alignment search tool
bp	base pairs
cDNA	complementary DNA; single-stranded DNA amplified from RNA
CH_4	methane
CO_2	carbon dioxide
CoA	coenzyme A
C_{org}	organic carbon
ddH_2O	deionised double destilled H_2O
DEPC-H_2O	DNase- and RNase-free H_2O pretreated with diethylepyrocarbonate (DEPC)
DNase	deoxyribonuclease
DNR	dissimatory nitrate reducers/reduction
dNTP	deoxyribonucleotide
DRNA	dissimatory reduction of nitrate to nitrite
EDTA	ethylenediaminetraacetic acid
Fd	ferredoxin
feeding guild	ecological category of earthworms based on feeding and burrowing habits
fw	fresh weight
GC	gas chromatography
H_2	dihydrogen
H_4MPT	tetrahydromethanopterin
H_4SPT	tetrahydrosarcinapterin
*Hha*I	endonuclease originally derived from *Haemophilus haemolyticus*
IPTG	isopropyl-*β*-D-thiogalactopyranoside
kb	kilo base pairs
M13-PCR	PCR to amplify fragments within the MCS of the pGEM-T cloning vector
*Mae*III	endonuclease originally derived from *Methanococcus aeolicus*
*Mbo*II	endonuclease originally derived from *Moraxella bovis*
Mcr	methyl-CoM reductase
MCS	multiple cloning site
MF	methanofuran
Mrt	isoenzme of the methyl-CoM reductase Mcr
n	number of replicates

N_2	dinitrogen
N_2O	nitrous oxide
n.a.	not applicable
n.d.	not determined
NAD^+	nicotinamide adenine dinucleotide
Nap	periplasmic nitrate reductase
Nar	membrane-bound nitrate reductase
NCBI	national center for biotechnology information
Nir	nitrite reductase
Nos	N_2O reductase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
OD	optical density
OTU	operational taxonomic unit
PAGE	polyacrylamide gel electrophoresis
PCA	principal component analysis
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PCR-H_2O	particle-free and autoclaved H_2O
pers. comm.	personal communication
ppb	parts per billon
ppm	parts per million
RNase	ribonuclease
RT-PCR	reverse transcription polymerase chain reaction
*Sau96*I	endonuclease originally derived from *Staphylococcus aureus* PS96
serum vial	butyl rubber stopped aluminium crimp sealed serum vial (150 to 1,000 ml)
SD	standard deviation
soil/substrate	interchangeably used terms for material usually ingested by earthworms
T	temperature
TAE	buffer solution containing Tris base, acetic acid, and EDTA
TBE	buffer solution containing Tris base, boric acid, and EDTA
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism (analysis)
v/v	volume per volume; volume fraction
X-Gal	5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside

1. INTRODUCTION

1.1. Nitrous oxide and methane as potent greenhouse gases

Nitrous oxide (N_2O) and methane (CH_4) are the two main greenhouse gases next to carbon dioxide (CO_2) causing the greenhouse gas effect (Alley *et al.* 2007, Forster *et al.* 2007). A part of the short-waved solar radiation passes through the atmosphere and reaches the surface where it is either reflected or absorbed. This absorption results in a heating of the earth´s surface and an emission of long-waved infrared radiation. A part of this radiation vanishes into outer space whereas another part gets absorbed by greenhouse gases and water vapor in the atmosphere. This absorbed energy causes the global warming (Alley *et al.* 2007, le Treut *et al.* 2007). This warming of the earth is primarily a natural effect that elevates the mean temperature on earth from - 19 °C (i.e., without the atmosphere and the greenhouse gas effect) to 14 °C which is essetial for life on earth (le Treut *et al.* 2007). However, this natural effect gets severely biased by anthropogenic activities releasing additional greenhouse gases into the atmosphere. About 60 % of the greenhouse gas effect is contributed to water vapor whereas the rest is caused by the greenhouse gases (Kiel & Trenberth 1997). Apart from water vapor, most of the greenhouse gas effect is attributed to CO_2 that is naturally predominantly released from biological respiration processes; in addition, CO_2 is released antropogenically from the burning of fossil fuels and forests, but also from changes in land use (Forster *et al.* 2007). Its concentration in the atmosphere increased significantly compared to pre-industrial times, i.e., around 1750, and is now around 379 ppm (Forster *et al.* 2007). CO_2 is assumed to be responsible for 77 % of the anthropogenically caused greenhouse gas effect; values for CH_4 and N_2O are 14 % and 8 %, respectively (Alley *et al.* 2007).

In a 100 year time frame, the global warming potential of CH_4 is 21-fold that of CO_2 (Forster *et al.* 2007). Its mean lifetime in the atmosphere is approximately 8 years (Denman *et al.* 2007). The concentration of CH_4 in the atmosphere increased from 715 ppb in 1750 to 1,774 ppb in 2005 with a reduced increase in the last decades (Alley *et al.* 2007, Forster *et al.* 2007) (Figure 1A). The majority of released CH_4, i.e., 70 % is of biogenic origin and is released from anoxic habitats, mainly wetlands and alimentary canals of ruminants and of invertebrates as termites (Denman *et al.* 2007, EPA 2010). To a lesser extent, terrestrial plants can release CH_4 under oxic conditions (Keppler *et al.* 2006). Abiogenic CH_4 is released from volcanoes, certain rocks, and fossil fuel and biomass burning (Denman *et al.* 2007, EPA 2010).

N_2O has a warming potential 310-fold higher than that of CO_2 in a 100 year time frame (Forster *et al.* 2007). The mean lifetime of N_2O in the atmosphere approximates 120 years. Its concentration increased from 270 ppb in 1750 to 319 ppb in 2005 (Forster *et al.* 2007) with an accelerated increase in the last decades (Figure 1B). Next to its warming potential, N_2O is also considered to be the major ozone depleting compound (Cicerone 1987, Ravishankara *et al.* 2009). Nearly 40 % of the emission of N_2O is of anthropogenic origin, e.g., industrial processes and fossil fuel and biomass burning (Alley *et al.* 2007, Forster *et al.* 2007, Schlesinger 2009). The major natural source of N_2O with approximately 70 % (Denman *et al.* 2007) are soils that are more and more influenced by human activity, especially by the application of fertilizers as ammonia (NH_4^+), nitrate (NO_3^-), and urea in areas with intense agricultural activity (Galloway 1998, EPA 2010).

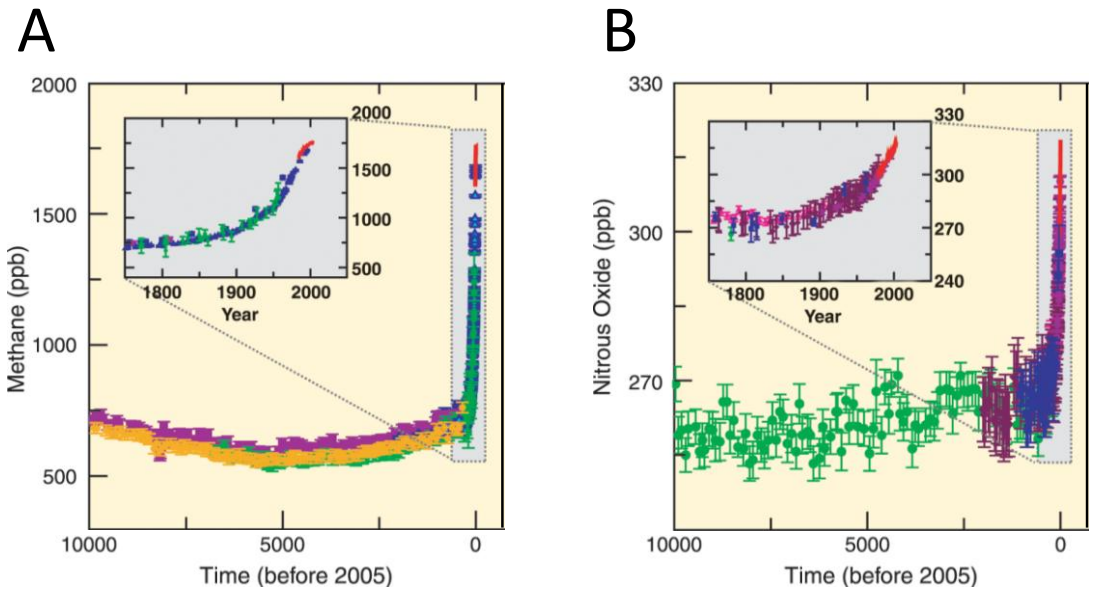


Figure 1: Atmospherical concentrations of the greenhouse gases CH_4 (A) and N_2O (B).
The figure displays the changes in concentrations of the greenhouse gases CH_4 in bbp (A) and N_2O in ppb (B) over the last 10,000 years with focus on the last approximately 200 years (expanded time scale in the inset picture). Modified from Alley *et al.* (2007).

1.2. Microbial processes involved in the formation of N_2O

The formation and emission of N_2O is mainly contributed to biotic rather than abiotic processes (Conrad 1995, EPA 2010). Microorganisms are considered as the main source of biotically produced N_2O (Conrad 1995, Hutchison 1995, Conrad 1996). By catalyzing the processes of denitrification, dissimilatory reduction of nitrate to ammonium (DRNA), and nitrification, these microorganisms are the main sources of soil-derived N_2O (Hutchison 1995, Conrad 1996, Bremner 1997, Zumft 1997, Colliver & Stephenson 2000, EPA 2010, Baggs

2011, Rütting *et al.* 2011) (Figure 2). In contrast, the assimilatory reduction of nitrate to nitrite via different forms of the assimilatory nitrate reductases (Nas) and the subsequent reduction to ammonium produce only minor amounts of N_2O (Kaspar & Tiedje 1981, Smith & Zimmerman 1981, Bleakley & Tiedje 1982, Smith 1982, Anderson & Levine 1986). The contribution of the dissimilatory reduction of nitrate to ammonium to the emission of N_2O is assumed to be negligible in soils but highly relevant in habitats that are mainly anoxic and rich of carbon compounds, such as the alimentary canal of vertebrates like cattle (Kaspar & Tiedje 1981, Tiedje 1988).

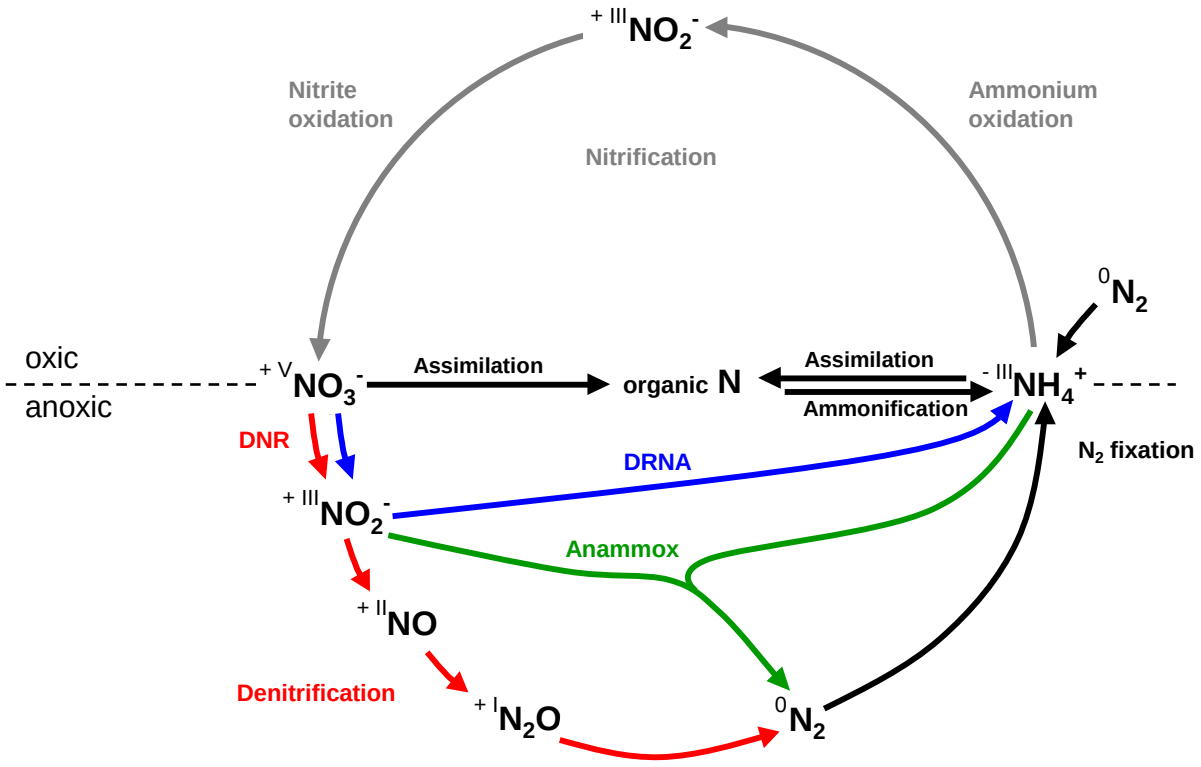


Figure 2: Major pathways of the prokaryotic nitrogen cycle.

The figure illustrates the prokaryotic conversions of N-compounds under oxic and anoxic conditions. Processes are indicated with different colors. Nitrification comprises the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and the oxidation of nitrite to nitrate (NO_3^-). DNR, dissimilatory nitrate reduction; DRNA, dissimilatory reduction of nitrate to ammonium; Anammox, anaerobic ammonium oxidation; NO, nitric oxide; N_2O, nitrous oxide ; N_2, dinitrogen; the fixation of N_2 to NH_4^+ can occur under oxic and anoxic conditions; Roman numerals indicate the redox state of the N atom. Except for denitrification, only typical substrates and end products of a reaction are displayed. Based on Schramm (2003), Zumft & Kroneck (2007), Jetten (2008), and Stein (2011).

1.2.1. Denitrification

1.2.1.1. Denitrification pathway and associated enzymes

Denitrification is one of the major processes in the nitrogen cycle producing dinitrogen (N_2) (Zumft 1997, Jetten 2008). Denitrification comprises the sequential reduction of nitrate to nitrite and to the gaseous compounds nitric oxide (NO), N_2O, and finally N_2 (often referred to as complete denitrification; Equation 1); the actual denitrification step is defined as the reduction of nitrogen oxides (nitrate and/or nitrite) to nitrogenous gases (N_2O and/or N_2) (Zumft 1997, Rudolf & Kroneck 2005, Shapleigh 2006) (Figure 2). Most denitrifiers are facultative aerobes that prefer dioxygen (O_2) as terminal acceptor as this reaction conserves more energy than complete denitrification (Shapleigh 2006). Under anoxic and microaerophilic conditions, denitrification enzymes are expressed and electrons (e^-) are transferred to nitrate, nitrite, NO, and N_2O creating an electrochemical gradient along the cytoplasmic membrane (Tiedje 1988, Zumft 1997, Baker *et al.* 1998). Energy is conserved via the generation of a proton motive force across the cytoplasmic membrane and the subsequent synthesis of ATP (Rudolf & Kroneck 2005, Kraft *et al.* 2011).

Equation 1: Single reactions of complete denitrification (Zumft 1997).

(a) $2\,NO_3^- + 4\,H^+ + 4\,e^- \rightarrow 2\,NO_2^- + 2\,H_2O$

(b) $2\,NO_2^- + 4\,H^+ + 2\,e^- \rightarrow 2\,NO + 2\,H_2O$

(c) $2\,NO + 2\,H^+ + 2\,e^- \rightarrow N_2O + H_2O$

(d) $N_2O + 2\,H^+ + 2\,e^- \rightarrow N_2 + H_2O$

In Gram-negative *Bacteria*, the four single reactions of denitrification (Equation 1) are catalyzed by enzymes associated with either the cytoplasmic membrane, or the periplasm (Kraft *et al.* 2011) (Figure 3). In Gram-positive *Bacteria* and *Archaea* that lack the periplasmic space, all four enzymes are suggested to be membrane-bound (Cabello *et al.* 2004, Suharti & de Vries 2005). For all *Bacteria* and *Archaea*, enzymes of denitrification are nitrate reductases, nitrite reductases, NO reductases, and N_2O reductases that catalyze reaction a, b, c, and d in Equation 1, respectively.

There exist two distinct classes of nitrate reductases with different localization and biochemical features (Stolz & Basu 2002, Ferguson & Richardson 2004, Philippot 2005, Richardson *et al.* 2007). Both enzymes contain a Mo-*bis*-molybdopterin guanine dinucleotide (Mo-*bis*-MGD) cofactor, Fe-S cluster, and *b*-type hemes (Philippot & Hojberg 1999, Richardson *et al.* 2007). The membrane-bound nitrate reductase (Nar) consists of three subunits and is encoded by *narGHI*. Nar can also function as a respiratory nitrate reductase

in non-denitrifying *Bacteria*, especially *Enterobacteriaceae* (Zumft 1997, Richardson *et al.* 2001, Kraft *et al.* 2011). The cytoplasmic domain of Nar consists of an α- (*narG*) and a β-subunit (*narH*) whereas the γ-subunit (*narI*) functions as a membrane anchor (Philippot & Hojberg 1999, Kraft *et al.* 2011). The α-subunit contains a 4Fe-4S cluster and the Mo-*bis*-MGD, and harbors the catalytic site of the nitrate reduction (Philippot & Hojberg 1999, Kraft *et al.* 2011). Electrons mainly derived from the oxidation of organic compounds, e.g., by NADH dehydrogenases are transferred to Nar and finally nitrate in the cytoplasm via usually ubiquinol located in the membrane (Richardson *et al.* 2007) (Figure 3). Here, two protons (H^+) are translocated across the membrane for each pair of electrons resulting in the generation a proton electrochemical gradient (Figure 3). Transmembrane transporters provide nitrate for the cytoplasmic Nar, e.g., a nitrate/nitrite antiporter that couples the translocation of nitrate into the cytoplasm with the translocation of nitrite into the periplasm (Richardson *et al.* 2007) (Figure 3). In *Archaea*, the catalytic subunit of Nar is located at the periplasmic site of the membrane-bound nitrate reductase (Martínez-Espinosa *et al.* 2007). Another membrane-bound nitrate reductase (NarZXY) that is highly similar to the NarGHI complex is known from *E. coli* (Philippot & Hojberg 1999). The heterodimeric periplasmic nitrate reductase (Nap) consists of two subunits and is encoded by *napAB*. The Mo-*bis*-MGD and a [4Fe-4S] cluster are located in the catalytic subunit encoded by *napA* (Richardson *et al.* 2007). The smaller subunit encoded by *napB* transfers electrons derived from the oxidation of the quinol pool to the catalytic subunit (Richardson *et al.* 2007). Other than with Nar, electron transfer to nitrate in Nap is not coupled to a direct generation of a proton motive force. Instead, nitrate reduction is coupled to free energy transduction via quinone reductases as the NADH dehydrogenase that generates a proton electrochemical gradient (Ellington *et al.* 2002) (Figure 3). *Bradyrhizobium japonicum* USDA110 is a widespread denitrifier and member of the *Rhizobiales* that possesses Nap instead of Nar (Delgado *et al.* 2003, Bedmar *et al.* 2005). Although most *Bacteria* possess only one nitrate reductase, several species, e.g., *Ralstonia* (redefined as *Wautersia*) *eutropha* and *Paracoccus denitrificans* possess both dissimilatory nitrate reductases, i.e., Nar and Nap (Warnecke-Eberz & Friedrich 1993, Sears *et al.* 1997, Richardson *et al.* 2001, Bru *et al.* 2007, Hartsock & Shapleigh 2011). However, Nar is slightly more abundant in environmental samples (Bru *et al.* 2007) and normally expressed under anaerobic growth conditions whereas Nap is also expressed and active in the presence of oxygen (Bell *et al.* 1990, Siddiqui *et al.* 1993, Shapleigh 2006). Other than the mainly energy-conserving function of Nar, Nap is rather assumed to regulate the redox state of the cell, is important for the transition of oxic to anoxic conditions, and/or for aerobic denitrification, i.e., the simultaneous use of nitrate and O_2 as electron acceptor by facultative microorganisms (Castillo *et al.* 1996, Zumft 1997, Hartsock &

Shapleigh 2011, Kraft *et al.* 2011, Zhang *et al.* 2012). In general, nucleotide sequences of Nar are more conserved than those of Nap (Sudesh & Cole 2007).

Two distinct classes of periplasmic nitrite reductases catalyze the reduction of nitrite to the gas NO; the copper-containing NirK and the heme cd_1-containing NirS encoded by *nirK* and *nirS*, respectively (Zumft 1997, Rinaldo & Cutruzzolá 2007). Whereas NirK and NirS are found in both *Bacteria* and *Archaea* (Zumft 1997, Kraft *et al.* 2011), there is no organism identified that harbors both nitrite reductases (Zumft 1997, Heylen *et al.* 2006, Shapleigh 2006, Rinaldo & Cutruzzolá 2007). Each subunit of the homotrimeric NirK contains two distinct Cu-centers, i.e., a type 1 and a type 2 Cu-center (Adman & Murphy 2001, Rinaldo & Cutruzzolá 2007). The type 2 Cu-center is the binding site for nitrite that is reduced to NO via electrons derived from the type 1 center. These electrons are delivered by electron carriers as *c*-type cytochromes that in turn get reduced by components of the respiratory chain in the cytoplasm membrane as the cytochrome bc_1 complex (Rinaldo & Cutruzzolá 2007) (Figure 3). Next to its main product NO, NirK can also produce small amounts (i.e., 3 to 6 %) of N_2O if NO accumulates (Rinaldo & Cutruzzolá 2007). In terms of molecular properties, NirK-type nitrite reductases are more heterogeneous than NirS-type nitrite reductases (Rinaldo & Cutruzzolá 2007). Each subunit of the homodimeric NirS contains a heme *c* and a unique heme d_1 (Cutruzzolá *et al.* 2003, Rinaldo & Cutruzzolá 2007, Kraft *et al.* 2011). Nitrite binds to the heme d_1 and is reduced to NO via an electron derived from the heme *c* that gets its electrons from soluble electron carriers, i.e., *c*-type cytochromes or Cu-proteins (Pearson *et al.* 2003) (Figure 3). Next to its main product NO, NirS can also catalyze the reactions of O_2 to H_2O, CO to CO_2, and NH_2OH to NH_3 to a small extent (Rinaldo & Cutruzzolá 2007).

Membrane-bound NO reductases are phylogenetically related to cytochrome oxidases (de Vries & Schröder 2002, de Vries *et al.* 2007) and can be primarily divided into three distinct groups; cNor, qNor, and qCu_ANor (de Vries & Schröder 2002, Zumft 2005, de Vries *et al.* 2007). The heterodimeric membrane-bound cNor consists of the heme-*c* containing subunit NorC (encoded by *norC*), and the heme-*b* and non-heme Fe containing subunit NorB (encoded by *norB*) where the catalytic site is localized. Electron donors are membrane or soluble *c*-type cytochromes and azurin or pseudoazurin (de Vries *et al.* 2007) (Figure 3). The NO reductase qNor consists of one subunit (NorB) containing heme-*c* and non-heme iron, and a N-terminal quinone oxidase. Thus, electrons are derived from reduced quinones only (Zumft 2005, de Vries *et al.* 2007). The heterodimeric qCu_ANor contains one non-heme Fe, two Cu atoms, two *b*-type hemes, and also a quinone oxidase per enzyme complex. Electrons are derived from quinoles and membrane-bound cytochromes c_{551} (Suharti & de Vries 2005, de Vries *et al.* 2007). For all three types of NO-reductases, energy is not conserved, i.e., protons are not translocated directly but by the preceding formation of reduced electron carriers only (de Vries *et al.* 2007). As NO is highly toxic for

microorganisms (Choi *et al.* 2006, Stein 2011), there also exist NO reductases of non-denitrifying organisms that only detoxify NO, like the flavorubredoxin-containing NorVW in *Escherichia coli* (Gomes *et al.* 2002, Rodinov *et al.* 2005).

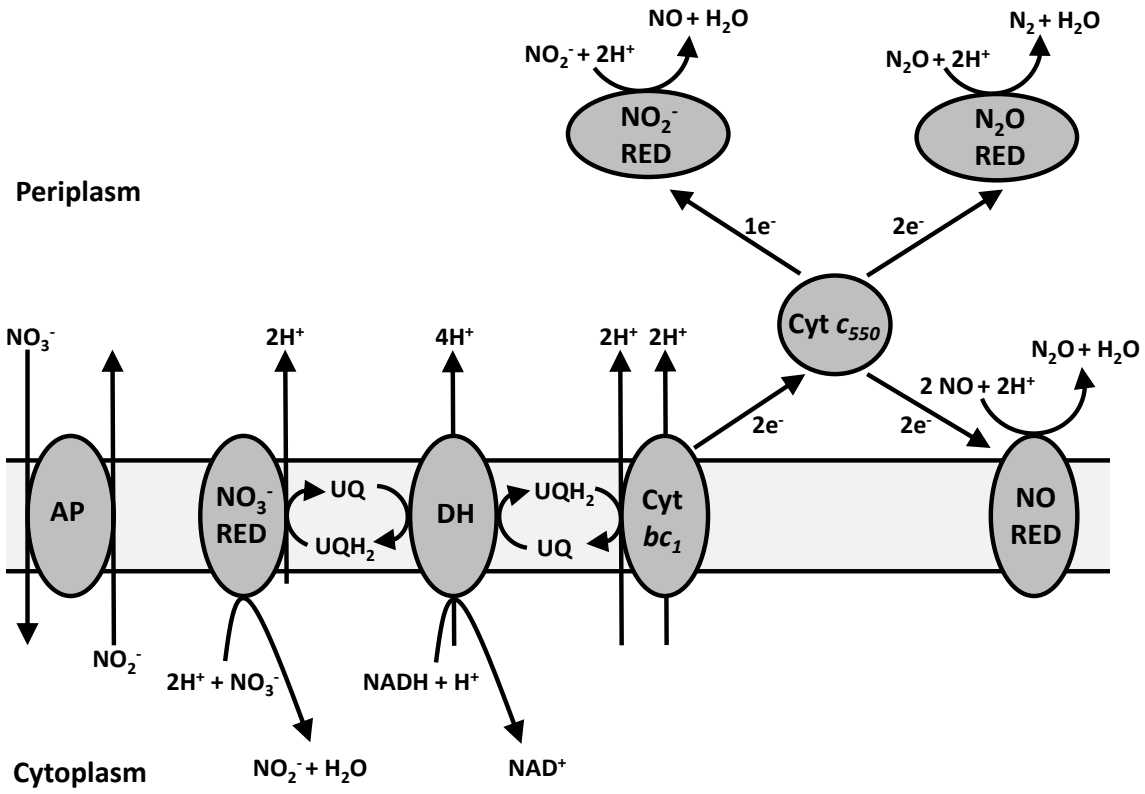


Figure 3: Denitrification and associated enzymes of the denitrifier *Paracoccus denitrificans*.
The figure illustrates the organization of respiratory elements involved in the denitrification of the Gram-negative *Paracoccus denitrificans* and the impact of proton translocation across the cytoplasmic membrane. Straight arrows indicate the translocation of protons (H^+), nitrate (NO_3^-) and nitrite (NO_2^-), and the direction of electron (e^-) flow; curved arrows indicate reactions. Abbreviations: AP, nitrate/nitrite antiporter; NO_3^- RED, nitrate reductase (Nar); UQ, ubiquinone; UQH_2, dihydroubiquinone; NAD^+, nicotinamide adenine dinucleotide; DH, NADH dehydrogenase; Cyt bc_1, cytochrome bc_1 complex; Cyt c_{550}, cytochrome c_{550}; NO_2^- RED, nitrite reductase (NirS); N_2O RED, N_2O reductase (NosZ); NO RED, NO reductase (cNor). Based on Shapleigh (2006), Richardson *et al.* (2007), and Strohm *et al.* (2007).

The most widespread and most thoroughly studied bacterial N_2O reductase is the homodimeric Z-type N_2O reductase (NosZ). It reduces N_2O to N_2 and is encoded by *nosZ* (Zumft & Körner 2007, Zumft & Kroneck 2007). A N_2O reductase is required for complete denitrification. However, also non-denitrifying microorganisms harbor NosZ to transfer electrons to N_2O and subsequently produce N_2 (Zumft 1997). NosZ is a periplasmic and membrane-bound enzyme in Gram-negative and Gram-positive *Bacteria*, respectively (Kraft

et al. 2011). Each NosZ monomer contains two copper centers, Cu_A and Cu_Z (Tavares *et al.* 2006, Zumft & Körner 2007, Zumft & Kroneck 2007, Kraft *et al.* 2011). Cu_Z is the catalytic site and is a [4Cu-4S] copper-sulphur cluster where the N_2O binds (Zumft & Körner 2007, Pauleta *et al.* 2013). Electrons enter the catalytic site via the binuclear Cu_A copper center. In Gram-negative *Bacteria*, NosZ receives its electrons from cytochrome *c* with an involvement of the cytochrome bc_1 complex which implies a coupling of proton transfer across the cytoplasmic membrane with the reduction of N_2O (Tavares *et al.* 2006, Zumft & Körner 2007, Kraft *et al.* 2011) (Figure 3). *Archaea* possess either a slightly modified Z-like N_2O reductase that is membrane-bound and receives electrons from quinol, or another, A-type designated N_2O reductase whose features are largely unresolved (Zumft & Körner 2007). *Wollinella succinogenes* possesses an H-type N_2O reductase (Zumft & Körner 2007) but is no classical denitrifier as it lacks an enzyme for the reduction of nitrite to NO (Zumft 1997). All N_2O reductases, i.e., Z-, A-, and H-type are encoded together with the associated genes *nosDFYL* (Zumft & Körner 2007). For the analysis of N_2O reductase activity, acetylene is applied to pure cultures or environmental samples. Acetylene inhibits the N_2O reductase resulting in an additional emission of N_2O that is then released instead of being reduced to N_2. Thus, the amount of N_2 produced by N_2O reductases can be assessed (Yoshinari & Knowles 1976).

1.2.1.2. Organisms and regulation of denitrification

Denitrifiers are phylogenetically and physiologically highly diverse and widespread in terrestrial and marine ecosystems (Gamble *et al.* 1977, Shirey & Sextone 1989, Zumft 1997, Shapleigh 2006, Kraft *et al.* 2011). *Alpha-*, *Beta-*, *Gamma-*, and *Epsilonproteobacteria*, *Firmicutes*, *Actinobacteria*, and *Archaea* are phyla that harbor most of the known denitrifiers (Zumft 1997, Philippot 2005, Shapleigh 2006). Other than for Gram-negative *Bacteria* as *Proteobacteria*, knowledge about denitrification in Gram-positive *Bacteria* and *Archaea* is still restricted (Shapleigh 2006, Martínez-Espinosa *et al.* 2007, Verbaendert *et al.* 2011b). Most denitrifiers are facultative heterotrophs that are able to utilize sugars and/or fatty acids as carbon and energy source, but are not capable of fermentations, i.e., no *Enterobacteriaceae* (Tiedje 1988, Zumft 1997, Shapleigh 2006). Autotrophic denitrifiers can use dihydrogen (H_2), ammonium (NH_4^+), nitrite, iron-II, or inorganic sulphur compounds as electron acceptors (Zumft 1997, Schwartz & Friedrich 2006, Shapleigh 2006). Most denitrifiers possess all four denitrification enzymes, i.e., for the complete denitrification. However, some lack a nitrate reductase, a NO reductase, or a N_2O reductase (Zumft 1997, Shapleigh 2006). Representative genera of Gram-negative denitrifiers with predominantly N_2 as end product are *Bradyrhizobium*, *Paracoccus*, *Pseudomonas*, *Brucella*, *Ralstonia* (*Wautersia*),

Rhodobacter, and *Sinorhizobium* (Zumft 1997, Shapleigh 2006). Representative Gram-positive denitrifiers within the *Firmicutes* and *Actinobacteria* with predominantly N_2O as end product belong to the genera *Bacillus*, *Paenibacillus*, *Micromonospora*, *Nocardia*, and *Streptomyces* (Zumft 1997, Shoun *et al.* 1998, Ihssen *et al.* 2003, Horn *et al.* 2005, Shapleigh 2006); Gram-positive denitrifiers with N_2 as end product appear in the genus *Bacillus* (Verbaendert *et al.* 2011a, Zhang *et al.* 2012) and within the *Bacteriodetes*/*Flavobacterium* branch (Horn *et al.* 2005). Next to *Bacteria* and *Archaea*, some denitrifying *Fungi imperfecti*, filamentous fungi, yeasts and *Foraminafera* are known (Bollag & Tung 1972, Bleakley & Tiedje 1982, Burth *et al.* 1982, Shoun *et al.* 1992, Zumft 1997, Risgaard-Petersen *et al.* 2006, Shapleigh 2006, Kraft *et al.* 2011).

Denitrification is mainly influenced by the environmental factors pH, temperature, water content, oxygen availability, nitrate availability, carbon availability, and the ratio of carbon to nitrogen (Tiedje 1988, Conrad 1996, van Cleemput 1998). Several factors influence the ratio of the emitted N_2O to N_2 (N_2O/N_2), e.g., the pH (Sahrawat & Keeney 1986), the growth phase (Baumann *et al.* 1996), and the ratio of the electron acceptors nitrate to the electron donors such as organic carbon (nitrate/C_{org}) (Davidson 1991, Kester *et al.* 1997). A low pH, an early growth phase, and a high nitrate/C_{org} ratio result in a higher N_2O/N_2 ratio (van Breemen & Feijtel 1990, Thomsen *et al.* 1994, Baumann *et al.* 1996, Stevens *et al.* 1998). A change from oxic to anoxic conditions can result in a transient accumulation of N_2O as the expression of denitrification genes is delayed (Baumann *et al.* 1996, Philippot *et al.* 2001).

Proteins involved in the transcriptional regulation of enzymes and other proteins of the denitrification pathway are highly diverse and regulation of denitrification can significantly differ between denitrifying species (van Spanning *et al.* 2007). Key factors are anoxia, nitrate and nitrite, and NO (Murai *et al.* 2000, Zumft 2002, van Spanning *et al.* 2007). Most denitrifiers can also use O_2 as electron acceptor, whose reduction to H_2O yields more energy than the complete reduction of nitrate to N_2 (Zehnder & Stumm 1988, Shapleigh 2006). Thus, O_2 is the preferred electron acceptor under oxic conditions and the transcription of enzymes for the denitrification pathway is often blocked when O_2 is present (Moir & Wood 2001, van Spanning *et al.* 2007). This threshold level for O_2 highly varies between different species of denitrifiers (John 1977, Bazylinski & Blakemore 1983, Shapleigh 2006). The two most important O_2 sensors are FixL and FNR (fumarate and nitrate reduction) proteins. In addition, the availability of nitrate and/or nitrite is crucial for denitrification and thus, nitrate and/or nitrite also function as signal molecules that activate the transcription of the nitrate reductase (van Spanning *et al.* 2007). For denitrifiers, three types of nitrate/nitrite sensing systems are known, i.e., NarXL, NarQP, and NarR with different affinities for nitrate and/or nitrite (Unden *et al.* 1995, van Spanning *et al.* 2007). Together with nitrite, NO is cytotoxic even in low concentrations (Choi *et al.* 2006, Stein 2011) and is therefore another key signal to activate

the expression of denitrification enzymes other than nitrate reductases (van Spanning *et al.* 2007). NNR (nitrite and NO gene regulator) and NorR are specific NO sensors. In addition, redox sensors are involved in the regulation in denitrifying *Rhodobacter* species (Zumft 2002, van Spanning *et al.* 2007).

1.2.2. Dissimilatory reduction of nitrate to ammonium

During the dissimilatory reduction of nitrate to ammonium, nitrate is reduced to nitrite and further reduced to ammonium (Sudesh & Cole 2007) (Figure 2). Other than denitrifiers, *Bacteria* that conduct dissimilatory reduction of nitrate to ammonium are frequently capable of fermentation processes (Tiedje 1988, Sudesh & Cole 2007, Kraft *et al.* 2011). Exemplary genera of strict anaerobes are *Clostridium*, *Desulfovibrio*, *Selenomonas*, and *Wollinella*, whereas those of facultatives are *Citrobacter*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Bacillus, Paenibacillus, Pseudomonas*, and *Escherichia coli* (Tiedje 1988, Simon 2002, Philippot 2005, Slepecky & Hemphill 2006, Rütting *et al.* 2011). The dissimilatory reduction of nitrate to ammonium and denitrification use nitrate as initial electron acceptor but there are no validly confirmed examples of *Bacteria* that conduct both processes. Thus, organisms conducting denitrification and dissimilatory reduction of nitrate to ammonium (Sudesh & Cole 2007) compete for the available nitrate. The reduction of nitrite to ammonium consumes six electrons whereas during the reduction of nitrite to N_2 only three electrons are consumed per molecule of nitrite (Equation 1). Thus, dissimilatory reduction of nitrate to ammonium is favored in anoxic habitats with high organic carbon contents and a low nitrate concentration, e.g., in the rumen where the fermentation of organic carbon compounds produces a high amount of electron equivalents that need to be re-oxidized (Cole & Brown 1980, Cole 1996, Sudesh & Cole 2007). However, there is evidence that dissimilatory reduction of nitrate to ammonium is not a strictly anaerobic process but can also occur in the presence of O_2 (Morley & Baggs 2010, Rütting *et al.* 2011) whereas completely anoxic environments might favor denitrification (Baggs 2011).

The dissimilatory reduction of nitrate to ammonium can occur in the cytoplasm, the periplasm, or both compartments, dependent on the bacterial species and the growth conditions (Sudesh & Cole 2007). The cytoplasmic pathway is conducted by a Nar-type nitrate reductase with its catalytic subunit encoded by *narG* (1.2.1.1) and either the monomeric NirB, or the dimeric NirB-NirD nitrite reductase (Sudesh & Cole 2007). The reduction of nitrite to ammonium occurs here without the conservation of energy but for the detoxification of nitrite and the regeneration of nicotinamide adenine dinucleotides (NAD^+) (Moreno-Vivián *et al.* 1999, Rütting *et al.* 2011). As species like *Mycobacterium tubercolosis* and *Streptomyces coelicolor* harbor two and three copies of *narG*, respectively, different

physiological roles of the different nitrate reductases are assumed but still largely unresolved (Sudesh & Cole 2007, Fischer *et al.* 2010). The periplasmic pathway is catalyzed by the membrane-bound nitrate reductase Nap (1.2.1.1) and the nitrite reductase NrfA; NrfA occurs either in a *nrfABCDEFG* or a *nrfHAIJ* operon (Sudesh & Cole 2007). Other than with Nir-type nitrite reductases, energy can be conserved with NrfA (Simon 2002, Stolz & Basu 2002, Sudesh & Cole 2007). Thus, the periplasmic dissimilatory reduction of nitrate to ammonium is called the respiratory dissimilatory reduction of nitrate to ammonium (Sudesh & Cole 2007). During the dissimilatory reduction of nitrate to ammonium, N_2O can be produced from nitrite via an unspecific reaction of the nitrate reductase, and/or NO is assumed to be an enzyme-bound intermediate that can subsequently be detoxified to N_2O (Tiedje 1988, Kraft *et al.* 2011, Vine & Cole 2011).

Next to denitrification and dissimilatory reduction of nitrate to ammonium, the sole reduction of nitrate to nitrite with subsequent accumulation of nitrite constitutes another way to dissimilate nitrate with representatives being called 'nitrite accumulators' (Rütting *et al.* 2011). However, the majority of these nitrite accumulators are assumed to be also capable of the further reduction of nitrite to ammonium although a distinct test for that is often lacking in standard tests during species descriptions (Dunn *et al*. 1979, Smith & Zimmerman 1981, Rütting *et al.* 2011). Therefore, dissimilatory reduction of nitrate to ammonium is hereafter separated from denitrification and referred to as 'dissimilatory nitrate reduction' with organisms conducting this process referred to as 'dissimilatory nitrate reducers' (DNR).

1.2.3. Molecular analysis of denitrifiers and dissimilatory nitrate reducers

Denitrifiers and dissimilatory nitrate reducers are widely distributed among prokaryotes (1.2.1.2, 1.2.2). Thus, molecular analyses based on the 16S rRNA genes are inapplicable. Instead, structural gene markers were developed that target enzymes involved in denitrification and dissimilatory nitrate reduction. Corresponding primers detect *narG* (Gregory *et al.* 2000, Philippot *et al.* 2002), *narH* (Petri & Imhoff 2000), *napA* (Flanagan *et al.* 1999), *nirK/nirS* (Braker *et al.* 1998, Hallin & Lindgren 1999), *nrfA* (Mohan *et al.* 2004), *norB* (Braker & Tiedje 2003), and *nosZ* (Scala & Kerkhof 1998, Rich *et al.* 2003).

As the reduction of nitrate to nitrite is the initial step for both denitrifiers and dissimilatory nitrate reducers (1.2.1.1, 1.2.2), nitrate reductases encoded by *narG* and *napA* detect both processes. In general, *narG* nucleotide sequences are more conserved than those of *napA* (Sudesh & Cole 2007) and Nap seems to be more relevant for dissimilatory nitrate reduction than Nar (Kraft *et al.* 2011). Up to now, the majority of studies used *narG* instead of *napA* to detect denitrifiers and dissimilatory nitrate reducers in the environment (e.g., Chèneby *et al.*

2003, Gregory *et al.* 2003, Mounier *et al.* 2004, Enwall *et al.* 2005, Deiglmayr *et al.* 2006, Palmer *et al.* 2012, Vilar-Sanz *et al.* 2013). To detect denitrifiers, the most frequently targeted genes are those of *nirK* and *nirS* (e.g., Braker *et al.* 2001, Prieme *et al.* 2003, Castro-Gonzáles *et al.* 2005, Hallin *et al.* 2006, Palmer *et al.* 2012, Vilar-Sanz *et al.* 2013) and *nosZ* (e.g., Rösch *et al.* 2002, Stres *et al.* 2004, Enwall *et al.* 2005, Horn *et al.* 2006a, Wüst *et al.* 2009b, Palmer *et al.* 2012, Vilar-Sanz *et al.* 2013). Whereas *narG* primers detect both Gram-negative and Gram-positive prokaryotes, those for *nirK*, *nirS*, and *nosZ* do not detect Gram-positive prokaryotes (Behrendt *et al.* 2010, Green *et al.* 2010, Verbaendert *et al.* 2011b).

1.2.4. Nitrification

Nitrification is the sequential oxidation of NH_4^+ to nitrite and then nitrate with O_2 as electron acceptor (Ferguson *et al.* 2007). This normally strictly aerobic process is conducted by two distinct groups of mostly autotrophic organisms, i.e., ammonium oxidizers and nitrite oxidizers (Ferguson *et al.* 2007). Bacterial ammonium oxidizers possess an ammonium monooxygenase to oxidize NH_4^+ to hydroxylamine (NH_2OH) that is oxidized to nitrite via a hydroxylamine oxidoreductase (Ferguson *et al.* 2007) (Figure 2). Bacterial ammonium oxidizers are *Gamma*- and *Betaproteobacteria* of the genera *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* (Schramm 2003, Prosser *et al.* 2007). In addition, ammonium oxidizing *Archaea* were discovered via molecular methods in mesophilic environments (Treusch *et al.* 2005, Prosser *et al.* 2007). However, there is still an ongoing debate about the significance of archaeal in comparison to bacterial ammonium oxidation (Prosser *et al.* 2007, Pratscher *et al.* 2011, Ward 2011). Nitrite oxidizers catalyze the oxidation of nitrite to nitrate with a nitrite oxidase and belong to the genera *Nitrospina*, *Nitrospira*, *Nitrobacter*, and *Nitrococcus*. Next to autotrophic nitrifiers, also heterotrophic nitrifiers are known (Schramm 2003, Prosser *et al.* 2007). There are no organisms known to harbor the enzymes for both processes of nitrification, i.e., ammonium oxidation and nitrite oxidation (Prosser *et al.* 2007) although their existence was postulated as a possible occurrence in nature (Costa *et al.* 2006). During ammonium oxidation, N_2O and NO can be produced as byproducts (Webster & Hopkins 1996, Bollmann & Conrad 1998, Wrage *et al.* 2001). Nitrifier denitrification is assumed to be an even more relevant source of N_2O released during ammonium oxidation. During this process, ammonium is oxidized to nitrite first, and subsequently reduced to NO, N_2O, and N_2 similar to denitrification (Schmidt *et al.* 2004, Kool *et al.* 2011). Nitrifier denitrification might be favored by a low pH and low oxygen conditions coupled with low organic carbon contents (Wrage *et al.* 2001, Kool *et al.* 2011). In addition, nitrification is influenced by the environmental factors of water content and ammonium availability (Tiedje 1988).

1.3. Methanogenesis as the main biotic source of CH_4

Methanogenesis is an anaerobic respiration process that is conducted by strictly anaerobic methanogenic *Archaea* belonging to the orders *Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales*, and *Methanosarcinales* within the *Euryarchaeota* (Bapteste *et al.* 2005, Hedderich & Whitman 2006, Liu & Whitman 2008, Sakai *et al.* 2008, Thauer *et al.* 2008). Methanogens have a limited substrate range, i.e., CH_4 is produced via hydrogenotrophic, acetoclastic, and metholytrophic *Archaea* (Hedderich & Whitman 2006, Liu & Whitman 2008). Only members of the genus *Methanocarcina* (order *Methanosarcinales*) are capable of all three CH_4-forming pathways (Liu & Whitman 2008). Methanogens cannot use most organic substances as carbohydrates, long-chain fatty acids, and alcohols, but rely on anaerobic microorganisms to produce the substrates needed for methanogenesis (Liu & Whitman 2008).

Equation 2: Exemplary reactions of hydrogenotrophic (a), acetoclastic (b), and methylotropic methanogenesis (c) (Liu & Whitman 2008, Thauer *et al.* 2008)

(a) $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$

(b) $CH_3COOH \rightarrow CH_4 + CO_2$

(c) $4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$

Hydrogenotrophic methanogens form CH_4 via the reduction of CO_2 with H_2 (Bapteste *et al.* 2005, Liu & Whitman 2008), but are often also able to utilize formate, with some species utilizing CO, ethanol, or 2-butanol (Liu & Whitman 2008). All six methanogenic orders of *Archaea* harbor hydrogenotrophic methanogens (Bapteste *et al.* 2005). In the hydrogenotrophic pathway (Equation 2a), CO_2 binds to methanofuran (MF) first and gets reduced to a formyl-group via ferredoxin (Fd) that is reduced by H_2 (Thauer *et al.* 2008) (Figure 4). The formyl-group in transferred to tetrahydromethanopterin (H_4MPT) and MF is released. In *Methanosarcina*, a modified H_4MPT is prevalent named tetrahydrosarcinapterin (H_4SPT) (Liu & Whitman 2008, Thauer *et al.* 2008). The formyl-group is dehydrated resulting in the formation of a methenyl-group that is subsequently reduced to a methylene-group and then to a methyl-group. These reductions are performed by the coenzyme F_{420} that is reduced by H_2 (Liu & Whitman 2008, Thauer *et al.* 2008). Next to methanogens, the fluorescent F_{420} appears only sporadically and sparsely among prokaryotes but was detected in the *Mycobacterium smegmatis* (*Actinobacteria*) in high abundances (Selengut & Haft 2010). The methyl-group is then transferred to a reduced coenzyme M (HS-CoM) and H_4MPT/H_4MSP is released. The final reduction and subsequent release of CH_4 is catalyzed by the methyl-CoM reductase with reduced coenzyme B (HS-CoB) (Liu & Whitman 2008,

Thauer *et al.* 2008) (Figure 4). With formate as substrate for hydrogenotrophic methanogens, four molecules of formate are oxidized resulting in the creation of reduction equivalents that are required to reduce CO_2 to CH_4 (Liu & Whitman 2008) as described avove.

Acetoclastic methanogens (Equation 2b) use acetate and belong to the order *Methanosarcinales* (genera *Methanosaeta* and *Methanosarcina*) (Hedderich & Whitman 2006, Liu & Whitman 2008). They split acetate and reduce the methyl-group to CH_4 whereas the carboxyl-group is oxidized to CO_2 (Liu & Whitman 2008). Other than species of *Methanosarcina*, those of *Methanosaeta* are strictly acetoclastic (Hedderich & Whitman 2006, Liu & Whitman 2008). Initially, acetate is activated to acetyl phosphate with ATP and then synthesized to acetyl-CoA with HS-CoA (Figure 4). With H_4MPT/H_4SPT, acetyl-CoA is split into methyl-H_4MPT/H_4SPT and CO-CoA by the CO dehydrogenase/acetyl-CoA synthase enzyme complex. The methyl-H_4MPT/H_4SPT is reduced to CH_4 as in the hydrogenotrophic pathway; CO-CoA is oxidized to CO_2 with electrons being transferred to oxidized Fd and later to protons with the creation of H_2 in a hydrogenase reaction (Liu & Whitman 2008, Thauer *et al.* 2008).

Methylotrophic methanogens (Equation 2c) belong to the *Methanosarcinales* and the genus *Methanosphaera* within the *Methanobacteriales*. Their substrates are methylated compounds as methanol, methylamines, and methylated sulphides, i.e., compounds with only one carbon atom (Bapteste *et al.* 2005, Liu & Whitman 2008) (Figure 4). During methylotrophic methanogenesis, the methylated substrates are transferred to HS-CoM forming methyl-CoM. One fraction is further reduced to CH_4 as with hydrogenotrophic and acetoclastic methanogenesis. The other fraction of methyl-groups is oxidized to CO_2 via a reversal hydrogenotrophic pathway to gain reduction equivalents (Liu & Whitman 2008) (Figure 4).

Methanogenesis is influenced by environmental factors as pH, temperature, water content, and the availability of C_{org} and is abundant in habitats with limiting concentrations of electron acceptors as O_2, nitrate, and sulphate (Conrad 1996, Segers 1998, Liu & Whitman 2008). Temperature influences fermentation processes and therefore the fermentation products H_2 and acetate, resulting in an influence on hydrogenotrophic and acetoclastic methanogenesis (Conrad 1996).

The methyl-CoM reductase appears as Mcr and its isoenzyme Mrt; their catalytic subunits are encoded by *mcrA* and *mrtA*, respectively (Gunsalus *et al.* 1987, Springer *et al*. 1995). As the methyl-CoM reductase catalyzes the final step for all three methanogenic pathways, i.e., for hydrogenotrophic, acetoclastic, and metholytrophic methanogens, *mcrA* is a frequently used structural gene marker to analyze the community of methanogens whereas *mrtA* is often co-amplified in lower numbers together with *mcrA* with commonly used primer systems (e.g., Lueders *et al.* 2001, Merilä *et al.* 2006, Hunger *et al.* 2011).

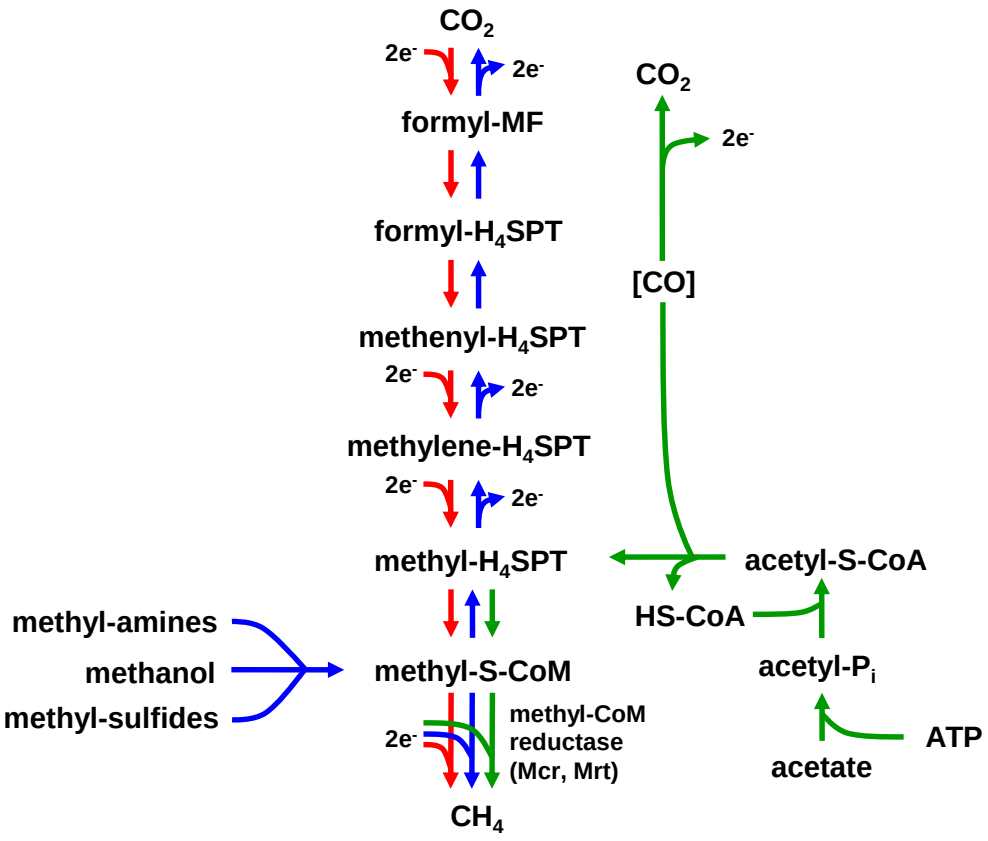


Figure 4: Diagram of the hydrogenotropic (red arrows), acetoclastic (green arrows), and methylotrophic (blue arrows) methanogenesis pathway, all three prevailing in *Methanosarcina*. The figure illustrates the three pathways of methanogenesis as indicated by red, green, and blue arrows for hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis, respectively. Electrons (e^-) are derived from reduction equivalents, primarily from H_2. For all three pathways, the final step is the reduction of methyl-CoM resulting in the relase of CH_4. This final step is catalyzed by a methyl-CoM reductase (Mcr and/or Mrt). In the hydrogenotropic pathway, CO_2 is reduced by e^- derived from H_2. Methylotropic methanogens utilize C_1 compounds, i.e., molecules with at least one methyl group. Here, one fraction is reduced to CH_4 whereas the rest is oxidized to CO_2 via the reversal hydrogenotrophic pathway to generate reduction equivalents. During acetoclastic methanogenesis, acetate is split into a methyl group and an enzyme-bound CO. The CO is oxidized to CO_2 to provide reduction equivalents for the reduction of the methyl group to CH_4. For all three pathways, an electrochemical gradient is generated for ATP synthesis. Whereas most methanogens possess only one of the three pathways, species of *Methanosarcina* possess all three. Abbreviations: MF, methanofuran; CoM, coenzyme M; CoA, coenzyme A; H_4SPT, tetrahydrosarcinapterin; P_i, inorganic P. Based on Bapteste *et al.* (2005), Welander & Metcalf (2005), and Liu & Whitman (2008).

1.4. Earthworms

1.4.1. Diversity, habitats and feeding guilds of earthworms

Earthworms belong to the subclass Oligochaeta (Annelida: Clitellata) and terrestrial species are often referred to as Crassiclitellata. There is an ongoing debate about the validity of the application of several superfamilies within the Crassiclitellata and about the number of overall earthworm families ranging from 15 to 21 in most recent taxonomy (Chang & James 2011). This re-arrangement of earthworm taxonomy took place especially in the last two decades with the additional application of molecular tools (Jamieson *et al.* 2002, Chang & James 2011, James & Davidson 2012). However, about 5,500 terrestrial earthworm species exist (Blakemore *et al.* 2007). Apart from some uncertainties in earthworm taxonomy, the most species-rich families are those of Megascolecidae (widespread in tropical, subtropical, and some temperate regions), Glossoscolecidae (tropical regions as Southern Africa, Southern Europe, and Latin America), and Eudrilidae (Central and West Africa) (Reynolds & Cook 1993, Edwards & Bohlen 1996, Omodeo 1998, Fragoso *et al.* 1999, Jamieson *et al.* 2002). In Europe and the remaining temperate northern hemisphere, the majority of earthworm species belongs to the family Lumbricidae with 39 species detectable in Germany (Graff 1983, Westheide & Rieger 2007). Earthworm species that can be typically found in German soils are *Lumbricus rubellus*, *Lumbricus terrestris*, *Aporrectodea caliginosa*, and *Octolasium lacteum* ranging from approximately 5 to 25 cm length (Graff 1983, Westheide & Rieger 2007; the current study). In Brazil, about 300 earthworm species are described with more than 1,000 species expected to exist there (Brown & James 2007). Most species are native, e.g., *Glossoscolex paulistus*, *Rhinodrilus alatus*, and *Pontoscolex corethrurus* (all Glossoscolecidae) whereas several species are exotic to Brazil, e.g., *Amynthas gracilis* (Megascolecidae) and *Eudrilus eugeniae* (Eudrilidae) (James & Brown 2006). Of the Brazilian earthworm species mentioned, *R. alatus* and *G. paulistus* are the largest ones reaching about 70 and 35 cm length, respectively (James & Brown 2006, Brown & James 2007; the current study); other species can exceed 1 m in length (Brown & James 2006).

Huge earthworm species, i.e., with approximately 30 cm length also exist in other countries, e.g., in New Zealand with the native *Octochaetus multiporus* (Lee 1959a, Springett *et al.* 1998). In New Zealand, species of the family Lumbricidae (e.g., *L. rubellus* and *Aporrectodea rosea*) were introduced by European settlers centuries ago and are now the most abundant earthworm family in anthropogenically treated areas like pastures and agricultural soils. In return, native species as *O. multiporus* were ousted into undisturbed areas such as forest soils (Lee 1985, Springett 1992, Springett *et al.* 1998).

Earthworms inhabit predominantly soils and the overlying litter layer, and most species form burrows (Lee 1985, Edwards & Bohlen 1996). Worldwide, earthworms are a major part

of the macrofauna in soil accounting for up to 90 % of the biomass of invertebrates there (Lee 1985). Apart from extremely cold, dry, and acidic areas, earthworms inhabit virtually all regions on earth (Lee 1985, Edwards & Bohlen 1996). For pasture and grassland soils, up to 2,000 earthworm individuals are reported per square meter, whereas numbers for deciduous forest soils, agricultural soils, and garden soils are lower with up to approximately 150, 300, and 500 individuals per square meter (Barley 1961, Lee 1985, Makeschin 1997). Due to the low pH and because needles of conifers are an unfavored diet for earthworms, these species mostly lack in coniferous forests (Hutha 1979, Hartmann *et al.* 1989).

According to their feeding and living habits, earthworms can be classified into ecological categories, hereafter referred to as 'feeding guilds', named epigeic, endogeic, and anecic (Bouché 1977, Barois *et al.* 1999). These three feeding guilds can be further described resulting in mixed expressions as epi-anecic and endo-anecic (Barois *et al.* 1999). Epigeic earthworms inhabit the litter and surface soil and feed on leaf litter. They do not form permanent burrows and therefore only ingest minor amounts of soil. Classical epigeic species are *L. rubellus* and *E. eugeniae* whereas *A. gracilis* is defined as epi-endogeic (Edwards & Bohlen 1996, Barois *et al.* 1999, James & Guimarães 2010, Brown GG *pers. comm*.). Endogeic earthworms live predominantly in the upper part of the mineral soil or in the rhizosphere where they form horizontal permanent burrows. They feed on minerals soil that is partly enriched with organic carbon. Representative endogeic species are *A. caliginosa*, *O. multiporus*, *P. corethrurus*, and *R. alatus* (Edwards & Bohlen 1996, Springett *et al.* 1998, Barois *et al.* 1999, James & Guimarães 2010). Anecic earthworms live in predominantly vertical permanent burrows of the mineral soil that can reach several meters of depth (Lee 1985, Edwards & Bohlen 1996). They feed on litter and soil resulting in a medium amount of ingested mineral soil. A classical anecic species is *L. terrestris* whereas *G. paulistus* is categorized as endo-anecic (Edwards & Bohlen 1996, Barois *et al.* 1999, James & Guimarães 2010, Brown GG *pers. comm*.). As epigeic earthworms are more often exposed to light than those of other feeding guilds, in common, most epigeic species are highly pigmented whereas this feature is rare in anecic species and mostly lacks for endogeic species (Bouché 1977, Edwards & Bohlen 1996, Barois *et al.* 1999).

1.4.2. Ecological relevance of earthworms

The outstanding beneficial influences of earthworms on soil fertility were already recognized by Charles Darwin in the 19th century who spent the last productive year of his life on the scientific investigation of earthworms (Darwin 1881). This influence is mainly attributed to the feeding and burrowing activities of earthworms (1.4.1). The part of the soil that is influenced by earthworms is called drilosphere (Brown *et al.* 2000, Brown & Doube

2004). Earthworms ingest high amounts of soil and organic material and therefore significantly contribute to the decomposition of organic matter in soils (Lee 1985, Judas 1992, Edwards & Bohlen 1996, Brown *et al.* 2000, Brown & Doube 2004, Curry & Schmidt 2007). In the earthworm alimentary canal, mucus is produced and mixed with the ingested material. Compared to the surrounding soil, the excreted material (i.e., earthworm casts) is enriched with soluble organic carbon, nitrate, ammonium, phosphorus, potassium, calcium, and magnesium, and often also display a higher water content (Scheu 1987, Tiwari *et al.* 1989, Brown *et al.* 2000, Brown & Doube 2004, Bityutskii *et al.* 2012). In earthworm casts, organic carbon is highly stabilized. These stabilized nutrients are now longer and better available for plants and heterotrophic organisms and are therefore enhancing and conserving soil fertility (Barley 1961, Edwards & Bohlen 1996, Brown *et al.* 2000, Brown *et al.* 2004). In this respect, epigeic earthworms as *E. eugeniae* (Eudrilidae), *Eisenia fetida* (Lumbricidae), and *P. excavatus* (Megascolecidae) display a high casting activity and are therefore used economically, i.e., for vermicomposting. Vermicomposting comprises the conversion of biodegradable matter by earthworms into nutrient enriched casts that are used as fertilizers afterwards (Gajalakshmi & Abbasi 2004). As its casts are often attached to the burrow walls, the earthworm also acts as vector for nutrients and makes them available in deeper soil layers that are normally nutrient-poor (Mansell *et al.* 1981, Edwards & Bohlen 1996, Brown *et al.* 2000, Brown *et al.* 2004). Most studies with crop and grass species under temperate and tropic conditions showed an increase in plant growth averaging about 50 % when earthworms were present (Brown *et al*. 1999, Scheu 2003, Eisenhauer & Scheu 2008, Laossi *et al.* 2009). In addition, the earthworm burrows are macro-pores in the soil that lead to an aeration and to a retention and therefore longer availability of water for plants (Lee 1985, Brown *et al.* 2000). Plant roots can also proliferate into earthworm castings and burrows (Darwin 1881, Wang *et al.* 1986, Logsdon & Linden 1992, Brown *et al.* 2000). In addition, earthworms are vectors or important promoters for the distribution and germination of plant seeds (Brown *et al*. 2004, Aira *et al*. 2009). Ingested seeds are often not digested but excreted in the casts leading to either an enhanced or a reduced ability of germination (Piearce *et al*. 1994, Brown *et al*. 2004, Eisenhauer *et al*. 2009). Earthworm tissue and casts contain substances, e.g., auxines and cytokines that can promote or at least influence the growth of plants (Graff & Makeschin 1980, Brown *et al.* 2004). These summarized beneficial influences on the fertility of soils and soil-dependent organisms generated the term 'ecosystem engineers' for earthworms (Jones *et al.* 1994, Lavelle *et al.* 1997, Jouquet *et al.* 2006).

1.4.3. Digestive system of earthworms and microorganisms as part of their nutrition

The digestive system of earthworms is most properly analyzed for species of the family Lumbricidae, e.g., *L. terrestris* and *E. fetida*, but seems to be transferable to other earthworm families (Edwards & Bohlen 1996, Breidenbach 2002). The alimentary canal consists of the mouth, pharynx, esophagus, crop, gizzard, gut (separted into foregut, midgut, and hindgut), and anus (Storch & Welsch 1999, Tillinghast *et al*. 2001, Breidenbach 2002 Westheide & Rieger 2007) (Figure 5). Mucus is secreted to the ingested material in the anterior part of the digestive system, i.e., especially in the pharynx and foregut region (Breidenbach 2002). This mucus represents a high energetic cost for earthworms and consists of sugars with a low molecular weight and with glyco-proteins (Martin *et al*. 1987, Trigo *et al*. 1999, Brown *et al.* 2000). Added calcium carbonate is most likely for the regulation of pH in the digestive tract (Edwards & Bohlen 1996, Breidenbach 2002). After the physical disruption of the ingested material by grinding effects of the thick and chitin containing wall of the gizzard, it gets further transferred into the gut via peristaltic contractions. Decomposition of organic matter is mainly conducted by enzymes (e.g., chitinases, cellulases, lipases, and proteases) supposably secreted by both the earthworm and ingested microbes (Lattaud *et al*. 1997, Brown *et al.* 2000, Prabha *et al.* 2007, Nozaki *et al*. 2009). The composition of enzymes is adapted to the feeding guild of the earthworm (Lattaud *et al*. 1998, Curry & Schmidt 2007). Absorption of nutrients is mainly conducted via the dorsally invaginated and thereby highly enlarged typhlosolis at the midgut region whereas undigested material is enveloped by the peritrophic membrane and finally excreted as casts (Edwards & Bohlen 1996, Breidenbach 2002). Gut passage time of the temperate *A. caliginosa* and *L. terrestris* is about less than 20 h (Parlé 1963, Wüst *et al.* 2009a) whereas that of tropic worms as *E. eugeniae* and *P. corethrurus* is in the range of 2 to 6 h (Mba 1982, Mba 1989). The earthworm alimentary canal lacks oxygen right from the beginning, i.e., the crop/gizzard region and is therefore called an anoxic microzone in aerated soils (Horn *et al*. 2003, Horn *et al*. 2006b, Drake & Horn 2007, Wüst *et al*. 2009a). Next to anoxia, the earthworm gut displays a low redox potential and displays a high water content, contains up to 4 mM nitrate and nitrite, ammonium, and a huge amount of organic carbon that is derived from ingested material, mucus, and their degradation products (Barois & Lavelle 1986, Daniel & Anderson 1992, Lattaud *et al*. 1997, Trigo & Lavelle 1999, Horn *et al*. 2003, Horn *et al*. 2006b, Drake & Horn 2007, Wüst *et al*. 2009a, Schmidt *et al.* 2011) (see 1.4.4). Earthworms also possess nephridia, more precisely metanephridia for the disposal of ions and other metabolic waste. Nephridia, of which there is often one per segment filter the coelom fluid and empty either into the gut lumen (i.e., enteronephry) or to the body surface (Edwards & Bohlen 1996, Storch & Welsch 1999, Breidenbach 2002, Brown & Doube 2004).

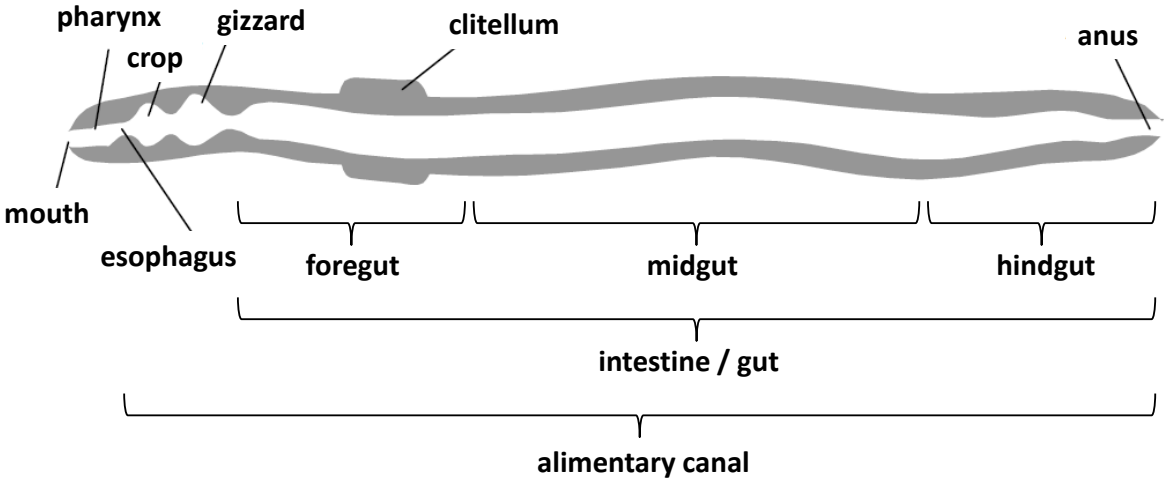


Figure 5: Diagram of the digestive system of an earthworm using the example of *L. terrestris*. The diagram displays a cross section from the anterior (left) to the posterior (right) end of the digestive system of the model organism *L. terrestris*. Modified from Horn *et al.* (2003) and Drake & Horn (2007).

Dependent of the feeding guild of an earthworm, its diet mainly consists of organic material in various stages of decay and different amonts of mineral soil (Lee 1985, Barois *et al.* 1999, Brown & Doube 2004, Curry & Schmidt 2007). Earthworms that ingest high amounts of soil, e.g., endogenous species often prefer mineral soil that is enriched with organic materials (Lee 1985, Barois *et al.* 1999, Brown & Doube 2004). Along with plant-derived organic material and soil, earthworms also ingest prokaryotes, fungi, algae, and protozoa (Brown & Doube 2004, Curry & Schmidt 2007). Large representatives of these microorganisms are disrupted during the grinding in the gizzard (Piearce & Philips 1980, Reddell & Spain 1991, Schönholzer *et al*. 1999, Wolter & Scheu 1999, Brown *et al*. 2000). Also substances secreted by the earthworm into the lumen of the digestive system can inhibit, kill or digest microorganisms, sometimes specifically distinct taxonomical groups (Khomyakov *et al*. 2007, Oleynik & Byzov 2008). It is also speculated that *Actinobacteria* that occur in high numbers in the earthworm gut (Furlong *et al*. 2002, Singleton *et al.* 2003, Knapp *et al.* 2009, Nechitaylo *et al.* 2010) produce antibiotics that influence the activity of other microbes (Ravasz *et al.* 1986, Krištůfek *et al.* 1993, Brown 1995, Masignani *et al.* 2006). Digested microorganisms can be a significant part of or even be essential for the earthworm´s nutrition (Miles 1963, Edwards & Fletcher 1988, Bonkowski & Schaefer 1997, Brown & Doube 2004). Thus, earthworms often preferentially feed on material rich in microorganisms as the plant rhizosphere (Cooke & Luxton 1980, Cooke 1983, Spain *et al*. 1990, Moody *et al*. 1995, Wolter & Scheu 1999, Brown & Doube 2004). However, the amount of microorganisms as part of in the earthworm´s diet is highly variable (Wolter & Scheu 1999, Brown & Doube 2004).

1.4.4. Microorganisms associated with the earthworm digestive system

Other than large microorganisms, fungal spores and the majority of *Bacteria* are too small to get disrupted and therefore often reach the gut lumen unharmed (Brown & Doube 2004). *Bacteria* from all major taxa were isolated from earthworm gut contents, e.g., *Actinobacteriales*, *Firmicutes*, *Cytophaga*/*Flavobacter*, *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobateria* (Citernesi *et al.* 1977, Ravasz *et al.* 1986, Krištůfek *et al.* 1990, Krištůfek *et al.* 1993, Toyota & Kimura 2000, Ihssen *et al.* 2003, Horn *et al*. 2005, Drake & Horn 2007, Brito-Vega & Espinosa-Victoria 2009, Byzov *et al.* 2009, Thakuria *et al*. 2010). Most of those microorganisms are classical soil microorganisms. Thus, there is no significant endogenous microbiota assumed to exist in the earthworm gut as detected by means of isolation and molecular techniques (Edwards & Bohlen 1996, Furlong *et al.* 2002, Horn *et al.* 2003, Singleton *et al.* 2003, Egert *et al.* 2004, Horn *et al.* 2006a, Wüst *et al.* 2009b). However, some filamentous *Bacteria* were detected attached to the gut wall of earthworms but seem to be also primarily derived from ingested material and of minor importance in respect of their abundance (Jolly *et al.* 1993, Thakuria *et al.* 2010). Outside the earthworm gut, i.e., in the coelom fluid of species of the family Lumbricidae uncultured *Bacteria* of the *Mollicutes* with an unknown relationship to its host were detected (Nechitaylo *et al.* 2009). In nephridia, i.e., in the excretion organs of earthworms species of several families, *Bacteria* are present, e.g., of the genera *Verminephrobacter* (*Betaproteobacteria*) and *Flexibacter* (*Bacteriodetes*) (Maziarski 1903, Schramm *et al*. 2003, Davidson & Stahl 2006, Davidson & Stahl 2008, Pinel *et al*. 2008, Davidson *et al*. 2010, Davidson *et al*. 2013). It is assumed that these *Bacteria* are beneficial for the earthworms and they are vertically transmitted via earthworm eggs (Davidson & Stahl 2006, Davidson & Stahl 2008, Davidson *et al*. 2010, Davidson *et al*. 2013).

In contrast to the earthworm gut, invertebrates as insects can harbor endogenous and highly adapted microorganisms in their gut or in specialized compartments, the bacteriocytes (Baumann *et al.* 2006). For instance, termite and cockroach guts are inherited by flagellates, *Bacteria*, and *Archaea* that assist in the breakdown of cellulose, produce acetate that is absorbed by the termite, and produce CH_4 (Schultz & Breznak 1978, Brune 2006, Köhler *et al*. 2012, Schauer *et al*. 2012). Symbiotic *Bacteria* and *Archaea* are estimated to inherit about 20 % of insect species. These symbionts can produce amino acids that are essential for the host anabolism or repellants against host predators whereas for most symbioses, the interaction between host and symbiont is still largely unresolved (Kellner 2002, Baumann *et al.* 2006, Weinert *et al*. 2007, Feldhaar & Gross 2009). However, the majority of microorganisms in the earthworm alimentary canal seems to be soil-derived and only transient.

Those microorganisms that survived the physical, chemical, and biological treatments of the digestive system of the earthworm alimentary canal, come upon conditions that can be highly beneficial for them, e.g., anoxia, a low redox potential, a high water content, nitrate, nitrite, ammonium, and a huge amount of organic carbon derived from the ingested material and the mucus produced by the earthworm (1.4.3). These conditions can highly promote the metabolism of *Bacteria* and lead to an up to 1,000-fold higher number of cultivable *Bacteria* in the earthworm gut compared to the surrounding soil. Such activated microorganisms are anaerobes in common, denitrifiers, dissimilatory nitrate reducers, nitrogen fixing *Bacteria*, but also aerobes in common, and nitrifiers (Parlé 1963, Ravasz *et al.* 1986, Krištůfek *et al.* 1990, Karsten & Drake 1995, Karsten & Drake 1997, Ihssen *et al.* 2003, Drake & Horn 2007, Byzov *et al.* 2009, Thakuria *et al*. 2010). In spite of the dramatically enhanced cultivable numbers of microorganisms, the numbers of living and total cells in the gut are only marginally higher, if at all (Krištůfek *et al.* 1992, Krištůfek *et al.* 1995, Schönholzer *et al.* 1999, Wolter & Scheu 1999, Schönholzer *et al.* 2002, Drake & Horn 2007). Thus, most studies indicate that microorganisms get highly activated by the gut transit instead of showing significant growth (Barois & Lavelle 1986, Daniel & Anderson 1992, Lavelle *et al.* 1995, Horn *et al.* 2003, Ihssen *et al.* 2003, Drake & Horn 2007). This activation of ingested, relatively inactive microorganisms in the earthworm alimentary canal is called the 'priming effect' and is highly beneficial for the microorganisms; it might be even essential for some microorganisms in respect of their live cycle (Brown *et al.* 2000, Bernard *et al*. 2012).

The most important carbon source for these gut microorganisms is the earthworm mucus. Its actual functions seem to be to mix and transport the rough ingested material through the gut without any injury of the earthworm gut tissue and/or to act as stimulatory substrate for the ingested microorganisms (Edwards & Bohlen 1996, Drake & Horn 2007). Microorganisms can utilize this easily available organic carbon leading to an activation of cell metabolism. These activated microorganisms can then assist to break down the ingested organic compounds in the gut content, mainly with anaerobic processes and also by the production of exoenzymes (Trigo *et al*. 1999, Brown *et al.* 2000, Curry & Schmidt 2007, Prabha *et al.* 2007, Nozaki *et al*. 2009). Indeed, in the anterior part of the digestive system, more than 100 mM of sugar equivalents can be detected that form the mucus polymer, e.g., maltose, galactose, fucose, glucose, and rhamnose, whereas at the posterior part, this concentration is below 10 mM (Wüst *et al.* 2009a) (Figure 6). In return to this decline of mucus, the concentration of typical fermentation products rises from the crop/gizzard to the midgut where about 30 mM of summarized organic acids are detectable, e.g., acetate, formate, butyrate, succinate, and lactate, concomitant with the presence of H_2 (Wüst *et al.* 2009a). The concentration of organic acids declines towards the hindgut where approximately 17 mM are present. Different types of fermentations appear from the anterior

to the posterior part of the alimentary canal, with *Clostridiaceae* and *Enterobacteriaceae* as the main fermenters (Wüst *et al.* 2009a, Schmidt *et al.* 2011, Wüst *et al.* 2011) (Figure 6). It is not completely resolved whether the earthworm assimilates its mucus again or only the degradation products. However, this symbiosis between the earthworm and its ingested microorganisms is called the 'mutualistic digestive system' of earthworms (Barois & Lavelle 1986, Lavelle *et al.* 1995, Trigo *et al.* 1999, Brown *et al.* 2000).

The amount of produced mucus is highest for endogeic earthworms and least for epigeic earthworms with anecic species displaying an intermediate production of mucus (Edwards & Bohlen 1996, Trigo *et al.* 1999, Brown *et al.* 2000). Endogeic species ingest huge amounts of mineral soil often poor in nutrients. Thus, it is supposed that species of this feeding guild have to provide more mucus than epigeic species as they have to prime ingested microorganisms to assist the earthworm with digestion (Trigo *et al.* 1999, Brown *et al.* 2000).

1.4.5. Emission of N_2O and CH_4 by earthworms

Earthworm species of the family Lumbricidae emit N_2O and N_2 *in vivo*, e.g., *L. terrestris*, *L. rubellus*, *A. caliginosa*, and *O. lacteum*. These nitrogenous gases were demonstrated to be not produced by the earthworm itself but by substrate-derived denitrifying *Bacteria* in the earthworm gut with the synergistic assistance of nitrate and nitrite dissimilating microorganisms (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Ihssen *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a) (Figure 6). The concentration of N_2O is highest in the core of the gut and the production of N_2O is distributed along the whole alimentary canal albeit with different detectable concentrations (Horn *et al.* 2003, Wüst *et al.* 2009a). In addition, the ratio between emitted N_2 and N_2O differs along the alimentary canal indicating the ratio between complete and incomplete denitrification also differs (Wüst *et al.* 2009a). Ammonium seems to be of minor importance in respect of the fate of nitrate in the earthworm gut (Ihssen *et al.* 2003). Next to the *in vivo* emission of N_2O from the earthworm, this invertebrate has also a significant effect on the emission of N_2O from soils by its castings and its behavior, i.e., by the mixing of organic material into the soil and by enhancing the release of gases produced in lower soil layers into the atmosphere by the earthworm burrows (Borken *et al.* 2000, Bertora *et al.* 2007, Rizhiya *et al.* 2007, Giannopoulos *et al.* 2010, Nebert *et al.* 2011, Augustenborg *et al.* 2012, Lubbers *et al.* 2013, Majeed *et al.* 2013). Summarized, the direct and indirect effects of earthworms, i.e., emission of N_2O by the earthworm as a whole and enhanced emissions of N_2O by its ecological strategy, respectively, may result in approximately 40 % of net emission of N_2O by soils inhabited by earthworms (Drake & Horn 2007, Lubbers *et al.* 2013). However, the *in vivo* emission of nitrogenous gases was demonstrated for species of the family Lumbricidae only.

The up to approximately 30 cm long *O. multiporus* (Megascolecidae) is the so far exclusive earthworm species not belonging to the family Lumbricidae that was analyzed for its potential to emit nitrogenous gases *in vivo*. This species displayed no emissions of N_2O. However, its dissected guts emitted N_2O under anoxic conditions when nitrite was added (Wüst *et al.* 2009b). Knowledge about other earthworm families completely lacks, as same as the influence of the earthworm family, feeding guild, and earthworm size on the emission of nitrogenous gases.

Methanogenesis needs anoxia and typical substrates as H_2/CO_2 or acetate to occur (1.3); all those conditions are prevailing in the earthworm gut (1.4.4). Thus, methanogenesis could occur in the earthworm gut resulting in the emission of CH_4. However, studies failed to demonstrate the *in vivo* emission of CH_4 by earthworms (Hornor & Mitchell 1981, Karsten & Drake 1997, Borken *et al.* 2000, Drake & Horn 2007, Šustr & Šimek 2009). Studies with the epigeic *Eisenia andrei* indicated that earthworms might alter the CH_4 production and oxidation rates, albeit the net CH_4 flux was unaffected by the presence of these earthworms in cattle-impacted soils (Bradley *et al.* 2012, Koubova *et al.* 2012). As all studies were restricted to species of the family Lumbricidae, knowledge about the emission of CH_4 by earthworms is highly limited and needs to be expanded to other earthworm families.

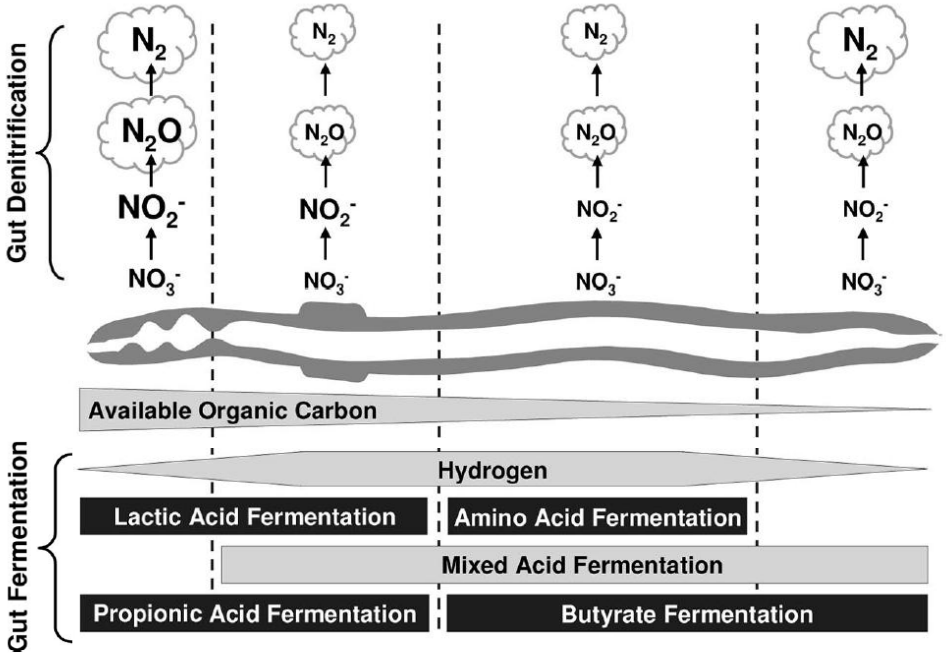


Figure 6: Hypothetical model of denitrification and fermentations along the alimentary canal of *L. terrestris* considering the availability of organic carbon and other *in situ* conditions.

The font size correlates with relative concentrations of nitrate, nitrite, N_2O, and N_2. The tapering off of shaded elements indicates that the item identified decreases in amount in the direction of the taper. Gases in indicated clouds represent the *in vivo* emission by the part of the worm, i.e., crop/gizzard, foregut, midgut, and hindgut from the anterior (left end) to the posterior part (right end) of the earthworm. Modified from Wüst *et al.* (2009b).

1.5. Hypotheses and Objectives

This dissertation examines the microbial processes of denitrification, dissimilatory nitrate reduction, and methanogenesis in the earthworm gut. It is known that ingested denitrifiers in the gut of small earthworms (family Lumbricidae) are responsible for the emission of N_2O, and that a large earthworm of the family Megascolecidae does not emit N_2O. It is thus far unknown if the earthworm family, size, feeding guild, and denitrifiers in the earthworm gut are factors that influence the emission of N_2O by earthworms. Of all earthworm species tested to date, studies have failed to demonstrate the emission of CH_4. Thus, the present study addressed the following hypotheses and objectives (indicated by arrows):

1. Earthworms emit denitrification-derived N_2O independent of their family, size, and feeding guild via ingested denitrifiers.
   - → Assessment of the emission of N_2O and N_2 by earthworms of different families, sizes, and feeding guilds.
   - → Comparative analysis of denitrifiers and dissimilatory nitrate reducers in the gut and corresponding soil of earthworm species displaying contrasting N_2O emission features.

2. The earthworm feeding guild affects the diversity and activity of ingested denitrifiers and dissimilatory nitrate reducers in the earthworm gut.
   - → Comparative analysis of denitrifiers and dissimilatory nitrate reducers on gene and partly on transcript level in the gut and corresponding soil of earthworm species representing contrasting feeding guilds.
   - → Isolation of denitrifiers from the gut of earthworm species representing contrasting feeding guilds.

3. Earthworms emit CH_4 via selectively activated, ingested methanogens.
   - → Assessment of the emission of CH_4 by different earthworm species.
   - → Comparative analysis of methanogens on gene and transcript level in the earthworm gut and substrate of a CH_4-emitting species.
   - → Enrichment of methanogens from the earthworm gut of a CH_4-emitting species.

2. MATERIALS AND METHODS

The current study aimed to analyze microbial dissimilatory nitrate reducing processes and methanogenesis in the gut of earthworms and their corresponding soil or other substrates upon which earthworms were maintained (e.g., compost). The terms 'soil' and 'substrate' are hereafter used interchangeably to describe material that was sampled along with earthworm species. For this purpose, a broad spectrum of methods was applied to gain a proper understanding of these processes, especially in the earthworm gut. Such methods included gas measurements of *in situ* material or treated with compounds enhancing predominantly denitrification and methanogenesis. Microorganisms conducting denitrification, dissimilatory nitrate reduction and methanogenesis were assessed with means of isolation or by analyzing genes indicative of these processes via cloning and pyrosequencing. Analyses were conducted with living earthworms, earthworm gut material, and soils/substrates from Brazil, Germany, and New Zealand.

2.1. Location and sampling of earthworms and their soils and other substrates

2.1.1. Brazil

2.1.1.1. Earthworms

Earthworms and soil samples were obtained in Brazil, Germany, and New Zealand (Table 1). In Brazil, specimens of ten earthworm species were sampled (Table 1, Figure 7). *Pontoscolex corethrurus* (Glossoscolecidae, native to Brazil, endogeic) was dug out from a 5 to 30 cm depth, and *Amynthas gracilis* (Megascolecidae, not native to Brazil, epi-endogeic) from the organic layer and upper 5 cm depth of a grassland soil (Substrate 4; Table 1, Figure 7) within the Esalq campus in Piracicaba. *Glossoscolex paulistus* (Glossoscolecidae, native to Brazil, endo-anecic) was sampled from a pasture soil near Assistência (Substrate 5; Table 1) with a flexible, thin steel cable with a thickened plastic tip and a crank handle at the end that was inserted into the surface burrow hole ot the earthworm. By pushing the cable further into the burrow and simultaneous winding, the earthworm was forced to escape through the other end of the U-turn-like burrow hole and was collected there. *Glossoscolex* sp. (Glossoscolecidae, native to Brazil, endogeic) was dug out from a swampy meadow nearby (Table 1, Figure 7). In March 2011, *Rhinodrilus alatus* (Glossoscolecidae, native to Brazil, endogeic) specimens were obtained by a private earthworm distributor in Assictência and previously sampled near Paraopeba whereas in September 2011, specimens were obtained by a private earthworm distributor near Boituya and were in the state of diapause, i.e., they

were inactive and their alimentary canal was empty (Table 1, Figure 7). *Eudrilus eugeniae* (Eudrilidae, not native to Brazil, epigeic), *Perionyx excavatus* (Megascolecidae, epigeic), *Dichogaster annae* (Acanthodrilidae, not native to Brazil, epigeic), and *Dichogaster* sp. (Acanthodrilidae, epigeic) were obtained from the Brazilian earthworm distributor Minhobox (Jiuz de Fora, Minas Gerais, Brazil) together with separate samples of Substrate 1 (Table 1, Figure 7). Substrate 1 consisted of commercially composted cow manure, i.e., cow manure that was periodically wetted and daily turned under aerated conditions for several weeks prior to introducing the earthworms to it. By this process, urine and urea were removed resulting in an odorless earthworm substrate that had the appearance of a rich soil. *E. eugeniae* specimens were additionally obtained by a private earthworm distributor near Boituya together with separate samples of Substrate 2 (Table 1, Figure 7). Substrate 2 consisted of commercially processed and composted sugar cane residues that had been stored for several weeks and wetted for several days before earthworms were introduced to it. Specimens of *E. eugeniae* and *Eisenia andrei* (Lumbricidae, not native to Brazil, epigeic) were obtained from a local earthworm distributor in Vinhedo together with separate samples of Substrate 3 (Table 1, Figure 7). Substrate 3 consisted of commercially processed sugar cane and was pre-processed the same way as Substrate 2 (see above). All soils/substrates and all earthworms on their natural soil/substrate were stored in the dark at approximately 15 °C before use. For some soils/substrates, general properties were determined by the Soil Analysis Laboratory of the University of São Paulo (http://www.solos.esalq.usp.br/, last visit 22.06.2013) from 500 g material (2.4.2) and are displayed later on (Table 15, Table 27). Earthworm species were identified with standard protocols (Righi 1990). All bracketed features of earthworm species mentioned in this paragraph are according to (Barois *et al.* 1999, James & Guimarães 2010, Brown GG *pers. comm*.).

2.1.1.2. Millipedes

Millipedes (Diplopoda) of the species *Gymnostreptus olivaceus* (approximately 1 g and 5 cm) of the family Spirostreptidae (identified by Fontanetti CS *pers. comm.*) were detected in the litter layer during the sampling of grassland soil (Substrate 4; Table 1) in September 2011.

Table 1: Earthworms sampled in Brazil (BRA), Germany (GER), and New Zealand (NZL), and their corresponding soil/substrate.

	Date of sampling	Species (family)	Location or distributor	Substrate/soil
BRA	11/2010, 03/2011	*Glossoscolex paulistus* (Glossoscolecidae)	Assistência, São Paulo (22°30´47´´S, 47°36´55´´W)	Pasture soil (Substrate 5)
	03/2011	*Glossoscolex* sp. (Glossoscolecidae)	Assistência, São Paulo (22°30´36´´S, 47°36´41´´W)	Meadow soil (Substrate 6)
	11/2010, 03/2011	*Pontoscolex corethrurus* (Glossoscolecidae)	Esalq campus, Piracicaba (22°42´22´´S, 47°38´02´´W)	Grassland soil (Substrate 4)
	11/2010, 03/2011	*Amynthas gracilis* (Megascolecidae)	Esalq campus, Piracicaba (22°42´22´´S, 47°38´02´´W)	Grassland soil (Substrate 4)
	03/2011	*Dichogaster annae* (Acanthodrilidae)	Earthworm distributor Minhobox	Comp. manu.[a] (Substrate 1)
	03/2011	*Dichogaster* sp. (Acanthodrilidae)	Earthworm distributor Minhobox	Comp. manu. (Substrate 1)
	03/2011, 09/2011	*Eudrilus eugeniae* (Eudrilidae)	Earthworm distributor Minhobox	Comp. manu. (Substrate 1)
	09/2011	*Eudrilus eugeniae* (Eudrilidae)	Local earthworm distributor, Boituya, São Paulo	Comp. sug.[b] (Substrate 2)
	09/2011	*Eudrilus eugeniae* (Eudrilidae)	Local earthworm distributor, Vinhedo, São Paulo	Comp. sug. (Substrate 3)
	03/2011, 09/2011	*Perionyx excavatus* (Megascolecidae)	Earthworm distributor Minhobox	Comp. manu. (Substrate 1)
	03/2011	*Rhinodrilus alatus* (Glossoscolecidae)	Local earthworm distributor, Paraopeba, Minas Gerais	Unknown soil (Substrate 7)
	09/2011	*Rhinodrilus alatus* (Glossoscolecidae)	Local earthworm distributor, Boituya, São Paulo	Comp. sug. (Substrate 2)
	09/2011	*Eisenia andrei* (Lumbricidae)	Local earthworm distributor, Vinhedo, São Paulo	Comp. sug. (Substrate 3)
GER	05/2007, 08/2008, 11/2008	*Lumbricus rubellus* (Lumbricidae)	Trafo Wiese, Bayreuth (49°55´41´´N, 11°31´50´´E)	Mineral soil, uppermost soil
	05/2007, 08/2008, 11/2008	*Lumbricus terrestris* (Lumbricidae)	Trafo Wiese, Bayreuth (49°55´41´´N, 11°31´50´´E)	Mineral soil, uppermost soil
	05/2007, 08/2008, 11/2008	*Aporrectodea caliginosa* (Lumbricidae)	Trafo Wiese, Bayreuth (49°55´41´´N, 11°31´50´´E)	Mineral soil, uppermost soil
	08/2008	*Octolasium lacteum* (Lumbricidae)	Trafo Wiese, Bayreuth (49°55´41´´N, 11°31´50´´E)	n.a.[c]
NZL	09/2008	*Octochaetus multiporus* (Megascolecidae)	Palmerston North (40°22´57´´S, 175°37´07´´E)	Forest soil

[a] composted cow manure (2.1.1.1).

[b] composted sugar cane residues (2.1.1.1).

[c] not applicable; only earthworms were sampled for isolation of denitrifiers (2.3.2.1).

See Table 3 for analyses conducted with the earthworms and soils/substrates.

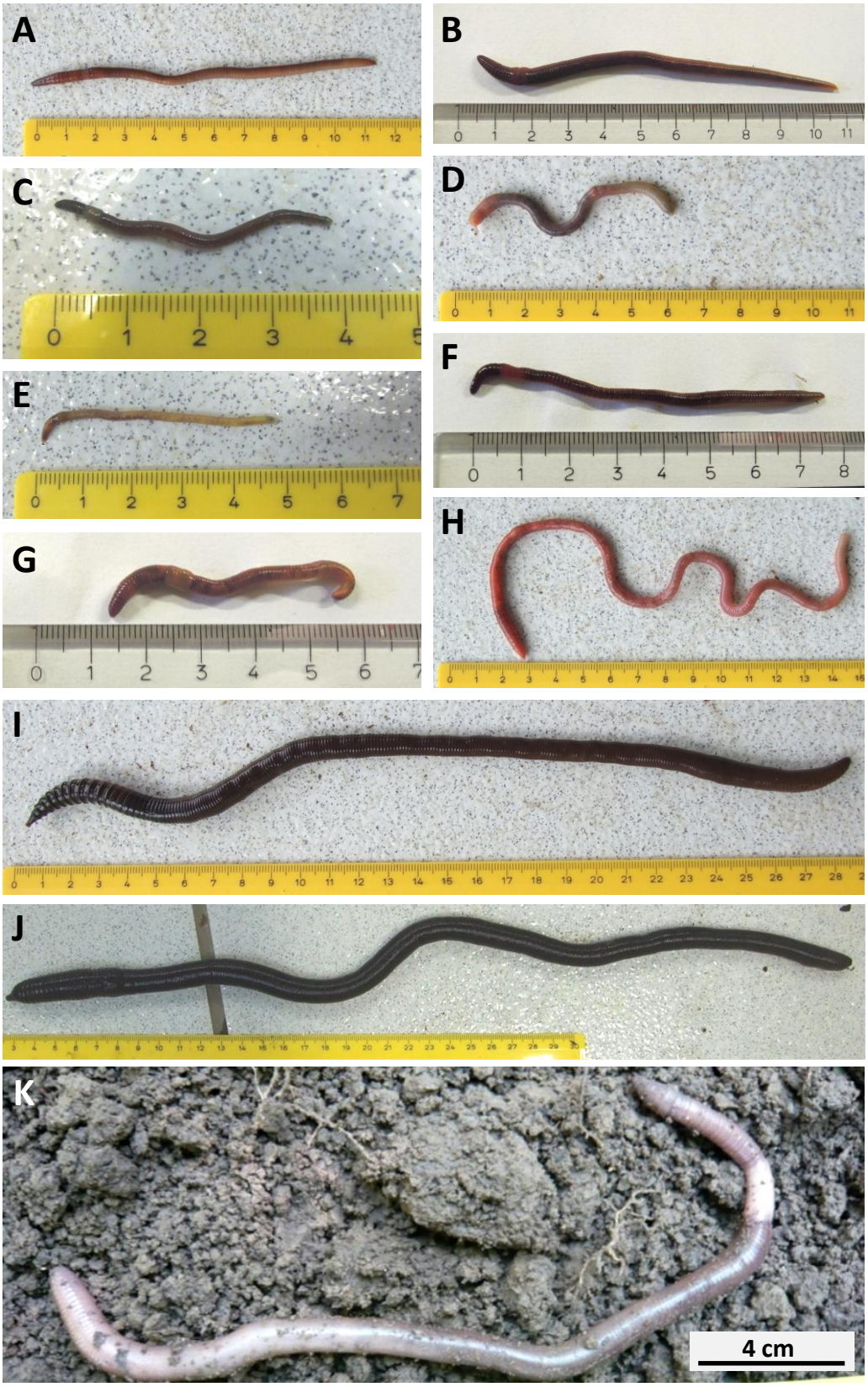


Figure 7: Analyzed earthworm species from Brazil (A to J) and New Zealand (K).

A, *Amynthas gracilis*; B, *Eudrilus eugeniae*; C, *Dichogaster annae*; D, *Pontoscolex corethrurus*; E, *Dichogaster* sp.; F, *Perionyx excavatus*; G, *Eisenia andrei*; H, *Glossoscolex* sp.; I, *Glossoscolex paulistus*; J, *Rhinodrilus alatus*; K, *Octochaetus multiporus* (picture K was modified from Wüst [2010]). The rulers are approximately 31 cm long and divided into cm.

2.1.2. Germany

For sampling in Germany, specimens of four earthworm species were dug out at the meadow Trafo Wiese near Bayreuth (Table 1). *Lumbricus rubellus* (Lumbricidae, epigeic) was sampled from the uppermost soil layer ('uppermost soil') whereas *Aporrectodea caliginosa* (Lumbricidae, endogeic) and *Octolasium lacteum* (Lumbricidae, endogeic) were sampled from a 5 to 20 cm depth, and *Lumbricus terrestris* (Lumbricidae, anecic) from a 10 to 35 cm depth of the mineral soil ('mineral soil') (Table 1). In addition, separate samples from the mineral soil (5 to 20 cm depth) were taken and the humid, air-exposed uppermost soil containing decaying organic material was also sampled. All soils and all earthworms on their natural soil samples were stored in the dark at approximately 15 °C for 1 h before use. Properties of the mineral soil of the Trafo Wiese (Table 2) were determined previously (Horn *et al.* 2003). Earthworm species were identified with standard protocols (Schaefer 2000) but not photographed. All bracketed features of earthworm species are according to (Barois *et al.* 1999).

Additional specimens of *L. terrestris* were purchased from a fishing shop (ANZO, Bayreuth, Germany). These earthworms were exclusively employed for an earthworm extract (2.3.1.1.17) that was used for the enrichment of methanogens from gut contents of *E. eugeniae* (2.3.2.3).

Table 2: Properties of the meadow Trafo Wiese (Germany) and the forest soil (New Zealand)[a].

	Trafo Wiese, Bayreuth, Germany (meadow)	Forest, Palmerston North, New Zealand (forest soil)
Land use type	Meadow	Forest
pH (H_2O)	6.8	6.7
Water content (%)	31.6	33.2
NH_4^+ (µmol l [water content]$^{-1}$)[b]	50.0	10.4
NO_3^- (µmol l [water content]$^{-1}$)[b]	280.0	73.5
NO_2^- (µmol l [water content]$^{-1}$)[b]	40.0	0.6
Total carbon (g (kg dw)$^{-1}$)	41	38
Total organic carbon (g (kg dw)$^{-1}$)	30	37
Total nitrogen (g (kg dw)$^{-1}$)	4	4
Reference	(Horn *et al.* 2003)	(Wüst *et al.* 2009b)

[a] displayed are the mean values of three to five replicates.

[b] based on the determined water content of the soil.

Abbreviations: dw, dry weight.

2.1.3. New Zealand

Octochaetus multiporus (Megascolecidae, native to New Zealand, endogeic; [Lee 1959a, Springett *et al.* 1998, Barois *et al.* 1999]) was dug out in a forest near Palmerston North, New Zealand (Wüst *et al.* 2009b) together with forest soil (Table 1, Figure 7). The forest soil and *O. multiporus* maintained on this soil were stored in the dark at approximately 4 °C before use. Properties of the forest soil (Table 2) were determined previously (Wüst *et al.* 2009b). *O. multiporus* was identified with standard protocols (Lee 1959b).

Table 3: Overview of experiments conducted with earthworms and soils/substrates from Brazil (BRA), Germany (GER), and New Zealand (NZL).

	Analysis	Method	Date of corresponding sampling of earthworms and soil/substrate	Cross reference (results)
BRA	N_2O and N_2 emissions	GC	11/2010, 03/2011, 11/2011	3.1.1.2
	narG, *nirK*, *nirS*, *nosZ*	pyrosequencing (g [a])	11/2010	3.1.1.3
	CH_4 emissions	GC	03/2011, 09/2011	3.2.2
	mcrA/*mrtA*	gene libraries (g + tc [b])	09/2011	3.2.3
	methanogens	enrichment	09/2011	3.2.4
	soil parameters	soil analysis	11/2010, 03/2011	3.1.1.2.3, 3.2.1.2
GER	*narG*	gene libraries (g)	05/2007	3.1.2.1.1.1
	narG	gene libraries (tc [c])	11/2008	3.1.2.1.1.1
	nosZ	gene libraries (g)	08/2008	3.1.2.1.1.2
	nosZ	gene libraries (tc)	11/2008	3.1.2.1.1.2
	narG,	T-RFLP (g + tc)	11/2008	3.1.2.1.1.1.3
	nosZ	T-RFLP (g + tc)	11/2008	3.1.2.1.1.2.3
	nirK, *nirS*	gene libraries (g)	10/2010	3.1.2.1.2
	denitrifiers	isolation	08/2008	3.1.2.2
NZL	*nosZ*	gene libraries (g)	08/2008	3.1.3.1

[a] analysis of gene sequences, i.e., from DNA.

[b] analysis of gene and transcript sequences, i.e., from DNA and complementary DNA (cDNA), respectively.

[c] analysis of transcript sequences, i.e., from cDNA.

Abbreviations: GC, gas chromatography; T-RFLP, terminal restriction fragment length polymorphism; gene libraries, construction of gene sequence libraries via cloning.

2.2. Experiments with living earthworms, their soils and substrates, dissected earthworm guts, and gut contents

All incubation experiments were conducted with material from and in Brazil (Table 3). The emission of nitrogenous gases and CH_4 was analyzed for living earthworms, their dissected guts, and their gut contents. In addition, similar experiments were conducted with selected earthworm soils/substrates. For all experiments, incubation was at room temperature (25 °C) in the dark and was conducted in sterile, gas-tight 120 ml or 500 ml butyl rubber stopped aluminium crimp sealed serum vials (hereafter termed 'serum vials') with a moderate overpressure to be able to take samples with syringes (2.6) that were flushed with 100 % argon before (Wüst *et al.* 2009b). Gas samples were transferred into 3 ml sterilized and pre-vacuumed Exetainer vials (Labco Ltd., High Wycombe, England; (2.4.1) and analyzed in Bayreuth, Germany via gas chromatography (GC) (2.4.1).

2.2.1. Incubation experiments with living earthworms

In Brazil, earthworm species and their substrates were tested for the emission of nitrogenous gases (*A. gracilis*, *D. annae*, *Dichogaster* sp., *E. andrei*, *E. eugeniae*, *G. paulistus*, *Glossoscolex* sp., *P. excavatus*, *P. corethrurus*, *R. alatus*; Substrates 1 to 7) and CH_4 (*A. gracilis*, *E. andrei*, *E. eugeniae*, *G. paulistus*, *Glossoscolex* sp., *P. excavatus*, *P. corethrurus*, *R. alatus;* Substrates 1 to 7) (Table 1, Table 3). After determination of the body length, earthworms were washed in sterilized water, dried with a paper towel, weighted, and transferred into sterile, gas-tight serum vials (Wüst *et al.* 2009b). *In vivo* emission of nitrogenous gases and CH_4 by earthworms (3 to 7 replicates; 1 specimen per replicate; 2 specimens per replicate for *E. andrei*; 10 specimens per replicate for *D. annae* and *Dichogaster* sp.) and soil/substrate (3 replicates each; 10 to 30 g fresh weight [fw] per replicate) were assessed under ambient air.

2.2.1.1. Additional N_2O emission experiments

Acetylene inhibits the N_2O reductase (Yoshinari & Knowles 1976). N_2O that normally gets reduced to N_2 in the last step of denitrification is therefore released as N_2O instead in the presence of acetylene (20 % v/v). Thus, the emission of N_2 by living earthworms and by soil/substrate was calculated as the difference in the amount of emitted N_2O in the presence of acetylene (20 % v/v) compared to the incubation without acetylene. For the analyses of the denitrification potential of an earthworm, *A. gracilis* and *G. paulistus* were wetted with

0.5 ml of a 2 mM sodium nitrite solution and incubated under ambient air with and without acetylene (20 % v/v) (Matthies *et al.* 1999, Wüst *et al.* 2009b). Nitrite is a precursor of denitrification-derived N_2O and utilized predominantly by denitrifiers (1.2.1.2), whereas nitrate is also used by dissimilatory and assimilatory nitrate reducers (1.2.2). As up to 4 mM of nitrite occur in the alimentary canal *of L. terrestris* (Wüst *et al.* 2009b), the concentration of nitrite applied in the experiment was similar to that assumed in the earthworm gut although analyses still lack for the alimentary canal of *G. paulistus* and *A. gracilis*.

2.2.1.2. Additional CH_4 emission experiments

For the emission of CH_4, earthworms and substrates were also incubated with 1.5 % H_2 and 0.4 % CO_2 (H_2/CO_2) to test the effect of additional H_2/CO_2, the substrates for hydrogenotrophic methanogens (1.3). Several earthworm species were also pre-incubated for 60 h on alternative substrates, i.e., on a substrate they were not sampled from or delivered with (Table 1). During this pre-incubation, the earthworm replaced the original gut content by the new substrate. Thus, the effect of the substrate on the emission of CH_4 by living earthworms could be tested. In this respect, *R. alatus* specimens from September 2011 obtained in diapause were pre-incubated on Substrate 1 with and without H_2/CO_2.

2.2.2. Incubation experiments with dissected earthworm guts

Dissected guts as a whole were analyzed for *G. paulistus* and *A. gracilis* and analyzed for the emission of nitrogenous gases only. Earthworms were washed in sterile water, sacrificed by brief exposure to 70 °C water and subsequently cooled down to room temperature (25 °C) again by brief exposure to 20 °C water. Guts were dissected under sterile and oxic conditions (Wüst *et al.* 2009b) (Figure 8). Earthworm guts (one and two guts per replicate for *G. paulistus* and *A. gracilis*, respectively) were transferred into sterile, gas-tight serum vials (Wüst *et al.* 2009b) that were previously and subsequently flushed with 100 % argon to gain anoxic incubation conditions. All incubations were with acetylene (20 % v/v) and with either 0.5 ml of a sterile-filtered (0.2 µm pore diameter) 2 mM sodium nitrite solution or 0.5 ml of sterile-filtered (0.2 µm pore diameter) and autoclaved ddH_2O (PCR-H_2O).

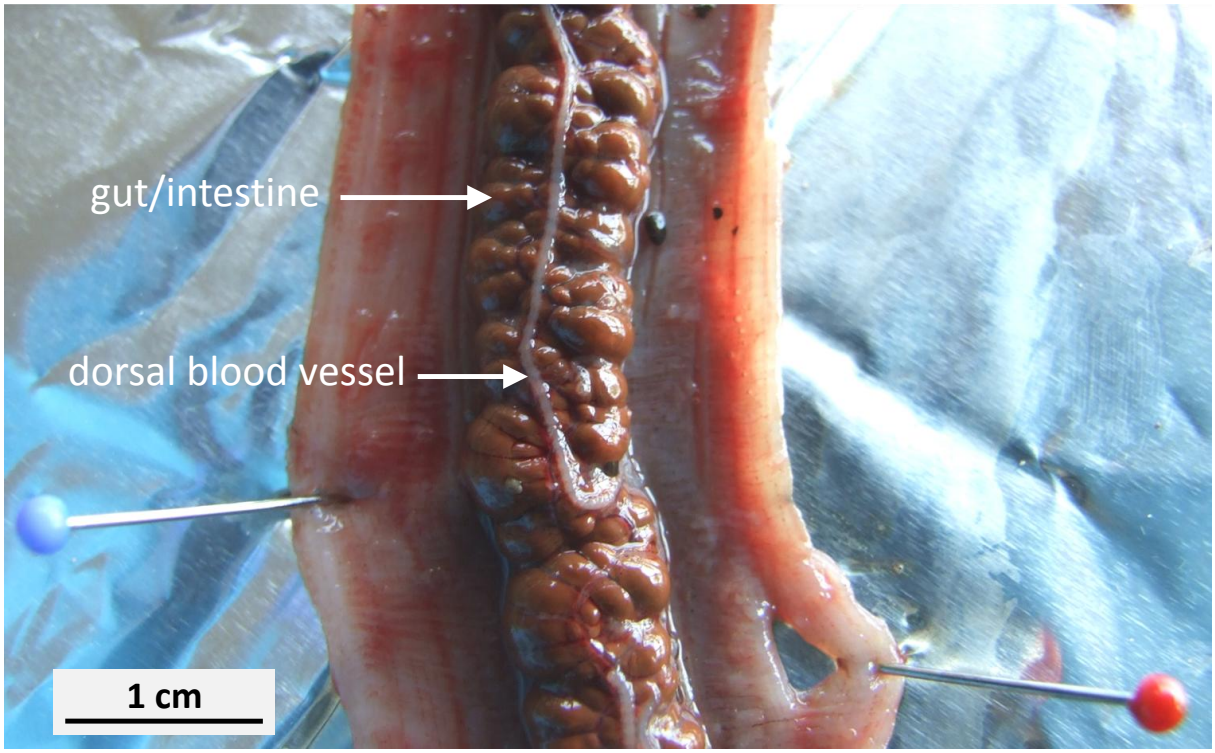


Figure 8: Dissected gut of *G. paulistus*.
Next to the brown gut/intestine, the white and empty dorsal blood vessel is visible.

2.2.3. Incubation experiments with earthworm gut contents

2.2.3.1. Brazil

2.2.3.1.1. Denitrification experiments

For *G. paulistus*, gut contents were prepared out of the dissected gut (2.2.2), homogenized, and 2 g per replicate was transferred into sterile, gas-tight 120 ml serum vials that were previously and subsequently flushed with 100 % argon to gain anoxic incubation conditions. All incubations were with acetylene (20 % v/v), and with either (1) 1.0 ml of PCR-H_2O, (2) 0.5 ml of PCR-H_2O and 0.5 ml of sterile-filtered (0.2 µm pore diameter) 2 mM sodium nitrite, or (3) solution 0.5 ml of sterile-filtered (0.2 µm pore diameter) 2 mM sodium nitrite and 0.5 ml of a sterile-filtered (0.2 µm pore diameter) solution of 4 mM glucose and 2 mM sodium acetate. The gut contents of *A. gracilis* could not be analyzed as there was no adequate number of specimens available.

2.2.3.1.2. Methanogenesis experiments

Specimens of *E. eugeniae* raised and maintained on Substrate 1 were washed in sterile water, sacrificed (2.2.2), and carefully squeezed out under sterile conditions and under a

permanent flow of 100 % argon to keep the gut content as anoxic as possible. 0.35 g per replicate was transferred into sterile, gas-tight 120 ml serum vials that were previously and subsequently flushed with 100 % argon to gain anoxic incubation conditions. Incubation vials were supplemented with either (i) 0.5 ml of sterile, anoxic ddH_2O (2.3.1.1.1), (ii) 0.5 ml of sterile, anoxic ddH_2O with 1.5 % H_2 and 0.4 % CO_2 in the headspace, or (iii) 1.5 % H_2 and 0.4 % CO_2 in the headspace with 0.5 ml of an anoxic, sterile-filtered (0.2 µm pore diameter) solution of BES, a metabolic inhibitor of methanogenesis (Gunsalus *et al.* 1978), yielding a final concentration of 30 mM BES.

2.2.4. Preparation of earthworm gut contents for other experiments

2.2.4.1. Brazil

Gut contents of *G. paulistus* and *A. gracilis* were obtained as described above (2.2.2) and, together with Substrate 5 and Substrate 4 subsequently freeze-dried (DuraDry, FTS Systems, Stone Ridge, NY, USA) for further use in Germany (2.5.1, 2.5.11). Substrate 1 and gut contents of *E. eugeniae* raised and maintained on Substrate 1 (2.2.2) were suspended in RNAlater (Qiagen, Hilden, Germany). This reagent stabilizes the nucleic acids and enables their transport at moderate temperatures (25 °C) for several days without significant degradation. In Bayreuth, dissolved Substrate 1 and gut contents of *E. eugeniae* were washed three times with RNase-free (2.6) 1 × phosphate-buffered saline (5.8 mM NaCl, 7.5 mM KCl, 14.2 mM Na_2HPO_4, 13.6 mM KH_2PO_4, pH 7.4; Green & Sambrook 2012) (centrifugation at 10,000 × g for 15 min) and subsequently used (2.5.1).

For the enrichment of methanogens from *E. eugeniae* raised and maintained on Substrate 1 (2.3.2.3), washed and sacrificed specimens were dissected (2.2.2) and gut contents, coelom fluid, and gut sections from the anterior part of the digestive system were vortexed in a 2 ml Eppendorf tube (Eppendorf, Hamburg, Germany) with phosphate buffer (2.3.1.1.2). Approximately 1 ml of the aqueous phase was used immediately as inoculum for the enrichment of methanogens (2.3.2.3).

2.2.4.2. Germany

Gut contents of *L. rubellus*, *L. terrestris*, *A. caliginosa*, and *O. lacteum* were obtained from earthworms that were washed with sterile water, sedated with CO_2, and sacrificed by brief immersion in 70 °C water or 70 % ethanol. Gut contents, mineral soil, and uppermost soil were cooled on ice until further use (2.3.2.1, 2.5.1). In addition, samples from the

crop/gizzard (Figure 5) of *L. terrestris* and *A. caliginosa* were prepared the same way and used for extraction of nucleic acids (2.5.1).

2.2.4.3. New Zealand

In New Zealand, gut contents of *O. multiporus* were obtained as described above (2.2.2) (Wüst *et al.* 2009b, Wüst 2010). In addition, forest soil from the location where *O. multiporus* was sampled from was also obtained for further use (2.5.1).

2.2.5. Millipedes

Millipedes of the species *Gymnostreptus olivaceus* (2.1.1.2) were tested for their *in vivo* emission of CH_4 in duplicate analyses for each experiment, i.e., with (2 specimens per replicate) and without (1 specimen per replicate) supplemental H_2/CO_2 (see 2.2.3.1.2).

2.3. Microbiological methods

2.3.1. Growth media and plates for cultivation and cloning

All anoxic solutions and anoxic media were prepared based on a modified Hungate techique (Hungate 1969, Daniel & Drake 1993).

2.3.1.1. Solutions

2.3.1.1.1. Anoxic water

For anoxic incubations, ddH_2O was boiled in Erlenmeyer flasks on a heating plate and subsequently transferred into serum vials previously flushed with N_2 (100 %). During the cooling down, the ddH_2O was continuously flushed with N_2 (100 %). A moderate overpressure was applied with 100 % N_2 for a better taking of samples afterwards. The solution was autoclaved and stored at 4 °C after cooling down.

2.3.1.1.2. Phosphate buffer

K_2HPO_4 x 3 H_2O	0.871 g
KH_2PO_4	0.540 g
Anoxic ddH_2O (2.3.1.1.1)	ad 1,000 ml

The anoxic phosphate buffer (Green & Sambrook 2012) was prepared with anoxic ddH_2O (2.3.1.1.1) in a serum vial that was flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with 100 % N_2 for a better taking of samples afterwards. The buffer was autoclaved and stored at 4 °C after cooling down. The oxic version of the phosphate buffer was made with normal, i.e., oxic ddH_2O and without gassing with N_2, and was also autoclaved and stored at 4 °C after cooling down.

2.3.1.1.3. Mineral salts DE-A

$(NH_4)_2SO_4$	0.3 g
K_2HPO_4 x 3 H_2O	0.435 g
KH_2PO_4	0.270 g
Anoxic ddH_2O (2.3.1.1.1)	ad 490 ml

The mineral salt solution DE-A (Atlas & Parks 2000) was prepared in a serum vial that was flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions.

2.3.1.1.4. Mineral salts DE-B

$MgSO_4$ x 7 H_2O	10.0 g
Anoxic ddH_2O (2.3.1.1.1)	ad 500 ml

The mineral salt solution DE-B (Atlas & Parks 2000) was prepared in a serum vial that was flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with 100 % N_2 for a better taking of samples afterwards. The solution was autoclaved and stored at 4 °C after cooling down.

2.3.1.1.5. Mineral salts ME

K_2HPO_4 x 3 H_2O	0.200 g
NH_4Cl	0.092 g
$MgCl_2$ x 6 H_2O	0.200 g
$CaCl_2$ x 2 H_2O	0.200 g
ddH_2O	ad 100 ml

The mineral salt solution ME (Wüst *et al.* 2009c) was prepared in a beaker. The solution was sterile filtered (0.2 µm pore diameter) into plastic tubes (50 ml) and stored at -20 °C.

2.3.1.1.6. Trace elements DE

$CaCl_2$ x 2 H_2O	1.0 g
$FeSO_4$ x 7 H_2O	0.5 g
$MnSO_4$ x H_2O	0.25 g
$CuSO_4$ x 5 H_2O	0.05 g
Na_2MoO_4 x 2 H_2O	0.05 g
HCl (0.1 N)	ad 500 ml

The trace element solution DE (Atlas & Parks 2000) was prepared in a serum vial that was flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with 100 % N_2 for a better taking of samples afterwards. The solution was autoclaved and stored at 4 °C after cooling down.

2.3.1.1.7. Trace elements ME

$CoCl_2$ x 2 H_2O	50 mg
$FeCl_2$ x H_2O	35 mg
$MnSO_4$ x H_2O	125 mg
$CuSO_4$ x 5 H_2O	5 mg
Na_2MoO_4 x 2 H_2O	5 mg
$CaCl_2$ x 2 H_2O	50 mg
$ZnCl_2$ x 2 H_2O	25 mg
$AlK(SO_4)_4$ x 12 H_2O	10 mg
H_3BO_3	5 mg
Na_2WO_4 x 2 H_2O	2.5 mg
$NiCl_2$ x 2 H_2O	10 mg
H_2SeO_3	25 mg
ddH_2O	ad 100 ml

The trace element solution ME (modified from Wüst *et al.* [2009c]) was prepared in a beaker. The solution was sterile filtered (0.2 µm pore diameter) into sterile plastic tubes (15 ml) and stored stored at - 20 °C.

2.3.1.1.8. Vitamins DE

4-aminobenzoic acid	4 mg
D+-biotin	2 mg
Nicotinic acid	10 mg
Ca-pantothenate	5 mg
Pyridoxine-HCl	15 mg
Folic acid	4 mg
Alpha lipoic acid	1 mg
Thiamine-HCl	10 mg
Cyanocobalamin	5 mg
Anoxic ddH_2O (2.3.1.1.1)	ad 100 ml

The vitamin solution DE (modified from Balch *et al.* [1979]) was prepared in a beaker that was flushed with 100 % N_2 before, and during the application of the components. The pH was adjusted to 6.8 - 7.0, and the solution was sterile filtered (0.2 µm pore diameter) into a sterile serum vial that was flushed with sterile N_2 (100 %) before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with sterile N_2 (100 %) for a better taking of samples afterwards. The solution was stored at 4 °C.

2.3.1.1.9. Vitamins ME-A

4-aminobenzoic acid	50 mg
D+-biotin	20 mg
Nicotinic acid	50 mg
Ca-pantothenate	50 mg
Pyridoxine-HCl	100 mg
Folic acid	20 mg
Alpha lipoic acid	50 mg
Thiamine-HCl	50 mg
Cyanocobalamin	50 mg
ddH_2O	ad 100 ml

The vitamin solution ME-A (Balch *et al.* 1979) was prepared in a beaker. The solution was sterile filtered (0.2 µm pore diameter) into plastic tubes (50 ml) and stored at - 20 °C.

2.3.1.1.10. Vitamins ME-B

Cyanocobalamin	50 mg
ddH_2O	ad 100 ml

The vitamin solution ME-B (Balch *et al.* 1979) was prepared in a beaker. The solution was sterile filtered (0.2 µm pore diameter) into plastic tubes (50 ml) and stored at - 20 °C.

2.3.1.1.11. Carbon sources

di-Na-succinate	0.900 g
Na-formate	0.227 g
Ethanol	195 µl
Na-acetate x 3 H_2O	0.454 g
Na-butyrate	0.367 g
Na-lactate	0.374 g
ddH_2O	ad 100 ml

The carbon sources for the isolation of denitrifiers from earthworm gut contents (2.3.2.1) contained compounds as fermentation products that were detected in the earthworm gut (Horn *et al.* 2003). The solution of carbon sources was prepared in a beaker that was flushed with 100 % N_2 before, and during the application of the components. The pH was adjusted to 6.8 - 7.0, and the solution was sterile filtered (0.2 µm pore diameter) into a sterile serum vial that was flushed with sterile N_2 (100 %) before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with sterile N_2 (100 %) for a better taking of samples afterwards. The solution was stored at 4 °C. Carbon sources had a concentration of 33.33 mM each in stock solution, and 0.2 mM each in the final medium/plates (2.3.2.1, 2.3.2.2).

2.3.1.1.12. Nitrite

KNO_2	4.26 g
ddH_2O	ad 100 ml

The nitrite stock solution (500 mM), was prepared in a beaker that was flushed with 100 % N_2 before, and during the application of the components. The pH was adjusted to 6.8 - 7.0, and the solution was sterile filtered (0.2 µm pore diameter) into a sterile serum vial that was flushed with sterile N_2 (100 %) before, during, and after the application of the

components to gain anoxic conditions. A moderate overpressure was applied with sterile N_2 (100 %) for a better taking of samples afterwards. The solution was stored at 4 °C.

2.3.1.1.13. Nitrate

KNO_3	5.06 g
ddH_2O	ad 100 ml

The nitrate stock solution (500 mM) was prepared in a beaker that was flushed with 100 % N_2 before, and during the application of the components. The pH was adjusted to 6.8 - 7.0, and the solution was sterile filtered (0.2 µm pore diameter) into a sterile serum vial that was flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with sterile N_2 (100 %) for a better taking of samples afterwards. The solution was stored at 4 °C.

2.3.1.1.14. Combined C-sources and vitamins

Carbon sources (2.3.1.1.11)	12 ml
Vitamins DE (2.3.1.1.8)	2 ml
Anoxic ddH_2O (2.3.1.1.1)	ad 100 ml

The solution of combined C-sources and vitamins was prepared in a beaker that was flushed with 100 % N_2 before, and during the application of the components. The pH was adjusted to 6.8 - 7.0, and the solution was sterile filtered (0.2 µm pore diameter) into a sterile serum vial that was flushed with sterile N_2 (100 %) before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with sterile N_2 (100 %) for a better taking of samples afterwards. The solution was stored at 4 °C.

2.3.1.1.15. Combined C-sources, vitamins, and nitrate

Carbon sources (2.3.1.1.11)	12 ml
Vitamins DE (2.3.1.1.8)	2 ml
Nitrate (2.3.1.1.13)	6 ml
Anoxic ddH_2O (2.3.1.1.1)	ad 100 ml

The solution of combined C-sources, vitamins, and nitrate was prepared in a beaker that was flushed with 100 % N_2 before, and during the application of the components. The pH

was adjusted to 6.8 - 7.0, and the solution was sterile filtered (0.2 µm pore diameter) into a sterile serum vial that was flushed with sterile N_2 (100 %) before, during, and after the application of the components to gain anoxic conditions. A moderate overpressure was applied with sterile N_2 (100 %) for a better taking of samples afterwards. The solution was stored at 4 °C.

2.3.1.1.16. Soil extract

For the enrichment and isolation of methanogens (2.3.2.3), 500 g earthworm-free Substrate 1 was combined with 500 ml ddH_2O, thoroughly mixed, and filtered and pressed through a clean, once laundered dishtowel into a serum vial. The extract was flushed with argon (100 %) for 2 h and autoclaved twice. The extract was stored at 4 °C.

2.3.1.1.17. Earthworm extract

For the enrichment and isolation of methanogens (2.3.2.3), 25 specimens of commercially obtained *L. terrestris* (2.1.2) were milled in a mixer (Waring commercial blender, Bender & Hobein, Zürich, Switzerland). The resulting mixture was centrifuged (3,000 × g, 10 min, 4 °C; 1-15K, Satorius, Sigma, Osterode am Harz, Germany). The supernatant was transferred into 2 ml Eppendorf tubes and again centrifuged (12,000 × g, 15 min, 4 °C). The clear, pink supernatant was diluted 1:10 with ddH_2O, sterile filtered (0.2 µm pore diameter) into a sterile plastic tube (15 ml), flushed with sterile argon (2.6) for 30 min, and stored at 4 °C.

2.3.1.2. Liquid media and media plates

2.3.1.2.1. DE/NO2 medium used for the isolation of denitrifiers

Mineral salts DE-B (2.3.1.1.4)	5 ml
Trace elements DE (2.3.1.1.6)	5 ml
Agar	7.5 g
Mineral salts DE-A (2.3.1.1.3)	ad 490 ml
Carbon sources (2.3.1.1.11)	6 ml
Vitamins DE (2.3.1.1.8)	1 ml
Nitrite (2.3.1.1.12)	3 ml

For the DE/NO2-medium used for the isolation of denitrifiers (2.3.2.1), the medium (modified from Atlas & Parks [2000]) was prepared in serum vials flushed with 100 % N_2

before, during, and after the application of the components to gain anoxic conditions. The pH was adjusted to 6.8 - 7.0, and the solution was autoclaved. After cooling down to approximately 70 °C, carbon sources, vitamins, and nitrite were added with syringes that were flushed with sterile N_2 (100 %) and mixed carefully to avoid bubbles in the medium. The medium was poured into sterile plastic Petri dishes in the oxygen-free chamber (100 % N_2). After solidification, the dishes were stored at room temperature in the oxygen-free chamber for at least 2 days before further use.

2.3.1.2.2. DE/N2O-medium used for the isolation of denitrifiers

Mineral salts DE-B (2.3.1.1.4)	5 ml
Trace elements DE (2.3.1.1.6)	5 ml
Agar	7.5 g
Mineral salts DE-A (2.3.1.1.3)	ad 496 ml
Carbon sources (2.3.1.1.11)	3 ml
Vitamins DE (2.3.1.1.8)	1 ml
Nitrite (2.3.1.1.12)	0.1 ml

For the DE/N2O-medium used for the isolation of denitrifiers (2.3.2.1), the medium (modified from Atlas & Parks [2000]) was prepared in serum vials flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. The pH was adjusted to 6.8 - 7.0, and the solution was autoclaved. After cooling down to approximately 70 °C, carbon sources, vitamins, and nitrite were added with syringes that were flushed with sterile N_2 (100 %) and mixed carefully to avoid bubbles in the medium. The medium was poured into sterile plastic Petri dishes in the oxygen-free chamber (100 % N_2). After solidification, the dishes were stored at room temperature in the oxygen-free chamber for at least 2 days before further use.

2.3.1.2.3. DE-plates to maintain isolates under oxic conditions

Yeast	0.5 g
Glucose	0.5 g
Agar	7.5 g
Mineral salts DE-B (2.3.1.1.4)	5 ml
Trace elements DE (2.3.1.1.6)	5 ml
Oxic mineral salts DE-A (2.3.1.1.3)	ad 493 ml
Carbon sources (2.3.1.1.11)	6 ml
Vitamins DE (2.3.1.1.8)	1 ml

Plates of the DE-medium were used to gain plate colonies for the growth experiments of potential denitrifiers (2.3.2.2). The medium (modified from Atlas & Parks [2000]) was prepared with mineral salt solution DE-A that was prepared with normal, i.e., oxic instead of anoxic ddH_2O. The pH was adjusted to 6.8 - 7.0, and the solution was autoclaved. After cooling down to approximately 70 °C, carbon sources and vitamins were added. The medium was poured into sterile plastic Petri dishes. After solidification, the dishes were stored at room temperature to get oxic conditions.

2.3.1.2.4. DE/ISO-medium for growth experiments with isolates

Yeast	0.06 g
Glucose	0.09 g
Mineral salts DE-B (2.3.1.1.4)	1 ml
Trace elements DE (2.3.1.1.6)	1 ml
Mineral salts DE-A (2.3.1.1.4)	ad 100 ml

The DE/ISO-medium was used for growth experiments under oxic and anoxic conditions (2.3.2.2). The anoxic medium (modified from Atlas & Parks [2000]) used to analyze growth under anoxic conditions (2.3.2.2.2) was prepared in serum vials flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. The pH was adjusted to 6.8 - 7.0, and the solution was autoclaved. After cooling down, 14 ml medium was transferred into sterile anoxic tubes (butyl rubber stopped aluminium crimp sealed glass tubes; 24 ml) that were flushed with sterile 100 % N_2 before and during the procedure. For each anoxic tube, 1.6 ml of combined C-sources and vitamins (2.3.1.1.14) was added.

The oxic medium used to analyze growth under oxic conditions (2.3.2.2.1) was prepared with mineral salt solution DE-A that was prepared with normal, i.e., oxic instead of anoxic ddH_2O. The medium was transferred into flasks and 1.6 ml of combined C-sources and vitamins (2.3.1.1.14) was added under oxic conditions. The flasks were sealed with an autoclaced air-permeable cellulose stopper and had an additional protuberance that enables the non-invasive the measurement of the optical density (OD; 2.3.2.4) during growth.

2.3.1.2.5. DE/ISO/NO3-medium for growth experiments with isolates

Yeast	0.06 g
Glucose	0.09 g
Mineral salts DE-B (2.3.1.1.4)	1 ml
Trace elements DE (2.3.1.1.6)	1 ml
Mineral salts DE-A (2.3.1.1.3)	ad 100 ml

The DE/ISO/NO3-medium was used for growth experiments under anoxic conditions with nitrate as electron acceptor (2.3.2.2.2). The anoxic medium (modified from Atlas & Parks [2000]) was prepared in serum vials flushed with 100 % N_2 before, during, and after the application of the components to gain anoxic conditions. The pH was adjusted to 6.8 - 7.0, and the solution was autoclaved. After cooling down, 14 ml medium was transferred into sterile anoxic tubes that were flushed with sterile 100 % N_2 before and during the procedure. For each anoxic tube, 1.6 ml of combined C-sources, vitamins, and nitrate (2.3.1.1.15) was added.

2.3.1.2.6. RUP-medium for the enrichment and isolation of methanogens

Yeast	0.015 g
Tryptone	0.015 g
Mineral salts ME (2.3.1.1.5)	1.5 ml
Trace elements ME (2.3.1.1.7)	0.6 ml
Vitamins ME-A (2.3.1.1.9)	30 µl
Vitamins ME-B (2.3.1.1.10)	3 µl
Resazurin (0.1 %)	0.3 ml
Bicarbonate	4.5 g
Soil extract (2.3.1.1.16)	1.5 ml
Cysteine solution (7.5 %)	1.2 ml
Na_2S solution (15 %)	0.6 ml
ddH_2O	ad 300 ml

For the RUP-medium (modified from Wüst *et al.* [2009c] and Bräuer *et al.* [2006]) used for the enrichment and isolation of methanogens (2.3.2.3), the medium was prepared in serum vials flushed with 100 % N_2 before, during, and after the application. Boiling ddH_2O was added to all components except for the soil extract, cysteine, Na_2S, and bicarbonate

($NaHCO_3$). After cooling down with a permanent flow of N_2, the soil extract, cysteine, Na_2S, and bicarbonate were added. The pH was adjusted to 7.1 with NaOH and a saturated solution of bicarbonate, and the medium was flushed with CO_2 (100 %). After another boiling and cooling down with a permanent flow of 100 % CO_2, the medium was filled into anoxic tubes (9 ml per 24 ml-vial) that were flushed with 100 % CO_2 before and during the procedure. For each anoxic tube, 8 ml of 100 % H_2 was added (the remaining gas phase consisted in the anoxic tube of CO_2) and the anoxic tubes were autoclaved. After cooling down, 0.1 ml of the earthworm extract (2.3.1.1.17) was added. The tubes were subsequently inoculated (2.3.2.3). For the very first enrichment step, i.e., the inoculum with material from *E. eugeniae* (2.3.2.3), a medium was used that contained no earthworm extract but the 20-fold amount of yeast and tryptone.

2.3.1.2.7. SOC medium

Tryptone	2 g
Yeast extract	0.5 g
NaCl solution (1 M)	1.0 ml
KCl solution (1 M)	0.25 ml
Mg^{2+} solution (2 M)	1.0 ml
Glucose solution (2 M)	1.0 ml
ddH_2O	ad 100 ml

Tryptone, yeast extract, NaCl and KCl were filled up with ddH_2O to approximately 95 ml and autoclaved. Sterile filtered (0.2 µm pore diameter) Mg^{2+} and glucose solutions were added, the pH was adjusted to 7.0 with sterile filtered solutions, and sterile filtered ddH_2O was added up to a final volume of 100 ml (Green & Sambrook 2012). Aliquots of the SOC medium were transferred into sterile 2 ml Eppendorf tubes and stored at - 20 °C.

2.3.1.2.8. LB (lysogeny broth) plates

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
ddH_2O	ad 1,000 ml

Tryptone, yeast extract, NaCl, and agar were filled up with ddH_2O to approximately 980 ml. The pH was adjusted to 7.0 and ddH_2O was added up to a final volume of 1,000 ml (Green & Sambrook 2012). After autoclaving, the medium was poured into sterile plastic Petri dishes and stored at 4 °C after solidification.

2.3.1.2.9. LB plates with ampicillin/IPTG/X-Gal

For blue/white screening of clones (2.5.9.3), 1 ml ampicillin (100 mg ml^{-1}), 1 ml isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 M), and 1.6 ml 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-Gal; 50 mg ml^{-1} in N,N´-dimethylformamide) solution was added to an autoclaved LB medium (2.3.1.2.8) after cooling down to approximately 60 °C. The medium was poured into sterile plastic Petri dishes, and the 'AIX plates' were stored at 4 °C after solidification.

2.3.2. Cultivation and growth experiments

2.3.2.1. Isolation of denitrifiers

The two experimental approaches to isolate denitrifiers from earthworm gut contents used nitrite and N_2O separately as main electron donors. These nitrogenous compounds are typical substrates for denitrifiers (1.2.1.1). Avoiding nitrate as electron acceptor aimed to predominantly isolate denitrifiers instead of dissimilatory nitrate reducers (1.2.2).

Dilution steps ranging from 10^{-2} to 10^{-4} of gut contents prepared from *L. rubellus*, *L. terrestris*, *A. caliginosa*, and *O. lacteum* (2.2.4.2) were conducted with anoxic phosphate buffer in anoxic tubes. In the oxygen-free chamber (100 % N_2), approximately 100 µl of the highest dilution step (10^{-4}) of the gut contents of each earthworm species was plated out on plates of the DE/NO2 and DE/N2O-medium.

The DE/NO2 medium was used to isolate denitrifiers with nitrite as electron donor, and therefore only contained nitrite (3 mM) as electron donor. Plates of this isolation approach were placed into anoxic jars (approximately 5 l; University of Bayreuth, Germany), flushed with 100 % argon, and incubated at 15 °C in the dark.

The DE/N2O-medium was used to isolate denitrifiers with N_2O as main electron donor, and contained minor amounts of nitrite (0.05 mM) only. Agar plates of this isolation approach were placed into anoxic jars and flushed with 100 % argon. N_2O (100 %) was added to the gas phase of the anoxic jar to a final concentration of approximately 10 mM. In addition, small amounts of NO (100 %) were added (approximately 100 nM). Nitrite and NO were needed in the approach with N_2O as main electron acceptor because nitrite and NO are

important signal molecules to activate the transcription and expression of denitrification genes, enzymes, and other signal proteins (1.2.1.2).

For both isolation approaches, agar plates were checked for growth of colonies in the oxygen-free chamber every 6 to 10 weeks. There were no visible differences of the prokaryotic colonies between the agar plates of the four earthworm species, or the two isolation approaches. Approximately 200 colonies were picked randomly, plated out on new agar plates containing the medium they were isolated from, and again incubated in anoxic jars as described above. This procedure was repeated three times. During the last plating out, a subsample of each of the remaining 159 colonies was dissolved in a small volume (20 µl) of anoxic phosphate buffer in 2 ml Eppendorf tubes. Polymerase chain reactions (PCRs; 2.5.7) amplifying the bacterial 16S rRNA gene were conducted (2.5.7.3). Isolate 201 and Isolate 208 were checked for the appearance of genes indicative of denitrification, i.e., *narG*, *napA*, *nirK*, *nirS*, and *nosZ* (2.5.7.2.2).

2.3.2.2. Growth experiments under oxic and anoxic condition with Isolate 201 and Isolate 208

For Isolate 201 and Isolate 208, basic physiological features were determined under oxic and anoxic conditions. Therefore, colonies of Isolate 201 and Isolate 208 isolated under anoxic conditions (2.3.2.1) were transferred to oxic DE-plates (2.3.1.2.3) containing the same substances as the plates used during their isolation (2.3.2.1) but no nitrite. Isolates were incubated under oxic conditions at 20 °C in the dark. Both isolates grew up to colonies with approximately 2 mm diameter within approximately 3 days. Colonies were used as inoculums for growth experiments under oxic (2.3.2.2.1) and anoxic conditions (2.3.2.2.2).

2.3.2.2.1. Growth under oxic conditions

Growth under oxic conditions was analyzed with medium DE/ISO in flasks that had an additional protuberance (2.3.1.2.4) that enables the non-invasive measurement of the OD during growth (2.3.2.4). The 24 ml-tubes used for the anoxic experiments (2.3.2.2.2) could not be used as the amount of oxygen in the tube was assumed to be insufficient to enable oxic growth for a sufficient period of time. 0.1 ml of a suspension of colonies (2.3.2.2) was injected and flasks were incubated for 48 hours to gain an active pre-culture. 0.1 ml of this pre-culture was used as inoculum for growth experiments, i.e., the measurement of the OD every hour over a period of 8 hours. All incubations were at 28 °C in the dark with a HT Infors Shaker (Infors, Bottmingen, Switzerland) at 150 rotations per minute.

2.3.2.2.2. Growth under anoxic conditions

Growth under anoxic conditions was analyzed in anoxic tubes that enable the direct measurement of the OD during growth (2.3.2.4). 0.1 ml of a suspension of colonies (2.3.2.2) was injected into anoxic tubes with DE/ISO/NO3-medium, i.e., a medium containing nitrate (2.3.1.2.5). Tubes were incubated for 48 hours to gain an active pre-culture. 0.1 ml of this pre-culture in DE/ISO/NO3-medium was used as inoculum for growth experiments, i.e., the measurement of the optical density OD over a period of 24 hours. These growth experiments under anoxic conditions, were conducted either with DE/ISO-medium to analyze anoxic growth without nitrate, or with DE/ISO/NO3-medium to analyze anoxic growth with nitrate. All incubations were at 28 °C in the dark with a HT Infors Shaker (Infors, Bottmingen, Switzerland) at 150 rotations per minute.

2.3.2.3. Enrichment and isolation of methanogens

Methanogens were enriched from a mixture of gut contents, coelom fluid and gut sections from the anterior part of the digestive system of *E. eugeniae* raised and maintained on Substrate 1. The enrichment was aimed to finally get methanogenic, archaeal isolates. Approximately 1 ml of the aqueous phase (2.2.4.1) was transferred into an anoxic tube with RUP-medium without earthworm extract but with the 20-fold amount of yeast and tryptone (2.3.1.2.6) with syringes that were flushed with sterile argon (100 %) before. After 4 weeks, CH_4 and H_2 were measured via GC (2.4.1) yielding approximately 5 % CH_4 and no detectable amounts of H_2 in the headspace (data not shown). Aliquots (1 ml) of this enrichment step (10^{-1} to 10^{-5}; dilution with RUP-medium) were used as inoculums for further enrichment steps with RUP-medium. After two additional transfers to new medium after 8 to 12 weeks each, GC measurements (2.4.1) were conducted after 50 days of incubation of the last enrichment step. Ratios between utilized H_2 and produced CH_4 were calculated in which a possible production of H_2 from fermentations during the incubation was disregarded.

An aliquot of the 10^{-5} dilution was used after 50 days for T-RFLP analysis with amplified *mcrA*/*mrtA* gene fragments (2.5.8) to check purity and phylogeny of enriched methanogens. Therefore, the aliquot (2 ml) was centrifuged (10,000 × g, 10 min, 4 °C), resuspended in PCR-H_2O, and subsequently used for PCR to amplify the fluorescence-labeled *mcrA*/*mrtA* fragments (2.5.8.1) for the T-RFLP analysis.

2.3.2.4. Optical density

The optical density (OD) was measured at 660 nm (OD_{660}) for growth experiments with Isolate 201 and Isolate 208 (2.3.2.2) in a photometer (Spectroquant Multy, Merck, Darmstadt,

Germany). A wavelength of more than 600 nm ensured to measure only the cell mass but no compounds as cytochromes and FeS clusters (Green & Sambrook 2012). The OD was normalized with a non-inoculated sample of the medium that was used in the corresponding experiment.

2.4. Analytical methods

2.4.1. Gas chromatography (GC)

The gases N_2O, CH_4, and H_2 were analyzed via gas chromatography (GC) with Hewlett Packard 5890 Series II gas chromatographs (Hewlett Packard, Palo Alto, CA, USA) in Bayreuth, Germany with conditions listed below (Table 5). All gas samples were taken with syringes (2.6) and flushed with 100 % sterile argon (2.6) before and after measurements from the incubation vials.

All gas samples derived from experiments in Brazil were stored in 3 ml sterilized and pre-vacuumed Exetainer vials (Labco Ltd., High Wycombe, England) for subsequent analysis in Bayreuth, Germany. Approximately 5 ml gas samples were injected into the vial resulting in an overpressure that enabled to take gas subsamples for injections into the GC (100 to 200 µl) later on. All vials were tested for leak tightness beforehand. Subsamples of these vials filled with different concentrations of N_2O, CH_4, and H_2 were tested if they would keep the gas concentration constant over a period of several weeks. It appeared that N_2O and CH_4 were highly storable in Exetainer vials for several weeks up to few months whereas H_2 appeared to leak through the rubber seal of the Exetainer vials (data not shown). Thus, only N_2O and CH_4 were measured from Exetainer vials filled with gas sampled in Brazil. Gas samples from enrichment cultures of methanogens were directly injected into the GC, i.e., without temporary storage in Exetainer vials and tested for CH_4 and also H_2.

Gas peaks were integrated with the Knauer IF2 and the EuroChrom software (both Knauer Advanced Scientific Instruments, Berlin, Germany). External gas standards with concentrations of 0.5 to 1,000 ppm were used to generate a straight calibration line (2.5.13.2). Overpressure was measured in the incubation vials before gas sampling and in the Exetainer vials if those vials were used to store gas samples before the injection into the GC. In addition, the current temperature (T_{curr}) and air pressure (p_{curr}) were measured to calculate the total amount of gas in the incubation vial.

The total amount of a certain gas (n_{total}) comprises the gaseous fraction (n_{gas}) and the fraction that is physically dissolved in the aqueous phase (n_{aqu}) (Equation 3). As there was no CO_2 analyzed, no chemically dissolved amounts of gases had to be considered.

Equation 3: Total amount of a gas.

$$n_{total} = n_{gas} + n_{aqu}$$

n_{total}, total amount of a gas; n_{gas}, gaseous fraction; n_{aqu}, fraction that is physically dissolved in the aqueous phase.

The amount of a certain gas in the gaseous fraction was calculated according to Equation 4. Here, the molar volume of a certain gas under the current conditions ($V_{curr, mol}$) was calculated with the ideal gas law (Equation 5).

Equation 4: Amount of gas in the gaseous fraction.

$$n_{gas} = V_{gas} \times \frac{c}{V_{curr,mol}} \times \frac{p_{curr} + p_{overp}}{p_{curr}}$$

n_{gas}, amount of a gas in the gaseous phase (µmol); V_{gas}, volume of the gaseous phase (ml); c, measured gas concentration (ppm); $V_{curr,mol}$, molar volume of a gas under the current conditions (ml); p_{curr}, current atmospheric pressure; p_{overp}, overpressure in the serum vials used for incubation (mbar).

Equation 5: Ideal gas law.

$$V_{curr,mol} = \frac{p_1 \times V_1 \times T_{curr}}{T_1 \times p_{curr}}$$

$V_{curr,mol}$, molar volume of a gas under the current conditions (ml); p_1, air pressure of standard conditions (mbar); V_1, molar volume under standard conditions (ml); p_{curr}, current atmospheric pressure; T_1, temperature of standard conditions (K); T_{curr}, current temperature (K).

Gases are also dissolved in the aqueous phase and therefore need to be added to the amount of gas in the gas phase (Equation 3). The variable Bunsen solubility coefficient (α) is dependent on the gas itself and the current temperature (Blachnik 1998; Equation 6).

Equation 6: Amount of physically dissolved gas in the aqueous phase.

$$n_{aqu} = V_{aqu} \times \frac{\mathrm{c}}{V_{curr,mol}} \times \propto \times \frac{p_{curr} + p_{overp}}{p_{curr}}$$

n_{aqu}, fraction that is physically dissolved in the aqueous phase; V_{aqu}, volume of the aqueous phase (ml); c, measured gas concentration (ppm); $V_{curr,mol}$, molar volume of a gas under the current conditions (ml); α, Bunsen solubility coefficient at the current temperature; p_{curr}, current atmospheric pressure; p_{overp}, overpressure in the serum vials used for incubation (mbar).

Table 4: Bunsen solubility coefficients of N_2O and CH_4 (Blachnik 1998).

	Bunsen solubility coefficient α (in H_2O)	
	20 °C	25 °C
N_2O	0.600	0.520
CH_4	0.032	0.029

Table 5: Parameters applied for GC analyses (Hewlett-Packard 5890 Series II).

Gases	N_2O	CH_4	H_2
Detector	Electron capture detector (ECD)	Flame ionization detector (FID)	Thermal conductivity detector (TCD)
Column	Poropak Q (80/100), 4 m × 1/8´´ (Supelco Bellefonte, PA, USA)	Molecular Sieve, 2 m × 1/2´´ (Alltech, Unterhaching, Germany)	Molecular Sieve, 2 m × 1/8´´ (Alltech, Unterhaching, Germany)
Carrier gas	95 % Ar, 5 % CH_4	100 % He	100 % Ar
Flow rate	20 to 40 ml min^{-1}	40 ml min^{-1}	33 ml min^{-1}
Oven temp.	60 °C	60 °C	50 °C
Injector temp.	150 °C	120 °C	150 °C
Detector temp.	300 °C	150 °C	175 °C
Injection vol.	100 to 200 µl	100 to 200 µl	100 to 200 µl
Retention time	3 to 4.5 min	1.7 min	0.6 min
Integration	Knauer IF2[a]	Knauer IF2	Knauer IF2
Software	EuroChrom[a,b]	EuroChrom	EuroChrom
Reference	(Karsten & Drake 1997)	(Küsel & Drake 1995)	(Daniel *et al.* 1990)

[a] Knauer Advanced Scientific Instruments, Berlin, Germany.

[b] version V3.05.

Abbreviations: temp., temperature; vol., volume.

2.4.2. Soil parameters

The soil parameters pH (determined in H_2O) and moisture content, and the concentrations of P, K, NH_4^+, NO_3^-, total organic material, total organic carbon, and total nitrogen were determined by standard techniques by the Soil Analysis Laboratory of the University of São Paulo (http://www.solos.esalq.usp.br/, last visit 22.06.2013) from 500 g material.

2.5. Molecular methods

2.5.1. Combined extraction of DNA and RNA

For soils/substrates and earthworm gut and crop/gizzard samples (2.2.4), the co-extraction of DNA and RNA was conducted by cell lysis and subsequent precipitation of nucleic acids (Griffiths *et al.* 2000). Extraction was conducted in Bayreuth, Germany (in Palmerston North, New Zealand for samples of *O. multiporus* and forest soil). Approximately 0.5 g was weighted into sterile 2 ml screw caps (VWR International, Darmstadt, Germany), 1 g sterilized (12 h at 200 °C) Zr beads (0.5 mm and 1.0 mm diameter, 50:50; Carl Roth, Karlsruhe, Germany), 0.5 ml extraction buffer (5 % CTAB, 350 mM NaCl, 120 mM potassium phosphate buffer containing KH_2PO_4 and K_2HPO_4, pH 8; pre-heated to 60 °C), and 0.5 ml phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) were placed into a FastPrep FP120 bead beater (Thermo Savant, Holbrook, USA) at 5.5 ms^{-1} for 2 × 30 s. Samples were subsequently cooled on ice and centrifuged (13,000 × g, 5 min, 4 °C; 1-15K, Satorius, Sigma, Osterode am Harz, Germany). Filter tips and RNase-free Eppendorf tubes were used. The supernatant was transferred into a new Eppendorf tube and 0.5 ml chloroform:isoamyl alcohol (24:1) were added, vortexed, and centrifuged (13,000 × g, 5 min, 4 °C). The supernatant was transferred into a new Eppendorf tube and twice the volume was applied of a precipitation buffer (30 % polyethylene glycol 6000, 0.1 M HEPES, pH 7.0) and vortexed. Nucleic acids precipitated at room temperature for 2 h and were pelleted (13,000 × g, 5 min, 4 °C), the pellet was washed twice with ethanol (70 %, - 20 °C) (13,000 × g, 1 min, 4 °C), dried at room temperature, and re-suspended in 30 to 50 µl of RNase-free ddH_2O (DEPC-H_2O; 2.6) with additional RNase inhibitor (Roche, Mannheim, Germany). Nucleic acids of *O. multiporus* and forest soil were subsequently freeze-dried (DuraDry, FTS Systems, Stone Ridge, NY, USA), sent to Bayreuth, Germany, and resuspended in 30 µl of DEPC-H_2O with additional RNase inhibitor (Roche, Mannheim, Germany). Quantity and quality were checked (2.5.4), and extracts were stored at - 80 °C.

2.5.2. Separation of DNA and RNA

2.5.2.1. Solid phase columns

The chromatographic separation was conducted via the Qiagen RNA/DNA Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer´s protocol ('Protocol for Isolation of Total RNA and Genomic DNA from Bacteria' for a total amount of RNA of less than 40 µg, starting with step 3). DNA or RNA was re-suspended in 30 to 50 µl of DEPC-H_2O with additional RNase inhibitor (Roche, Mannheim, Germany) if RNA was resuspended. Nucleic acids of *O. multiporus* and forest soil were subsequently freeze-dried (DuraDry, FTS Systems, Stone Ridge, NY, USA), sent to Bayreuth and re-suspended in 30 µl of DEPC-H_2O with additional RNase inhibitor (Roche, Mannheim, Germany). Quantity and quality were checked (2.5.4) and extracts were stored at - 80 °C.

2.5.2.2. Enzymatic digestion

An enzymatic digestion with DNase and RNase yielded RNA and RNA, respectively. 30 µl co-extract of nucleic acids were incubated with 3.4 µl reaction buffer (10×; Fermentas, St. Leon-Roth, Germany) and 1 µl DNase I (1 U μl^{-1}; Fermentas, St. Leon-Roth, Germany) at 37 °C for 1 h in a TGradient thermo cyclers (Biometra, Göttingen, Germany) to obtain RNA. 30 µl co-ectract of nucleic acids were incubated with 1 µl RNase A (10 U μl^{-1}; Fermentas, St. Leon-Roth, Germany) at room temperature for 30 min to obtain DNA. Both enzymatic reactions were stopped by precipitation of the nucleic acids (2.5.3.1). Optionally, quantity and quality were checked (2.5.4), and extracts were stored at - 80 °C.

2.5.3. Precipitation and purification of nucleic acids

2.5.3.1. Precipitation with isopropanol and sodium chloride

Extracted nucleic acid and PCR products were purified by precipitation with isopropanol and NaCl (Green & Sambrook 2012). 0.7 volumes of isopropanol (100 %, - 20 °C) and 0.1 volume of NaCl (5 M) were consecutively added to one volume of the sample, and incubated for at least 10 h at - 20 °C. Nucleic acids were precipitated by centrifugation (18,000 × g, 45 min, 4 °C). The pellet was washed with ethanol (70 %, - 20 °C) (13,000 × g, 1 min, 4 °C), dried at room temperature, and re-suspended in 30 to 50 µl of DEPC-H_2O. An RNase inhibitor (Roche, Mannheim, Germany) was added if RNA was precipitated. Optionally, quantity and quality were checked (2.5.4), and extracts were stored at - 80 °C.

2.5.3.2. Gel extraction

Amplicons, i.e., PCR products (2.5.7) for T-RFLP analysis (2.5.8.1) and for the construction of gene libraries (2.5.9) and for pyrosequencing (2.5.11) were purified by extraction out of the agarose gel (1 %; 2.5.5) according the manufacturer´s protocol (Montage Gel Extraction Kit, Merck Millipore, Billerica, MA, USA). The amplicon of the desired length was cut out of the agarose gel under UV-light (2.5.5) and placed into the Eppendorf tube. DNA in the resulting eluate was optionally checked (2.5.4.1), and extracts were stored at - 80 °C. For agarose gel electrophoresis (2.5.5), a buffer with 0.1 instead of 1.0 mM ethylenediaminetraacetic acid (EDTA) (2.5.5) was used (Merck Millipore, Billerica, MA, USA) as high amounts of EDTA block enzymatic reactions which can be conducted with the resulting eluate.

2.5.3.3. Filter plates

Amplicons treated with mungbean nuclease (2.5.8.2) were purified with Millipore PCR_{96} Cleanup Plates (Merck Millipore, Billerica, MA, USA) for approximately 15 min with a suction pump (KNF Neuberger, Balterswill, Switzerland) according to the manufacturer´s protocol. PCR products were washed twice with 100 µl PCR-H_2O and subsequently re-suspended in 10 to 15 µl PCR-H_2O. DNA concentration was checked (2.5.4.2), and extracts were further used for T-RFLP analysis (2.5.8.3).

2.5.4. Check of nucleic acids for purity and quantity

2.5.4.1. NanoDrop

Concentration and purity of nucleic acids was determined wit a ND1000 spectrophotometer (NanoDrop Technology, Wilmington, NC, USA) at 230, 260, and 280 nm wavelength. The absorption at 260 nm (A_{260}) is indicative of nucleic acids. A A_{260}/A_{280} ratio between 1.6 and 2.0 is indicative of nucleic acid with few contamination of proteins or phenol (Green & Sambrook 2012). A A_{260}/A_{230} ratio > 1.0 is indicative of few contamination with humic substances (Tsutsuki & Kuwatsuka 1979). All samples of the current study displayed few contaminations (data not shown) and were therefore used for further experiments.

2.5.4.2. PicoGreen and RiboGreen

Prior to T-RFLP (2.5.8) and reverse transcription of RNA into complementary DNA (cDNA; 2.5.6), the concentration of nucleic acids was determined spectrometrically via a fluorescence-based method that is less sensitive to interference by contaminants and more applicable for low concentrations, i.e., 0.1 to 5 ng μl^{-1} than NanoDrop (2.5.4.1) (Green & Sambrook 2012). In microtiter plates, Quant-iT-PicoGreen (for DNA) and Quant-iT-RiboGreen (for RNA) (both Invitrogen, Carlsbad, CA, USA) was added to the samples according to the manufacturer´s protocol and measured with a FLx800 Microplate Fluorimeter (BioTek, Bad Friedrichshall, Germany). Evaluation was with the software Gen5 (BioTek, Bad Friedrichshall, Germany). To calibrate the method, a straight calibration line was produced with standardized concentrations of DNA or RNA delivered by the manufacturer.

2.5.4.3. Test of RNA for contamination with DNA

RNA was checked for DNA contamination (i.e., possible DNA residues from the coextract [2.5.1]) via a PCR (2.5.7) amplifying a fragment of the bacterial 16S rRNA gene (primers 27F and 907RM; 2.5.7.3). All RNA fractions were tested negative for the occurrence of 16S rRNA gene fragments, i.e., there was no visible band on the agarose gel (2.5.5) whereas the positive control of the PCR (*E. coli* JM 109 cell material from cloning experiments [2.5.9.3]) displayed a very distinct band (data not shown). Thus, all RNA samples were treated as DNA-free and used for transcription into cDNA (2.5.6).

2.5.5. Agarose gel electrophoresis

Nucleic acids can be separated according to their size in a horizontal agarose gel electrophoresis (Aaij & Borst 1972, Green & Sambrook 2012). Agarose (Agarose low EEO, Applichem, Darmstadt, Germany) was applied to a 1 × TAE buffer (40 mM Tric-HCl, 20 mM acetate, 1 mM EDTA, pH 8) at a concentration of 1 % (w/v) and heated. Prior to pouring into a rack, liquid ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenenthridinium bromide) was added (approximately 0.1 mg ml^{-1}). 5 µl of the sample and 1 µl 6 × Blue Orange Loading Dye (Promega, Madison, WI, USA) were applied to the gel that was placed in the gel rack and poured with 1 × TAE buffer. For higher volumes, these amounts were up-scaled. A size standard was also applied (MWM-1, 200 to 1,000 bp, Bilatec, Viernheim, Germany). Accoding to the gel size, the electrophoretical separation was at 50 to 120 V for 20 to 60 min (Power-Pak 3000, BioRad, Richmond, CA, USA). Bands of nucleic acids were detected via the ethidium bromide when UV-light was applied (UVT-20M, Herolab, Wiesloch, Germany),

and a photo was taken (Canon PowerShot G5, Canon, Krefeld, Germany) with the associated software (RemoteCapture). Via the size standard, the size of the DNA (genomic or amplicon) or RNA could be estimated, and possible undesired bands, i.e., of the non-expected size (compare Table 6) could be detected.

2.5.6. Reverse transcription of RNA into cDNA

The reverse transcriptase transcribes RNA into a RNA-cDNA-hybrid (RT-PCR) that can be used for PCRs whereas PCRs cannot be conducted with RNA. According to the manufacturer´s protocol, RNA (50 to 300 ng per 20 µl reaction volume) was transcribed into cDNA via reverse transcriptase (SuperScript Vilo cDNA Synthesis Kit [3.1.2.1.1] or SuperScript III First-Strand Synthesis Kit [3.2.3]; both Invitrogen, Carlsbad, CA, USA) with a pre-incubation with 1 µl Random Hexamer Primers (50 ng μl^{-1}) for 10 min at 25 °C, 120 min at 42 °C, and 5 min at 85 °C with a thermo cycler (PeqStar; PEQLAB Biotechnologie, Erlangen, Germany). As reagents of the RT reaction mix might be inhibitory to subsequent enzymatic reactions, cDNA was precipitated with isopropanol and NaCl (2.5.3.1).

2.5.7. Polymerase chain reaction (PCR)

Genes and gene fragments can be amplified in high numbers from a matrix DNA or cDNA (cDNA is not explicitly mentioned in the following) via the polymerase chain reaction (PCR). A PCR cycle is divided into the denaturation step that separates double stranded DNA, the annealing step where the primers attach to the complementary region on the matrix DNA, and the elongation, where the polymerase synthesizes a DNA strand from dNTPs (Eppendorf, Hamburg, Germany) complementary to that of the matrix DNA starting from the attached primer. Theoretically, the amount of DNA products (amplicons) doubles every cycle, i.e., it grows exponentially (Saiki *et al.* 1988).

2.5.7.1. PCR primers, composition, and protocols

All PCRs were conducted via the thermo cyclers TGradient (Biometra, Göttingen, Germany), PeqStar, and Primus 96 advanced (both PEQLAB Biotechnologie, Erlangen, Germany). All primers of the current study were synthesized by Biomers (Ulm, Germany). For T-RFLP analyses, primers (Table 6) were preceded by the fluorescence dye DY681.

Table 6: Properties of primers used in the current study.

Primer[a]	Sequence (5´ → 3´)[d]	Target gene[e]	Reference
narG1960F[b,c]	TAY GTS GGS CAR GAR AA	*narG*	Philippot *et al*. 2002
narG2650R[c]	TTY TCR TAC CAB GTB GC	*narG*	Philippot *et al*. 2002
V16	GCN CCN TGY MGN TTY TGY GG	*napA*	Flanagan *et al*. 1999
V17	RTG YTG RTT RAA NCC CAT NGT CCA	*napA*	Flanagan *et al*. 1999
F1aCu[c]	ATC ATG GTS CTG CCG CG	*nirK*	Hallin & Lindgren 1999
R3Cu[c]	GCC TCG ATC AGR TTGT GGT T	*nirK*	Hallin & Lindgren 1999
Cd3aF[c]	GTS AAC GTS AAG GAR ACS GG	*nirS*	Throbäck *et al*. 2004
R3cd[c]	GAS TTC GGR TGS GTC TTG A	*nirS*	Throbäck *et al*. 2004
nosZF[b,c]	CGC TGT TCI TCG ACA GYC AG	*nosZ*	Rich et al. 2003
nosZR[c]	ATG TGC AKI GCR TGG CAG AA	*nosZ*	Rich et al. 2003
nosZ661F	CGG CTG GGG GCT GAC CAA	*nosZ*	Scala & Kerkhof 1999
nosZ1773R	ATR TCG ATC ARC TGB TCG TT	*nosZ*	Scala & Kerkhof 1999
mcrAF[b]	TAY GAY CAR ATH TGG YT	*mcrA* and *mrtA*	Springer *et al*. 1995
mcrAR	ACR TTC ATN GCR TAR TT	*mcrA* and *mrtA*	Springer *et al*. 1995
27F	AGA GTT TGA TCM TGG CTC	16S rRNA gene[f]	Lane 1991
1492R	GGT TAC CTT GTT ACG ACT T	16S rRNA gene	Lane 1991
907RM	CCG TCA ATT CMT TTG AGT TT	16S rRNA gene	Muyzer *et al*. 1998
M13uniF	TGT AAA ACG ACG GCC AGT	MCS pGEM-T[g]	Messing 1983
M13uniR	CAG GAA ACA GCT ATG ACC	MCS pGEM-T	Messing 1983

[a] forward and reverse primer for the first and second line of a primer couple, respectively. 907RM is also a reverse primer. If not indicated otherwise, primers were used for the construction of gene libraries via cloning (2.5.9) or for the molecular analysis of isolates (2.3.2.1).

[b] for T-RFLP analysis (2.5.8), primer was preceded by the fluorescence dye DY681 (Biomers GmbH, Ulm, Germany).

[c] for pyrosequencing (2.5.11), primer was preceded by a 6 bp-long barcode, i.e., ACACAC for gut contents of *G. paulistus*, ACGAGC for pasture soil, ACAGTC for gut contents of *A. gracilis*, and ACGCTC for grassland soil.

[d] M=A/C, R= A/G, H=A/T/C, W=A/T, K=G/T, D=G/A/T, Y=C/T, S=G/C, B=G/C/T, N=A/T/G/C, I=Inosine (according to IUPAC, International Union of Pure and Applied Chemistry).

[e] Expected sizes of amplified gene fragments: *narG*, 0.7 kb; *napA*, 1.0 kb; *nirK*, 0.5 kb; *nirS*, 0.4 kb; *nosZ* (nosZF/nosZR), 0.7 kb; *nosZ* (nosZ661F/nosZ1773R), 1.1 kb; *mcrA*/*mrtA*, 0.5 kb; 16S rRNA (27F/907RM), 0.9 kb; 16S rRNA (27F/1492R), 1.4 kb.

[f] the primer targets bacterial 16S rRNA genes only.

[g] multiple cloning site within the pGEM-T vector (2.5.9.1).

Most PCRs were conducted with a reaction volume of 50 µl (Table 7). However, volume was up- or down-scaled to 25, 50, 75, or 100 µl according to the requirements. For each PCR other than the M13-PCR (2.5.7.4), a negative control (PCR-H_2O instead of DNA, cDNA, or cell mass) and a positive control (2.5.7.2, 2.5.7.3) were prepared. The PCR were only used if there was no visible band in the agarose gel (2.5.5) of the expected size in the negative control. A gene was estimated as not detectable if the positive control of the PCR yielded amplicons of the expected size (2.5.5) whereas PCRs of the samples with different amounts of DNA, cDNA, or cell mass yielded no amplicons of the expected size even with different concentrations of PCR reagents, i.e., Mg^{2+}, primers, BSA and DNA polymerase.

Table 7: Chemical composition of the PCR reactions.

	PCR		
	Structural genes[h]	16S rRNA gene	M13 vector insert
PCR buffer (10 ×; Bilatec[a])[b]	-	-	5 µl
Mg^{2+} (25 mM; 5Prime[c] or Bilatec)	2 µl	2 µl	5 µl
BSA (10 mg ml^{-1})	2 µl	-	-
dNTPs (2 mM, Eppendorf)	-	-	5 µl
5Prime master mix (2.5 ×; 5Prime)[d]	20µl	20µl	-
Forward primer (10 µM or 100 µM)[e]	2 µl	2 µl	1 µl
Forward primer (10 µM or 100 µM)[e]	2 µl	2 µl	1 µl
Taq polymerase (5 U $µl^{-1}$; Bilatec)	-	-	0.2 µl
DNA or cDNA[f]	2 µl or cell mass	2 µl or cell mass	1 µl or cell mass
PCR-H_2O[g]	ad 50 µl	ad 50 µl	ad 50 µl

[a] Bilatec, Viernheim, Germany.

[b] buffer (10 ×): 0.8 M Tris-HCl (pH 9.4 - 9.5), 0.2 M $(NH_4)_2SO_4$, 0.2 % (w/v) Tween-20.

[c] 5Prime, Hamburg, Germany.

[d] master mix (2.5 ×): Taq DNA polymerase (0.06 U $µl^{-1}$), 125 mM KCl, 75 mM Tris-HCl (pH 8.4), 4 mM Mg^{2+}, 0.25 % Nonidet-P40, 500 µM of each dNTP, stabilizers.

[e] concentration was 100 µM for *narG*, *napA*, *nirK*, *nirS*, and *mcrA*/*mrtA*; concentration of primers for all other amplifications was 10 µM.

[f] for transcript analyses of *narG*, *nosZ*, and *mcrA*, cDNA was used; all other reactions were done with DNA or cell mass as template; concentration of DNA or cDNA in solution approximated 10 ng $µl^{-1}$.

[g] particle-free and autoclaved H_2O.

[h] i.e., *narG*, *napA*, *nirK*, *nirS*, *nosZ*, and *mcrA*/*mrtA*.

The purpose of a PCR is mentioned in the header and legend of a table displaying the PCR programs (Table 8, Table 9, Table 10). Some genes were amplified with the same primers but different programs. This is due to the fact that programs were optimized specifically for every sample of DNA or cDNA to gain the best results.

Table 8: PCR programs to amplify *narG*, *napA*, *nirK*, and *nirS* fragments.

		Primer combination				
		narG1960F/ narG2650R [a]	narG1960F/ narG2650R [c]	V16/ V17[d]	F1aCu/ R3Cu[d]	Cd3aF/ R3cd[d]
#	Step	T (°C) / time (min)				
1	Initial denaturation	95/8	95/8	94/5	95/10	95/10
2	Denaturation	95/1	-	94/1	95/1	95/1
3	Annealing	56/1	-	60/1	58/1	58/1
4	Elongation	72/2	-	72/1	72/2	72/2
5	Cycle: Steps 2 to 4	8 ×	-	10 ×	8 ×	8 ×
		↓ - 0.5 °C [b]		↓ - 0.5 °C	↓ - 0.5 °C	↓ - 0.5 °C
6	Denaturation	95/1	95/1	94/1	95/1	95/1
7	Annealing	52/1	58/1	55/1	54/1	54/1
8	Elongation	72/2	72/2	72/1	72/2	72/2
9	Cycle: Steps 6 to 8	35 ×	45 ×	30 ×	35 ×	35 ×
10	Terminal elongation	72/10	72/10	72/10	72/10	72/10
11	Storage	8/∞	8/∞	8/∞	8/∞	8/∞

[a] analyses with isolates (2.3.2.1), and earthworms from Germany (2.2.4.2) on gene level.

[b] 'touch down'; annealing temperature was lowered 0.5 °C per cycle.

[c] analyses with earthworms from Germany (2.2.4.2) on transcript level.

[d] analyses with isolates (2.3.2.1), and earthworms from Germany (2.2.4.2).

Abbreviations: T, temperature.

Table 9: PCR programs to amplify *nosZ* and 16S rRNA gene fragments, and regions of the pGEM-T cloning vector.

		Primer combination				
		nosZF/ nosZR[a]	nosZ661F/ nosZ1773R[c]	27F/ 1492R[d]	27F/ 907RM[f]	M13uniF/ M13uniR[g]
#	Step	T (°C) / time (min)				
1	Initial denaturation	95/8	95/8	95/10	95/8	95/8
2	Denaturation	95/1	95/1	95/1	95/1	-
3	Annealing	58/1	60/1	40/1	40/1	-
4	Elongation	72/2	72/2	72/3	72/2	-
5	Cycle: Steps 2 to 4	12 × ↓ - 0.5 °C[b]	8 × ↓ - 0.5 °C	6 × ↑ + 0.5 °C[e]	5 ×	-
6	Denaturation	95/1	95/1	95/1	95/1	95/1
7	Annealing	52/1	56/1	43/1	50/2	58/1
8	Elongation	72/2	72/2	72/3	72/2	72/2
9	Cycle: Steps 6 to 8	30 ×	30 ×	30 ×	30 ×	30 ×
10	Terminal elongation	72/10	72/10	72/10	72/10	72/10
11	Storage	8/∞	8/∞	8/∞	8/∞	8/∞

[a] analyses with isolates (2.3.2.1), and with earthworms from Germany (2.2.4.2) on gene and transcript level.

[b] 'touch down'; annealing temperature was lowered 0.5 °C per cycle.

[c] analyses with *O. multiporus* from New Zealand (2.2.4.3).

[d] analyses with isolates (2.3.2.1).

[e] annealing temperature was elevated 0.5 °C per cycle

[f] check of RNA for contamination with DNA (2.5.4.3).

[g] amplification of the insert of the pGEM-T vector for subsequent sequencing (2.5.9.3).

Table 10: PCR programs to amplify *narG*, *nirK*, *nirS*, and *nosZ* gene fragments for pyrosequencing.

		Primer combination
		narG1960F/narG2650R, F1aCu/R3Cu, Cd3aF/R3cd, nosZF/nosZR[a]
#	Step	T (°C) / time (min)
1	Initial denaturation	95/8
2	Denaturation	95/1
3	Annealing	60 - 67/1[b]
4	Elongation	72/1
5	Cycle: Steps 2 to 4	10 ×
		↓ - 0.5 °C[c]
6	Denaturation	95/1
7	Annealing	55 - 62/1[b]
8	Elongation	72/1
9	Cycle: Steps 6 to 8	40 ×
10	Terminal elongation	72/10
11	Storage	8/∞

[a] analyses with earthworms from Brazil for pyrosequencing (2.5.11); conditions were identical for all four primer pairs, but only one primer pair was applied per PCR reaction. Primers were preceded by a 6 bp-long barcode, i.e., ACACAC for gut contents of *G. paulistus*, ACGAGC for pasture soil, ACAGTC for gut contents of *A. gracilis*, and ACGCTC for grassland soil.

[b] replicate PCR reaction were performed at different annealing temperatures, and products of the correct size were pooled (2.5.11.1) to detect the maximum diversity.

[c] 'touch down'; annealing temperature was lowered 0.5 °C per cycle.

2.5.7.2. Structural genes

Fragments of the structural genes *narG*, *napA*, *nirK*, *nirS*, *nosZ* (two primer pairs; Table 6), and *mcrA*/*mrtA* were amplified for the creation of gene libraries (2.5.9), pyrosequencing (2.5.11), T-RFLP analyses (2.5.8), and to gain gene sequences from isolates (2.3.2.1) (Table 3). DNA (10 ng μl^{-1}) derived from the gut contents of *A. caliginosa* (2.5.1) served as positive control for the amplification of all genes indicative of denitrification as this sample always yielded best amplification results for all PCRs conducted. DNA (10 ng μl^{-1}) derived from the fen Schlöppnerbrunnen (Fichtelgebirge, Germany) served as positive control the amplification of *mcrA*/*mrtA*.

2.5.7.2.1. Brazilian earthworms, soils/substrates, and enrichment cultures

For gut contents of *G. paulistus* and for its pasture soil, and for gut contents of *A. gracilis* and its grassland soil (2.2.4.1), sequences of *narG*, *nirK*, *nirS*, and *nosZ* (*nosZ* primers according to Rich *et al.* 2003; 2.5.7.1) were amplified from DNA samples (2.5.2) with primers that were preceded by a 6 bp-long barcode. Each forward and reverse primer of a gene was preceded with a tag according to the sample is was derived from, i.e., ACACAC for gut contents of *G. paulistus*, ACGAGC for its pasture soil, ACAGTC for gut contents of *A. gracilis*, and ACGCTC for its grassland soil. Gene fragments of the correct size verified by agarose gel electrophoresis (2.5.5) were purified via gel extraction (2.5.3.2), subsequently precipitated (2.5.3.1), and concentration was measured (2.5.4.2). Amplicons were used for pyrosequencing (2.5.11).

For gut contents of *E. eugeniae* and for its Substrate 1 (2.2.4.1), sequences of *mcrA*/*mrtA* were amplified from DNA and cDNA samples (2.5.1, 2.5.6). Gene fragments of the correct size verified by agarose gel electrophoresis (2.5.5) were purified via gel extraction (2.5.3.2), subsequently precipitated (2.5.3.1), and concentration was measured (2.5.4.2). Amplicons were used for the construction of gene libraries via cloning (2.5.9).

For a sample from the enrichment experiment of methanogens from gut contents of *E. eugeniae* (2.3.2.3) sequences of *mcrA*/*mrtA* were amplified from pelleted cell material with primers preceded by the fluorescence dye DY681 (Biomers GmbH, Ulm, Germany; Table 6). Gene fragments of the correct size were verified by agarose gel electrophoresis (2.5.5), purified via gel extraction (2.5.3.2), subsequently precipitated (2.5.3.1), and concentration was measured (2.5.4.2). Amplicons were used for T-RFLP analysis (2.5.8).

2.5.7.2.2. German earthworms, soils, and isolates

For gut contents of *A. caliginosa*, *L. terrestris*, and *L. rubellus* (2.2.4.2), mineral soil and uppermost soil, sequences of *narG* and *nosZ* (*nosZ* primers according to Rich *et al.* 2003; Table 6) were amplified from DNA (2.5.2) and cDNA (2.5.6) samples. For *narG* transcripts, a PCR protocol with slightly different conditions (Table 8) was appllied to yield best results. In addition, *narG* and *nosZ* gene sequences were amplified from crop/gizzard samples of *A. caliginosa* and *L. terrestris* (2.2.4.2) for T-RFLP analysis (2.5.8). For gut contents of *A. caliginosa* and *L. terrestris*, and for mineral soil and uppermost soil, amplicons of *nirK* and *nirS* were amplified from DNA. All PCRs for *narG* and *nosZ* were with normal primers, i.e., for the creation of gene libraries (2.5.9) and with primers preceded by the fluorescence dye DY681 (Biomers GmbH, Ulm, Germany) for T-RFLP analysis (2.5.8.1). PCRs of *nirK* and *nirS* were with DNA and non-labeled primers only (Table 6). All gene fragments were

checked for the correct size via agarose gel electrophoresis (2.5.5), purified via gel extraction (2.5.3.2), and concentration was measured (2.5.4.1). Isolate 201 and Isolate 208 were checked for the appearance of genes indicative of denitrification, i.e., *narG*, *napA*, *nirK*, *nirS*, and *nosZ* (2.5.7.2.2). PCR products were checked for the correct size (2.5.5), purified via gel extraction (2.5.3.2), and the concentration was measured (2.5.4.1).

2.5.7.2.3. New Zealand earthworm and soil

For gut contents of *O. multiporus* and for forest soil, sequences of *nosZ* (*nosZ* primers according to Scala & Kerkhof 1999) were amplified from DNA (2.5.2). Gene fragments were checked for the correct size via agarose gel electrophoresis (2.5.5), purified via gel extraction (2.5.3.2) and concentration was measured (2.5.4.1).

2.5.7.3. 16S rRNA gene

Fragments of the bacterial 16S rRNA gene were amplified to gain gene sequences from isolates (2.3.2.1) and to test RNA extracts for the contamination with DNA (2.5.4.3) (Table 3). Cell mass of *E. coli* JM 109 cell derived from cloning (2.5.9.3) served as positive control for the amplification of the 16S rRNA gene. RNA extracts were tested as described above (2.5.4.3). The 159 isolates (2.3.2.1) were tested for the amplification of the 16S rRNA gene to analyze if novel species were isolated. PCRs were conducted from dissolved cell material (2.3.2.1). Gene fragments were checked for the correct size via agarose gel electrophoresis (2.5.5) and the resulting 151 samples were sent for sequencing without further purification (2.5.10).

2.5.7.4. Clone inserts of the pGEM-T vector (M13-PCR)

The M13-PCR was used to amplify a cloned insert from bacterial clones drived during the construction of gene libraries via cloning (2.5.9.3). Cell material dissolved in 20 µl PCR-H_2O was used as template for PCR. The primer pair M13uniF/M13uniR targets the the flanking region of the multiple cloning site (MCS) of the vector used (pGEM-T) (Green & Sambrook 2012). This PCR results in an amplification of either a DNA fragment containing the flanking vector region of the MCS and the inserted amplicon (insert size plus approximately 150 bp length; 'insert positive clone'), or the flanking vector region of the MCS only (fragment size about 150 bp; 'insert negative clone'). M13-PCR products were checked for the correct size via agarose gel electrophoresis (2.5.5), and insert positive clones were either purified first (2.5.3.3) or directly sent for sequencing (2.5.10).

2.5.8. Terminal restriction fragment length polymorphism (T-RFLP) analysis

The terminal restriction fragment length polymorphism (T-RFLP) analysis is a fingerprinting technique to compare microbial communities and also identify abundant taxa (Lui *et al.* 1997, Thies 2007). Amplicons that are fluorescence labeld at the forward primer are generated from a microbial community and digested by an endonuclease (restriction enzyme) the cuts the double stranded DNA at a specific and often palindromic restriction site. Dependent of the gene sequence, this digestion yields DNA fragments of different sizes, ideally also different terminal restriction fragments (T-RFs) that contain the fluorescence dye. Denatured and single stranded fragments are applied to a denaturing polyacrylamide gel electrophoresis (PAGE) where the fragments are separated according to their size. The T-RFs are detected at the end of the polyacrylamide gel by their fluorescence dye. Via an analysis *in silico*, species or OTUs can be affiliated with T-RFs of a defined length via sequence library or nucleotide database derived reference sequences that were *in silico* digested with the same restriction enzyme to determine their T-RF.

2.5.8.1. Amplification of fluorescence-labeled PCR products

Amplicons were generated with forward primers that were preceded by the fluorescence dye DY681 (Biomers GmbH, Ulm, Germany) from *narG* and *nosZ* gene and transcript sequences from gut contents and crop/gizzard contents of earthworms from Germany (2.2.4.2) and from *mcrA*/*mrtA* sequences from the enrichment experiment of methanogens from gut contents of *E. eugeniae* (2.3.2.3). PCR products of the correct size were purified via gel extraction (2.5.5), perecipitated (2.5.3.1), dissolved in PCR-H_2O, and their concentration was determined (2.5.4.1).

2.5.8.2. Digestion with mung bean endonuclease

During PCR, single stranded can be generated within an amplicon by premature termination of the DNA polymerase during the elongation step. As the digestion enzyme essentially needs double stranded regions to cut, these PCR errors can lead to an omission, i.e., no cut of the terminal restriction site but therefore a cut at another restriction site. These 'pseudo T-RFs' can significantly bias the T-RFLP analysis (Egert & Friedrich 2003). Thus, a digestion with an endonuclease that specifically cuts single stranded regions on the amplicon avoids this bias.

Purified amplicons (2.5.8.1) were incubated according to the manufacturer´s protocol with mung bean nuclease (10 U μl^{-1}, New England Biolabs, Frankfurt/Main, Germany) according to their concentration. Digestion was stopped by purification with filter plates (2.5.3.3).

2.5.8.3. Digestion with restriction enzymes

The restriction enzymes *Ban*I (5'→3' recognition and restriction site: G'GYRCC), *Hha*I (GCG'C), *Mbo*II (GAAGA(N)$_8$'), *Mae*III ('GTNAC), and *Sau96*I (G'GNCC) (all New England Biolabs, Frankfurt/Main, Germany) were used for different analyses. For each purified sample, triplicate digestions were conducted to create technical triplicates.

For *narG* gene analysis of German earthworms and soils (3.1.2.1.1.1.3), *Ban*I was used. For *narG* transcript analysis of German earthworms and soils, *Mae*III was used as results with *Ban*I could not be evaluated as the fragment sizes of the T-FRs were too small. For *nosZ* gene and transcript analysis of German earthworms and soils (3.1.2.1.1.2.3), *Hha*I was used. All these digestions were according to the manufacturer´s protocol but with 3 units per digestion that was conducted for 16 hours. For *mcrA*/*mrtA* gene analysis of methanogens from the enrichment experiment (3.2.4), a double digestion was performed, i.e., with *Mbo*II and *Sau96*I in the same reaction. This double digestion was performed according to the manufacturer´s protocol but with 2,5 and 3 units per digestion for *Mbo*II and *Sau96*I, respectively. All enzymatic digestions were stopped according to the manufacturer´s protocol. Concentrations were determined with PicoGreen (2.5.4.2) and samples were adjusted to a concentration of approximately 1 ng μl^{-1} with PCR-H_2O.

For *narG*, *nosZ*, and *mcrA*/*mrtA*, aligned sequences (2.5.12.9) derived from sequence libraries (3.1.2.1.1.1.1, 3.1.2.1.1.2.1, and 3.2.3, respectively) were *in silico* digested in MEGA 4.0 (Kumar *et al.* 2008), i.e., the length of a T-RF that was expected from the digestion with a certain restriction enzyme was calculated from the recognition site of the restriction enzyme. Thus, all restriction enzymes available from New England Biolabs (Frankfurt/Main, Germany) were tested beforehand to get the best separation and resolution of OTUs. This information was lateron used to affiliate the T-RFs from the PAGE to certain OUTs (2.5.8.5).

2.5.8.4. Denaturing polyacrylamide gel electrophoresis (PAGE)

The denaturing polyacrylamide gel electrophoresis was performed on a NEN 4300 DNA Analyzer (Licor, Lincoln NE, USA). Glass gel plates (Boroflat glass plates, 25 cm × 25 cm × 0.5 cm) were cleaned with ddH_2O, ethanol (70 %) and isopropanol (80 %). A bind silane solution (1:1 bind silane plusOne, GE Healthcare, Piscataway, MD, USA; 10 %

acetate) was applied as a thin film at the uppermost area of the plates to stabilize the gel pockets. Spacers (0.2 mm) separated the two plates. For the polyacrylamide gel, 15 g urea (Roche Pharma, Reinach, Switzerland) was mixed with 3.75 ml of a 40 % acrylamide-bis-solution (37.5:1, 2.6 % C; BioRad, Hercules, CA, USA), 5 ml 5 × TBE buffer (450 mM Tris, 450 mM H_3Bo_3, 10 mM EDTA (pH 8), and 9.25 ml ddH_2O. The solution was sterile-filtered (pore size 0.2 µm) to exclude un-dissolved salts. The application of 175 µl ammonium persulfate (440 mM) and 17 µl ultra-pure N,N,N,N-tetramethylethylendiamine (Invitrogen, Karlsruhe, Germany) started the polymerization reaction. The gel was immediately poured between the two gel plates, the comb (48 lanes) was inserted, and the polymerization was for approximately 45 minutes at room temperature. Afterward, the plates were placed into the DNA Analyzer, the buffer tanks were added, and the upper and lower tank was filled with 1 × TBE buffer. The comb was removed, residual urea was flushed out of the gel pockets with 1 × TBE buffer, and it was strictly avoided to flush bubbles into the pockets. A pre-run was performed for 25 minutes at 1,200 V and 45 °C. In the meantime, 2 µl T-RFLP samples and a size standard (µ-STEP-24a, 50 - 700 bp; Microzone, Haywards Heath, UK) were mixed with 2 µl Stop-Solution (Licor, Lincoln, NE, USA), denaturated for 3 minutes at 94 °C on a TGradient thermo cycler (Biometra, Göttingen, Germany), and placed on ice at once. Approximately 0.7 to 0.3 µl sample and standard were loaded into the gel pockets, respectively. The gel electrophoresis was performed for 4 hours at 1,200 V and 45 °C.

2.5.8.5. Analysis of T-RF profiles

Gel images were analyzed with GelQuest (version 2.6.3; Sequentix, Klein Raden, Germany). According to their absolute fluorescence, peak areas were attributed to T-RFs. Relative fluorescences were calculated for each lane as the absolute amount of DNA applied into the gel pockets varied. Relative fluorescences enable the comparison of different samples. To exclude background jitter and T-RFs of insignificant abundance, only T-RFs with a minimum relative abundance of at least 3 % in at least one sample were used for further analyses; their summarized fluorescence was reset as 100 %. The average (2.5.13.1) of technical triplicates is displayed. The relative abundances of the T-RFs were also used for the principal component analysis (2.5.13.5), i.e., to display relative differences between the libraries. T-RFs were affiliated to OTUs according to their *in silico* calculated T-RFs (2.5.8.3) allowing a minor tolerance of the *in silico* calculated and from the gel measured T-RF length.

For each T-RFLP analysis, gene sequences were amplified from one to three M13-clones of a known sequence and treated the same way as the environmental samples. Thus, digestion with the particular restriction enzyme could be checked for completeness. As there was always more than 90 % of the relative fluorescence of a M13-clone assigned to the *in*

silico calculated T-RF, all digestions were estimated as complete and appropriate for further analyses.

2.5.9. Construction of gene sequence libraries via cloning

Gene libraries were constructed by inserting PCR products into a cloning vector (ligation; 2.5.9.1), introducing these vectors into competent cells (transformation; 2.5.9.2), testing grown colonies of bacterial clones for the existence of the vector with the right insert (blue/white screening), and sequencing of amplicons derived from insert positive clones (2.5.9.3) (Green & Sambrook 2012).

Gene libraries were constructed from the following inserts (genes and transcripts, each earthworm species, and each soil/substrate separately): *narG* and *nosZ* genes and transcripts derived from the gut contents of *A. caliginosa*, *L. terrestris*, *L. rubellus*, and from mineral soil and uppermost soil (2.5.7.2.2); *nirS* genes derived from the gut contents of *L. terrestris*, and of mineral soil (2.5.7.2.2); *nosZ* genes derived from the gut contents of *O. multiporus*, and from forest soil (2.5.7.2.3); *mcrA*/*mrtA* genes and transcripts derived from the gut contents of *E. eugeniae*, and from Substrate 1 (2.5.7.2.1).

2.5.9.1. Ligation

A linerized pGEM-T vector (pGEM-T Vector System II, Promega, Madison, WI, USA) of approximately 3,000 bp length with a single 3'-terminal thymidine-overhang at both ends within the MCS was used. Thus, self-linearization was prevented and the insertion of PCR products was favored as the DNA polymerase preferentially but not always creates a single 5'-terminal adenosine-overhang at both ends of the amplicon (Mülhardt 2009). The vector contains a gene encoding for a protein for a resistance against the antibiotic ampicillin. The MCS of the vector is located within the *lacZ* operon that encodes for a β-galactosidase. An insertion of a gene fragment at the MCS therefore interrupts the *lacZ* operon resulting in an inactive β-galactosidase (Green & Sambrook 2012). This feature is used later on (2.5.9.3). According to the manufacturer´s protocol, a molar insert to vector ratio of 1:1 is suggested. However, the ligation can be successful for ratios ranging between 1:8 and 8:1, and ratios used in the current study ranged from 1:2 to 6:1. The calculation of the amount of insert that had to be applied using a given molar insert to vector ratio was calculated according to Equation 7.

Equation 7: Molar insert to vector ratio.

$$insert\ (ng) = \frac{vector\ (ng) \times insert\ size\ (bp)}{vector\ size\ (bp)} \times (molar\ insert\ to\ vector\ ratio)$$

$insert\ (ng)$, amount of insert necessary for ligation at a given molar insert to vector ratio; $vector\ (ng)$, amount of vector used for ligation reaction (25 ng for 5 µl reaction); $insert\ size\ (bp)$, size of the insert in bp; $vector\ size\ (bp)$, size of the pGEM-T vector in pb.

Purified PCR products (2.5.7.2) whose concentration was determined (2.5.4.1) and whose amount for the ligation was calculated (Equation 7) were used for the ligation reaction (Table 11) with a T4 DNA ligase. The reaction was incubated in a water bath at room temperature (20 to 25 °C) that was incubated overnight in the refrigerator (4 °C) allowing the reaction mix to cool down to 4 °C and thereby slowly transcend the optimal temperature for ligation. After ligation, the vector and the inserted gene fragment are circulized to a plasmid.

Table 11: Chemical composition of the ligation reaction.

Component	Volume
2 × Rapid Ligation Buffer (Promega[a])[b]	2.5 µl
pGEM-T vector (50 ng μl^{-1})	0.5 µl
PCR product, i.e., insert	0.5 - 1.5 µl
T4 DNA ligase (3 Weiss units μl^{-1})	0.5 µl
PCR-H_2O	ad 5 µl

[a] Promega, Madison, WI, USA.

[b] buffer (2 ×): 60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM dithiothreitole, 2 mM ATP, 10 % (v/v) polyethylene glycol.

2.5.9.2. Transformation

Competent cells of *Escherichia coli* JM 109 (Promega, Madison, WI, USA) that were stored at - 80 °C and cooled on ice directly before use, were applied to insert the generated vector plasmids (2.5.9.1). 50 µl of competent cells were transferred into ice-cooled Eppendorf tubes, gently mixed with 2 µl of the finished ligation reaction, and incubated for 30 min on ice. After a heat-shock for exact 50 seconds in a water bath with 42 °C, cells were immediately put back on ice for 2 min. 950 µl of SOC-medium (2.3.1.2.7) was added, gently mixed, and the transformation reaction was incubated for 90 min at 37 °C on a gently shaking (300 rpm) thermo-mixer (Eppendorf, Hamburg, Germany). Cells were gently centrifuged (1000 × g, 10 min, room temperature), spread over AIX-plates (2.3.1.2.9), and incubated overnight at

37 °C in the dark. The ampicillin in the agar plates ensure that only *E. coli* cells can grow that inherit an uptaken vector plasmid with the ampicillin resistance gene.

2.5.9.3. Blue/white screening

Blue/white screening was applied to check if grown bacterial insert clones from the transformation (2.5.9.2) posses a vector with an insert or a self-ligated vector only (Green & Sambrook 2012). IPTG in the AIX-plates (2.3.1.2.9) induces the expression of the *lacZ* operon located at the MCS of the vector plasmids. Its product, the β-galactosidase converts the colorless X-Gal (an analogue of lactose) into a product that turns into dark blue when exposed to oxygen. Thus, cells of white colonies possess a vector without an insert (intact MCS and therefore β-galactosidase) whereas dark blue colonies possess a vector with an insert (interrupted MCS and therefore inactive β-galactosidase). However, for small inserts (i.e., valid for all inserts used in the current study) into the MCS, a β-galactosidase with a reduced activity can be expressed resulting in light blue colonies that gain their color often after a longer period of time. Thus, AIX-plates were stored in the refrigerator (4 °C) for several hours prior to the blue/white screening and both dark blue and light blue colonies were picked, i.e., they were dissolved in 20 µl PCR-H_2O and frozen for 1 hour (- 20 °C). Afterwards, a M13-PCR (2.5.7.4) was conducted. PCR products of insert positive clones with the expected size, i.e., the gene fragment size (Table 6) plus additional approximately 150 bp vector rest were either purified first (2.5.3.3) or directly sent for sequencing (2.5.10).

2.5.10. Sequencing

M13-PCR products of insert positive clones from the construction of gene libraries via cloning (2.5.9) were either purified first (2.5.3.3) or sent for sequencing without purification; 16S rRNA genes and genes indicative of denitrification derived from bacterial isolates (2.5.7.2.2, 2.5.7.3) were sent for sequencing without purification. Sequencing (based on Sanger *et al.* [1977]; Sanger sequencing) was conducted by Marogen (Kumchun-ke, Seoul, South Korea). M13-PCR products were sequenced with the forward primer (M13uniF, Table 6), genes indicative of denitrification derived from bacterial isolates were sequenced with the according forward primer (Table 6), and 16S rRNA genes derived from bacterial isolates were sequences with the forward primer only (27F, Table 6; all 151 sequences derived from isolates) or additionally with the reverse primer (1492R, Table 6; Isolate 201, Isolate 208, Isolate 403, Isolate 823, and Isolate ISO4).

2.5.11. Barcoded amplicon pyrosequencing

Pyrosequencing (Hymen 1988, Ronaghi *et al.* 1998) is a molecular tool that enables a higher throughput of sequences albeit concomitant with a shorter read length (up to date, 300 to 500 bp) than for the classical Sanger sequencing (800 to 1,000 bp) (Metzker 2005). Pyrosequencing is based on the measurement of inorganic phosphate which is released during the synthesis of a DNA strand ('sequencing by synthesis') and which is proportionally converted into visible light by enzymatic reactions (Ronaghi *et al.* 1998, Metzker 2005).

During the 454 GS-FLX Titanium pyrosequencing, a pyrosequencing technique of which a modified protocol (2.5.11.1, 2.5.11.2) was applied in the current study, two approximately 30 bp long sequencing adapters are ligated to each of the both ends of the amplicon of the desired gene. In an emulsion-PCR, stochastically one amplicon is included in a separated reaction volume together with a capture bead that is coated with complementary strands of the adapters. Single stranded amplicons can bind to the bead-bound adapters and are subsequently multiplied in this separated reaction volume via a PCR. Beads coated with copies of the original amplicon are then transferred into picolitre reactors (Margulies *et al.* 2005) where the actual 'sequencing by synthesis' reaction occurs, i.e., a double stranded amplicon gets sequenced from the single stranded and bead-bound amplicon via a DNA polymerase. The four different dNTPs are sequentially washed over the picolitre reactors. If a nucleotide gets incorporated into the growing double stranded amplicon, pyrophosphate (compare 'pyrosequencing') is released. This pyrophosphate reacts to ATP together with an adenosine-5'-phosphosulphate catalyzed by the ATP-surfurylase. ATP and luciferin react to oxoluciferin and thereby emit a light quantum. Non-used dNTPs and ATP are degraded by an apyrase, and the next dNTP is washed over the picolitre reactors. A photo detector recognizes the light that is emitted if a dNTPs is incorporated. If two or more dNTPs of the same type are incorporated next to each other, the intensity of the emitted light is higher. This is also the most error-prone feature of the pyrosequencing as the brightness of the emitted light not always exactly correlated with the amount of incorporated nucleotides, i.e., the amount of dNTPs of the same type occurring next to each other is misinterpreted (Gilles *et al.* 2011).

2.5.11.1. Amplification of barcoded amplicons

In the current study, a strategy was applied (modified from Palmer *et al.* [2012]) that is based on previous studies with amplicon pyroseqencing (Huber *et al.* 2007, Iwai *et al.* 2010, Will *et al.* 2010). Here, the adapter used for the pyrosequencing reaction with approximately 30 bp length was not preceded to PCR primers prior to the PCR. Instead, primers were preceded by a 6 bp-long barcode to enable the identification of the origin of a sequence from

pooled amplicons (see below). This modified pyrosequencing procedure was applied as a PCR with primers with a short barcode only was assumed to yield less unspecific PCR products than a PCR with primers with the relatively long adapter (Palmer *et al.* 2012). The adapters were ligated to the amplicons lateron (2.5.11.2).

Amplicons of *narG*, *nirK*, *nirS*, and *nosZ* were generated with primers that were preceded by a 6 bp-long barcode (Table 6). After purification (2.5.3.2), precipitation (2.5.3.1), and quantification (2.5.4.2), similar amounts of amplicons from gut contents and soils were pooled for each gene. Possible damages of the DNA during amplification and treatment under the UV-light for gel extraction (2.5.3.2) as thymidine dimers were eliminated via a PreCR Repair Mix (New England Biolabs, Frankfurt/Main, Germany) according to the manufacturer´s protocol. Precipitated (2.5.3.1) amplicon mixtures were sent to the Genomics Laboratory (Göttingen, Germany).

2.5.11.2. Ligation of adapters and subsequent pyrosequencing

Sequencing adapters A (CGT ATC GCC TCC CTC GCG CCA TCA G) and B (CTA TGC GCC TTG CCA GCC CGC TCA G) were ligated to the barcode-tagged amplicons by workers of the Genomics Laboratory (Göttingen, Germany). All other downsteam reactions as described above (2.5.11) were conducted and pyrosequencing was done with a Roche GS-FLX 454 pyrosequencer and GS-FLX Titanium series reagents (Roche, Mannheim, Germany) according to the manufacturer´s instructions.

2.5.12. Sequence analysis

2.5.12.1. Calculation of cutoff values to define operational taxonomic units (OTUs) from nucleotide and amino acid sequences

When analyzing sequences of functional genes instead of 16S rRNA genes, it is crucial to define an empiric cutoff value that defines if two sequences are probably derived from two different species or belong to the same species or OTU (Purkhold *et al.* 2000). Published values were used for analyzing *narG* and *nosZ* (Palmer *et al.* 2009), and *mcrA*/*mrtA* (Hunger *et al.* 2011). Values for *nirK* and *nirS* were not available and therefore calculated *in silico* prior to gene sequence analyses according to published procedures (Palmer *et al.* 2009). This method gives an estimate of the minimal number of OTUs, i.e., the true species-level diversity might be significantly higher (Palmer *et al.* 2009).

For *nirK* and *nirS*, nucleotide sequences were retrieved from the NCBI (National Center for Biotechnology Information; http://ncbi.nlm.org, last visit 22.06.2013) that approximately

comprise the region within the gene that is amplified with the primer pairs F1aCu/R3Cu (Hallin & Lindgren 1999; Table 6) and Cd3aF/R3cd (Throbäck *et al.* 2004; Table 6), respectively. In addition, the 16S rRNA gene of the corresponding organism the *nirK* or *nirS* gene was derived from was also downloaded, i.e., the whole gene, or a region that can be amplified with the primer pair 27F/1492R (Lane 1991; Table 6) of at least approximately 1,000 bp. If an organism harbored two distinct copies of a *nirK* or *nirS* gene, both copies were used. Some organisms harbored multiple copies of the 16S rRNA gene. However, these 16S rRNA gene copies were highly similar (> 99.5 %) or identical. Thus, only the longest sequences of a multiple 16S rRNA gene sequence of an organism was used. All sequence couples used for the analysis of *nirK* and *nirS* are listed in Table A 3 and in Table A 4, respectively. In the following, the description of the procedure is restricted to *nirK* only, but was conducted for both *nirK* and *nirS* separately.

Sequence alignments of *in silico* translated *nirK* amino acid sequences and of 16S rRNA nucleotide sequences were conducted with CLUSTALW (Thompson *et al.* 1994) that is implemented in MEGA 4.0 (Kumar *et al.* 2008) and were manually refined. The amino acid or nucleotide base difference per site (D) was calculated for pairwise comparisons of all *nirK* and 16S rRNA gene fragments. The similarity (S) was calculated as 1 - D (Equation 8). The similarity of *nirK* derived from amino acid- and nucleic acid-based comparisons was plotted against the similarity of the corresponding 16S rRNA gene. For both nucleotide and amino acid correlation plots, similarity values of sequence couples with a 16S rRNA gene similarity of ≥ 97 % (a conservative species-level cutoff; Stackebrandt 2006, Stackebrandt & Ebers 2006), were selected. Within these truncated data points, cutoff values were calculated that cover ≥ 90 % (i.e., 90 % quantile) of the remaining data points (according to Palmer *et al.* [2009]). Phylogenetic trees were calculated based on nucleotide sequences for *nirK* and the corresponding 16S rRNA gene fragments from a p-distance matrix (2.5.12.9). Selected clusters were highlighted in the phylogenetic tree of both, *nirK* and 16S rRNA gene sequences.

Equation 8: Similarity.

$$S = 1 - D$$

S, similarity; D, difference between two nucleotide or amino acid sequences with $0 \leq D \leq 1$.

2.5.12.2. Sequences derived from cloning and from isolates

All sequences or OTU representative sequences of a library generated for gene libraries via cloning (2.5.9) and from bacterial isolates (2.3.2.1) via sequencing by Macrogen

(Kumchun-ke, Seoul, South Korea; 2.5.10), were imprted into MEGA 4.0 (Kumar *et al.* 2008). For M13-PCR-derived sequences, all residual vector sequences of the MCS were deleted. Sequences were analyzed in BLAST (Altschul *et al.* 1990). Sequences comprising not the expected gene or possible chimeric sequences were discarded for further analyses. A chimeric sequences was defined as a sequence that was closely related to two or more, i.e., instead of one reference sequences as determined by BLAST. For sequences comprising the expected gene (denoted as 'valid'), cultured and uncultured closest related sequences were retrieved from BLAST and added to MEGA 4.0. All sequences were analyzed starting with the corresponding forward primer. Nucleotides at the end of a sequence read did often not meet the criteria of a good quality sequence (Macrogen, Kumchun-ke, Seoul, South Korea) and were therefore deleted.

For sequences of functional genes, i.e., sequences other than 16S rRNA genes, valid sequences and their related sequences were translated *in silico* into their amino acid sequences, checked for raster mutations, aligned with CLUSTALW (Thompson *et al.* 1994) that is implemented in MEGA 4.0, and manually refined. From these sequence data, phylogenetic trees (2.5.12.9) and diversity estimators were calculated (2.5.12.4, 2.5.12.5, 2.5.12.6, 2.5.12.7, 2.5.12.8).

For the 16S rRNA gene fragments, 141 valid sequences were retrieved from 151 sequenced amplicon samples (2.5.7.3). For Isolate 201, Isolate 208, Isolate 403, Isolate 823, and Isolate ISO4, overlapping regions of the 16S rRNA gen fragment retrieved from the sequencing with both the forward and the reverse primer from each isolate were combined to one 16S rRNA gene fragment. All 16S rRNA gene fragment sequences were checked for their similarity to cultured organisms to analyze if novel species were isolated (2.3.2.1).

2.5.12.3. Pyrosequencing-derived data

Nucleotide sequences derived from the pyrosequencing (2.5.11) were sorted according to their barcodes and primers. For each gene, i.e., *narG*, *nirK*, *nirS*, and *nosZ* sequences with at least 350 bp length were clustered with JAGUC2, a software that uses pairwise comparison of input sequences to generate distance matrices and sequence clusters, i.e., OTUs from these matrices (http://wwwagak.informatik.uni-kl.de/JAguc, last visit 22.06.2013) (Nebel *et al.* 2011). OTUs were generated according to the DNA-based species-level cutoff values, i.e., 67 % for *narG* (Palmer *et al.* 2009), 83 % for *nirK* (3.1.1.3.1.1), 82 % for *nirS* (3.1.1.3.1.2), and 80 % for *nosZ* (Palmer *et al.* 2009). These alignments applied (i.e., using pairwise comparison of input sequences) are assumed to yield a smaller overestimation of the real diversity in the samples occurring by errors during PCR and sequencing than expected from alignments with multiple comparisons and/or algorithms with complete linkage

(Quince *et al.* 2009, Sun *et al.* 2009, Huse *et al.* 2010, Kunin *et al.* 2011, Palmer *et al.* 2012). The overall error rate of 454 GS-FLX Titanium pyrosequencing is about 1.07 % (Gilles *et al.* 2011) what is far below the cutoff values used to define OTUs from sequence data (see above). Thus, other than for analyses with 16S rRNA gene sequences with a cutoff value of about 97 %, PCR and sequencing errors were not anticipated to significantly affect the diversity of OTUs detected from sequences of the current study.

For *narG* and *nosZ*, sequences starting with the forward primer were used; for *nirK* and *nirS*, forward and reverse reads could be combined resulting in sequences covering nearly the complete amplicon. For each of the four genes, representative sequences of each OTU were imported into MEGA 4.0 (Kumar *et al.* 2008), analyzed in BLAST (Altschul *et al.* 1990), and cultured and uncultured closest related sequences were retrieved from BLAST and added to the OTU representatives in MEGA 4.0. This procedure was conducted for sequences comprising the expected gene only (denoted as 'valid'). Sequences comprising not the expected gene or possible chimeric sequences were discarded for further analyses (denoted as 'invalid'). A chimeric sequences was defined as a sequence that was closely related to two or more, i.e., instead of one reference sequences as determined by BLAST. Sequences derived from the four different origins, i.e., gut contents of *G. paulistus*, pasture soil, gut contents of *A. gracilis*, and grassland soil were identified via their 6 bp long sequence tag (Table 6). Valid OTU representatives and their related sequences were translated *in silico* into their amino acid sequences, checked for raster mutations, aligned with CLUSTALW (Thompson *et al.* 1994) that is implemented in MEGA 4.0, and manually refined. From these sequence data, phylogenetic trees (2.5.12.9) and diversity estimators were calculated (2.5.12.5, 2.5.12.6, 2.5.12.7, 2.5.12.8) and libraries were checked for their relative differences (2.5.13.3), and for significant differences (2.5.13.4).

2.5.12.4. DOTUR

For all sequences of insert positive clones derived for the construction of gene libraries via cloning (2.5.9), a p-distance-based distance matrix of amino acid sequences *in silico* translated from nucleotide sequences was generated (2.5.12.9). This matrix was used as an input file for DOTUR-1.53 ([Schloss & Handelsman 2005]; now implemented in MOTHUR, http://www.mothur.org, last visit 22.06.2013) [Schloss *et al.* 2009]) to define OTUs according to their species-level cutoff values, i.e., 59 % for *narG* (Palmer *et al.* 2009), 87 % for *nirS* (3.1.1.3.1.2), 86 % for *nosZ* (Palmer *et al.* 2009), and 86 % for *mcrA*/*mrtA* (Hunger *et al.* 2011). In the output file, all sequences of a defined OTU were displayed. From these data, coverage (2.5.12.6) and rarefraction curves (2.5.12.5) were calculated. In addition, the richness and diversity estimators Chao1, ACE, Bootstrap, Jackknife (all 2.5.12.7), Shannon-

Weaver, and Simpson index (both 2.5.12.8) were retrieved as output files from DOTUR-1.53, and the Evenness (2.5.12.8.2) and reciprocal Simpson index (2.5.12.8.3) were calculated from these indices for all gene and transcript sequences except for *mcrA*/*mrtA*.

2.5.12.5. Rarefaction analysis

The rarefaction analysis allows the comparison of sequence libraries with a different amount of samples sequences. The drawn calculated number of OTUs after n sequences (Equation 9) is called rarefaction curve (Hulbert 1971, Heck *et al.* 1975, Magurran 2004). Flat and plateauing rarefaction curves indicate that no more OTUs and few more OTUs are expected from additionally sampled sequences, respectively. In contrast, a steeply rising rarefaction curve indicates that the sampled amount of sequences is still insufficient to cover the OTUs expected in a library. Rarefaction curves were calculated from OTU and sequence data derived from pyrosequencing (2.5.11) and DOTUR analysis (2.5.12.4). However, as the coverage (2.5.12.6) yields similar information and can be displayed in a table, figures of calculated rarefaction curves are not displayed in the current study.

Equation 9: Hulbert equation.

$$S_n = \sum_{i=1}^{S}\left[1 - \frac{\binom{N - N_i}{n}}{\binom{N}{n}}\right]$$

S_n, expected number of OTUs in a sample with n individuals; S, total number of OTUs; N, total number of sequences; n, standardized sample size with $n \leq N$; i, continuous index that runs from 1 to S.

2.5.12.6. Coverage

The coverage of a gene library indicates how many OTUs were detected by the sampling in comparison to the total amount of OTUs expected (Good 1985). The coverage C was calculated with Equation 10 (Schloss & Handelsman 2005) from OTU and sequence data derived from pyrosequencing (2.5.11) and DOTUR analysis (2.5.12.4).

Equation 10: Coverage.

$$C = \left[1 - \left(\frac{N_{OTUs,min}}{N_{Seq,total}}\right)\right] \times 100$$

C, coverage (%); $N_{OTUs,min}$, number of OTUs that occur once; $N_{Seq,total}$, total number of OTUs in a gene library.

2.5.12.7. Richness estimators Chao1, ACE, Bootstrap, and Jackknife

The richness estimators Chao1 (Equation 11; Chao 1984, Hill *et al.* 2003, Magurran 2004), ACE (Equation 12; Chao & Lee 1992, Chao *et al.* 1993, Magurran 2004), Bootstrap (Equation 13; Smith & van Belle 1984), and Jackknife (Equation 14; Heltshe & Forrester 1983, Magurran 2004) indicate the expected diversity, i.e., number of OTUs from a library with a restricted amount of sampled sequences. These estimators were calculated via DOTUR-1.53 (2.5.12.4) for sequences derived from sequence libraries (2.5.9) or independently for OTU and sequence data from pyrosequencing (2.5.11). For most analyses, the richness of a library was calculated as the average (2.5.13.1) of some or all of these four richness estimators as indicated in the corresponding table legend.

Equation 11: Chao1.

$$S_{Chao1} = S_{obs} + \left(\frac{n_1 \times (n_1 - 1)}{2 \times (n_2 + 1)}\right)$$

S_{Chao1}, expected number of OTUs; S_{obs}, observed number of OTUs; n_1, number of OTUs that occur exactly once; n_2, number of OTUs that occur exactly twice.

Equation 12: ACE.

$$S_{ACE} = S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{n_1}{C_{ACE}} \times \gamma^2_{ACE}$$

$$C_{ACE} = 1 - \frac{n_1}{N_{rare}}$$

$$N_{rare} = \sum_{i=1}^{10} (i \times n_i)$$

$$\gamma^2_{ACE} = max\left[\frac{S_{rare}}{C_{ACE}} \times \frac{\sum_{i=1}^{10}[i \times (i-1) \times n_1]}{N_{rare} \times (N_{rare} - 1)} - 1\right]$$

S_{ACE}, expected number of OTUs; S_{abund}, number of OTUs that occur at least 10 times; S_{rare}, number of OTUs that occur 10 times or less often; n_1, number of OTUs that occur exactly once; C_{ACE}, sample abundance coverage estimator; n_i, number of OTUs that occur exactly i-fold; γ^2_{ACE}, estimated coefficient of variation of the $n_i$for rare OTUs; ; N_{rare}, total number of sequences in rare OTUs; i, continuous index that runs from 1 to 10.

Equation 13: Bootstrap.

$$S_{Boot} = S_{obs} + \sum_{i=1}^{S_{obs}} \left(1 - \frac{S_i}{N}\right)^{\mathrm{N}}$$

S_{Boot}, expected number of OTUs; S_{obs}, observed number of OTUs; S_i, number of sequences in the i^{th} OTU; i, continuous index that runs from 1 to S_{obs}; N, total number of sequences analyzed.

Equation 14: Jackknife.

$$S_{Jack} = S_{obs} + n_1 \times \left(\frac{N-1}{N}\right)$$

S_{Jack}, expected number of OTUs; S_{obs}, observed number of OTUs; n_1, number of OTUs that occur exactly once; N, total number of sequences analyzed.

2.5.12.8. Diversity indices

The Shannon-Weaver (2.5.12.8.1) and the reciprocal Simpson diversity index (2.5.12.8.3) are estimators that indicate the diversity and relative abundance of single OTUs within a community or sequence library instead of only indicating the number of expected OTUs as with the richness indicators Chao1, ACE, Bootstrap, and Jackknife (2.5.12.7). The Shannon-Weaver and the reciprocal Simpson diversity index were calculated via DOTUR-1.53 (2.5.12.4) for sequences derived from sequence libraries (2.5.9) or independently for OTU and sequence data from pyrosequencing (2.5.11).

2.5.12.8.1. Shannon-Weaver index

The Shannon-Weaver diversity index H´ estimates if the diversity of a library is high (high values for H´) or low (low values for H´). This index is comparable with other libraries only when the amount of analyzed sequences is similar (Hill *et al.* 2003, Magurran 2004). Calculation was with Equation 15 (Shannon & Weaver 1949).

Equation 15: Shannon-Weaver index.

$$H' = -\sum_{i=1}^{S_{obs}} \frac{\mathrm{S_i}}{\mathrm{N}} \times \ln \frac{S_i}{N}$$

H', Shannon-Weaver diversity index; S_{obs}, observed number of OTUs; S_i, number of sequences in the i^{th} OTU; i, continuous index that runs from 1 to S_{obs}; N, total number of sequences analyzed.

2.5.12.8.2. Evenness

The Evenness E (Equation 16) is an estimation if OTUs are distributed evenly in a library (E approximates 1), or if one OTU or few OTUs dominate (E approximates 0) (Pielou 1977, Magurran 2004). This estimator is less biased by a varying amount of analyzed sequences between compared libraries than the Shannon-Weaver index (2.5.12.8.1).

Equation 16: Evenness.

$$E = \frac{H'}{\ln S_{obs}}$$

E, Evenness with $0 \leq E \leq 1$; H', Shannon-Weaver diversity index; S_{obs}, observed number of OTUs.

2.5.12.8.3. Reciprocal Simpson index

The reciprocal Simpson richness estimator 1/D (Equation 17) indicates if the diversity of a library is high (high values for 1/D) or low (low values for 1/D) (Simpson 1949, Magurran 2004). The reciprocal index was used instead of the original index, as only with the reciprocal index, a higher value indicates a higher diversity.

Equation 17: Reciprocal Simpson index.

$$\frac{1}{D} = \frac{N \times (N-1)}{\sum_{i=1}^{S_{obs}} [S_i \times (S_i - 1)]}$$

$\frac{1}{D}$, Simpson diversity index; S_{obs}, observed number of OTUs; S_i, number of sequences in the i^{th} OTU; i, continuous index that runs from 1 to S_{obs}; N, total number of sequences analyzed.

2.5.12.9. Calculation of phylogenetic trees

Phylogenetic trees were calculated with MEGA 4.0 (Kumar *et al.* 2008) or ARB (Ludwig *et al.* 2004). Nucleic acid sequences of structural genes and the 16S rRNA gene were imported into the program. For the analysis of structural gene markers, reference sequences of cultured und uncultured organisms determined by a BLAST (Altschul *et al.* 1990) search were added for sequences from the current study. For sequences derived from cloning experiments (2.5.9), representative sequences of each OTU were analyzed with BLAST. For sequences derived from pyrosequencing (2.5.11), representative sequences of each OTU exceeding 1 % relative abundance in a library were analyzed with BLAST. Sequences were *in silico* translated into amino acid sequences, checked for the correct orientation and for raster mutations. Sequences were aligned with the CLUSTALW algorithm (Thompson *et al.* 1994) implemented in MEGA 4.0 or with ARB. Alignements were manually refined. A p-distance-based distance matrix was used to calculate phylogenetic trees in MEGA 4.0 utilizing the neighbor-joining method (Saitou & Nei 1987) and the pairwise sequence comparison (pairwise deletion option). For *mcrA*/*mrtA* sequences (2.5.7.2.1), a Dayhoff-corrected neighbor-joining phylogenetic tree was calculated in ARB (Ludwig *et al.* 2004). For all analyses, the percentage of replicate trees in which the taxa clustered together in the bootstrap test (10,000 replicates) is displayed next to the branches (Felsenstein 1985). For the analysis of *mcrA*/*mrtA* sequences, additional phylogenetic trees based on alternative algorithms (maximum likelihood [Jukes-Cantor or Dayhoff correction] and maximum parsimony) were calculated, and trees were compared to confirm tree topology (indicated by nodes in the displayed neighbor-joining tree; Figure 40). Additional information is displayed in the figure legends.

For the evaluation of cutoff values to define OTUs (2.5.12.1), phylogenetic trees were calculated from 16S rRNA sequences and from nucleotide sequences instead of amino acid sequences of the corresponding *nirK* or *nirS* sequences as described above, i.e., a p-distance-based distance matrix was used to calculate phylogenetic trees utilizing the neighbor-joining method (Saitou & Nei 1987) and the bootstrap test (10,000 replicates) (Felsenstein 1985).

For the principal coordinate analysis (2.5.13.3), phylogenetic trees were calculated from condensed datasets of the four libraries gut *G. paulistus*, soil *G. paulistus*, gut *A. gracilis*, and soil *A. gracilis* as described below (2.5.13.3). A p-distance-based distance matrix was used to calculate phylogenetic trees utilizing the neighbor-joining method (Saitou & Nei 1987).

2.5.13. Statistical analyses

2.5.13.1. Average, standard deviation, and standard error

Arithmetic mean (average; $\bar{x}$), standard deviation (S; in the following text of the current study, SD is applied as abbreviation for standard deviation), and standard error ($S_{\bar{x}}$,) were calculated with Equation 18, Equation 19, and Equation 20, accordingly (Sachs 1999, Precht *et al.* 2005). If not indicated otherwise, results are displayed as $\bar{x} \pm S$.

Equation 18: Average.

$$\bar{x} = \frac{1}{n} \times \sum_{i=1}^{n} x_i$$

$\bar{x}$, average; n, number of values; i, continuous index that runs from 1 to n.

Equation 19: Standard deviation.

$$S = \sqrt{\frac{1}{n-1} \times \left[\sum_{i=1}^{n} (x_i - \bar{x})^2\right]}$$

S, standard deviation; ; n, number of values; $\bar{x}$, average; i, continuous index that runs from 1 to n.

Equation 20: Standard error.

$$S_{\bar{x}} = \frac{S}{\sqrt{n}}$$

$S_{\bar{x}}$, standard error; S, standard deviation; ; n, number of values.

2.5.13.2. Regression

Regression lines were calculated with Excel 2007 (Microsoft, Redmond, WA, USA) to gain straight calibration lines for GC measurements.

2.5.13.3. Principal coordinate analysis (PCoA)

A principal coordinate analysis (PCoA) was conducted with *narG*, *nirK*, and *nosZ* sequences derived from pyrosequencing (2.5.11). This method is also called the classical multidimensional scaling. Its input is a matrix with dissimilarities between its pairs and it produces a displayable coordinate matrix with a minimum loss of variance information (McCune & Grace 2002, Borg & Groenen 2005). Condensed datasets of all four libraries (i.e., gut *G. paulistus*, soil *G. paulistus*, gut *A. gracilis*, and soil *A. gracilis*) were created with *narG*, *nirK*, and *nosZ* sequences using representative sequences of OTUs exceeding 1 % relative abundance in at least one library. Each representative sequence of an OTU in a given library was multiplied with the amount of sequences that was detected there. A phylogenetic tree was calculated from this dataset (2.5.12.9) and used as input file for the analysis with FASTUNIFRAC (http://bmf2.colorado.edu/fastunifrac, last visit 22.6.2013; Lozupone & Knight 2005, Hamady *et al.* 2010). This method was applied to display relative differences between the four libraries for each gene analyzed.

2.5.13.4. Significance test

The phylogentic trees calculated from condensed datasets of all four pyrosequencing-derived libraries (i.e., gut *G. paulistus*, soil *G. paulistus*, gut *A. gracilis*, and soil *A. gracilis*) generated for the PCoA (2.5.13.3), were used as input file for the analysis with FASTUNIFRAC (http://bmf2.colorado.edu/fastunifrac, last visit 22.06.2013; Lozupone & Knight 2005, Hamady *et al.* 2010). This method was applied to calculate if the diversity of two libraries is significantly different.

2.5.13.5. Principal component analysis (PCA)

A principal component analysis (PCA) was conducted with T-RF patterns of *narG* and *nosZ* genes and transcripts (2.5.8.5) to display relative differences between libraries. It is the simplest version of the eigenvestor-based multivariate analysis and explains the variance of data in a low-dimensional space. It converts a set of possibly correlated variables into a smaller or the same amount of variables that are linearly uncorrelated, called principal components (PC). The first PC (PC1) covers the largest variance, i.e., it accounts for the most variability in the dataset, followed by PC2, and so on (McCune & Grace 2002, Borg & Groenen 2005, Abdi & Williams 2010). The relative abundances of all displayed T-RFs (3.1.2.1.1.1.3, 3.1.2.1.1.2.3) were used as input file for the software RAPIDMINER (http://rapid-i.com, last visit 22.06.2013; Mierswa *et al.* 2006).

2.5.14. Deposition of sequences and metafiles in public databases

Published sequences obtained in the current study are available from the EMBL nucleotide sequence database (European Molecular Biology Laboratory; http://www.embl.de, last visit 22.06.2013). For sequences retrieved from analyses with earthworms and soils/substrates from Germany and Brazil, the accession numbers are listed in Table 12.

Table 12: Accession numbers of sequences deposited in public sequence databases.

Gene (origin of samples; cross reference)	Accession numbers[c]
narG (Germany; 3.1.2.1.1.1)	FN859458 – FN859704
narG (Brazil; 3.1.1.3.2)[a]	HE802107 – HE802120
nirK (Brazil; 3.1.1.3.3)[a]	HE802121 – HE802144
nosZ (Germany; 3.1.2.1.1.2)	FN859705 – FN859774, FN859874 – FN859960
nosZ (Brazil; 3.1.1.3.5)[a]	HE802145 – HE802168
narG, *nirK*, *nirS*, *nosZ* (Brazil; 2.5.12.3)[b]	ERP001284

[a] reference sequences used in the current study from all OTUs exceeding 1 % relative abundance.

[b] complete amplicon sequence meta file retrieved from pyrosequencing.

[c] the ERP001284 meta file is available from the ENA Short Read Archive whereas all other, single sequences are available from the EMBL nucleotide sequence database.

2.6. Chemicals, gases, and labware

Deionised double destilled water (ddH_2O) was produced with a Seralpur Pro 90 CN ultrapure water purification system (Seral Erich Alhäuser, Ransbach-Baumbach, Germany) with a conductivity of less than 0.055 µS cm^{-1}. PCR-H_2O was prepared by sterile-filtration (pore diameter 0.2 µm) and autoclaving (121 °C, 1 bar, 20 min) of PCR-H_2O. RNase- and DNase-free water (DEPC-H_2O) was produced by the application of diethylepyrocarbonate (DEPC, 0.1 % v/v) to ddH_2O, an incubation at 37 °C for 3 hours (shaking at 200 rpm), and a subsequent autoclaving. Small volumes of sterile gases were produced by autoclaving in serum vials whereas constant flushing with sterile gases was achieved by flushing the gas through an autoclaved 1 ml syringe that was padded with cotton batting.

Syringes (BD Biosciences, Heidelberg, Germany) with a 14- to 20-gauge needle (BD Microlane 3, BD Biosciences, Heidelberg, Germany) were used to take gas samples and apply substances to anoxic tubes (butyl rubber stopped aluminium crimp sealed glass tubes; 24 ml) or serum vials (butyl rubber stopped aluminium crimp sealed serum vials; 150 to 1,000 ml).

If not indicated otherwise, all chemicals, gases (Table 13), and labware were obtained from Applichem (Darmstadt, Germany), BioRad (Hercules, CA, USA), Carl Roth (Karlsruhe, Germany), Eppendorf (Hamburg, Germany), Fluka (Buchs, Switzerland), Rießner (Lichtenfels, Germany), and Sigma Aldrich (Steinheim, Germany).

Table 13: Gases and their purity.

	Ar	CH_4	CO_2	H_2	He	N_2	N_2O	NO
Purity	4.8	3.5	technical	5.0	4.6	5.0	4.5	5.0

2.7. Contribution of other workers to this dissertation

If not indicated otherwise, samplings, experiments, and evaluations were conducted by myself. Individuals who significantly contributed to information presented in this dissertation are identified below. Results from the current study that were already published in peer-reviewed journals (Depkat-Jakob *et al.* 2010, Depkat-Jakob *et al.* 2012, Depkat-Jakob *et al.* 2013) are presented and discussed in a way that is similar to how the information was presented in these publications.

2.7.1. Denitrification and dissimilatory nitrate reduction

2.7.1.1. Earthworms from Brazil

All earthworms, soils, and substrates were sampled or purchased (2.1.1) by myself or together with Prof. George G. Brown (Embrapa Florestas, Colombo, Brazil). Earthworm species were identified by Prof. George G. Brown. Gas emission experiments (2.2), DNA extractions (2.5.1), and amplifications of genes (2.5.7.1) were conducted by myself. Subsequent barcoded amplicon pyrosequencing (2.5.11) was conducted together with Katharina Palmer (University of Bayreuth), and partly by the Genomics Laboratory (Göttingen, Germany). Soil properties (2.4.2) were determined by the Soil Analysis Laboratory of the University of São Paulo, Brazil.

2.7.1.2. Earthworms from Germany

2.7.1.2.1. *narG* and *nosZ* gene and transcript studies

Samplings of earthworms and soils (2.1.2), and extractions of DNA and RNA (2.5.1) were conducted by myself. Clone libraries (2.5.9) and T-RFLP-analyses (2.5.8) of *narG* transcripts were generated by Maik Hilgarth starting with RNA provided by myself during his bachelor thesis at the Department of Ecological Microbiology that was elaborated and supervised by myself (Hilgarth 2009). Thereafter, *narG* transcript sequences and T-RFLP patterns were re-evaluated and analyzed by myself for dissertation, together with the sequences for *narG* genes, *nosZ* genes, and *nosZ* transcripts that were generated during my diploma thesis at the Department of Ecological Microbiology (Depkat-Jakob 2009).

2.7.1.2.2. *nirK* and *nirS* studies

Sampling of earthworms and soils (2.1.2), and extractions of DNA and RNA (2.5.1) were conducted together with Julia Gebelein during her bachelor thesis at the Department of Ecological Microbiology (Gebelein 2011) that was elaborated and supervised by myself. Julia Gebelein tested DNA and cDNA samples for the detectability of *nirK* and *nirS* genes, and generated *nirK* and *nirS* gene sequence libraries (2.5.9) that were analyzed by her. Thereafter, *nirS* sequences were re-evaluated and analyzed by myself for dissertation.

2.7.1.2.3. Isolation of denitrifiers from earthworm guts

All bacterial strains were isolated (2.3.2.1), and 16S rRNA gene fragments were amplified (2.5.7.3) and evaluated by myself. Sarah Muszynski conducted basic physiological analyses with Isolate 201 and Isolate 208 (2.3.2.2) during her bachelor thesis at the Department of Ecological Microbiology that was elaborated and supervised by myself (Muszynski 2012). Sarah Muszynski generated additional sequences of 16S rRNA gene fragments of more than 1,000 bp size for Isolate 201, Isolate 208, Isolate 403, Isolate 823, and Isolate ISO4 (2.5.12.2), and tested Isolate 201 and Isolate 208 for the appearance of gene markers for denitrification and dissimilatory nitrate reduction (2.5.7.2.2). Results were re-evaluated and analyzed by myself for dissertation.

2.7.1.3. Earthworms from New Zealand

Earthworms and soils were sampled (2.1.3), and DNA was extracted from earthworm gut contents and soils (2.5.1) by Pia K. Wüst during her PhD thesis at the Department of

Ecological Microbiology (Wüst 2010). All *nosZ* gene fragments were amplified (2.5.7.2.3) and clone sequences were sent for sequencing by Pia K. Wüst. Thereafter, sequence analyses, diversity analyses, and phylogenetic analyses were conducted by myself.

2.7.2. Methanogenesis

All earthworms, soils, and substrates were sampled or purchased (2.1.1) by myself or together with Prof. George G. Brown (Embrapa Florestas, Colombo, Brazil). Earthworm species were identified by Prof. George G. Brown. All gas experiments (2.2) were conducted by myself. Gene and transcript sequence libraries of *mcrA*/*mrtA* (2.5.7.2.1, 2.5.9) were generated together with Sindy Hunger (Department of Ecological Microbiology). Soil properties (2.4.2) were determined by the Soil Analysis Laboratory of the University of São Paulo, Brazil. Sequence analyses of *mcrA*/*mrtA* (2.5.12) were conducted by Sindy Hunger. Sequences were used for the calculation of relative distributions by myself. Inoculation and first enrichment steps for the isolation of methanogens from the gut of *E. eugeniae* (2.3.2.3) were conducted by myself. Consecutive enrichment steps with serial dilutions (2.3.2.3) including the results displayed were conducted together with and predominantly by Sindy Hunger. Concomitant T-RFLP analyses of *mcrA*/*mrtA* genes (2.5.8.1) were conducted by Linda Hink (Department of Ecological Microbiology) but elaborated and supervised by myself.

3. RESULTS

3.1. Emission of nitrogenous gases by earthworms and analysis of associated microorganisms in the earthworm gut

3.1.1. Earthworms from Brazil

Small earthworm species belonging to the family Lumbricidae from Germany and New Zealand representing all three feeding guilds are known to emit denitrification-derived nitrogenous gases, i.e., N_2O and N_2 (1.4.5). The large *O. multiporus* from New Zealand (Megascolecidae) emits no N_2O *in vivo* although its gut displays a high denitrification potential (Wüst *et al.* 2009b). Thus, knowledge about the emission of nitrogenous gases by earthworms is restricted to the family Lumbricidae and one representative of the family Megascolecidae. The influence of the earthworm size and feeding guild on the release of nitrogenous gases and on the diversity and activation of ingested denitrifiers and dissimilatory nitrate reducers remains largely unresolved. Thus, earthworms of different families, feeding guilds, and sizes were sampled near Piracicaba (State of São Paulo, Brazil) along with their soils/substrates (2.1.1), and analyzed for the emission of N_2O and N_2 (2.2). Earthworm species with contrasting gas emission, ecological, taxonomical, and physiological features were analyzed for genes indicative of denitrification and dissimilatory nitrate reduction (2.5.11).

3.1.1.1. Earthworm species sampled in Brazil

Altogether, ten earthworm species were sampled that represent five different families and were of different sizes and different feeding guilds (2.1.1.1); the worms were obtained along with their soils/substrates (2.1.1.1, Table 14, Table 1). Analyzed families and corresponding species were Glossoscolecidae (*Glossoscolex paulistus*, *Glossoscolex* sp., *Pontoscolex corethrurus*, and *Rhinodrilus alatus*), Megascolecidae (*Amynthas gracilis* and *Perionyx excavatus*), Acanthodrilidae (*Dichogaster annae* and *Dichogaster* sp.), Eudrilidae (*Eudrilus eugeniae*), and Lumbricidae (*Eisenia andrei*) (Table 14). The species *D. annae*, *Dichogaster* sp., *E. andrei*, *E. eugeniae*, and *R. alatus* were purchased from an earthworm distributor or earthworm collector along with soil/substrate (2.1.1.1). *G. paulistus*, *Glossoscolex* sp., and *R. alatus* are in the following termed as large, all other species as small species.

3.1.1.2. Emission of N_2O and N_2 by earthworms and soils

3.1.1.2.1. *In vivo* emission of N_2O and N_2 by earthworms and soils

When incubated under ambient air (2.2.1), earthworm species of the families Glossoscolecidae, Megascolecidae, Acanthodrilidae, and Eudrilidae emitted nitrogenous gases *in vivo*; *E. andrei* (Lumbricidae) did not (Table 14). Altogether, seven species belonging to endogeic and epigeic feeding guilds emitted *in vivo* N_2O up to 10.7 nmol N_2O per g fresh weight (nmol N_2O [g fw]$^{-1}$) by *A. gracilis* after 9 h of incubation (Table 14). The small *E. andrei* did not emit N_2O whereas all other small species did. The two large *Glossoscolex* species did not emit N_2O whereas the very large *R. alatus* did (Table 14). Thus, N_2O emissions were prevalent and absent for both large and small sized earthworm species of different earthworm families and different feeding guilds, indicating that one of these factors alone appears not to be the determining factors for the emission of N_2O.

Acetylene inhibits the N_2O reductase (Yoshinari & Knowles 1976). Thus, applying acetylene to a denitrifying community results in the emission of additional N_2O that would be converted to N_2 if N_2O reductase was not inhibited. This allows the calculation of N_2 being normally produced by N_2O reductases. If determined, all earthworm species emitting N_2O also emitted N_2. In addition, *G. paulistus* emitted minor amounts of N_2. *R. alatus* emitted the highest amounts of N_2, i.e., 67.2 nmol N_2 (g fw)$^{-1}$ after 6 h of incubation. *Dichogaster* sp. and *P. corethrurus* were the only two species that emitted greater amounts of N_2O than of N_2 (Table 14). More often than not, the emission of N_2O by earthworms was essentially higher than by the corresponding soil or substrate. For all soils and substrates tested, N_2 emissions exceeded N_2O emissions and were therefore the main nitrogenous gas released (Table 14).

Table 14: Emission of N_2O and N_2 by living earthworms, soils, composted cow manure, and composted sugarcane residues.

						Gas emission after 5h (03/2011), 6h (09/2011), or 9h (11/2010) (nmol [g fw]$^{-1}$)			
						N_2O		N_2[e]	
Material (feeding guild[a])	Sampling date	Earthworm substrate	Length (cm)	Weight (g)	*n*[b]	Mean[c]	SD[d]	Mean	SD
Earthworms									
Amynthas gracilis (epi-endogeic)	11/2010	Grassland soil	7.0-11.6	0.8-1.5	3	10.7	(5.3)	n.d.[f]	
	03/2011	Grassland soil	8.2-12.9	2.3-5.0	3	0.6	(0.4)	7.7	(8.7)
Dichogaster annae (epigeic)	03/2011	Composted cow manure	4.3-5.2	0.13-0.14	3	0.4	(0.6)	2.0	(0.7)
Dichogaster sp. (epigeic)	03/2011	Composted cow manure	4.0-5.0	0.13-0.14	3	1.7	(0.6)	0.1	(2.9)
Eisenia andrei (epigeic)	09/2011	Composted sugarcane 1	4.6-6.8	0.35-0.74	5	-0.2	(0.5)	n.d.	
Eudrilus eugeniae (epigeic)	03/2011	Composted cow manure	11.5-18.3	2.5-3.3	3	4.1	(1.3)	13.3	(2.1)
	09/2011	Composted cow manure	7.8-12.5	0.9-1.5	5	0.4	(0.8)	n.d.	
	09/2011	Composted sugarcane 2	8.9-12.7	1.3-2.0	5	6.1	(6.6)	n.d.	
Glossoscolex paulistus (endo-anecic)	11/2010	Pasture soil	23.3-31.4	16.1-27.2	3	-0.3	(0.2)	n.d.	
	03/2011	Pasture soil	20.7-33.7	14.1-26.3	7	-0.1	(0.2)	0.2	(0.4)
Glossoscolex sp. (endogeic)	03/2011	Meadow soil	20.3-29.3	2.9-4.4	3	-0.1	(0.4)	0.0	(0.6)
Perionyx excavatus (epigeic)	03/2011	Composted cow manure	10.7-17.0	1.3-3.3	3	1.4	(0.5)	6.8	(4.3)
Pontoscolex corethrurus (endogeic)	11/2010	Grassland soil	5.9-11.0	0.6-1.2	3	11.6	(2.8)	n.d.	
	03/2011	Grassland soil	6.4-12.8	0.7-1.6	3	5.5	(3.7)	3.9	(8.7)
Rhinodrilus alatus (endogeic)	03/2011	Unknown soil	38.5-62.8	30.3-43.7	3	1.9	(0.2)	67.2	(29.2)

Sequel to Table 14.

Material	Sampling date	n[b]	Gas emission after 5h (03/2011), 6h (09/2011), or 9h (11/2010) (nmol [g fw]$^{-1}$)			
			N_2O		N_2[e]	
			Mean[c]	SD[d]	Mean	SD
Substrate of earthworms						
Grassland soil (Substrate 4)	03/2011	3	0.1	(0.0)	1.4	(0.3)
Pasture soil (Substrate 5)	03/2011	3	0.1	(0.1)	3.2	(2.2)
Meadow soil (Substrate 6)	03/2011	3	0.0	(0.0)	1.3	(0.5)
Unknown soil (Substrate 7)	03/2011	3	3.2	(1.8)	5.2	(7.3)
Composted cow manure (Substrate 1)	03/2011	3	-0.1	(0.1)	6.6	(1.6)
	09/2011	3	-0.1	(0.0)	n.d.	
Composted sugarcane 1 (Substrate 2)	09/2011	3	0.4	(0.3)	n.d.	
Composted sugarcane 2 (Substrate 3)	09/2011	3	1.0	(0.9)	n.d.	

[a] according to James and Guimarães (2010), Barois *et al.* (1999), and Brown GG (*pers. comm.).*

[b] *n*, number of replicates (one specimen per replicate; for *D. annae* and *Dichogaster* sp., ten specimens were used per replicate).

[c] Mean, average of replicate values.

[d] SD, standard deviation.

[e] N_2, denitrification-derived N_2 was calculated as the difference of N_2O of incubations with and without acetylene (20 % vol/vol).

[f] n.d., not determined.

[g] Enumeration of substrates according to Table 1.

There were two different substrates composed of composted sugarcane, i.e., composted sugarcane 1 and composted sugarcane 2.

Abbreviations: fw, fresh weight.

Modified from Depkat-Jakob *et al.* (2013).

3.1.1.2.2. Selection of earthworm species with contrasting features

The large *G. paulistus* (Glossoscolecidae; up to 34 cm long and 27 g; sampled from pasture soil) representing the endo-anecic feeding guild (Table 14) emitted no N_2O *in vivo* and only minor amounts of N_2; the small *A. gracilis* (Megascolecidae; up to 13 cm long and 5 g; sampled from grassland soil) representing the epi-endogeic feeding guild (Table 14) emitted *in vivo* high amounts of N_2O and N_2 in a relatively linear manner (Figure 9). Thus, these two species and their soils were selected for analyses of soil properties, denitrification capacities, and molecular detection of denitrifiers and dissimilatory nitrate reducers via genetic markers.

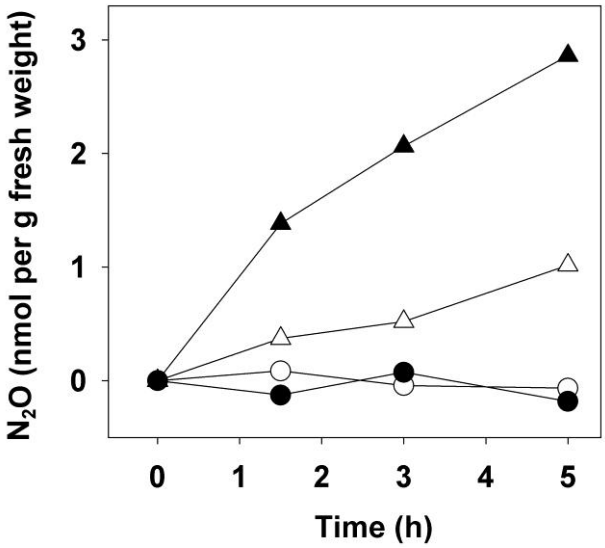


Figure 9: *In vivo* emission of N_2O by representative specimens of *G. paulistus* and *A. gracilis*. Earthworms were sampled in March 2011. Triangles, *A. gracilis*; circles, *G. paulistus*; empty symbols, headspace was ambient air; filled symbols, headspace was ambient air + acetylene (20 % v/v); see methods parts (2.2.1) and (2.2.1.1) for further information. Modified from Depkat-Jakob *et al.* (2013).

3.1.1.2.3. Properties of the soils *G. paulistus*, *A. gracilis* and *P. corethrurus* were sampled from

Both soils, i.e., pasture soil for *G. paulistus* and grassland soil for *A. gracilis* and *P. corethrurus* were slightly acidic. The grassland soil showed higher concentrations for all compounds measured, i.e., ammonia, nitrate, total organic carbon, total organic material, and total nitrogen (Table 15). However, differences were not fundamental and both soils contained nitrate, the electron acceptor used by both denitrifiers and dissimilatory nitrate reducers (Zumft 1997).

Table 15: Properties of soils sampled along with *G. paulistus*, *A. gracilis*, and *P. corethrurus*.

Material	pH (H_2O)	NH_4^+ (mg [kg fw]$^{-1}$)	NO_3^- (mg [kg fw]$^{-1}$)	Total organic carbon (g [kg fw]$^{-1}$)	Total organic material (g [kg fw]$^{-1}$)	Total nitrogen (g [kg fw]$^{-1}$)
Pasture soil[a]	6.2	15	14	14	20	1.23
Grassland soil[b]	6.5	24	24	26	44	2.27

[a] *G. paulistus* was sampled from this soil; Substrate 5 (Table 1).

[b] *A. gracilis* and *P. corethrurus* were sampled from this soil; Substrate 4 (Table 1).

Modified from Depkat-Jakob *et al.* (2013).

3.1.1.2.4. Effect of nitrite on the *in vivo* emission of N_2O and N_2 by *G. paulistus* and *A. gracilis*

It is known that applying nitrite to living earthworms, guts, or gut contents can significantly stimulate the emission of denitrification-derived nitrogenous gases and can be used to determine the denitrification potential of earthworms (Matthies *et al.* 1999, Wüst *et al.* 2009b). Wetting of *G. paulistus* with nitrite (2.2.1.1) resulted in an emission of approximately 5 nmol N_2O (g fw)$^{-1}$ and 10 nmol N_2 (g fw)$^{-1}$ in a 5 h-incubation whereas untreated specimens displayed no and minor emission of N_2O and N_2, respectively (Figure 10A, Table 14). This demonstrates that *G. paulistus* had the potential do denitrify. Nitrite greatly stimulated the emission of nitrogenous gases by *A. gracilis* resulting in approximately 92 nmol N_2O (g fw)$^{-1}$ and 33 nmol N_2 (g fw)$^{-1}$ in a 5 h-incubation (Figure 10B). Thus, on a per g fresh weight basis, *A. gracilis* emitted about one order of magnitude more nitrogenous gases than *G. paulistus* did when wetted with nitrite.

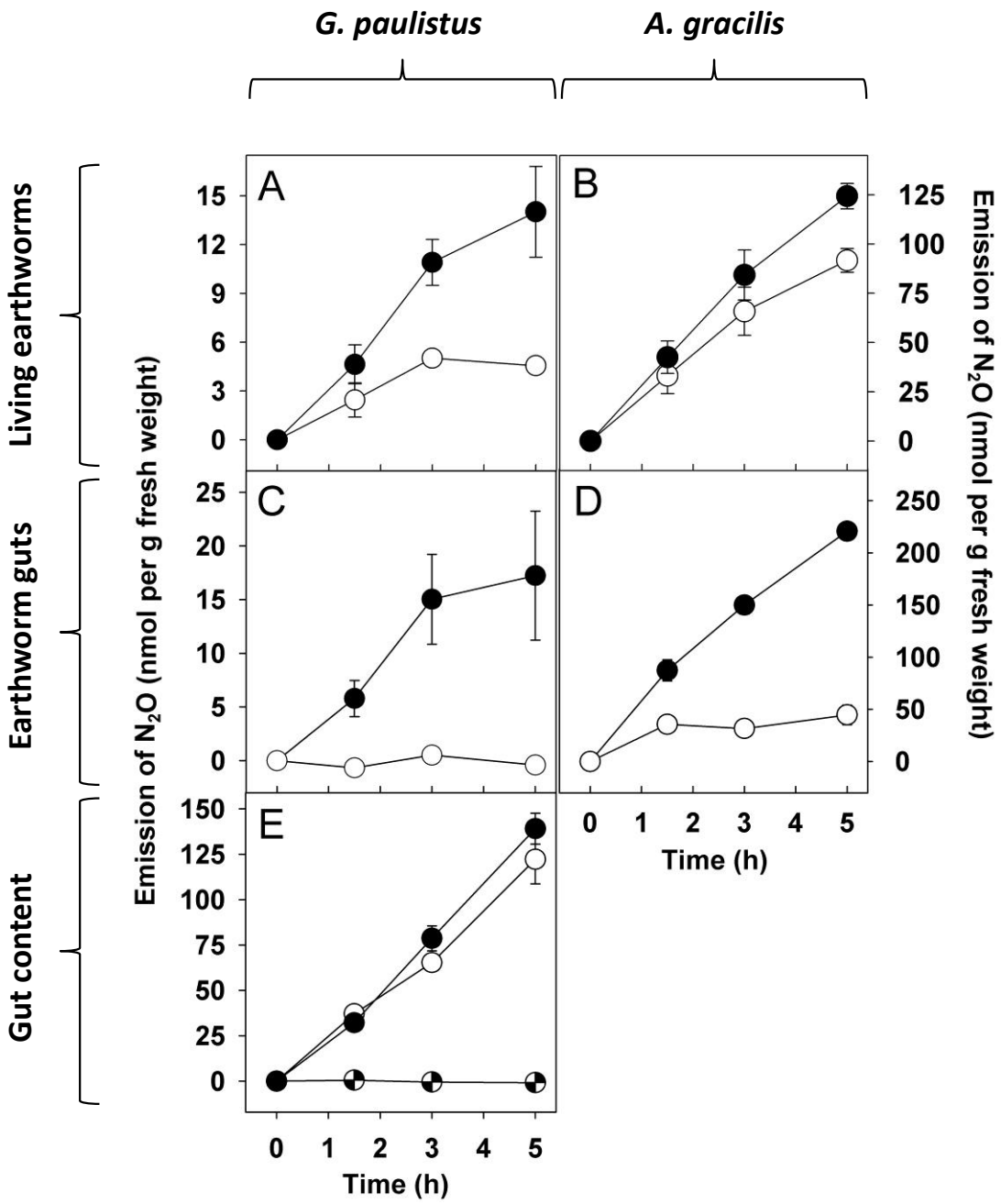


Figure 10: Emission of N_2O by living earthworms, dissected earthworm guts, and gut contents. A, B: gas phase was ambient air; empty circles, earthworm + nitrite; filled circles, earthworm + nitrite + acetylene. C, D: gas phase was argon and acetylene; empty circles, dissected gut; filled circles, dissected gut + nitrite. E: gas phase was argon and acetlene; checked circles, earthworm gut content; empty circles, earthworm gut content + nitrite; filled circles, earthworm gut content + nitrite + glucose + acetate. See methods part (2.2) for detailed information. Specimens for A to D were sampled in November 2010, specimens for E were sampled in March 2011. Experiments were conducted in triplicates; error bars indicate standard deviations but are not always visible due their diminutive size. Modified from Depkat-Jakob *et al.* (2013).

3.1.1.2.5. Emission of N_2O by dissected guts of *G. paulistus* and *A. gracilis*

The dissected gut of *G. paulistus* (2.2.2) emitted no N_2O when incubated under an argon atmosphere containing 20 % acetylene (v/v) only. Additional nitrite resulted in an emission of approximately 17 nmol N_2O (g fw)$^{-1}$ in a 5 h-incubation (Figure 10C). In contrast, the dissected gut of *A. gracilis* emitted approximately 45 nmol N_2O (g fw)$^{-1}$ when incubated under an argon atmosphere containing 20 % acetylene (v/v) only, and approximately 221 nmol N_2O (g fw)$^{-1}$ when incubation was with additional nitrite (Figure 10D). Again, on a per g fresh weight basis, *A. gracilis* emitted about one order of magnitude more nitrogenous gases than *G. paulistus* did when wetted with nitrite.

3.1.1.2.6. Emission of N_2O by gut contents of *G. paulistus*

As with whole guts (Figure 10C), gut contents of *G. paulistus* (2.2.3) emitted no N_2O when incubated under an argon atmosphere containing 20 % acetylene (v/v) only. Additional nitrite resulted in a nearly linear production of N_2O up to approximately 122 nmol N_2O (g fw)$^{-1}$ in a 5 h-incubation (Figure 10E). Incubation with supplemental glucose and acetate next to nitrite did not significantly increase the emission of N_2O (Figure 10E), indicating that denitrification in the gut of *G. paulistus* was not limited for the carbon sources applied. The gut content of *A. gracilis* could not be analyzed because a sufficient number of specimens was not available.

3.1.1.3. Analysis of gene markers indicative of denitrification and dissimilatory nitrate reduction in the gut contents and soils of *G. paulistus* and *A. gracilis*

A. gracilis displayed a high *in vivo* emission of nitrogenous gases whereas *G. paulistus* did not emit significant amounts of nitrogenous gases. However, the guts of these two species had the capacity to denitrify, albeit the capacity of guts to denitrify was greater for *A. gracilis* than for *G. paulistus* (Figure 10). Thus, their gut contents and corresponding soils (2.2.4) were analyzed for the occurrence and composition of genes indicative of denitrification and dissimilatory nitrate reduction (2.5.8, 2.5.12). The analyzed genes encode for the enzyme or a subunit of a dissimilatory nitrate reductase (*narG*), nitrite reductases (*nirK* and *nirS*), and N_2O reductase (*nosZ*) catalyzing the denitrification pathway (Zumft 1997) (1.2.1.1).

3.1.1.3.1. Criteria for assigning *nirK* and *nirS* sequences to operational taxonomic units

When analyzing sequences of functional genes instead of 16S rRNA genes, it is crucial to define an empiric cutoff value that defines if two sequences are probably derived from two different species or belong to the same species or OTU (Purkhold *et al.* 2000). Values used for analyzing *narG* and *nosZ* were as published (Palmer *et al.* 2009). Values for *nirK* and *nirS* were not available and were therefore calculated *in silico* (2.5.12.1) prior to gene analyses in the earthworm gut and soil.

3.1.1.3.1.1. Phylogenetic correlation plots and comparative tree topologies of *nirK* and corresponding 16S rRNA genes

For *nirK*, phylogenetic correlation plots were constructed with 74 *nirK* sequences together with 73 corresponding 16S rRNA sequences (*Pseudomonas palustris* TIE-1 contained two copies of *nirK*) for both *nirK* gene and *in silico* translated *nirK* amino acid sequences (2.5.12.1, Figure 11). Linearity between 16S rRNA gene similarity and both *nirK* gene and amino acid similarity was particularly apparent for a 16S rRNA gene similarity of about ≥ 90 % (Figure 11). Some distantly related organisms (i.e., with a 16S rRNA gene similarity between 78 % and 83 %) carried highly similar *nirK* genes (i.e., their *nirK* gene and amino acid sequences were 90 % to 100 % identical). This feature was more pronounced for amino acid sequences (Figure 11B) than for gene sequences (Figure 11A).

Of all organisms with a ≥ 97 % 16S rRNA gene similarity, 90 % had a *nirK* similarity of ≥ 83 % (Figure 11A) and a *nirK in silico* translated amino acid sequence similarity of ≥ 91 % (Figure 11B). Thus, 83 % was defined as a cutoff value to create *nirK* gene sequence species-level OTUs, i.e., a dissimilarity of two *nirK* gene sequences of 17 %. For *nirK* amino acid sequences, this cutoff value was 91 %, i.e., a dissimilarity of 9 % between two *nirK* amino acid sequences. Both cutoff values are conservative estimates that indicate a minimum amount of species-level OTUs that can be expected.

Comparison of 16S rRNA gene phylogeny and *nirK* gene phylogeny showed that some taxa were separated in both phylogenetic trees (e.g., clusters 2 to 5) whereas other taxa were separated in the 16S rRNA tree only but clustered together in the *nirK* gene tree (e.g., cluster 1 and clusters 6 to 9) (Figure 12). The two *nirK* gene copies of *Pseudomonas palustris* TIE-1 clustered closely together in the phylogenetic tree (Figure 12B).

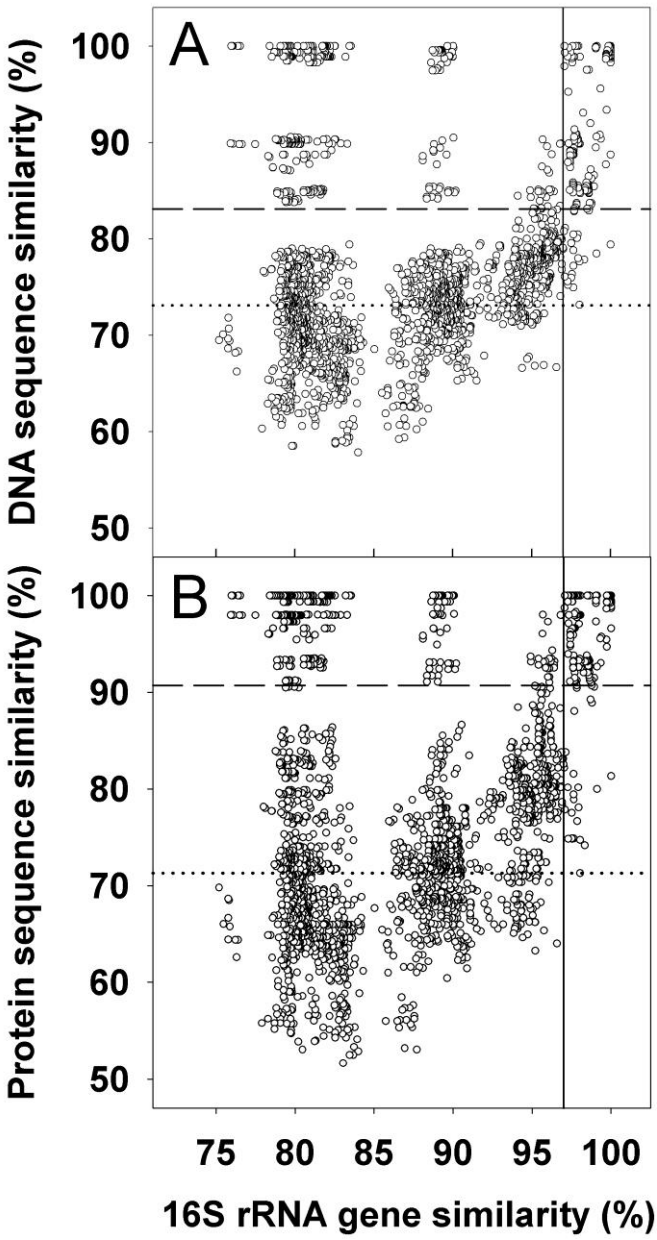


Figure 11: Phylogenetic correlation plots of gene (A) and *in silico* translated amino acid sequence (B) similarities of *nirK* versus 16S rRNA gene similarity.

Dotted vertical lines show the similarity values, below which two sequences always had less than 97 % 16S rRNA gene sequence similarity. Dashed vertical lines show the 90 % quantile of pairwise sequence comparisons with a 16S rRNA gene sequence similarity of at least 97 % (i.e., threshold similarity). The solid vertical lines show the 97 % 16S rRNA gene similarities. Modified from Depkat-Jakob *et al.* (2013).

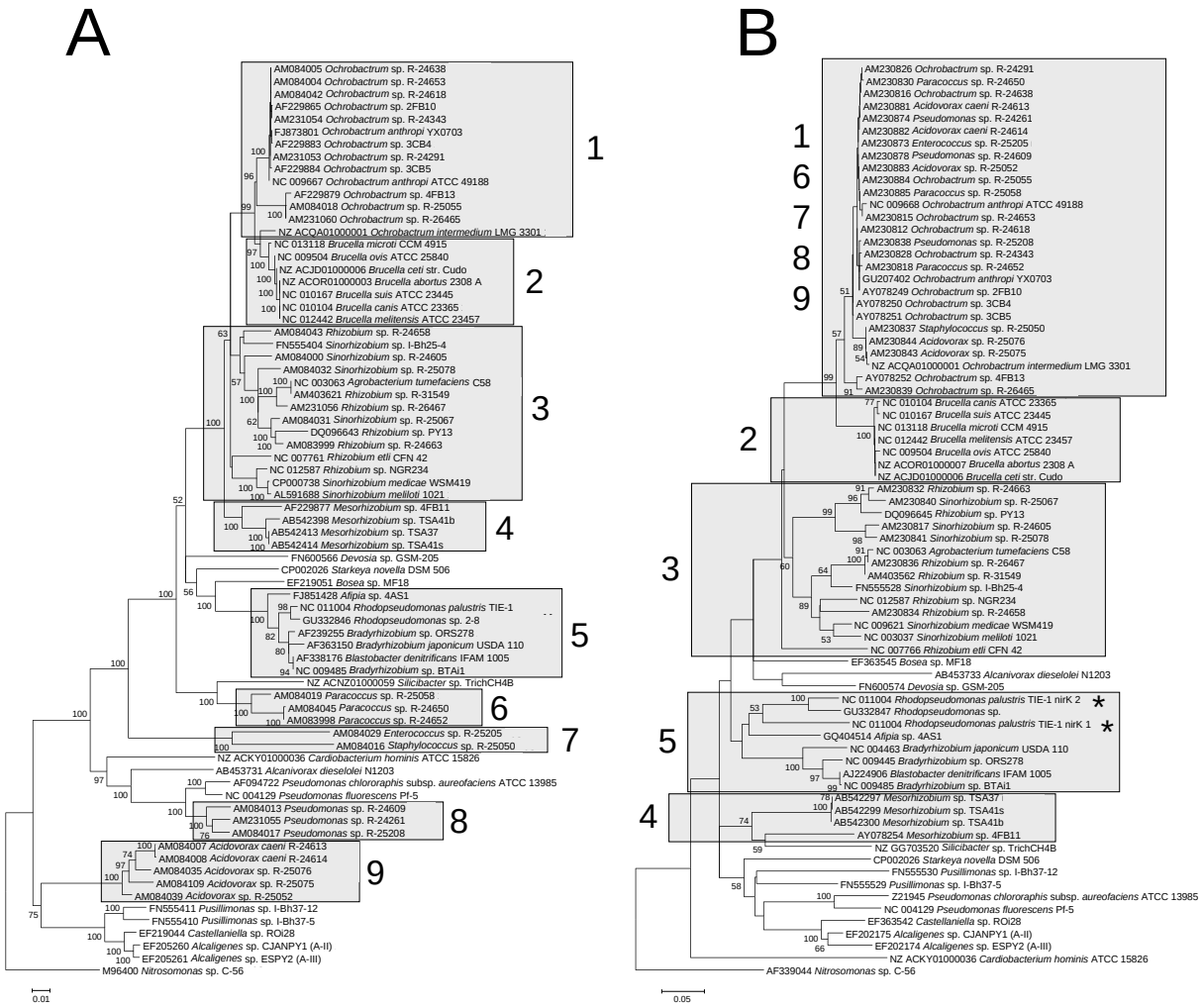


Figure 12: Comparison of 16S rRNA gene (A) and *nirK* (B) phylogenies of different species.
Neighbor-joining trees of 16S rRNA gene (A) and *nirK* gene (B) sequences fragments were constructed. The percentage of replicate trees the associated taxa clustered together in the bootstrap test (10,000 replicates), are shown at the node of two branches (values below 50 % are not displayed). Numbers indicate the clustering of representative taxa in both trees. The 16S rRNA gene based taxa 1 and 6 to 9 cluster together in the *nirK* based tree. The asterisks indicate the two *nirK* copies of *Rhodopseudomonas palustris* TIE-1. The bars represent an estimated sequence dissimilarity of 0.01 (A) and 0.05 (B).

3.1.1.3.1.2. Phylogenetic correlation plots and comparative tree topologies of *nirS* and corresponding 16S rRNA genes

For *nirS*, phylogenetic correlation plots were constructed with 96 *nirS* sequences together with 95 corresponding 16S rRNA sequences (*Thauera* sp. 27 contained two copies of *nirS*) for both *nirS* gene and *in silico* translated *nirS* amino acid sequences (2.5.12.1, Figure 13). Linearity between 16S rRNA gene similarity and both *nirS* gene and amino acid

similarity was particularly apparent for a 16S rRNA gene similarity of about ≥ 90 % (Figure 13). The amount of distantly related organisms (i.e., with a 16S rRNA gene similarity ≤ 85 %) carrying highly similar *nirS* genes (i.e., their *nirS* sequences were 90 % to 100 % identical) was negligible for both *nirS* gene and amino acid sequences (Figure 13).

Of all organisms with a ≥ 97 % 16S rRNA gene similarity, 90 % had a *nirS* similarity of ≥ 82 % (Figure 13A) and a *nirS in silico* translated amino acid sequence similarity of ≥ 87 % (Figure 13B). Thus, 82 % was defined as a cutoff value to create *nirS* gene sequence species-level OTUs, i.e., a dissimilarity of two *nirS* gene sequences of 18 %. For *nirS* amino acid sequences, this cutoff value was 87 %, i.e., a dissimilarity of 13 % between two *nirS* amino acid sequences. Both cutoff values are conservative estimates that indicate a minimum amount of species-level OTUs that can be expected.

Comparison of 16S rRNA gene phylogeny and *nirS* gene phylogeny showed that some taxa were completely separated in both phylogenetic trees (e.g., clusters 2, 3, and 5) whereas other taxa were separated in the 16S rRNA tree only but clustered together in the *nirS* gene tree (e.g., clusters 1, 7, and 9) (Figure 14). Single sequences of some taxa clustered together in the 16S rRNA gene based tree but were split in the *nirS* gene tree (e.g., clusters 4 to 6) (Figure 14B). The two *nirS* gene copies of *Thauera* sp. 27 were placed in two distinct clusters (clusters 4a and 4c) (Figure 14B).

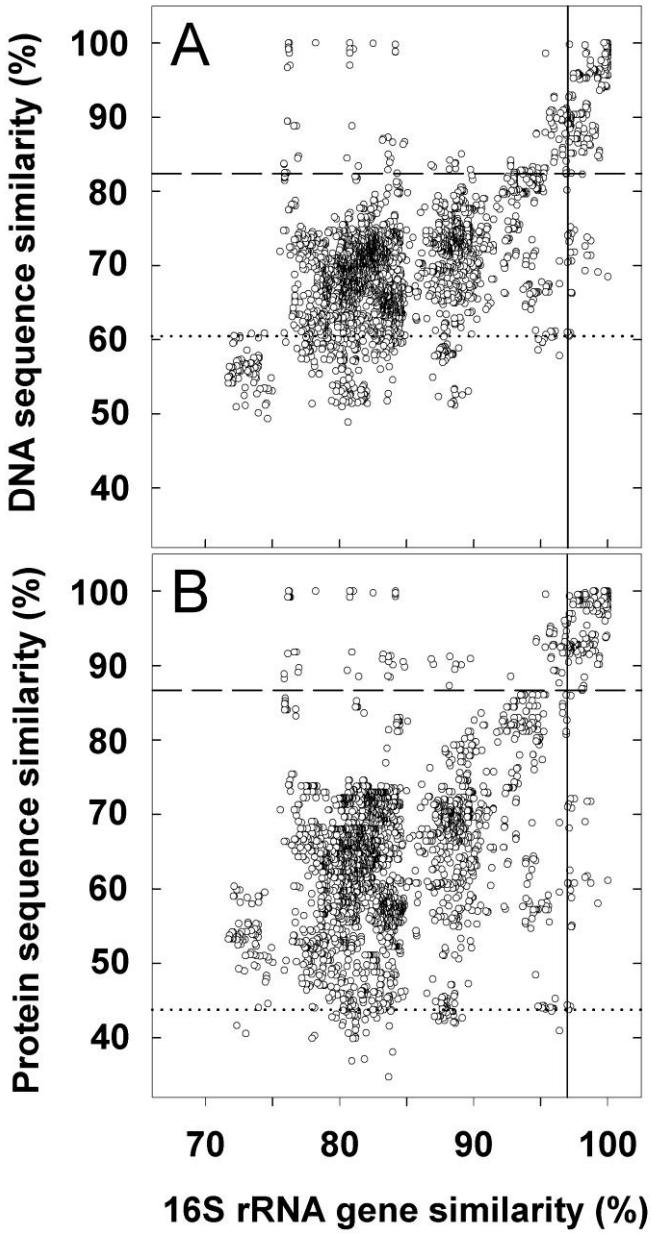


Figure 13: Phylogenetic correlation plots of gene (A) and *in silico* translated amino acid sequence (B) similarities of *nirS* versus 16S rRNA gene similarity.

Dotted vertical lines show the similarity values, below which two sequences always had less than 97 % 16S rRNA gene sequence similarity. Dashed vertical lines show the 90 % quantile of pairwise sequence comparisons with a 16S rRNA gene sequence similarity of at least 97 % (i.e., threshold similarity). The solid vertical lines show the 97 % 16S rRNA gene similarities.

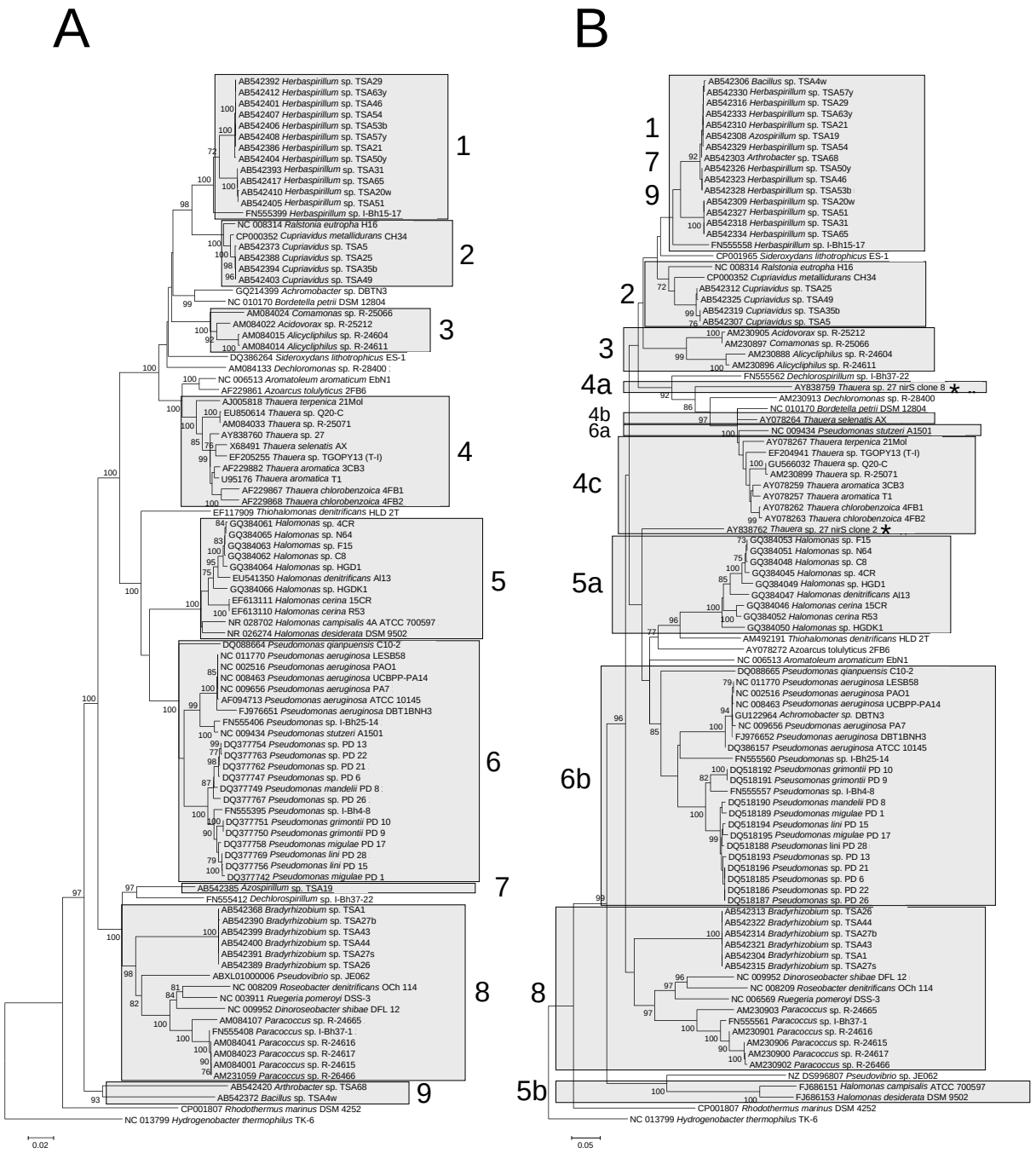


Figure 14: Comparison of 16S rRNA gene (A) and *nirS* (B) phylogenies of different species.

Neighbor-joining trees of 16S rRNA gene (A) and *nirS* gene (B) sequences were constructed. The percentage of replicate trees the associated taxa clustered together in the bootstrap test (10,000 replicates), are shown at the node of two branches (values below 50 % are not displayed). Numbers indicate the clustering of representative taxa in both trees; some 16S rRNA gene based taxa are split and therefore indicated with a, b, and c after the number in the *nirS* based tree.The asterisks indicate the two *nirS* copies of *Thauera* sp. 27. The bars represent an estimated sequence dissimilarity of 0.02 (A) and 0.05 (B).

3.1.1.3.2. Denitrifiers and dissimilatory nitrate reducers detected via *narG* in gut contents and soils of *G. paulistus* and *A. gracilis*

For gut contents and soils of *G. paulistus* and *A. gracilis*, genes were amplified from extracted DNA (2.5.7.2.1) and used for barcoded amplicon pyrosequencing (2.5.11). Coverage (2.5.12.6) and diversity (2.5.12.7, 2.5.12.8) were calculated with gene sequences (2.5.12.3) whereas phylogenetic analyses (2.5.12.9) were calculated with *in silico* translated amino acid sequences.

3.1.1.3.2.1. Barcoded amplicon pyrosequencing and diversity analysis of *narG*

For *narG*, altogether 7,809 valid sequences were retrieved, yielding 15, 28, 27, and 17 OTUs at a species-level cutoff of 67 % (i.e., on nucleotide sequence level) for the libraries from gut contents of *G. paulistus*, gut contents of *A. gracilis*, soil of *G. paulistus*, and soil of *A. gracilis*, respectively (Table 16). The species-level cutoff value for *narG* of 67 % is a very conservative estimate; the real number of species represented by *narG* sequences is assumed to be higher. However, the cutoff value used displays the minimum amount of OTUs and was the standard of comparison between the analyzed samples. Coverages ranged between 99.0 % and 100 %, indicating that sampling was sufficient. Estimated richness was highest in gut contents of *A. gracilis* with 34 OTUs, whereas for the other libraries, estimated richness was only slightly higher (gut of *G. paulistus* and soil of *A. gracilis*) or the same (soil of *G. paulistus*) as already sampled (Table 16). Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) of gut-derived *narG* sequences were always higher than those of the corresponding soil. This is indicative of a more broad than selective stimulation of soil-derived nitrate reducers in the earthworm gut.

Table 16: Sequence qualities, OTUs, coverages, and diversity indices of *narG* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*.

	Sequences				Diversity indices			
Library[a]	Valid[b]	Invalid[c]	OTUs[d]	C[e] (%)	Chao1[f]	H´[g]	E[h]	1/D[i]
Gut *GP*	405	16	15	99.0	17	1.66	0.61	3.85
Gut *AG*	1,486	57	28	99.7	34	1.78	0.53	3.07
Soil *GP*	4,985	45	27	100	27	1.49	0.45	2.79
Soil *AG*	933	22	17	99.6	19	1.39	0.49	2.79

[a] *GP*, *G. paulistus*; *AG*, *A. gracilis*.

[b] Sequences encoding for the desired gene as verified by BLAST analysis. Potential chimeras were excluded.

[c] Sequences that were discarded for further analyses as encoding not for the desired gene or representing potential chimeras.

[d] Species-level OTUs of valid sequences. Cutoff value was 67 %.

[e] Coverage.

[f] Chao1 richness estimator.

[g] Shannon diversity index.

[h] Evenness.

[i] Reciprocal Simpson diversity index.

See the methods part for further information (2.5.12).

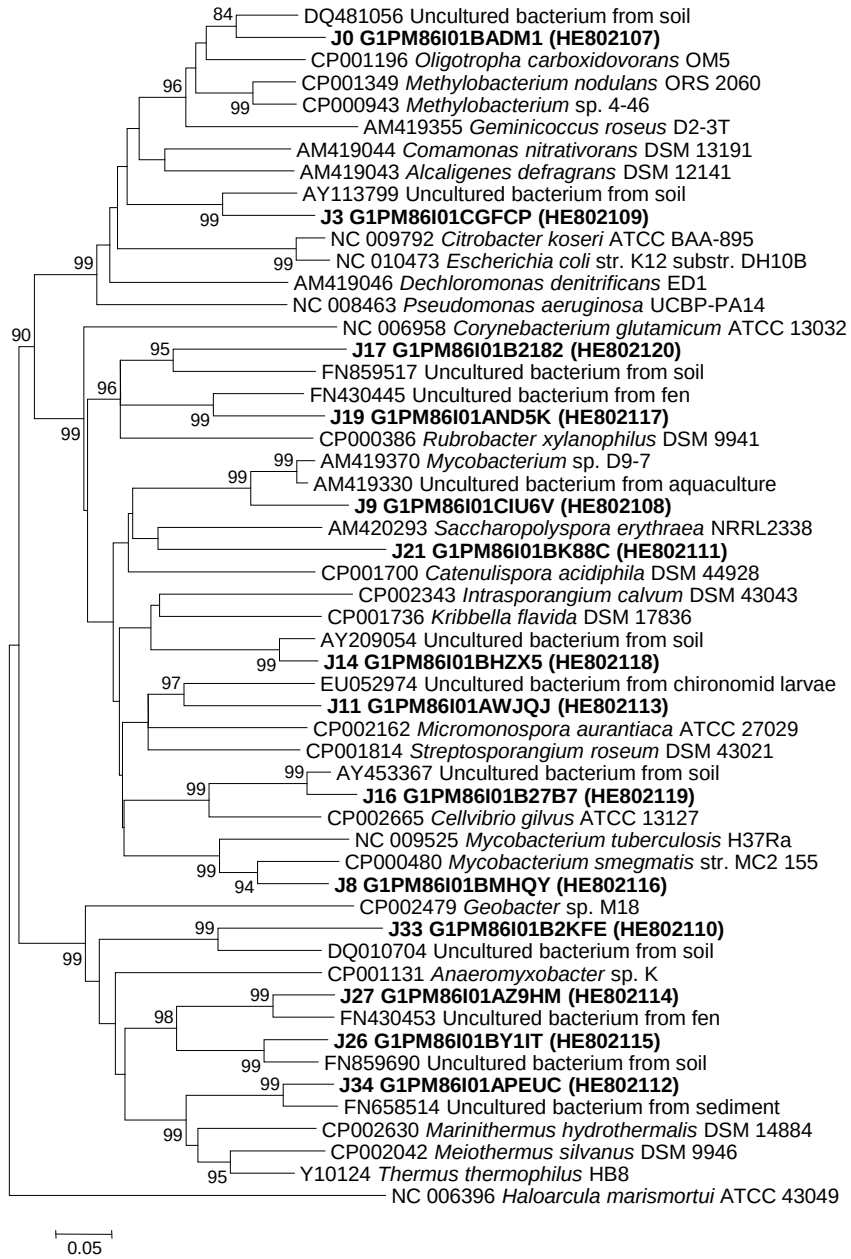
Modified from Depkat-Jakob *et al.* (2013).

3.1.1.3.2.2. Phylogenetic analysis of *narG*

The *narG* sequences of OTU 1 were most closely affiliated with *Methylobacterium* sp. 4-46 and *Oligotropha carboxidovorans* OM5, both members of the *Bradyrhizobiaceae* within the *Rhizobiales* (Garrity *et al.* 2005, Sadowsky & Graham 2006). Related sequences were abundantly detected in the libraries derived from the gut contents of *G. paulistus*, gut contents of *A. gracilis*, and soil of *G. paulistus*. Sequences derived from the soil of *A. gracilis* were predominantly affiliated with OTU 4 that was distantly related to *Anaeromyxobacter* sp. K (Figure 15, Figure 16). Sequences related to *Mycobacterium* sp. D9-7 (OTU 2) and *Saccharopolyspora erythraea* NRRL2338 (OTU 5), both members of the *Actinobacteria*, were dominant in both gut content and soil-derived libraries of *G. paulistus*, but did not exceed 1 % relative abundance in the libraries from gut content and soil of *A. gracilis*. Sequences related to *Micromonospora aurantiaca* (OTU 7) were abundantly detected in the gut contents of *A. gracilis* but were virtually absent in all other libraries (Figure 15, Figure 16).

Most *narG* sequences occurred in OTUs that contained both soil-derived and gut-derived sequences, except for the quantitatively minor OTUs 10 and 13 (Figure 15). This indicates that the majority of gut-derived sequences originated from ingested soil *Bacteria*.

Altogether, gut and soil-derived *narG* sequences of *G. paulistus* were more similar to each other than to gut and soil-derived sequences of *A. gracilis* (Figure 16, Figure 17). Despite of the detected differences in *narG* diversity, differences between *narG* libraries were not significant except for the soil library of *A. gracilis* that showed significant differences to all other libraries (Table A 1).



OTU	Relative abundance of speciel-level OTUs (%)			
	Gut *GP*	Gut *AG*	Soil *GP*	Soil *AG*
1	42	55	56	27
3	1	2	14	>0
14	0	1	>0	>0
11	>0	2	>0	1
2	14	1	13	1
5	24	1	9	>0
12	0	1	>0	>0
7	0	10	>0	>0
13	>0	1	>0	0
10	3	>0	1	0
4	10	7	2	53
8	3	2	1	5
9	>0	1	1	5
6	0	11	>0	6
	98	95	99	98

Figure 15: Phylogenetic neighbor-joining tree of representative *narG* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*, and related sequences.

The phylogenetic tree is based on *in silico* translated amino acid sequences. OTUs that accounted for at least 1 % in at least one library are shown with one representative sequence (bold, with accession numbers in parentheses). The percentage of replicate trees the associated taxa clustered together in the bootstrap test (10,000 replicates), are shown at the node of two branches (values below 80 % are not displayed). The table shows the relative distribution of sequences in each OTU (>0 indicates that at least one sequence was detected but its relative abundance was below 1%). Numbers at the bottom of the table indicated the sums of percentages of sequences for each library covered by the OTUs shown in the tree. Differences between the sum and the combined percentage of the individual OTU percentages for one library are due to the rounding off of values. *GP*, *G. paulistus*; *AG*, *A. gracilis*. The libraries Gut *GP*, Gut *AG*, Soil *GP*, and Soil *AG* contain 405, 1,486, 4,985, and 933 sequences, respectively. The enumeration of OTUs corresponds with those in the figure displaying the relative distribution of *narG* OTUs. The bar indicates a 0.05 estimated change per amino acid. Modified from Depkat-Jakob *et al.* (2013).

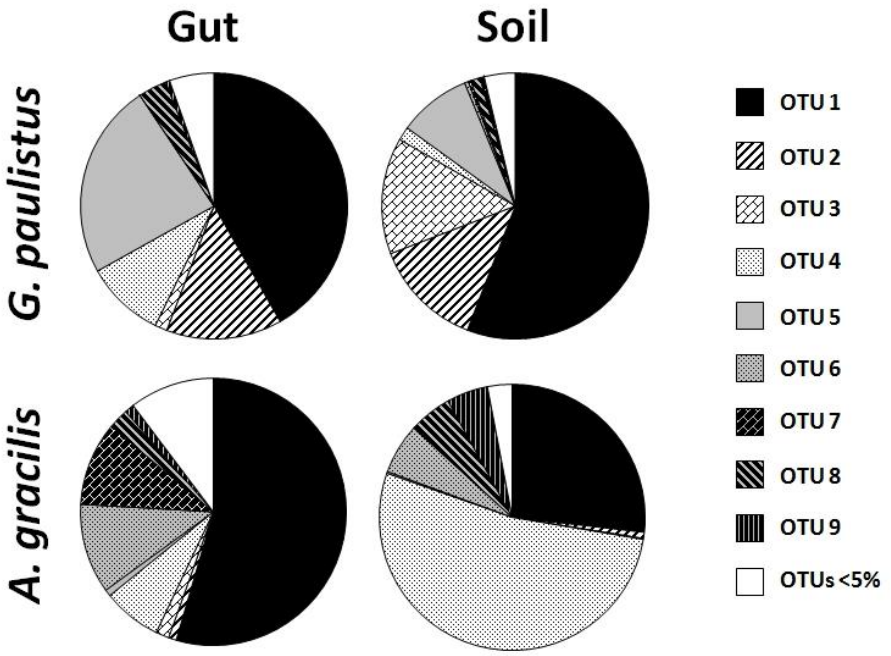


Figure 16: Relative distribution of *narG* OTUs from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.

G. paulistus and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. OTUs were calculated from *narG* sequences. OTU numbers at the right correspond with those in the phylogenetic tree of *in silico* translated *narG* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies. Based on Depkat-Jakob *et al.* (2013).

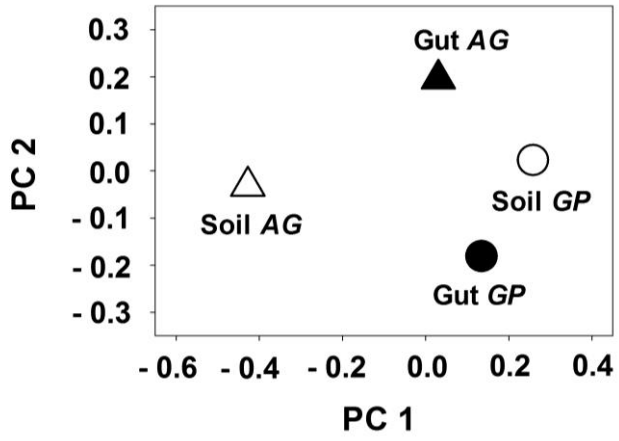


Figure 17: FastUniFrac principle coordinate analysis of *narG* sequences from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.
This analysis was used to display relative differences between the four different *narG* gene libraries. *G. paulistus* and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. A variance of 94.9 % is covered by x-axis (PC 1, 74.7 %) and y-axis (PC 2, 20.2 %). *GP*, *G. paulistus*; *AG*, *A. gracilis*. Modified from Depkat-Jakob *et al.* (2013).

3.1.1.3.3. Nitrite reducers detected via *nirK* in gut contents and soils of *G. paulistus* and *A. gracilis*

3.1.1.3.3.1. Barcoded amplicon pyrosequencing and diversity analysis of *nirK*

For *nirK*, altogether 29,894 valid sequences were retrieved, yielding 194, 244, 189, and 154 OTUs at a species-level cutoff of 83 % (i.e., on nucleotide sequence level) for the libraries from gut contents of *G. paulistus*, gut contents of *A. gracilis*, soil of *G. paulistus*, and soil of *A. gracilis*, respectively (Table 17). Coverages ranged between 98.3 % and 99.8 %, indicating that sampling was sufficient. Estimated richness was always higher than already sampled, and was highest in gut of *A. gracilis* (283 OTUs) (Table 17).

Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) of gut-derived *nirK* sequences were always higher than those of the corresponding soil, with sequences derived from the gut and soil of *A. gracilis* showing higher values than those derived from gut and soil of *G. paulistus* (Table 17). This is indicative of a more broad than selective stimulation of soil-derived *nirK* nitrite reducers in the earthworm gut, and a soil-derived higher diversity in *A. gracilis* than in *G. paulistus*.

Table 17: Sequence qualities, OTUs, coverages, and diversity indices of *nirK* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*.

	Sequences				Diversity indices			
Library	Valid	Invalid	OTUs[a]	C (%)	Chao1	H´	E	1/D
Gut *GP*	6,868	66	194	99.2	236	2.52	0.48	4.09
Gut *AG*	3,911	118	244	98.3	283	3.77	0.59	16.59
Soil *GP*	13,773	144	189	99.5	245	1.48	0.28	1.82
Soil *AG*	5,342	51	154	99.8	230	2.20	0.44	3.03

[a] Species-level OTUs of valid sequences. Cutoff value was 83 %.

See legend of Table 16 for further information.

Modified from Depkat-Jakob *et al.* (2013).

3.1.1.3.3.2. Phylogenetic analysis of *nirK*

Detected *nirK* sequences of OTU 1 were most closely related to *Bradyrhizobium* sp. BTAi1, and were most abundant in libraries from both the gut content and soil of *G. paulistus* with 47 % and 74 % relative abundance, respectively (Figure 18, Figure 19). In addition, sequences related to *Sinorhizobium* sp. NP1 and *Achromobacter cycloclastes* (OTU 6), *Bradyrhizobium japonicum* (OTU 11), and *Rhizobium etli* CFN (OTU 9) were abundant in the gut of *A. gracilis*. OTU 2 that was distantly related to *Pseudomonas fluorescens* Pf-5, represented 6 % of the sequences from the gut of *A. gracilis*, and was predominant in the soil library of *A. gracilis* (Figure 18, Figure 19).

The vast majority of detected *nirK* sequences was affiliated with taxa that showed a good correlation between 16S rRNA and *nirK* gene similarity (Figure 12, Figure 18). Thus, phylogeny detected via the *nirK* genes is very likely to display the correct phylogeny, i.e., the phylogeny based on the 16S rRNA genes.

All *nirK* OTUs contained both soil-derived and gut-derived sequences. This indicates that the gut-derived sequences originated from ingested soil *Bacteria* (Figure 18).

Altogether, gut and soil-derived *nirK* sequences of *G. paulistus* were similar to each other but were significantly different from both *A. gracilis* gut and soil-derived sequences. In contrast, gut and soil-derived *nirK* sequences of *A. gracilis* differed significantly from each other and were also significantly different from both *G. paulistus* gut and soil-derived sequences (Figure 20, Table A 1).

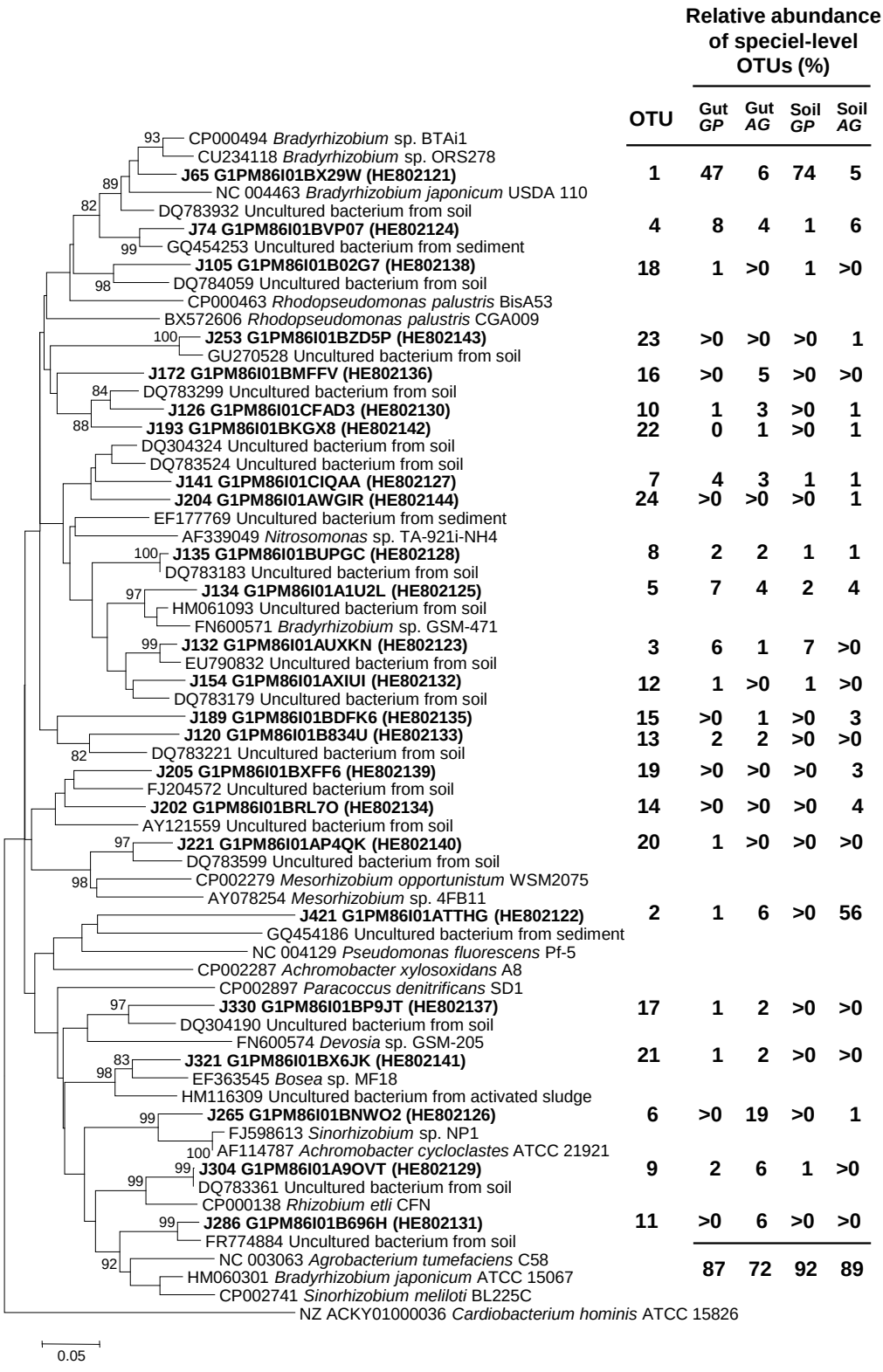


Figure 18: Phylogenetic neighbor-joining tree of representative *nirK* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*, and related sequences.

The libraries Gut *GP*, Gut *AG*, Soil *GP*, and Soil *AG* contain 6,868, 3,911, 13,773, and 5,342 sequences, respectively. The enumeration of OTUs corresponds with those in the figure displaying the relative distribution of *nirK* OTUs. The bar indicates a 0.05 estimated change per amino acid. See the legend of Figure 15 for further information. Modified from Depkat-Jakob *et al.* (2013).

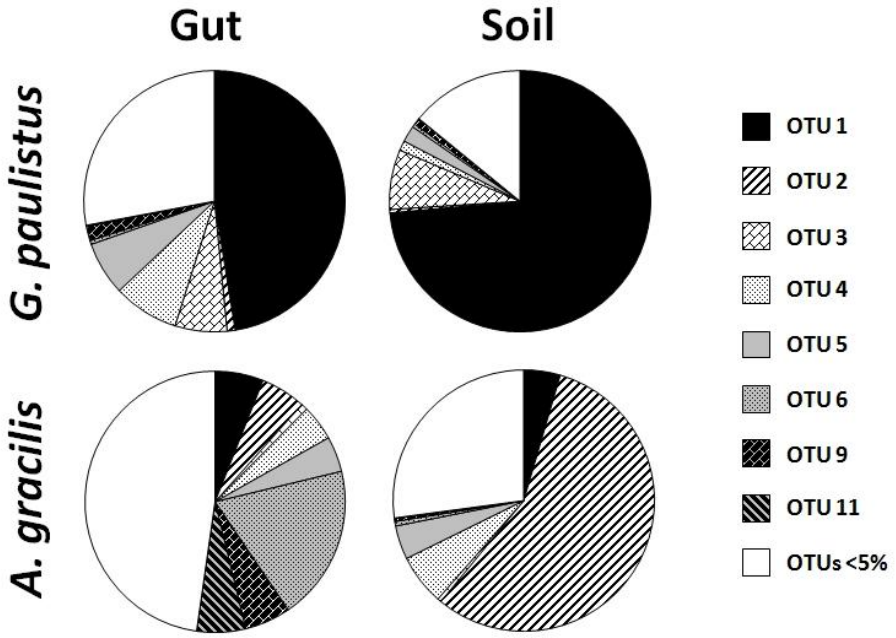


Figure 19: Relative distribution of *nirK* OTUs from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.

G. paulistus and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. OTUs were calculated from *nirK* sequences. OTU numbers at the right correspond with those in the phylogenetic tree of *in silico* translated *nirK* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies. Based on Depkat-Jakob *et al.* (2013).

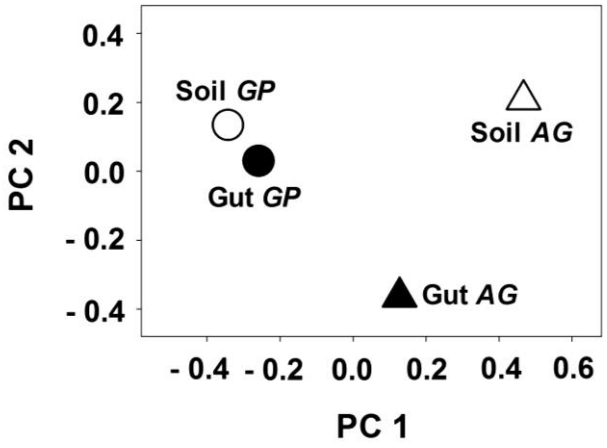


Figure 20: FastUniFrac principle coordinate analysis of *nirK* sequences from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.

This analysis was used to display relative differences between the four different *nirK* gene libraries. *G. paulistus* and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. A variance of 98.3 % is covered by x-axis (PC 1, 67.0 %) and y-axis (PC 2, 31.3 %). *GP*, *G. paulistus*; *AG*, *A. gracilis*. Modified from Depkat-Jakob *et al.* (2013).

3.1.1.3.4. Nitrite reducers detected via *nirS* in gut contents and soils of *G. paulistus* and *A. gracilis*

3.1.1.3.4.1. Barcoded amplicon pyrosequencing and diversity analysis of *nirS*

For *nirS*, altogether 12,401 valid sequences were retrieved, yielding 61, 38, 55, and 66 OTUs at a species-level cutoff of 82 % (i.e., on nucleotide sequence level) for the libraries from gut contents of *G. paulistus*, gut contents of *A. gracilis*, soil of *G. paulistus*, and soil of *A. gracilis*, respectively (Table 18). Coverages ranged between 98.7 % and 99.7 %, indicating that sampling was sufficient. Estimated richness was always higher than already sampled, and was highest in gut of *A. gracilis* (91 OTUs) (Table 18).

Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) of *nirS* sequences were slightly higher in the gut of *G. paulistus* than its soil, whereas most diversity indices of sequences derived from the gut of *A. gracilis* were lower than those of its soil (Table 18). This indicates that stimulation of soil-derived *nirS* nitrite reducers in the gut of *A. gracilis* is restricted to a smaller fraction rather than applied to all ingested *nirS* nitrite reducers, whereas stimulation of *nirS* nitrite reducers in the gut of *G. paulistus* is more unspecific.

Table 18: Sequence qualities, OTUs, coverages, and diversity indices of *nirS* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*.

	Sequences				Diversity indices			
Library	Valid	Invalid	OTUs[a]	C (%)	Chao1	H´	E	1/D
Gut *GP*	3,974	26	61	99.4	86	1.73	0.42	3.02
Gut *AG*	1,437	7	38	98.7	91	2.09	0.57	4.91
Soil *GP*	4,366	34	55	99.7	64	1.60	0.40	2.76
Soil *AG*	2,624	29	66	99.3	79	2.35	0.56	6.21

[a] Species-level OTUs of valid sequences. Cutoff value was 82 %.

See legend of Table 16 for further information.

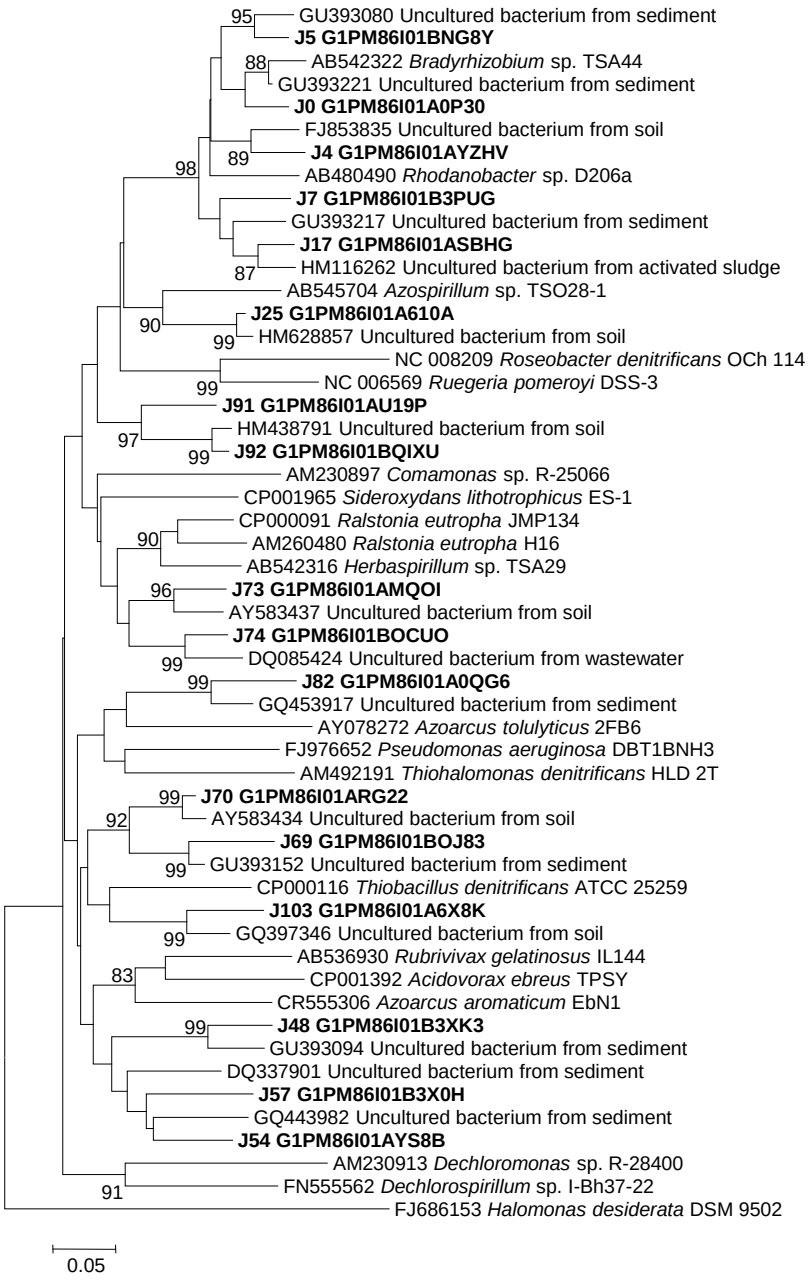
3.1.1.3.4.2. Phylogenetic analysis of *nirS*

The *nirS* sequences of OTU 1 and OTU 2 were most closely related to *Bradyrhizobium* sp. TSA44, and were most abundant in all libraries with together about 75 % and 78 % in the gut and soil of *G. paulistus*, respectively. In the gut and soil of *A. gracilis*, these two OTUs accounted for 61 % and 36 %, respectively (Figure 21, Figure 22). Other abundant OTUs were related to *Herbaspirillum* sp. TSA29 (OTU 2) and distantly related to *Azoarcus aromaticum* EbN1 (OTU 3) (Figure 21, Figure 22).

The vast majority of detected *nirS* sequences was affiliated with taxa that showed a good correlation between 16S rRNA and *nirS* gene similarity (Figure 14, Figure 21). Thus, phylogeny detected via the *nirS* genes is very likely to display the correct phylogeny, i.e., the phylogeny based on the 16S rRNA genes.

All *nirS* OTUs contained both soil-derived and gut-derived sequences. This indicates that the gut-derived sequences originated from ingested soil *Bacteria* (Figure 21).

Altogether, gut and soil-derived *nirS* sequences of *G. paulistus* were highly similar to each other and were also similar to those from the gut of *A. gracilis*. Sequences derived from the soil of *A. gracilis* were most similar to those from the gut of *A. gracilis*, and were most different from those from both gut- and soil-derived sequences of *G. paulistus*. (Figure 21, Figure 22).



OTU	Relative abundance of speciel-level OTUs (%)			
	Gut *GP*	Gut *AG*	Soil *GP*	Soil *AG*
1	53	29	56	5
2	22	32	22	31
10	1	1	1	>0
7	2	3	4	2
6	1	5	1	17
12	2	1	>0	>0
9	1	1	2	>0
8	2	1	1	>0
4	5	6	3	8
16	>0	1	>0	>0
15	>0	1	1	>0
11	>0	1	>0	3
14	>0	1	>0	2
13	1	1	1	>0
3	4	7	2	13
17	>0	>0	>0	1
5	4	6	4	7
	97	97	98	93

Figure 21: Phylogenetic neighbor-joining tree of representative *nirS* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*, and related sequences.

The libraries Gut *GP*, Gut *AG*, Soil *GP*, and Soil *AG* contain 3,974, 1,437, 4,366, and 2,624 sequences, respectively. The enumeration of OTUs corresponds with those in the figure displaying the relative distribution of *nirS* OTUs. The bar indicates a 0.05 estimated change per amino acid. See the legend of Figure 15 for further information.

Figure 22: Relative distribution of *nirS* OTUs from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.

G. paulistus and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. OTUs were calculated from *nirS* sequences. OTU numbers at the right correspond with those in the phylogenetic tree of *in silico* translated *nirS* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies.

3.1.1.3.5. Denitrifiers detected via *nosZ* in gut contents and soils of *G. paulistus* and *A. gracilis*

3.1.1.3.5.1. Barcoded amplicon pyrosequencing and diversity analysis of *nosZ*

For *nosZ*, altogether 3,822 valid sequences were retrieved, yielding 34, 23, 14, and 53 OTUs at a species-level cutoff of 80 % (i.e., on nucleotide sequence level) for the libraries from gut contents of *G. paulistus*, gut contents of *A. gracilis*, soil of *G. paulistus*, and soil of *A. gracilis*, respectively (Table 19). Coverages ranged between 97.3 % and 99.3 %, indicating that sampling was sufficient despite of the reduced amount of sequences. Estimated richness was highest in the soil of *A. gracilis* with 72 OTUs, whereas for the other libraries, estimated richness was only slightly higher (gut of *G. paulistus* and gut of *A. gracilis*) or the same (soil of *G. paulistus*) as already sampled (Table 19).

Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) of gut-derived *nosZ* sequences were higher than those of the corresponding soil for *G. paulistus*, whereas values for gut- and soil-derived sequences of *A. gracilis* were highly similar (Table 19). This indicates that stimulation of soil-derived denitrifiers in the gut of *G. paulistus* is restricted to a smaller fraction rather than applied to all denitrifiers.

Table 19: Sequence qualities, OTUs, coverages, and diversity indices of *nosZ* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*.

	Sequences				Diversity indices			
Library	Valid	Invalid	OTUs[a]	C (%)	Chao1	H´	E	1/D
Gut *GP*	791	19	34	99.0	39	2.17	0.62	4.82
Gut *AG*	258	8	23	97.3	28	2.23	0.71	5.95
Soil *GP*	180	2	14	98.3	14	1.55	0.59	2.90
Soil *AG*	2,593	18	53	99.3	72	2.29	0.58	6.08

[a] Species-level OTUs of valid sequences. Cutoff value was 80 %.

See legend of Table 16 for further information.

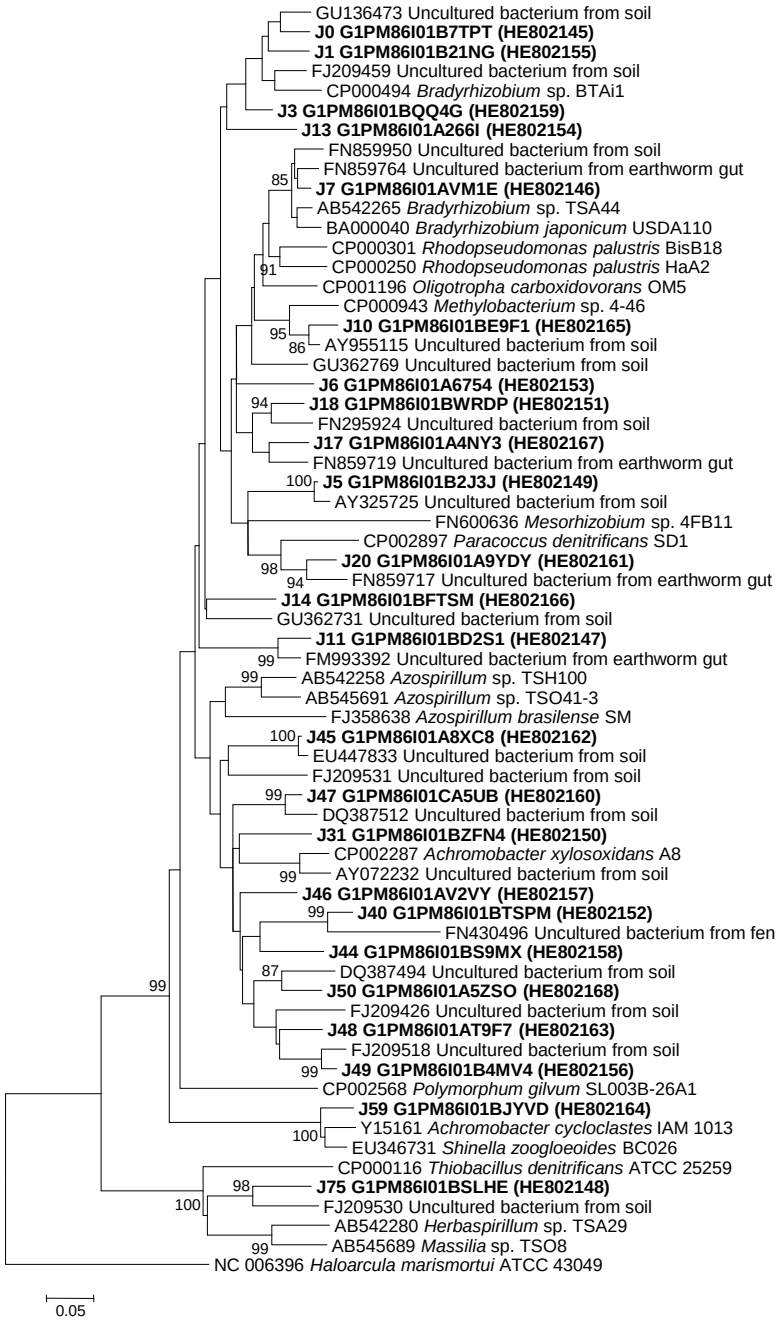
Modified from Depkat-Jakob *et al.* (2013).

3.1.1.3.5.2. Phylogenetic analysis of *nosZ*

The *nosZ* sequences of OTU 1 and OTU 2 were most closely related to species of the genus *Bradyrhizobium* (*Rhizobiales*), and were together most abundant in all libraries, especially in the gut and soil of *G. paulistus* (Figure 23, Figure 24). Only in the gut of *A. gracilis*, OTU 5 was dominant (33 %), and was related to *Paracoccus denitrificans* SD1. OTU 4 was exclusively detected in the soil of *A. gracilis* and was affiliated with *Thiobacillus denitrificans* and *Herbaspirillum* sp. TSA29 (Figure 23, Figure 24).

Most *nosZ* OTUs detected in the earthworm gut were also found in the corresponding soil, except for the quantitatively minor OTUs 14, 18, 19, 21, 22, and 23 (Figure 23). This indicates that the majority of gut-derived sequences originated from ingested soil *Bacteria*.

Altogether, gut- and soil-derived *nosZ* sequences of *G. paulistus* were highly similar to each other but were significantly different from both *A. gracilis* gut- and soil-derived sequences. In contrast, gut- and soil-derived *nosZ* sequences of *A. gracilis* differed significantly from each other and were also significantly different from both *G. paulistus* gut- and soil-derived sequences (Figure 25, Table A 1).



OTU	Relative abundance of speciel-level OTUs (%)			
	Gut *GP*	Gut *AG*	Soil *GP*	Soil *AG*
1	39	13	56	29
11	0	3	0	2
15	1	1	9	>0
10	6	0	5	1
2	20	19	16	20
21	0	2	0	0
9	1	2	1	3
7	0	1	0	4
23	0	2	0	0
5	4	33	1	1
17	>0	5	1	>0
22	1	>0	0	>0
3	4	0	1	15
18	2	0	0	>0
16	1	2	1	>0
6	0	4	0	4
13	3	0	1	1
8	7	2	7	1
14	4	2	1	0
24	>0	0	1	0
19	2	0	0	0
12	0	0	0	2
20	0	6	0	>0
4	0	0	0	11
	95	97	99	94

Figure 23: Phylogenetic neighbor-joining tree of representative *nosZ* sequences from gut contents and soils of *G. paulistus* and *A. gracilis*, and related sequences.

The libraries Gut *GP*, Gut *AG*, Soil *GP*, and Soil *AG* contain 791, 258, 180, and 2,593 *nosZ* sequences, respectively. The enumeration of OTUs corresponds with those in the figure displaying the relative distribution of *nosZ* OTUs. The bar indicates a 0.05 estimated change per amino acid. See the legend of Figure 15 for further information. Modified from Depkat-Jakob *et al.* (2013).

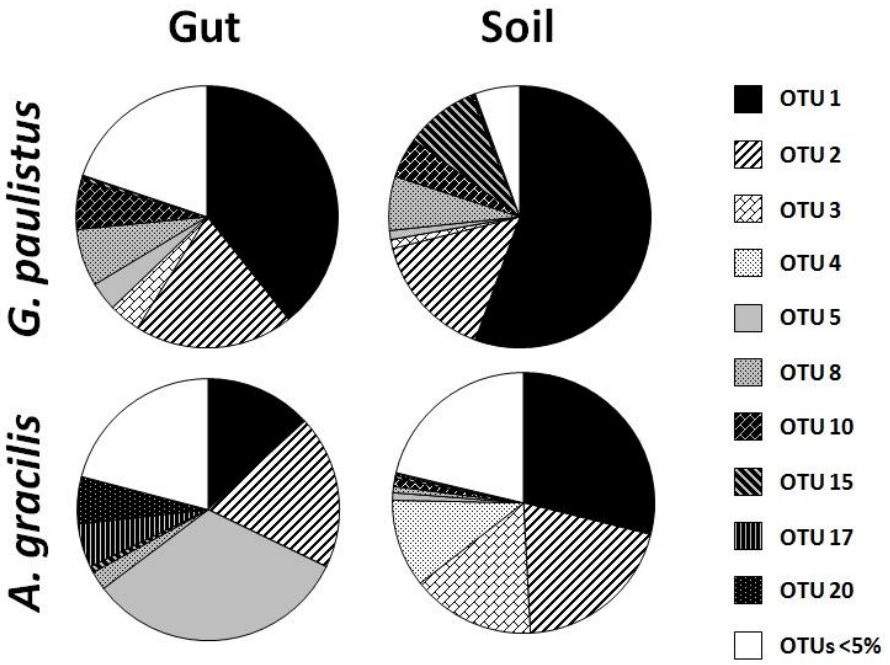


Figure 24: Relative distribution of *nosZ* OTUs from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.

G. paulistus and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. OTUs were calculated from *nirS* sequences. OTU numbers at the right correspond with those in the phylogenetic tree of *in silico* translated *nosZ* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies. Based on Depkat-Jakob *et al.* (2013).

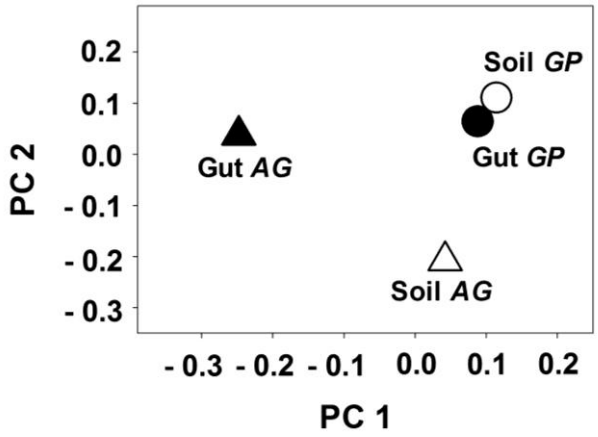


Figure 25: FastUniFrac principle coordinate analysis of *nosZ* sequences from gut contents of *G. paulistus* and *A. gracilis*, and from their corresponding soils.

This analysis was used to display relative differences between the four different *nosZ* gene libraries. *G. paulistus* and *A. gracilis* were sampled from pasture soil and grassland soil, respectively. A variance of 94.2 % is covered by x-axis (PC 1, 55.0 %) and y-axis (PC 2, 39.2 %). *GP*, *G. paulistus*; *AG*, *A. gracilis*. Modified from Depkat-Jakob *et al.* (2013).

3.1.2. Earthworms from Germany

Earthworms from Germany have been thoroughly studied for their ability to emit denitrification-derived N_2O (1.4.5). However, a limited amount of information is available on denitrifiers in earthworms based on the analysis of structural genes of the denitrification pathway, i.e., only *nosZ* of pooled gut contents of several species has been analyzed (Horn *et al.* 2006a). Furthermore, when this dissertation research was initiated, information about gene transcripts and the influence of the earthworm feeding guild on dissimilatory nitrate reducer and denitrifier communities in the gut was unavailable. Thus, earthworm species of the family Lumbricidae representing three different feeding guilds were sampled along with the soil or material they were detected in from the grassland 'Trafowiese' near Bayreuth, Germany (Table 1, Table 2). *Aporectodea caliginosa* (endogeic feeding guild) and *Lumbricus terrestris* (anecic) were sampled from and along with mineral soil (2.1.2). *Lumbricus rubellus* (epigeic) was sampled from and along with the moist uppermost soil layer and overlying decaying organic material, together denoted as uppermost soil (2.1.2). In addition, for the isolation approach only (2.3.2.1), *Octolasium lacteum* (endogeic) was sampled from mineral soil (2.1.2, 2.2.4.2).

3.1.2.1. Analysis of gene markers indicative of denitrification and dissimilatory nitrate reduction in gut contents and soils of earthworms from Germany

Gut contents (2.2.4.2) and corresponding soils were analyzed for the appearance and composition of genes indicative of denitrification and dissimilatory nitrate reduction (2.5.7.2.2). The analyzed genes encode for the enzyme or a subunit of a dissimilatory nitrate reductase (*narG*), nitrite reductases (*nirK* and *nirS*), and N_2O reductase (*nosZ*) (1.2.3).

3.1.2.1.1. Gene and transcript analysis of *narG* and *nosZ*

Nucleic acids from earthworm gut contents of the endogeic *A. caliginosa*, the anecic *L. terrestris*, and the epigeic *L. rubellus*, and from mineral soil and uppermost soil were extracted (2.5.1). DNA and RNA were separated and reverse transcription (2.5.6) yielded cDNA from DNA-free RNA (2.5.4.3). Partial *narG* and *nosZ* gene and transcript sequences could be amplified from every sub-sample from both DNA- and cDNA-derived samples and were used for sequence libraries (2.5.7.2.2) and T-RFLP analyses (2.5.8). Diversity and phylogeny analyses (2.5.12) were conducted with *in silico* translated amino acid sequences of *narG* and *nosZ* gene and transcript sequences.

3.1.2.1.1.1. Denitrifiers and dissimilatory nitrate reducers detected via *narG* in gut contents and corresponding soils of *A. caliginosa*, *L. terrestris*, and *L. rubellus*

3.1.2.1.1.1.1. Cloning and diversity analysis of *narG* genes and transcripts

139 *narG* gene and 108 *narG* transcript sequences were retrieved from earthworm gut contents and soil samples, and *in silico* translated amino acid sequences yielded 9 species-level OTUs (Table 20, Figure 26). The species-level cutoff value for *narG* of 59 % is a very conservative estimate as the OTUs 3 and 7 contain several different species based on a 16S rRNA gene phylogeny (Figure 26). Thus, the real number of species represented by *narG* sequences is assumed to be higher than 9. However, the cutoff value used displays the minimum amount of OTUs and was the standard of comparison between the analyzed samples. For *narG* genes, 57 and 82 sequences were retrieved from gut- and soil-derived samples, respectively, yielding 5 OTUs each, together 8 OTUs (Table 20, Figure 26). For *narG* transcripts, 82 and 26 sequences were retrieved from gut- and soil-derived samples, respectively, yielding 3 OTUs each, together 4 OTUs (Table 20, Figure 26).

Table 20: Estimated genotypes, coverage, and diversity indices of *in silico* translated *narG* gene and transcript amino acid sequences from gut contents of earthworms and from soils.[a]

Library	No. of sequences	OTUs[b]	Coverage (%)	Diversity indices			
				Rich-ness	H´[c]	Even-ness	1/D[d]
DNA, guts	57	5	100	5 ± 0	1.30	0.81	2.95
DNA, soils	82	5	100	5 ± 0	1.16	0.72	2.33
cDNA, guts	82	3	100	3 ± 0	0.55	0.50	1.45
cDNA, soils	26	3	100	3 ± 0	1.06	0.97	3.00
DNA, total	139	8	99	8 ± 0	1.41	0.68	2.67
cDNA, total	108	4	100	4 ± 0	0.82	0.59	1.80

[a] All calculations were carried out with DOTUR-1.53 and based on amino acid sequences (2.5.12.4).

[b] *narG* at a species-level cutoff value of 59 %.

[c] H´, Shannon-Weaver diversity index.

[d] 1/D, reciprocal Simpson diversity index.

See the methods part for further information (2.5.12).

Modified from Depkat-Jakob *et al.* (2010).

Coverages for all *narG* libraries ranged between 99.3 % and 100 %; libraries were therefore sampled sufficiently with the cutoff used (Table 20). Estimated richness of *narG* gene and transcript sequences was not higher than of sequences already sampled (Table 20). Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) for *narG* gene sequences were slightly higher for gut-derived than for soil-derived sequences. Diversity indices of *narG* transcripts were lower than those of *narG* genes, and were lower for gut-derived than for soil-derived sequences (Table 20).

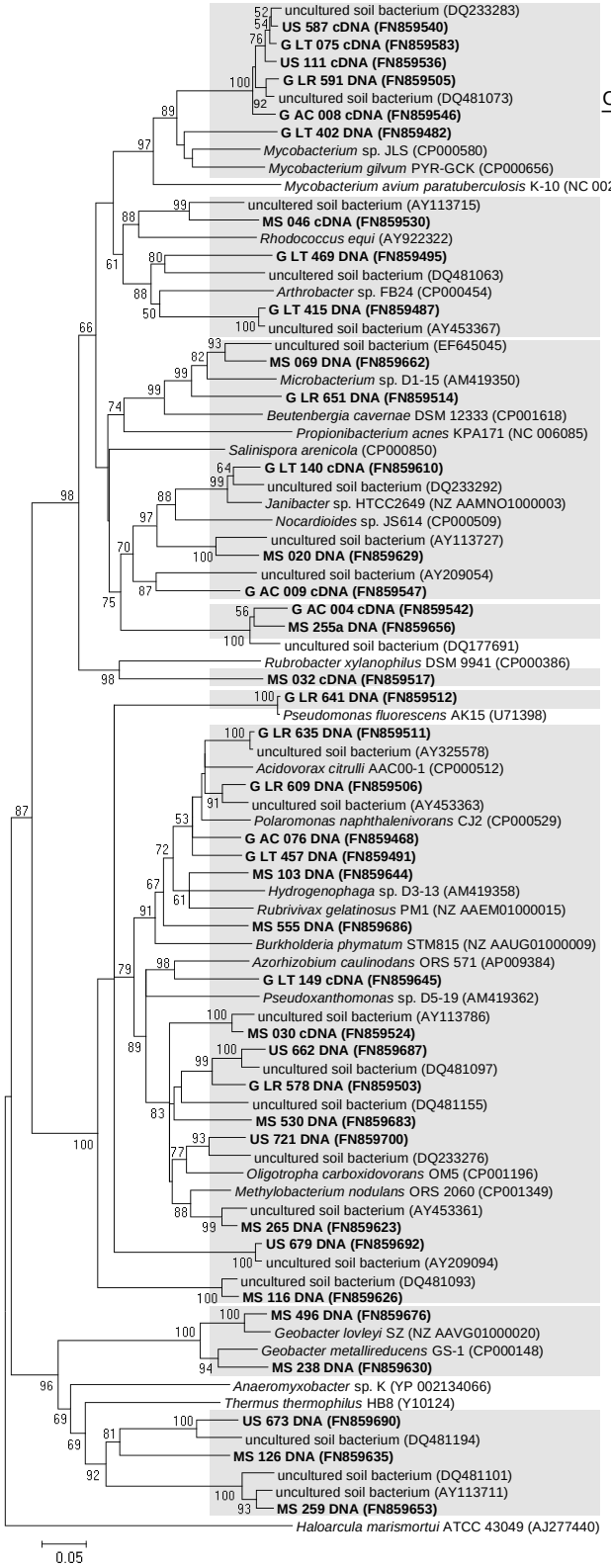
3.1.2.1.1.1.2. Phylogenetic analysis of *narG* genes and transcripts

Detected *narG* sequences of OTUs 1 to 5 were affiliated with Gram-positive genera, i.e., *Actinobacteria* (e.g., *Mycobacterium*, *Arthrobacter*, and *Microbacterium*). OTUs 6 to 9 were affiliated with Gram-negative genera, i.e., *Proteobacteria* (*Alphaproteobacteria*: *Oligotropha* and *Methylobacterium*; both members of the *Rhizobiales* harboring several denitrifiers [Zumft 1997, Shapleigh 2006]; *Betaproteobacteria*: e.g., *Acidovorax* and *Rubrivivax*; *Gammaproteobacteria*: e.g., *Pseudomonas*; and *Deltaproteobacteria*: e.g., *Geobacter*) and *Thermus* (Figure 26). For most *narG* sequences, the highest similarity was shared with uncultured soil *Bacteria* (Figure 26).

Soil-derived *narG* gene sequences were predominantly affiliated with OTUs of Gram-negative *Bacteria* (e.g., OTUs 7 to 9) with minor affiliation of OTUs of *Actinobacteria* (e.g., OTUs 1 to 4). Gut-derived sequences were mainly affiliated with members of the *Rhizobiales* (i.e., *Methylobacterium* and *Oligotropha* within OTU 7), but showed stronger affiliation with *Actinobacteria*-related OTUs (e.g., OTUs 1 to 4) than the soil did (Figure 26, Figure 27).

33 % of *L. rubellus*-derived sequences were affiliated with OTU 7 whereas for the other four libraries, 59 % to 67 % of sequences were affiliated with this OTU (Figure 26). Detected *narG* transcripts derived from earthworm guts and from uppermost soil were predominantly affiliated with *Actinobacteria*, especially with OTU 1 most closely affiliated with the genus *Mycobacterium* (Figure 26, Figure 27). In contrast, *narG* transcripts from mineral soil were predominantly affiliated with OTU 7 (*Proteobacteria*, especially *Rhizobiales*), OTU 2, and OTU 3 (both *Actinobacteria*) (Figure 26).

Most *narG* sequences occurred in OTUs that contained both soil- and gut-derived sequences, and were most closely related to uncultured soil *Bacteria* (Figure 26, Figure 27), indicating that the majority of gut-derived sequences originated from ingested soil *Bacteria*.



	Relative abundance of species-level OTUs (%)									
	DNA					cDNA				
	Earthworms			Soils		Earthworms			Soils	
OTU	AC	LT	LR	MS	US	AC	LT	LR	MS	US
1	5	12	22	2	6	81	80	100	6	88
2	23	18	0	6	11	5	0	0	17	0
3	14	0	22	6	11	10	10	0	33	0
4	0	6	17	5	0	5	5	0	0	13
5	0	0	0	0	0	0	2	0	5	0
6	0	0	6	0	0	0	0	0	0	0
7	59	65	33	63	67	0	3	0	39	0
8	0	0	0	8	0	0	0	0	0	0
9	0	0	0	11	6	0	0	0	0	0

Figure 26: Phylogenetic neighbor-joining tree of representative *narG* gene and transcript sequences retrieved from earthworm gut contents and soils, and related *narG* sequences.

Sequences from this study are bold with accession numbers in parentheses. Tree is based on *in silico* translated amino acids. The percentage of replicate trees the associated taxa clustered together in the bootstrap test (10,000 replicates), are shown at the node of two branches (values below 50 % are not displayed). The bar indicates a 0.05 estimated change per amino acid. The table shows the relative distribution of sequences in each cluster (i.e., OTU; shaded text) as calculated with DOTUR-1.53. Relative abundances of a library may not add up to 100% due to roundings. Abbreviations: G, gut; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil. *A. caliginosa* and *L. terrestris* were sampled from mineral soil, *L. rubellus* was sampled from uppermost soil. Absolute number of sequences retrieved from DNA was 22, 17, 18, 64, and 18 for AC, LT, LR, MS, and US, respectively. Absolute number of sequences retrieved from cDNA was 21, 59, 2, 18, and 8 for AC, LT, LR, MS, and US, respectively. Modified from Depkat-Jakob *et al.* (2010).

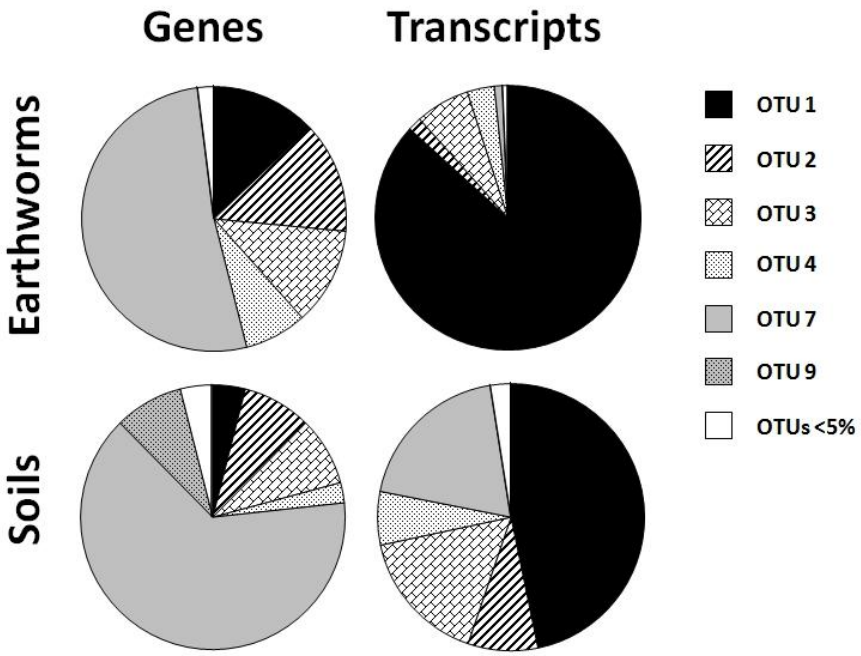


Figure 27: Relative distribution of *in silico* translated *narG* gene and transcript OTUs from combined earthworm gut content and from corresponding soil libraries.

OTUs were calculated from *in silico* translated amino acid sequences of *narG* sequences. OTUs were calculated from *in silico* translated amino acid sequences of *narG* sequences. The sequences of the three earthworm gut libraries (*A. caliginosa, L. terrestris*, and *L. rubellus*) and the two soil libraries (mineral and uppermost soil) were combined, respectively. Sequences retrieved from DNA (genes) and cDNA (transcripts) are displayed. Numbering of OTUs at the right side correspond with that in the phylogenetic tree of *in silico* translated *narG* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies.

3.1.2.1.1.1.3. T-RFLP analysis of *narG* genes and transcripts

T-RFLP analysis with *narG* genes yielded 13 T-RFs (Figure 28A). The crop/gizzard- and gut-derived T-RFs of the three earthworm species were rather similar than dissimilar compared to each other, and were also highly similar to those of the uppermost soil (Figure 28A, Figure 29A). Most of the T-RFs had a length of 91 bp (primarily indicative of OTUs 2 and 3), 104 bp (primarily indicative of OTU 3), and 458 bp (OTU 7); one abundant but unaffiliated T-RF had a length of 632 bp (Figure 28A, Figure 26). T-RFs of the mineral soil were highly different from those of the earthworm guts and uppermost soil (Figure 29A), and were mostly affiliated with OTU 7 (168 bp, 190 bp, 243/245 bp, and 469 bp), i.e., with *Proteobacteria*/*Rhizobiales*; and a low relative fluorescence was represented by T-RFs with 91 bp and 104 bp indicative of the OTUs 2 and 3, i.e., *Actinobacteria* (Figure 28A). Although displaying high similarities to other earthworm- and uppermost soil-derived samples, crop/gizzard- and gut-derived T-RFs of the endogeic (i.e., predominantly feeding on soil) *A. caliginosa* were affiliated with both *Actinobacteria*- and *Proteobacteria*/*Rhizobiales*-related OTUs (Figure 28A) and were therefore placed between the mineral soil and the group of the two other earthworms plus uppermost soil in the PCA (Figure 29A).

T-RFLP analysis with *narG* transcripts had to be conducted with a different restriction enzyme than that used for *narG* genes (2.5.8.3) and yielded 15 T-RFs (Figure 28B). As with *narG* gene T-RFLP analysis, earthworm gut- and uppermost soil-derived T-RFs were highly similar to each other (Figure 28B, Figure 29B). Most of those T-RFs were affiliated with *Actionbacteria* (i.e., the T-RFs with 357bp, >640 bp, and 477 bp were affiliated with OTU 1, OTU 3, and OTU 4, respectively), with a predominance of T-RF with 357 bp length affiliated with OTU 1 and the genus *Mycobacterium* (Figure 28B, Figure 26). The composition of T-RFs of the mineral soil was distinct from all other samples and dominated by those affiliated with *Proteobacteria/Rhizobiales*, i.e., OTU 7 (127 bp), rather than those affiliated with *Actinobacteria* (i.e., T-RFs with 357bp, >640 bp, and 477 bp), with several T-RF that could not be affiliated with an OTU (e.g., 583 bp, 569 bp, 560 bp, and 159 bp) (Figure 28B). The effect of the earthworm feeding guild on the detected *narG* transcripts was small but detectable as the T-RF with 573 bp length (OTU 7, *Proteobacteria/Rhizobiales*) dominant in the mineral soil showed highest detectability in the endogeic *A. caliginosa* compared to the other earthworm species (Figure 28B, Figure 29B), and the T-RF with 477 bp length (OTU 4, *Actinobacteria*) showed strong relative fluorescence in the gut of *L. rubellus* only (Figure 28B).

In summary, T-RFLP analysis strongly confirmed the results derived from *narG* gene and transcript libraries showing that sequences affiliated with *Actinobacteria* were predominant in earthworm guts and in the moist uppermost soil, whereas *Proteobacteria/Rhizobiales*-related sequences were predominant in the mineral soil. These trends were even more pronounced

on *narG* transcript level than on gene level with most sequences affiliated with the genus *Mycobacterium*. In addition, an effect of the earthworm feeding guild on the *narG* gene and transcript sequences was detected.

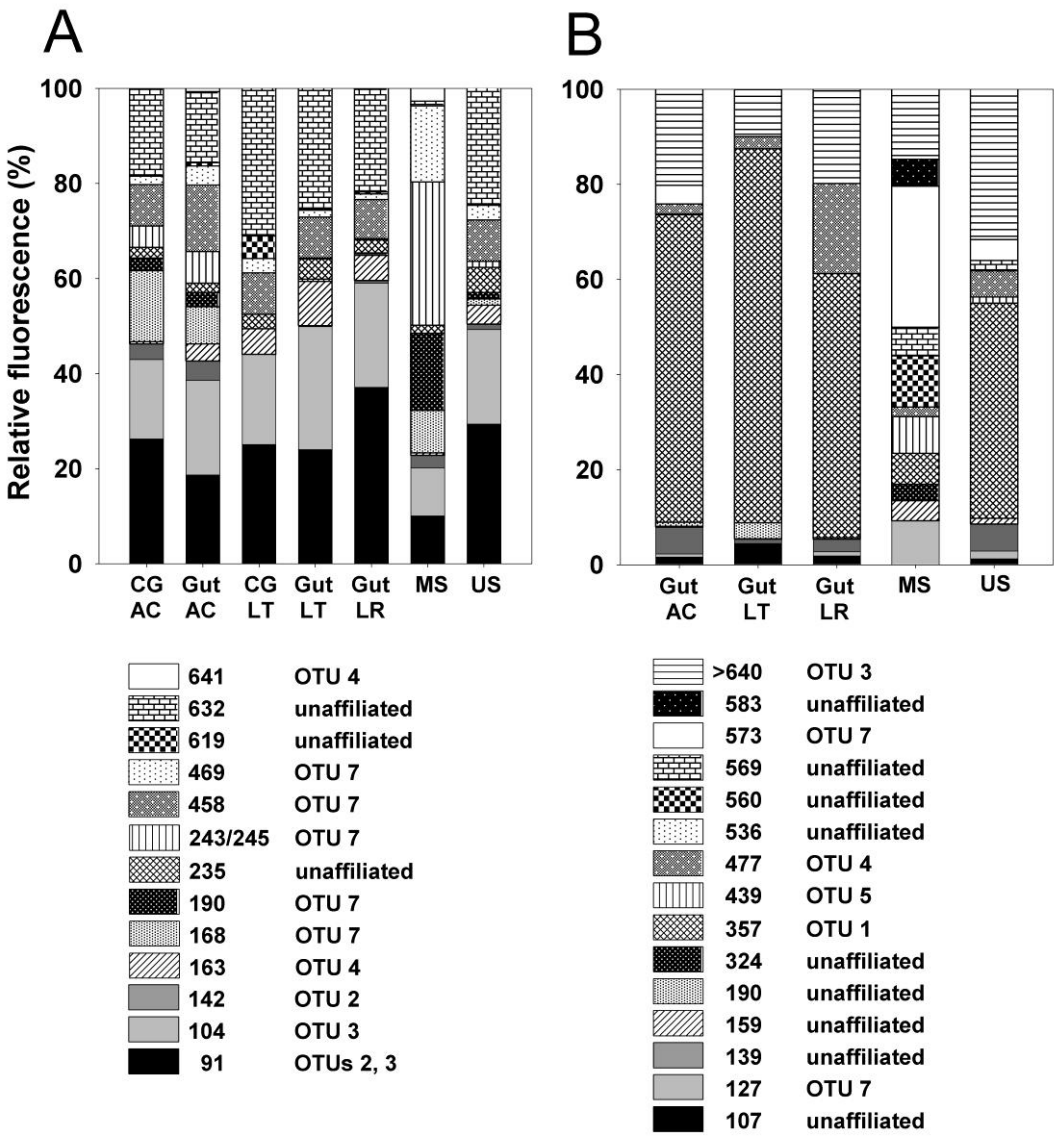


Figure 28: *narG* gene and transcript T-RFLP patterns from earthworm gut contents and soils. Displayed are the relative fluorescences of T-RFs exceeding 3 % in at least one sample; their sums were set as 100 %. A, genes (DNA-derived; digestion was with *Ban*I). Shown are mean values (*n*=3). B, transcripts (cDNA-derived; digestion was with *Mae*III). Abbreviations: CG, crop/gizzard (if lacking, no samples were available); AC, *A. caliginosa*; LT, *L. terrestris*; MS, mineral soil; US, uppermost soil. *A. caliginosa* and *L. terrestris* were sampled from mineral soil, *L. rubellus* was sampled from uppermost soil. Caption shows the lengths of the T-RFs as measured in bp and the *in silico* affiliated OTUs containing the highest numbers of T-RF sequences in a descending order; some T-RFs could not be affiliated with an OTU. Modified from Depkat-Jakob *et al.* (2010).

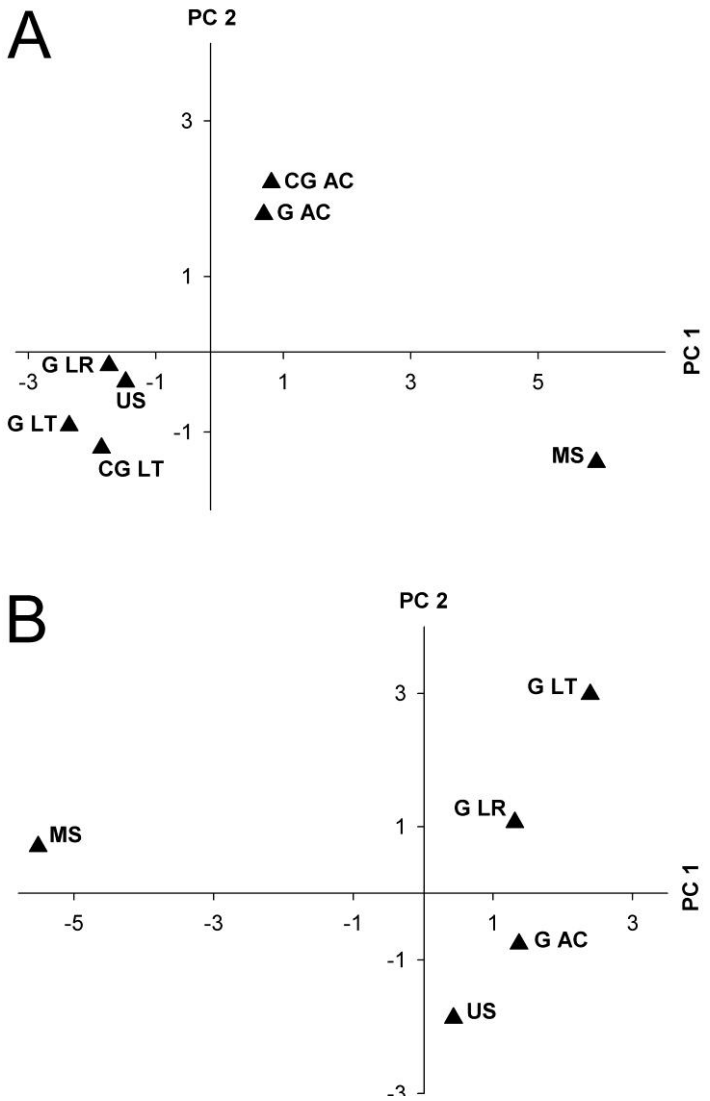


Figure 29: Principal component analysis of *narG* gene and transcript T-RFs.

A, *narG* genes (DNA-derived). A variance of 84.7% is covered by x-axis (PC 1, 67.9 %) and y-axis (PC 2, 16.8 %). B, *narG* transcripts (cDNA-derived). A variance of 92.6 % is covered by x-axis (PC 1, 67.8 %) and y-axis (PC 2, 24.8 %). Gene and transcript analysis could be not be conducted together in one analysis as the same restriction enzymes differed in the underlying T-RFLP analyses. Abbreviations: G, gut; CG, crop/gizzard; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil. *A. caliginosa* and *L. terrestris* were sampled from mineral soil, *L. rubellus* was sampled from uppermost soil. Modified from Depkat-Jakob *et al.* (2010).

3.1.2.1.1.2. Denitrifiers detected via *nosZ* in the gut and corresponding soil of *A. caliginosa, L. terrestris*, and *L. rubellus*

3.1.2.1.1.2.1. Cloning and diversity analysis of *nosZ* genes and transcripts

89 *nosZ* gene and 68 *nosZ* transcript sequences were retrieved from earthworm gut contents and soil samples, respectively, and *in silico* translated amino acid sequences yielded together 26 species-level OTUs at a species-level cutoff value for of 86 % (Table 20, Figure 30). For *nosZ* genes, 70 and 19 sequences were retrieved from gut- and soil-derived samples, respectively, yielding 11 and 8 OTUs, respectively, together 15 OTUs (Table 20, Figure 30). For *nosZ* transcripts, 39 and 29 sequences were retrieved from gut- and soil-derived samples, respectively, yielding 9 and 13 OTUs, respectively, together 18 OTUs (Table 20, Figure 30).

Table 21: Estimated genotypes, coverage, and diversity indices of *in silico* translated *nosZ* gene and transcript amino acid sequences from gut contents of earthworms and from soils.

Library	No. of sequences	OTUs[a]	Coverage (%)	Diversity indices			
				Rich-ness	H´	Even-ness	1/D
DNA, guts	70	11	99	12 ± 1	1.53	0.64	2.75
DNA, soils	19	8	84	10 ± 1	1.84	0.88	6.33
cDNA, guts	39	9	92	11 ± 1	1.74	0.79	4.72
cDNA, soils	29	13	72	20 ± 3	2.08	0.81	5.64
DNA, total	89	15	96	17 ± 1	1.78	0.66	3.23
cDNA, total	68	18	84	41 ± 13	2.15	0.74	5.68

[a] *nosZ* at a species-level cutoff value of 86 %.
See legend of Table 20 for further information.
Modified from Depkat-Jakob *et al.* (2010).

Coverages for all *nosZ* libraries ranged between 72.4 % and 98.6 % (Table 20). As coverages of *nosZ* gene and *nosZ* transcript libraries derived from earthworm guts were both higher than 92 %, sampling was estimated as sufficent for further analyses (Table 20). Estimated richness of *nosZ* gene sequences was only slightly higher than already sampled (Table 20). Estimated richness of *nosZ* transcript sequences was significantly higher than the sampled OTU number for soil-derived and all *nosZ* transcripts, whereas the estimated

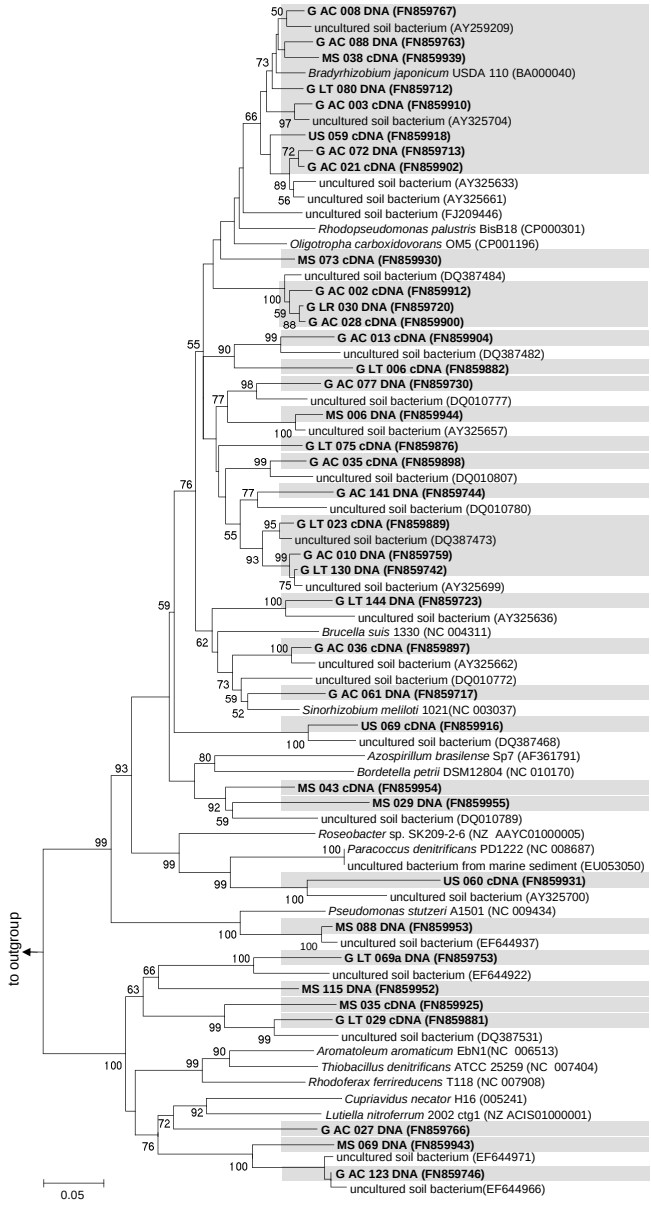
richness of gut-derived *nosZ* transcript sequences war only slightly higher than already sampled (Table 20).

Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) for *nosZ* gene and transcript sequences were always higher for soil-derived than for gut-derived sequences. Diversity indices for *nosZ* transcripts were lower than those of *nosZ* genes, and were lower for gut-derived than for soil-derived sequences (Table 20). This indicates that stimulation of soil-derived denitrifiers in the earthworm gut is restricted to a smaller fraction rather than applied to all ingested denitrifiers.

3.1.2.1.1.2.2. Phylogenetic analysis of *nosZ* genes and transcripts

Detected *nosZ* sequences were affiliated with genera belonging to *Alphaproteobacteria* (e.g., *Bradyrhizobium*, *Rhodopseudomonas*, and *Oligotropha;* all *Rhozobiales*), *Betaproteobacteria* (e.g., *Bordetella*, and *Cupriavidus*), and *Gammaproteobacteria* (*Pseudomonas stutzeri*) but were most closely related to sequences of uncultured soil *Bacteria* (Figure 30). Gut- and uppermost soil-derived *nosZ* gene sequences were mostly affiliated with OTU 1 (*Bradyrhizobium japonicum* as closest relative), and, in addition, gut-derived sequences were affiliated with OTU 11 (uncultured soil bacterium as closest relative) (Figure 30, Figure 31). *nosZ* gene sequences from mineral soil were distributed more evenly in several OTUs (Figure 30). Gut-derived *nosZ* transcript sequences were mainly distributed in OTU 1 and OTU 3 (both with *Bradyrhizobium japonicum* as closest relative) (Figure 30, Figure 31). Mineral soil-and uppermost soil-derived *nosZ* transcripts were distributed more evenly in several OTUs (Figure 30, Figure 31) indicating that stimulation of soil-derived denitrifiers in the earthworm gut is restricted to a smaller fraction rather than applicaple for all ingested denitrifiers.

Most *nosZ* sequences occurred in OTUs that contained both soil- and gut-derived sequences, and were most closely related to uncultured soil *Bacteria* (Figure 30, Figure 31), indicating that the majority of gut-derived sequences originated from ingested soil *Bacteria*.



	Relative abundance of species-level OTUs (%)									
	DNA					cDNA				
	Earthworms			Soils		Earthworms			Soils	
OTU	AC	LT	LR	MS	US	AC	LT	LR	MS	US
1	55	65	50	29	60	30	11	43	45	33
2	0	0	0	0	0	4	0	0	15	0
3	0	0	33	0	40	30	33	57	5	11
4	0	0	0	0	0	13	0	0	0	0
5	0	0	0	0	0	0	11	0	0	0
6	3	0	0	0	0	0	0	0	0	0
7	0	3	0	14	0	0	0	0	0	0
8	0	0	0	0	0	0	11	0	0	0
9	0	0	0	0	0	4	0	0	0	0
10	3	3	0	0	0	0	11	0	0	0
11	15	13	17	7	0	9	11	0	5	11
12	3	3	0	0	0	0	0	0	5	0
13	0	0	0	0	0	9	0	0	0	11
14	3	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	11
16	0	0	0	0	0	0	0	0	5	0
17	0	0	0	7	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	11
19	0	0	0	14	0	0	0	0	0	11
20	3	6	0	0	0	0	0	0	5	0
21	0	0	0	7	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	5	0
23	0	0	0	0	0	0	11	0	0	0
24	6	0	0	0	0	0	0	0	0	0
25	6	0	0	7	0	0	0	0	0	0
26	3	6	0	14	0	0	0	0	10	0

Figure 30: Phylogenetic neighbor-joining tree of representative *nosZ* gene and transcript sequences retrieved from earthworm gut contents and corresponding soils, and related *nosZ*.

The outgroup was *Holarcula marismortui* ATCC 43049 (AY5962197). Absolute number of sequences retrieved from DNA was 33, 31, 6, 14, and 5 for AC, LT, LR, MS, and US, respectively. Absolute number of sequences retrieved from cDNA (transcripts) was 23, 9, 7, 20, and 9 for AC, LT, LR, MS, and US, respectively. See legend of Figure 26 for further information. Modified from Depkat-Jakob *et al.* (2010).

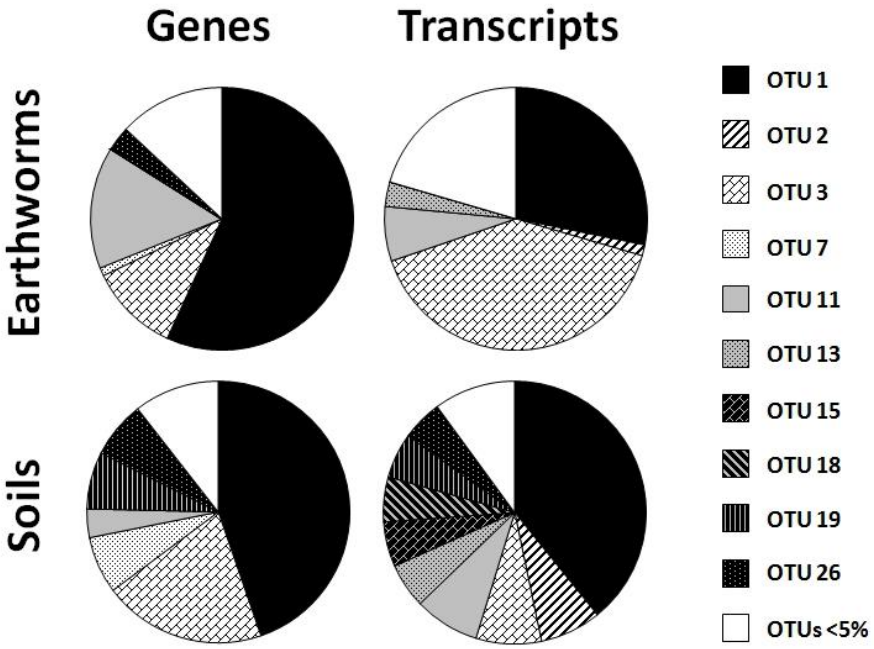


Figure 31: Relative distribution of *in silico* translated *nosZ* gene and transcript OTUs from combined earthworm gut contents and from corresponding soil libraries.

OTUs were calculated from *in silico* translated amino acid sequences of *nosZ* sequences. The sequences of the three earthworm gut libraries (*A. caliginosa, L. terrestris*, and *L. rubellus*) and the two soil libraries (mineral and uppermost soil) were combined, respectively. Sequences retrieved from DNA (genes) and cDNA (transcripts) are displayed. Numbering of OTUs at the right side correspond with that in the phylogenetic tree of *in silico* translated *nosZ* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies.

3.1.2.1.1.2.3. T-RFLP analysis of *nosZ* genes and transcripts

T-RFLP analysis with *nosZ* genes and transcripts was conducted with the same restriction enzyme and yielded 8 and 19 T-RFs, respectively (Figure 32A). Earthworm- and soil-derived *nosZ* gene sequences showed highly similar patterns (Figure 32A) of their most abundant T-RFs (e.g., 352 to 357 bp, 452/454 bp, 148 bp, 52/53/54 bp, and 47 bp) all affiliated with OTUs 1, 3, and 11 (Figure 32A, Figure 33). The *nosZ* transcript sequences generated nearly the same T-RFs but showed a more uneven distribution of the T-RFs resulting in more pronounced differences between the samples (Figure 32A, Figure 33).

As detectable with *nosZ* gene sequences, crop/gizzard samples were highly similar to their corresponding gut sample with *A. caliginosa* and *L. terrestris*. However, crop/gizzard samples always showed a higher abundance for the T-RFs 47 bp, 52/53/54 bp, and 65 bp than gut samples did (Figure 32A).

The earthworm feeding guild influenced the detected *nosZ* gene T-RFs as only the gut sample of the endogeic *A. caliginosa* showed a T-RF with 148 bp that was also present in

both soil samples and that lacks in both other earthworms (Figure 32A, Figure 33). In addition, the gut samples of the anecic *L. terrestris* and the epigeic *L. rubellus* showed a rising abundance of the T-RFs 47 bp (OTUs 3 and 1), 52/53/54 bp (OTUs 1, 3, and 11), and 65 bp (unaffiliated), other than *A. caliginosa* (Figure 32A, Figure 33). On *nosZ* transcript level, feeding guild-related differences were even stronger as the T-RF with 52/54 bp length showed a relative abundance of 15 %, 28 %, and 60 % in the gut of *A. caliginosa*, *L. terrestris*, and *L. rubellus*, respectively, and the T-RF with 352 to 357 bp length showed a relative abundance of 61 %, 33 %, and 2 % in the gut of *A. caliginosa*, *L. terrestris*, *and L. rubellus*, respectively (Figure 32A). In addition, the gut of *L. rubellus* showed the highest abundance of the T-RF with 47 bp length and, only here, a T-RF with 258 bp (OTU 23) appeared, separating the gut T-RFLP patterns of *L. rubellus* strongly from those of the two other earthworms (Figure 33).

Both soil samples were highly similar on *nosZ* gene level but displayed major differences on *nosZ* transcript level where the uppermost soil showed a higher similarity to the gut samples of *A. caliginosa* and *L. terrestris* whereas in the mineral soil, the T-RF with 47 pb length showed the highest abundance of all samples (Figure 32A, Figure 33).

In summary, T-RFLP analysis strongly confirmed the results derived from *nosZ* gene and transcript libraries showing a high detectability of sequences affiliated with *Rhizobiales*, especially with *Bradyrhizobium japonicum*. On gene and, to a greater extent on transcript level, the earthworm feeding guild had a strong influence on the detected *nosZ* sequences.

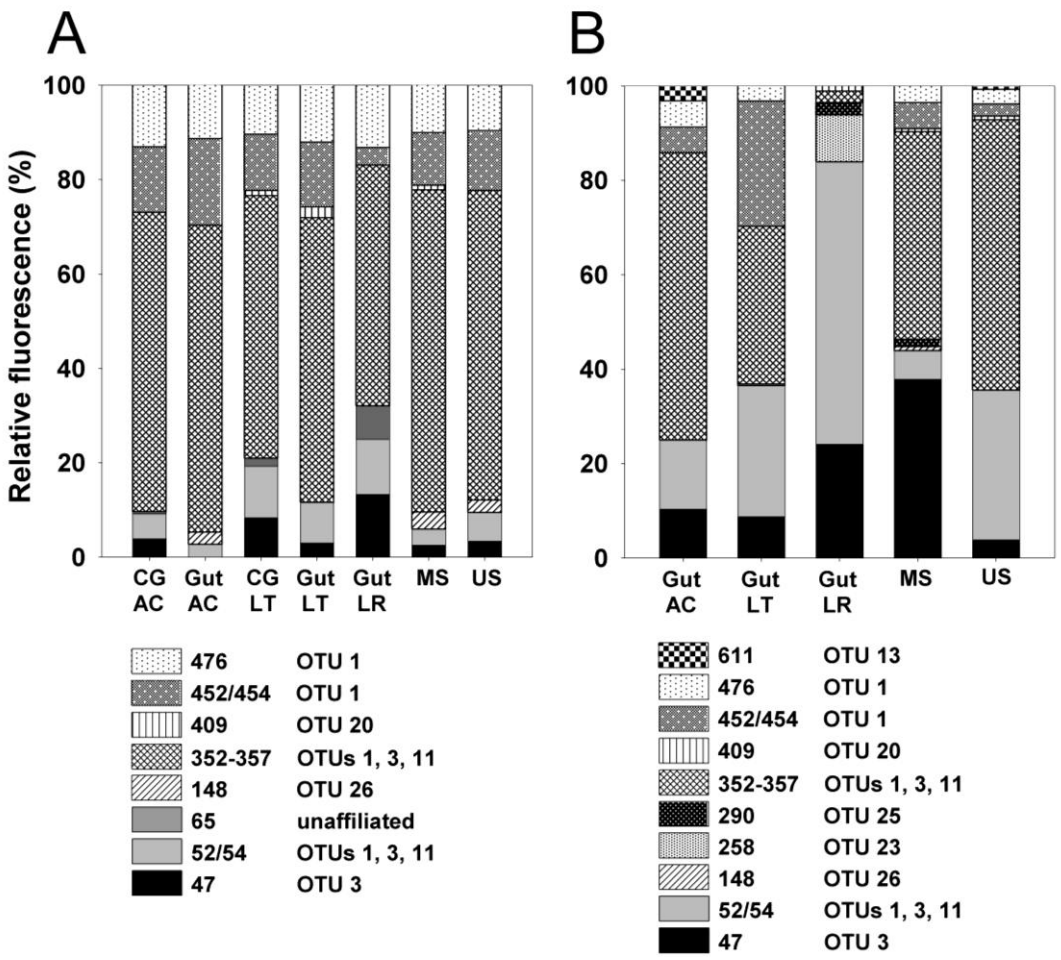


Figure 32: *nosZ* gene and transcript T-RFLP patterns from earthworm gut contents and soils. Displayed are the relative fluorescences of T-RFs exceeding 3 % in at least one sample; their sums were set as 100 %. Shown are mean values (*n*=3). A, genes (DNA-derived; digestion was with *Hha*I); B, transcripts (cDNA-derived; digestion was with *Hha*I). Abbreviations: CG, crop/gizzard (if lacking, no samples were available); AC, *A. caliginosa*; LT, *L. terrestris*; MS, mineral soil; US, uppermost soil. *A. caliginosa* and *L. terrestris* were sampled from mineral soil, *L. rubellus* was sampled from uppermost soil. Caption shows the lengths of the T-RFs as measured in bp and the *in silico* affiliated OTUs containing the highest numbers of T-RF sequences in a descending order; some T-RFs could not be affiliated with an OTU. Modified from Depkat-Jakob *et al.* (2010).

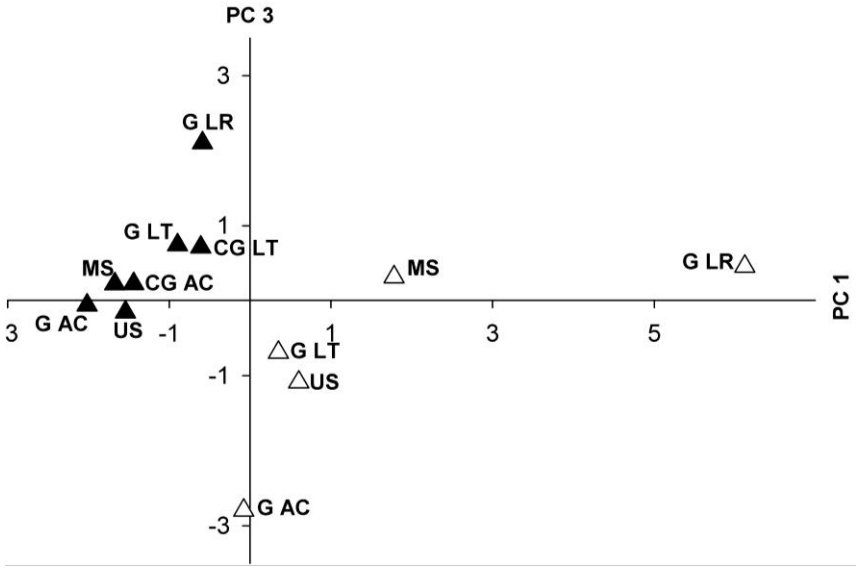


Figure 33: Principal component analysis of *nosZ* gene and transcript T-RFs.

A variance of 57.3 %is covered by x-axis (PC 1, 44.6 %) and y-axis (PC 3, 12.7 %). Gene and transcript analysis could be conducted together in one analysis as the same restriction enzyme was used for both underlying T-RFLP analyses. Filled symbols, DNA-derived samples; open symbols, cDNA-derived samples (transcripts). Abbreviations: G, gut; CG, crop/gizzard; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil. *A. caliginosa* and *L. terrestris* were sampled from mineral soil, *L. rubellus* was sampled from uppermost soil. Modified from Depkat-Jakob *et al.* (2010).

3.1.2.1.2. Gene analysis of *nirK* and *nirS* in gut contents and soils of *L. terrestris* and *A. caliginosa*

Amplification of *nirK* and *nirS* gene fragments was conducted with DNA derived from earthworm gut contents of *L. terrestris* and *A. caliginosa* (Table 1, 2.5.7.2.2). In addition, the mineral and uppermost soil was analyzed.

3.1.2.1.2.1. Nitrite reducers detected via *nirK* in gut contents and soils of *L. terrestris* and *A. caliginosa*

Amplification of *nirK* gene fragments was successful and yielded DNA fragments of the expected size for all samples (i.e., gut contents and soils of *L. terrestris* and *A. caliginosa*). However, cloning of the purified gene fragments yielded only few sequence clones, and sequencing of a subset of representative clones and subsequent BLAST analysis (2.5.12.2) revealed that sequences displayed no similarities to *nirK*. An optimization of PCR conditions

was estimated as a promising approach to overcome this problem but was canceled due to a limitation of time.

3.1.2.1.2.2. Nitrite reducers detected via *nirS* in gut contents and soils of *L. terrestris* and *A. caliginosa*

Amplification of *nirS* gene fragments was successful and yielded DNA fragments of the expected size for all samples (i.e., gut contents and soils of *L. terrestris* and *A. caliginosa*). However, cloning yielded a reasonable number of clones for the gut contents and soil of *L. terrestris* only. Thus, *nirS* sequence libraries were created for the gut contents and soil of *L. terrestris*.

3.1.2.1.2.2.1. Cloning and diversity analysis of *nirS* from the gut contents and soil of *L. terrestris*

97 *nirS* gene sequences were retrieved from the gut of *L. terrestris* and its corresponding soil, and *in silico* translated amino acid sequences yielded together 16 species-level OTUs (Table 20, Figure 34). 28 and 69 sequences were retrieved from the gut of *L. terrestris* and its soil, respectively; these sequences yielded 8 and 14 OTUs, respectively (Table 20, Figure 34).

Table 22: Estimated genotypes, coverage, and diversity indices of *in silico* translated *nirS* amino acid sequences from gut contents of *L. terrestris* and from its corresponding soil.[a]

				Diversity indices			
Library	No. of sequences	OTUs[b]	Coverage (%)	Rich-ness	H´[c]	Even-ness	1/D[d]
Gut	28	8	82	14 ± 4	1.36	0.65	2.64
Soil	69	14	88	22 ± 4	1.43	0.54	2.23

[a] All calculations were carried out with DOTUR-1.53 and based on amino acid sequences (2.5.12.4)

[b] *nirS* at a species-level cutoff value of 87 %.

[c] H´, Shannon-Weaver diversity index.

[d] 1/D, reciprocal Simpson diversity index.

See the methods part for further information (2.5.12).

Coverages for the gut and soil libraries were 82 % and 88 %, respectively (Table 20). Thus, it is anticipated that more OTUs would be obtained if more sequences had been analyzed. This was confirmed by the estimated richness of 14 and 22 OTUs for the gut library and soil library, respectively (Table 20).

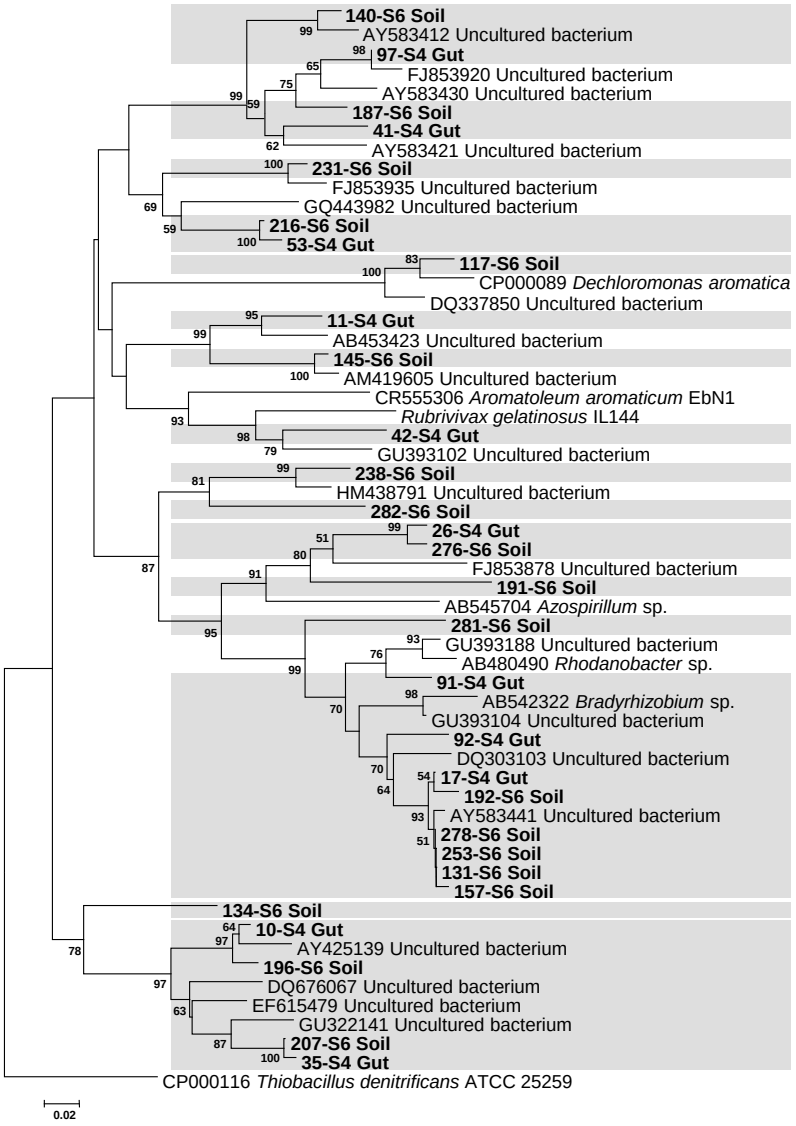
Diversity indices (i.e., Shannon-Weaver index, Evenness, and reciprocal Simpson index) for *nirS* gene sequences were similar to slightly higher for gut-derived than for soil-derived sequences (Table 20). This is indicative of a broad stimulation of soil-derived *nirS* nitrite reducers in the gut of *L. terrestris*.

3.1.2.1.2.2.2. Phylogenetic analysis of *nirS* from gut contents and soil of *L. terrestris*

The *nirS* sequences from both earthworm gut and soil of *L. terrestris* were predominantly affiliated with the genus *Bradyrhizobium* (OTU 14), and to a lower extent with uncultured soil *Bacteria* distantly related to *Thiobacillus denitrificans* (OTU 16) (Figure 34, Figure 35). Other OTUs were of minor abundance. Differences between gut- and soil-derived *nirS* sequences were small. However, gut-derived sequences showed a lower abundance of OTU 14 and a higher abundance of OTU 16 compared to those derived from soil (Figure 34, Figure 35).

Most *nirS* sequences occurred in OTUs that contained both soil- and gut-derived sequences (Figure 34, Figure 35), and were most closely related to uncultured soil *Bacteria*. This indicates that the majority of gut-derived sequences originated from ingested soil *Bacteria*.

The vast majority of detected *nirS* sequences was affiliated with taxa that showed a good correlation between 16S rRNA and *nirS* gene similarity (Figure 14, Figure 34). Thus, phylogeny detected via the *nirS* genes is very likely to display the correct phylogeny, i.e., the phylogeny based on the 16S rRNA genes.



OTU	Relative abundance of speciel-level OTUs (%) Gut	Soil
1	4	4
2	7	4
3	0	1
4	4	3
5	0	1
6	4	0
7	0	3
8	4	0
9	0	1
10	0	1
11	4	1
12	0	1
13	0	1
14	61	67
15	0	1
16	14	7

Figure 34: Phylogenetic neighbor-joining tree of *in silico* translated *nirS* amino acid sequences from gut contents of *L. terrestris*, its corresponding soil, and related sequences.

The phylogenetic tree is based on representative *in silico* translated amino acid sequences. Sequences from this work are bold. The percentage of replicate trees the associated taxa clustered together in the bootstrap test (10,000 replicates), are shown at the node of two branches (values below 50 % are not displayed). The table shows the relative distribution of sequences in each OTU as calculated with DOTUR-1.53. Differences between the sum and the combined percentage of the individual OTU percentages for one library are due to the rounding off of values. Gut, sequences derived from gut content of *L. terrestris*; Soil, sequences derived from the corresponding soil of *L. terrestris* (mineral soil). The libraries Gut *GP* and Soil *GP* contain 28 and 69 sequences, respectively. The enumeration of OTUs corresponds with those in the figure displaying the relative distribution of *nirS* OTUs. The bar indicates a 0.02 estimated change per amino acid.

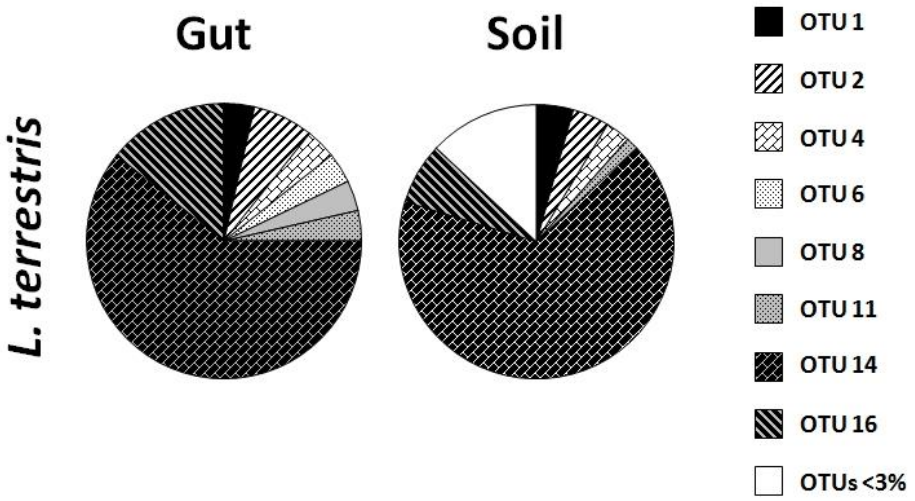


Figure 35: Relative distribution of *in silico* translated *nirS* amino acid sequences from gut contents of *L. terrestris* and from its corresponding soil.

L. terrestris was sampled from mineral soil. OTUs were calculated from *in silico* translated *nirS* sequences. OTU numbers at the right correspond with those in the phylogenetic tree of *in silico* translated *nirS* sequences; OTUs below 3 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies.

3.1.2.2. Isolation of potential denitrifiers from gut contents of earthworms of the family Lumbricidae representing different feeding guilds

Next to molecular methods, the isolation of denitrifying *Bacteria* (2.3.2.1) is an important additional technique to reveal the organisms that are responsible for the release of nitrogenous gases in the earthworm gut. The isolation approach of this work used nitrite and N_2O as added electron acceptors together with typical fermentation products detectable in the earthworm gut as carbon and energy source (2.3.2.1). Omitting nitrate aimed to isolate predominantly targeted denitrifiers instead of dissimilatory nitrate reducers. For inoculum, diluted gut contents of the earthworm species *L. rubellus*, *A. caliginosa*, *O. lacteum,* and *L. terrestris* were used representing the epigeic, endogeic, endogeic, and anecic feeding guild, respectively. Inoculums were applied to anoxic agar plates (2.3.1.2.1, 2.3.1.2.2) and incubated under anoxic conditions (2.3.2.1).

3.1.2.2.1. Summary and taxonomic analysis of bacterial isolates

For *Bacteria* isolated with nitrite and N_2O, 57 and 84 partial 16S rRNA genes were successfully sequenced, respectively (Table 23). Both isolation approaches yielded *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacilli*, and *Actinobacteria*, whereas few *Bacteriodetes* were detected with N_2O as added electron acceptor only (Table 23). The most frequently isolated bacterial strains for both isolation approaches were *Ensifer*, *Bacillus*, and *Paenibacillus*, whereas *Aeromonas* and *Pseudomonas* were only abundant within *Bacteria* isolated with N_2O (Table 23).

Both electron acceptor and earthworm species influenced the composition of the isolates detected. Isolation with nitrite yielded more *Bacilli* and *Actinobacteria* whereas isolation with N_2O yielded more *Gammaproteobacteria*, especially *Pseudomonas* and *Aeromonas* (Table 23). Within the *Bacteria* isolated with nitrite, the earthworm species was of minor influence for detected *Alphaproteobacteria*, whereas *L. rubellus*-derived isolates showed, for instance, a stronger affiliation with *Gammaproteobacteria* than isolates derived from the other earthworm species. Few *Bacilli* were derived from the gut of *L. rubellus* but were highly abundant in the gut of *A. caliginosa* (here, 53 % of all isolates were affiliated with *Paenibacillus*) (Table 23). Within the *Bacteria* isolated with N_2O, these trends were very similar compared to those of the *Bacteria* isolated with nitrite. However, the appearance of *Pseudomonas* in the gut of *L. rubellus* within the *Bacteria* isolated with N_2O was even more pronounced compared to those isolated with nitrite and to the other earthworm species (for *L. rubellus,* 56 % of all isolates were affiliated with *Pseudomonas*) (Table 23).

In summary, the most abundantly isolated taxa were *Ensifer*, *Aeromonas*, *Pseudomonas*, *Bacillus*, and *Paenibacillus*. The electron acceptor used (i.e., nitrite or N_2O) had an effect on the diversity of the detected isolates. Also the earthworm species, i.e., the feeding guild, influenced the diversity and composition of the detected isolates.

For the majority of isolates, the similarity of the 16S rRNA gene was > 98 % compared to species that were already described (Table A 2). However, the 16S rRNA gene fragment of Isolate 201 showed approximately 97 % similarity to its closest relative. Isolate 201 was therefore selected for detailed analysis of its 16S rRNA gene together with the Isolates 208, 403, 823 and ISO4.

Table 23: Bacterial isolates from gut contents of *L. rubellus*, *A. caliginosa*, *L. terrestris*, and *O. lacteum* isolated under anoxia with nitrite or N_2O as terminal electron acceptor.[a]

			NO_2^-				N_2O			
	NO_2^-	N_2O	*LR*	*AC*	*LT*	*OL*	*LR*	*AC*	*LT*	*OL*
Number of isolates sequenced	57	84	10	17	13	17	18	19	27	20
Alphaproteobacteria (%)	25	21	30	24	15	29	17	21	22	25
Aminobacter (%)	0	1	0	0	0	0	0	0	4	0
Bosea (%)	0	4	0	0	0	0	6	5	4	0
Ensifer (%)	25	15	30	24	15	29	11	16	15	20
Mesorhizobium (%)	0	1	0	0	0	0	0	0	0	5
Gammaproteobacteria (%)	7	33	30	0	8	0	83	11	30	15
Aeromonas (%)	4	13	20	0	0	0	22	11	11	10
Buttiauxella (%)	2	0	0	0	8	0	0	0	0	0
Erwinia (%)	0	2	0	0	0	0	6	0	4	0
Pantoea (%)	0	1	0	0	0	0	0	0	4	0
Pseudomonas (%)	2	17	10	0	0	0	56	0	11	5
Bacilli (%)	63	42	20	76	69	71	0	63	44	55
Bacillus (%)	42	29	20	24	62	59	0	42	41	25
Paenibacillus (%)	21	13	0	53	8	12	0	21	4	30
Bacteriodetes (%)	0	2	0	0	0	0	0	0	4	5
Flavobacterium (%)	0	1	0	0	0	0	0	0	0	5
Flexiacter (%)	0	1	0	0	0	0	0	0	4	0
Actinobacteria (%)	5	1	20	0	8	0	0	5	0	0
Cellulomonas (%)	2	0	0	0	8	0	0	0	0	0
Oerskovia (%)	4	1	20	0	0	0	0	5	0	0

[a] Nitrite or N_2O was added as electron acceptor to isolate denitrifiers instead of dissimilatory nitrate reducers on agar plates. NO was added to the incubation with N_2O in small amounts. Carbon sources consisted of fermentation products typically detectable in the earthworm gut. See methods part (2.3.2.1) for detailed information. 16S rRNA gene fragments of ca. 600 to 800 bp were used for sequencing with primer 27F. *LR*, *AC*, *LT*, *OL*: isolation source was gut content of *L. rubellus*, *A. caliginosa*, *L. terrestris*, and *O. lacteum*, respectively. Differences between the sum and the combined percentage of the individual percentages of one column are due to the rounding off of values.

3.1.2.2.2. Physiological and genetic characterization of selected bacterial isolates

For Isolate 201 and Isolate 208 (both from gut contents of *A. caliginosa* and isolated with nitrite), a 1320 bp and a 1268 bp 16S rRNA gene fragment was sequenced successfully, respectively (Table 24). The next related type strains of Isolate 201 and Isolate 208 were *Mycoplana ramosa* DSM7292 and *Paenibacillus borealis* KK19, respectively, with a 16S

rRNA gene similarity of 97.7 % and 99.0 %, respectively (Table 24). The 16S rRNA gene fragments of Isolate 403, Isolate 823, and Isolate ISO4 shared 99.8 %, 100 %, and 99.0 % similarity with that of *Paenibacillus borealis* 15, *Bacillus drentensis +Y73*, and *Pantoea agglomerans* HDDMN03, respectively (Table A 2). Thus, other than Isolate 208, Isolate 403, Isolate 823, and Isolate ISO4, Isolate 201 was assumed to represent a potentially novel species as the species level cutoff-value of 97 % for 16S rRNA genes is a very conservative species-cutoff estimate only (Stackebrandt 2006, Stackebrandt & Ebers 2006).

Isolate 201 and Isolate 208 were selected for basic physiological analyses and genes indicative of denitrification and dissimilatory nitrate reduction (i.e., *narG*, *napA*, *nirK*, *nirS*, *nosZ*) after transfer from anoxic to oxic agar plates (2.3.2.1, 2.3.2.2). Under oxic conditions without nitrate (2.3.2.2.1), the OD of cultures of Isolate 201 and Isolate 208 increased 0.43 and 0.75 OD units, respectively, within 7 h (an increase of OD is hereafter referred to as 'growth') (Table 24). Isolate 208 displayed a moderate growth under anoxic conditions without nitrate (i.e., the OD increased 0.33 OD units) whereas isolate 201 did not grow under these conditions. Supplemental nitrate (2.3.2.2.2) had no apparent effect on the moderate anaerobic growth of Isolate 208 but greatly stimulated the anaerobic growth of Isolate 201 whose OD increased 0.73 OD units (Table 24).

Table 24: Physiological and genetic features of two bacterial isolates from gut contents of *A. caliginosa* isolated under anoxia with nitrite as electron acceptor.[a]

			Growth condition[c]			
Isolate	Next related type strain (accession number)[b]	Similarity	oxic - NO_3^-	anoxic - NO_3^-	anoxic + NO_3^-	Nitrate reductase[d]
201	*Mycoplana ramosa* DSM7292 (EU022308)	97.7 %	+ +	o	+ +	*napA*
208	*Paenibacillus borealis* KK19 (AJ011322)	99.0 %	+ +	+	+	*narG*

[a] During isolation procedure on agar plates, nitrite was added as electron acceptor. Carbon sources consisted of fermentation products typically detectable in the earthworm gut. See methods part (2.3.2.1) for detailed information.

[b] Next related species type strain and its accession number as based of the similarity of the 16S rRNA gene fragment of an isolate compared to that of the next related species type strain. Gene fragment size was 1320 and 1268 bp for Isolate 201 and Isolate 208, respectively.

[c] Isolates were incubated in an oxic or anoxic liquid medium containing yeast with and without nitrate. Growth was defined as an increase of OD determined for 7 h at 660 nm wavelength. o, increase of OD < 0.02; +, 0.30 < increase of OD < 0.40; + + , increase of OD > 0.40.

[d] Isolates were tested for the occurrence of *napA*, *narG*, *nirK*, *nirS*, and *nosZ*. Only amplicons of *napA* (Isolate 201) and *narG* (Isolate 208) were detected and sequenced.

Screening of isolates for denitrification and dissimilatory nitrate reduction marker genes (2.5.7.2.2) revealed that Isolate 201 and Isolate 208 harbored *napA* and *narG* (both encoding for a nitrate reductase), respectively. The *napA* of Isolate 201 was most closely related to that of *Agrobacterium tumefaciens* C58, whereas the *narG* of Isolate 208 was most closely related to that of *Paenibacillus terrae* HPL-003 (Table 24). Other genes (i.e., *nirK*, *nirS*, and *nosZ*) were not detected or sequences could not be affiliated with the target gene.

3.1.3. Earthworms from New Zealand

Preceding to the present study, earthworms from New Zealand (2.1.3) had been tested by other workers for their ability to release denitrification-derived N_2O and N_2 (Wüst *et al.* 2009b) It was shown that the introduced *L. rubellus* (Lumbricidae) emitted N_2O whereas the native *O. multiporus* (Megascolecidae) did not although both earthworm guts displayed a high denitrification potential (Wüst *et al.* 2009b). For these earthworms and soils, *nosZ* gene fragments had been studied revealing a predominance of sequences related to *Bradyrhizobium* and *Rhodopseudomonas* (both *Rhizobiales*) (Wüst *et al.* 2009b). The present study used DNA from the gut content and soil (i.e., forest soil) of the large, endogeic *O. multiporus* from this preceding work (2.2.4.3) to analyze *nosZ* gene fragments amplified with a different primer system (Table 6) than used before (Wüst *et al.* 2009b).

3.1.3.1. Denitrifiers detected via *nosZ* in gut contents and soil of *O. multiporus*

In comparison to the preceding study in New Zealand (Wüst *et al.* 2009b) and to all other analyses of *nosZ* in this dissertation (3.1.1.3.5, 3.1.2.1.1.2), a different *nosZ* primer system was used (Scala & Kerkhof 1998; Table 6), i.e., a larger gene fragment at altering primer positions and primer sequences was amplified.

3.1.3.1.1. Cloning and diversity analysis of *nosZ*

60 and 47 *nosZ* gene fragment sequences were retrieved from the earthworm gut contents and the corresponding soil of *O. multiporus*, respectively, yielding each 11 species-level OTUs at a species-level cutoff value for of 86 % for *in silico* translated amino acid sequences (Table 25, Figure 36). The number of OTUs in the preceding *nosZ* study was lower with 8 and 7 OTUs for *O. multiporus* and forest soil, respectively (Table 25; Wüst *et al.* 2009b).

Coverages for all *nosZ* libraries ranged between 93.0 % and 93.3 %, and sampling was therefore estimated as sufficient for further analyses (Table 25). This was confirmed by the estimated richness that was the same and one OTU higher than for the sequences already sampled for the forest soil and *O. multiporus,* respectively (Table 25).

Diversity indices (i.e., Shannon-Weaver index and Evenness) were slightly higher for *O. multiporus* than for the forest soil. This indicates that stimulation of soil-derived denitrifiers in the earthworm gut is restricted to a smaller fraction rather than applied to all ingested denitrifiers.

Table 25: Diversity of *in silico* translated *nosZ* amino acid sequences retrieved from earthworm gut contents of *O. multiporus* and from its corresponding forest soil.[a]

	Library	
	O. multiporus	Forest soil
Sequences	60	57
Coverage (%)	93.3	93.0
OTUs (the current study)[b]	11	11
OTUs (Wüst *et al.* 2009b)[c]	8	7
Chao1 richness	12	11
H´[d]	1.29	1.38
Evenness	0.54	0.57

[a] All calculations were carried out with DOTUR-1.53 and based on amino acid sequences (2.5.12.4).

[b] *nosZ* at a species-level cutoff value of 86 %.

[c] *nosZ* sequences with a different primer system (Rich *et al*. 2003) were retrieved from the same extract of nucleic acids as used for this study, and were also analyzed with DOTUR-1.53 at a *nosZ* at a species-level cutoff value of 14 % dissimilarity of amino acid sequence.

[d] H´, Shannon-Weaver diversity index.

3.1.3.1.2. Phylogenetic analysis of *nosZ*

Detected *nosZ* sequences were predominantly affiliated with OTU 2 whose sequences are closely related to *Rhodopseudomonas*, *Flavobacterium*, and *Dechloromonas*. OTU 1 was affiliated with *Bradyrhizobium* and accounted for 20 % and 5 % of all sequences derived from *O. multiporus* and forest soil, respectively. OTU 8, 12, and 14 showed a relative abundance of at least 10 % in one library and were most closely affiliated *nosZ* sequences of uncultured soil *Bacteria* (Figure 36, Figure 37).

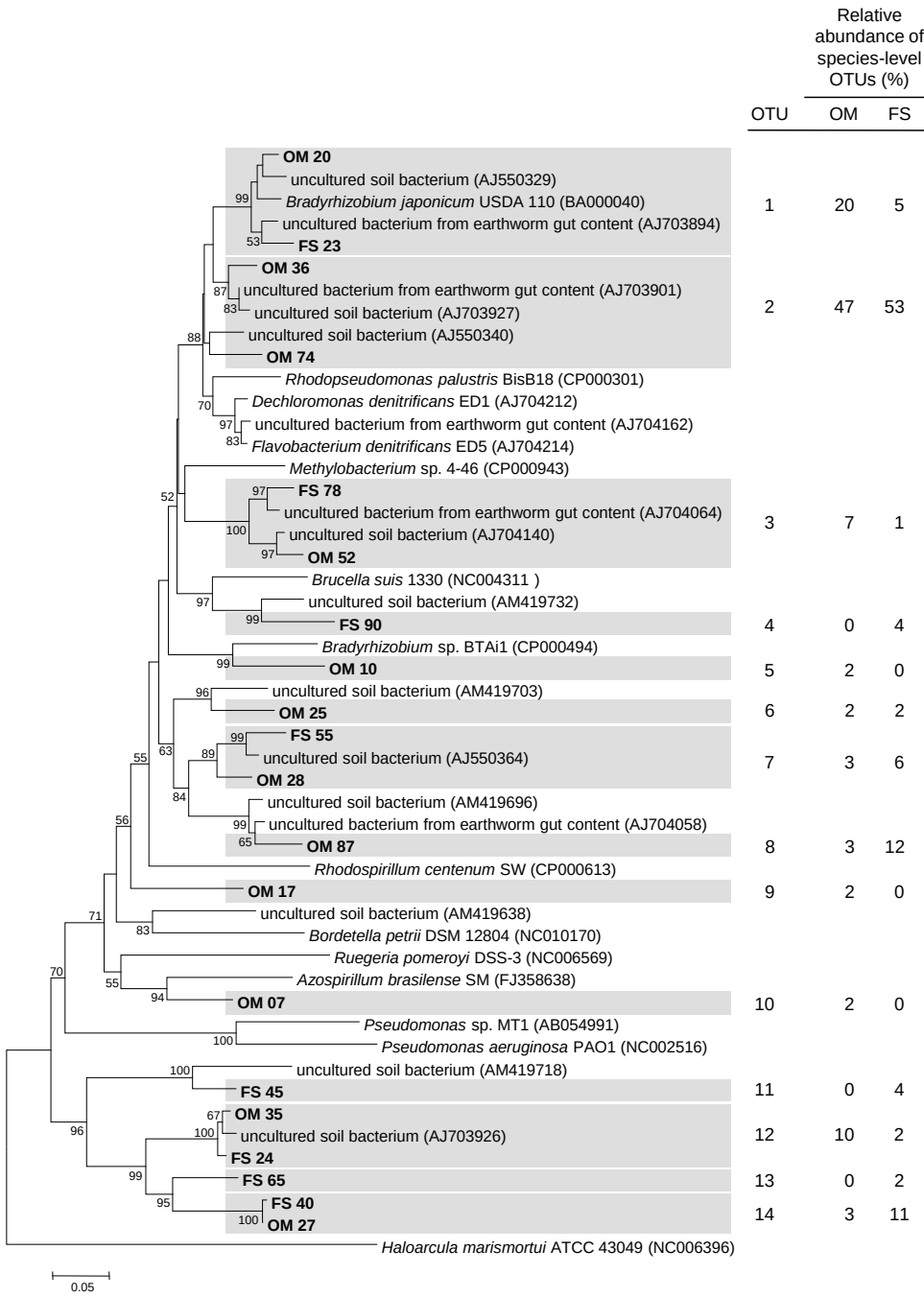


Figure 36: Phylogenetic neighbor-joining tree of *in silico* translated *nosZ* sequences from gut contents of *O. multiporus* and from its corresponding forest soil, and related *nosZ* sequences. Sequences from this study are bold. Tree is based on translated amino acids. Values next to the branches show the percentages of replicate neighbor-joining trees in the bootstrap test (10,000 bootstraps) in which the associated taxa clustered together (values below 50 % are masked). The bar indicates a 0.05 estimated change per amino acid. The table shows the relative distribution of sequences in each cluster (i.e., OTU; shaded text) as calculated with DOTUR-1.53. Relative abundances of a library may not add up to 100 % due to roundings. Abbreviations: OM, *O. multiporus*; FS, forest soil. Representative sequences were used for each OTU.

Most *nosZ* sequences occurred in OTUs that contained both soil- and gut-derived sequences; OTU 5, 9, and 10 were present in the gut only but were of minor abundance, i.e., 2 % each (Figure 36, Figure 37). Most sequences were most closely related to uncultured soil *Bacteria* (Figure 36), indicating that the majority of gut-derived sequences originated from ingested soil *Bacteria*.

Figure 37: Relative distribution of OTUs of *in silico* translated *nosZ* sequences retrieved from the gut contents of *O. multiporus* and from its corresponding forest soil.
OTUs were calculated from *in silico* translated *nosZ* sequences and OTU numbers at the right correspond with those in the phylogenetic tree of *in silico* translated *nosZ* sequences; OTUs below 5 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies.

3.2. Emission of CH_4 by earthworms and analysis of associated microorganisms in the earthworm gut

The emission of CH_4 by earthworms has not been previously observed, and research to date that has examined this potential has been restricted to members of the family Lumbricidae (Karsten & Drake 1995, Šustr & Šimek 2009). Thus, eight earthworm species of different families, sizes, and feeding guilds were sampled in Brazil (2.1.1.1) along with their soils and substrates, analyzed for the emission of CH_4 (2.2), and for genes indicative of methanogenesis, i.e., *mcrA*/*mrtA* (2.5.7.2.1).

3.2.1. Earthworms and substrates sampled for analysis

3.2.1.1. Origin of earthworm species and substrates

Eight earthworm species were analyzed that represented five different families and were of different sizes and different feeding guilds; the worms were obtained along with their soils/substrates (Table 1, Table 26). Analyzed families and corresponding species were Glossoscolecidae (*G. paulistus*, *Glossoscolex* sp., *P. corethrurus*, and *R. alatus*), Megascolecidae (*A. gracilis* and *P. excavatus*), *Eudrilidae* (*E. eugeniae*), and Lumbricidae (*E. andrei*) (Table 26).

Table 26: Origin of earthworms and earthworm soils and substrates in Brazil selected for the assessment of the emission of CH_4.

Substrate	Type	Earthworms[a]	Origin	Sampling 2011
S1	Composted cow manure	*E. eugeniae*, *P. excavatus*	Minhobox	March, September
S2	Processed sugarcane residue	*E. eugeniae*	Earthworm distributor	September
S3	Processed sugarcane residue	*E. eugeniae*, *E. andrei*	Earthworm distributor	September
S4	Grassland soil	*A. gracilis*, *P. corethrurus*	Piracicaba, São Paulo, Brazil	March, September
S5	Pasture soil	*G. paulistus*	Assistência district, São Paulo, Brazil	March
S6	Soil from a swampy meadow	*Glossoscolex* sp.	Assistência district, São Paulo, Brazil	March
S7	Soil obtained with worms	*R. alatus*	Paraopeba, Minas Gerais, Brazil	March

[a] Earthworms were originally obtained on these substrates. *R. alatus* was also obtained in September 2011, but was in diapause, i.e., its gut was not filled with any substrate. See Figure 38A for information on which different substrates worms were subjected to.

Modified from Depkat-Jakob *et al.* (2012).

The species *E. andrei*, *E. eugeniae*, and *R. alatus* were purchased from an earthworm distributor or earthworm collector along with their substrate; *E. eugeniae* was obtained on three substrates from three different distributors (Table 26). In addition to *R. alatus* obtained with Substrate 7 in March 2011(Table 26), *R. alatus* specimens were also obtained in diapause, i.e., without gut content in September 2011 (2.1.1.1). *G. paulistus*, *Glossoscolex* sp., and *R. alatus* are in the following termed as large, all other species as small species.

3.2.1.2. Substrate properties

For Substrates 1 to 4, soil properties were determined (2.4.2). Concerning pH, moisture, ammonium, nitrate, phosphorus, and potassium concentrations, total organic material, and total nitrogen, values were always highest for Substrate 1 (processed cow manure), followed by Substrate 2 (residues from processed sugarcane), Substrate 3 (residues from processed sugarcane), and substrate 4 (grassland soil) (Table 27). Thus, Substrate 4 was identified as the driest and 'poorest' substrate, i.e., the substrate with least nutrients of all four substrates analyzed.

Table 27: Properties of selected substrates of eartworms analyzed for the emission of CH_4.[a]

Substrate	pH (H_2O)[b]	Moisture (%)	NH_4^+ [c]	NO_3^- [c]	P[c]	K[c]	Total organic material[d]	Total nitrogen[d]
S1[e]	8.0	76	95	60	2,276	164	594	14.84
S2[f]	7.5	59	90	56	1,751	34	414	14.71
S3[g]	7.6	56	64	28	270	91	283	10.92
S4[h]	6.5	22	28	13	9	8	40	3.36

[a] Substrates were obtained and analyzed in September 2011 (2.4.2).

[b] pH was measured in H_2O.

[c] Concentration in mg (kg fresh weight)$^{-1}$.

[d] Concentration in g (kg fresh weight)$^{-1}$.

[e] Processed cow manure.

[f] Residues from processed sugarcane.

[g] Residues from processed sugarcane.

[h] Grassland soil.

Modified from Depkat-Jakob *et al.* (2012).

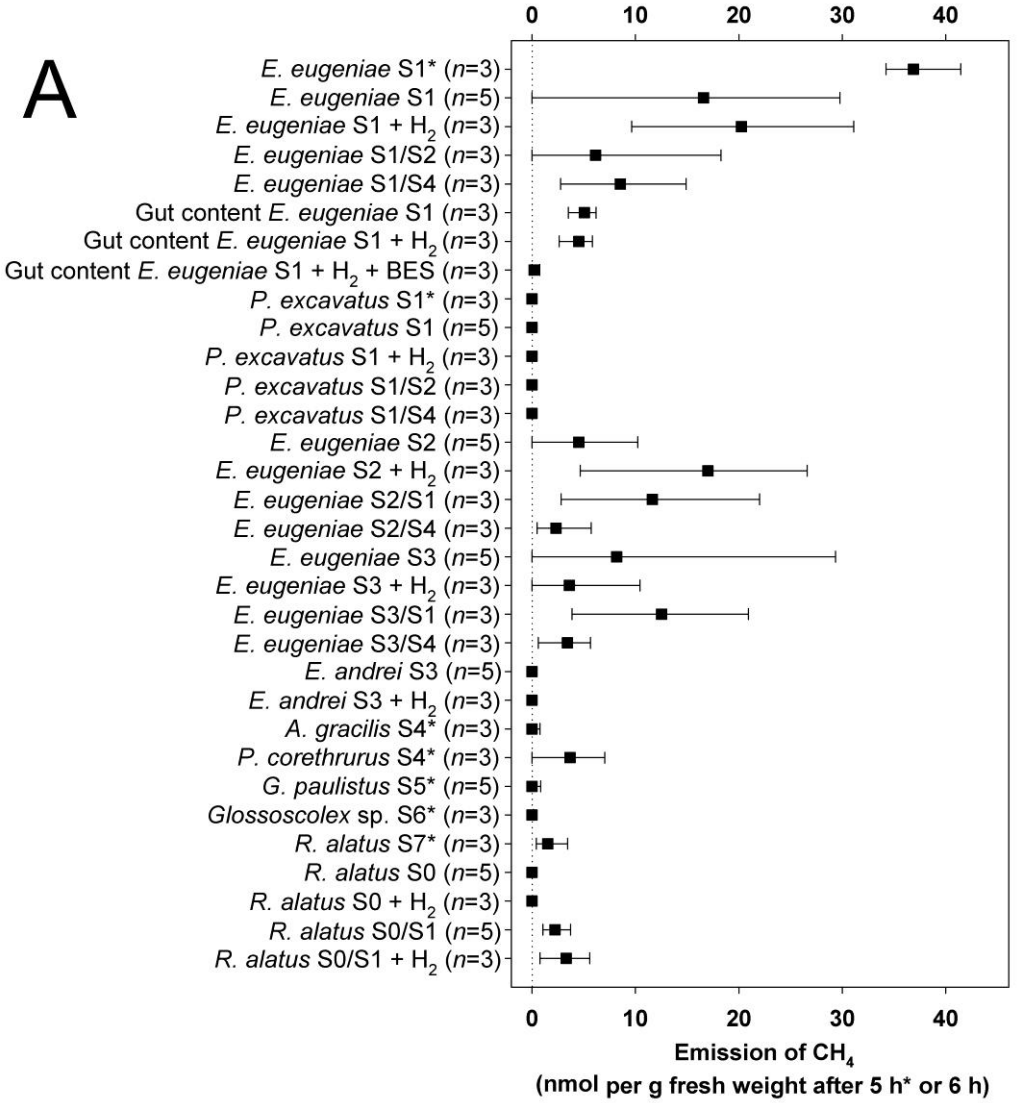
3.2.2. Emission of CH_4 by earthworms and their substrates

3.2.2.1. Emission of CH_4 by earthworms raised and maintained on their substrates

E. eugeniae displayed the highest emissions of CH_4 (2.2.1) independent of the substrate the earthworms were raised on, i.e., Substrate 1, 2, or 3. Emissions of CH_4 were up to 41 and 30 nmol CH_4 (g fw)$^{-1}$ after a 5 and 6 h incubation when raised on Substrate 1 (i.e., composted cow manure), respectively, up to 10 nmol CH_4 (g fw)$^{-1}$ after 6 h when raised on Substrate 2 (i.e., residues from processed sugarcane), and up to 29 nmol CH_4 (g fw)$^{-1}$ after 6 h when raised on Substrate 3 (i.e., residues from processed sugarcane) (Figure 38A). Emissions of CH_4 were relatively linear with approximately 5 nmol CH_4 (g fw)$^{-1}$ h^{-1} for *E. eugeniae* raised on Substrate 1 (Figure 38A, Figure 39). Most specimens of *E. eugeniae* emitted CH_4 whereas some specimens completely lacked CH_4 emission although raised on the same substrate and with comparable length and weight (Figure 38A). Anoxically incubated gut contents of *E. eugeniae* raised on Substrate 1 also emitted CH_4. However, CH_4 emissions by gut contents were significantly lower than those of living earthworms on a per g fresh weigh basis (Figure 38A). Gut contents emitted no CH_4 when incubated with 2-bromoethane sulfonate (BES), a metabolic inhibitor of methanogenesis (Gunsalus *et al.* 1978) (Figure 38A).

Supplemental H_2 and CO_2 (H_2/CO_2) in the headspace of living earthworms did not stimulate the emission of CH_4 by *E. eugeniae* raised on Substrate 1, whereas CH_4 emissions by specimens raised on Substrate 2 and 3 were enhanced and reduced with supplemental H_2/CO_2, respectively. Supplemental H_2/CO_2 did not stimulate the emission of CH_4 by gut contents of *E. eugeniae*.

P. excavatus and *E. andrei* showed no emission of CH_4 although these species were raised on the same substrates as *E. eugeniae*, i.e., Substrate 1 and 3, respectively (Figure 38A, Table 26). Supplemental H_2/CO_2 had no effect on *P. excavatus* and *E. andrei* concerning the emission of CH_4 (Figure 38A). *G. paulistus*, *Glossoscolex* sp., and *A. gracilis*, emitted no CH_4. Also *R. alatus* obtained without gut contents emitted no CH_4 (Figure 38A). *P. corethrurus* sampled from Substrate 4 emitted up to 7 nmol CH_4 (g fw)$^{-1}$ after 5 h, *R. alatus* obtained on Substrate 7 emitted up to 4 nmol CH_4 (g fw)$^{-1}$ after 5 h (Figure 38A). *G. paulistus* and *Glossoscolex* sp. emitted no CH_4 (Figure 38A). Of the three earthworm species emitting CH_4, *E. eugeniae* (Eudrilidae) and *P. corethrurus* (Glossoscolecidae) were small whereas *R. alatus* (Glossoscolecidae) was the largest earthworm of all species sampled. Thus, the emission of CH_4 was independent of the earthworm size and family.


A
E. eugeniae S1* (n=3)
E. eugeniae S1 (n=5)
E. eugeniae S1 + H2 (n=3)
E. eugeniae S1/S2 (n=3)
E. eugeniae S1/S4 (n=3)
Gut content E. eugeniae S1 (n=3)
Gut content E. eugeniae S1 + H2 (n=3)
Gut content E. eugeniae S1 + H2 + BES (n=3)
P. excavatus S1* (n=3)
P. excavatus S1 (n=5)
P. excavatus S1 + H2 (n=3)
P. excavatus S1/S2 (n=3)
P. excavatus S1/S4 (n=3)
E. eugeniae S2 (n=5)
E. eugeniae S2 + H2 (n=3)
E. eugeniae S2/S1 (n=3)
E. eugeniae S2/S4 (n=3)
E. eugeniae S3 (n=5)
E. eugeniae S3 + H2 (n=3)
E. eugeniae S3/S1 (n=3)
E. eugeniae S3/S4 (n=3)
E. andrei S3 (n=5)
E. andrei S3 + H2 (n=3)
A. gracilis S4* (n=3)
P. corethrurus S4* (n=3)
G. paulistus S5* (n=5)
Glossoscolex sp. S6* (n=3)
R. alatus S7* (n=3)
R. alatus S0 (n=5)
R. alatus S0 + H2 (n=3)
R. alatus S0/S1 (n=5)
R. alatus S0/S1 + H2 (n=3)
0 10 20 30 40
Emission of CH4
(nmol per g fresh weight after 5 h* or 6 h)

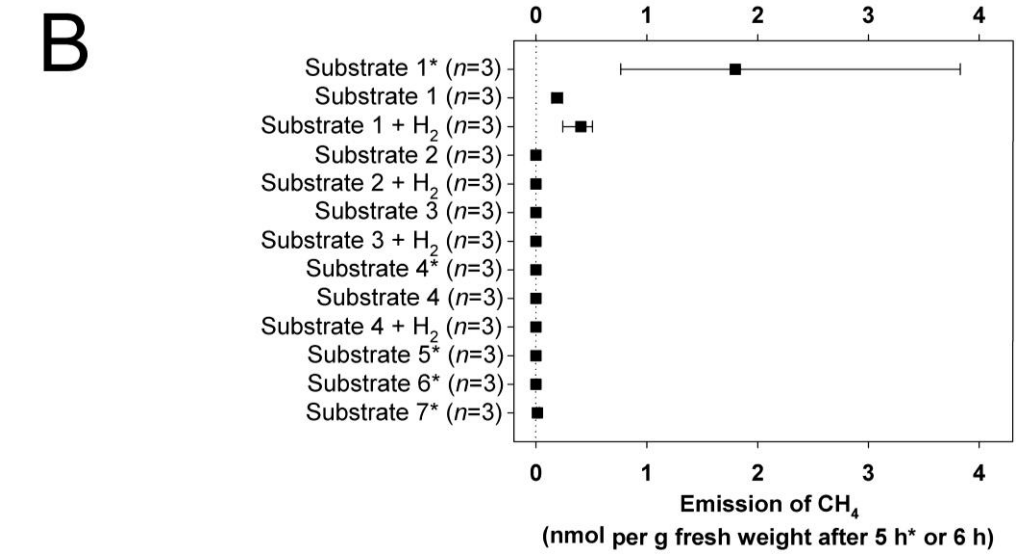

B
Substrate 1* (n=3)
Substrate 1 (n=3)
Substrate 1 + H2 (n=3)
Substrate 2 (n=3)
Substrate 2 + H2 (n=3)
Substrate 3 (n=3)
Substrate 3 + H2 (n=3)
Substrate 4* (n=3)
Substrate 4 (n=3)
Substrate 4 + H2 (n=3)
Substrate 5* (n=3)
Substrate 6* (n=3)
Substrate 7* (n=3)
0 1 2 3 4
Emission of CH4
(nmol per g fresh weight after 5 h* or 6 h)

Figure 38: Emission of CH_4 by living earthworms and gut contents (A) and earthworm substrates (B).

Results marked with and without an asterisk are from the sampling in March 2011 (5-h incubation) and September 2011 (6-h incubation), respectively. Filled squares show mean values, lines show lowest and highest single values. Abbreviations: *n*, number of replicates; S, substrate; first number after S, indicate the substrate on which worms were raised and maintained (e.g., S1 is Substrate 1); second S and accompanying number, indicate the substrate to which the worms were transferred and maintained for 60 h prior to assay (e.g., S1/S2 indicates that worms raised on Substrate 1 but transferred to and maintained on Substrate 2 prior to assay); H_2, indicates that the headspace contained approximately 1.5 % H_2 and 0.4 % CO_2; BES, indicates that the assays were supplemented with BES yielding a final concentration of 30 mM; S0, worms were received in diapause (i.e., without gut content); S0/S1, worms were received in diapause without gut content and incubated on Substrate 1 for 60 h prior to assay. One worm (two worms for *E. andrei*) or 10 g soil per replicate. Modified from Depkat-Jakob *et al.* (2012).

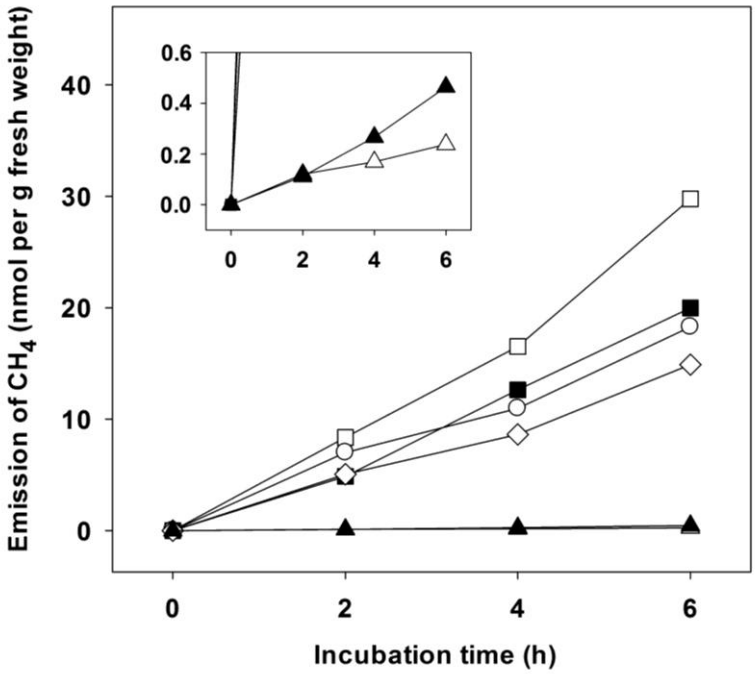


Figure 39: Emission of CH_4 by representative specimens of *E. eugeniae* and Substrate 1 under different incubation conditions.

Symbols: squares, *E. eugeniae* raised and maintained on Substrate 1; circles, *E. eugeniae* raised on Substrate 1 and transferred onto substrate 2 for 60 h prior to assay; diamonds, *E. eugeniae* raised on Substrate 1 and transferred onto substrate 4 for 60 h prior to assay; inset displays minor emission of CH_4 by Substrate 1 in nmol per g fresh weight within 6 h of incubation; triangles, Substrate 1; empty symbols, headspace was ambient air; filled symbols, headspace was ambient air supplemented with approximately 1.5 % H_2 and 0.4 % CO_2;); see methods parts (2.2.1) and (2.2.1.2) for further information. Modified from Depkat-Jakob *et al.* (2012).

All substrates except for Substrate 1 lacked the emission of CH_4 (Figure 38B). Substrate 1 emitted minor amounts of CH_4 compared to *E. eugeniae* raised on this substrate, i.e., Substrate 1 emitted approximately 20- and 90-fold less CH_4 than the average emission of *E. eugeniae* on a per g fresh weight basis in March and August 2011, respectively (Figure 38B). Supplemental H_2/CO_2 in the headspace marginally enhanced the emission of CH_4 by substrate 1 but had no effect on all other substrates (Figure 38B, Figure 39).

3.2.2.2. Emission of CH_4 by earthworms subjected to different substrates

Earthworms were subjected to substrates different from those they were raised on to analyze the effect of the substrate on the emission of CH_4. Earthworms were kept for 60 h on the new substrate and repeated exchange of the gut content was verified by the amount and different color of the earthworm casts detected (2.2.1.2).

E. eugeniae specimens raised on Substrate 1, 2, and 3 maintained their ability to emit CH_4 when subjected to Substrate 2, 1, and 1, respectively. Subjected to Substrate 4, emission of CH_4 was also maintained, albeit strongly reduced for *E. eugeniae* specimens raised on Substrate 1, 2, and 3 (Figure 38A). *P. excavatus* raised on Substrate 1 maintained its inability to emit CH_4 when subjected to Substrate 2 and 4 (Figure 38A). *R. alatus* emitted up to 4 nmol CH_4 (g fw)$^{-1}$ after 6 h when subjected to Substrate 1, and up to 6 nmol CH_4 (g fw)$^{-1}$ after 6 h with additional H_2/CO_2 in the headspace (Figure 38A).

3.2.3. Methanogens in gut contents and Substrate 1 of *E. eugeniae* detected via the structural gene markers *mcrA* and *mrtA*

Emissions of CH_4 were highest for the earthworm species *E. eugeniae* and were also detectable for its substrate, i.e., Substrate 1 (Table 26). Thus, DNA, and DNA-free RNA were extracted (2.5.1) from both gut contents of *E. eugeniae* raised on Substrate 1 and from Substrate 1. Sequence libraries were constructed from DNA, and from cDNA derived from DNA-free RNA (2.5.6, 2.5.4.3) for the structural gene markers *mcrA* and *mrtA* encoding for a subunit of the methyl-CoM reductase and its isoenzyme, respectively.

94 gene sequences (including 5 *mrtA* sequences) and 94 *mcrA* transcript sequences were retrieved from gut contents of *E. eugeniae*. 87 gene sequences (including 2 *mrtA* sequences) and 92 *mcrA* transcript sequences were retrieved from Substrate 1. For all four

libraries, coverage exceeded 97 % at a species-level cutoff of 86 % (Hunger *et al.* 2011). Altogether, 11 *mcrA* OTUs and one *mrtA* OTU were detected (Figure 40).

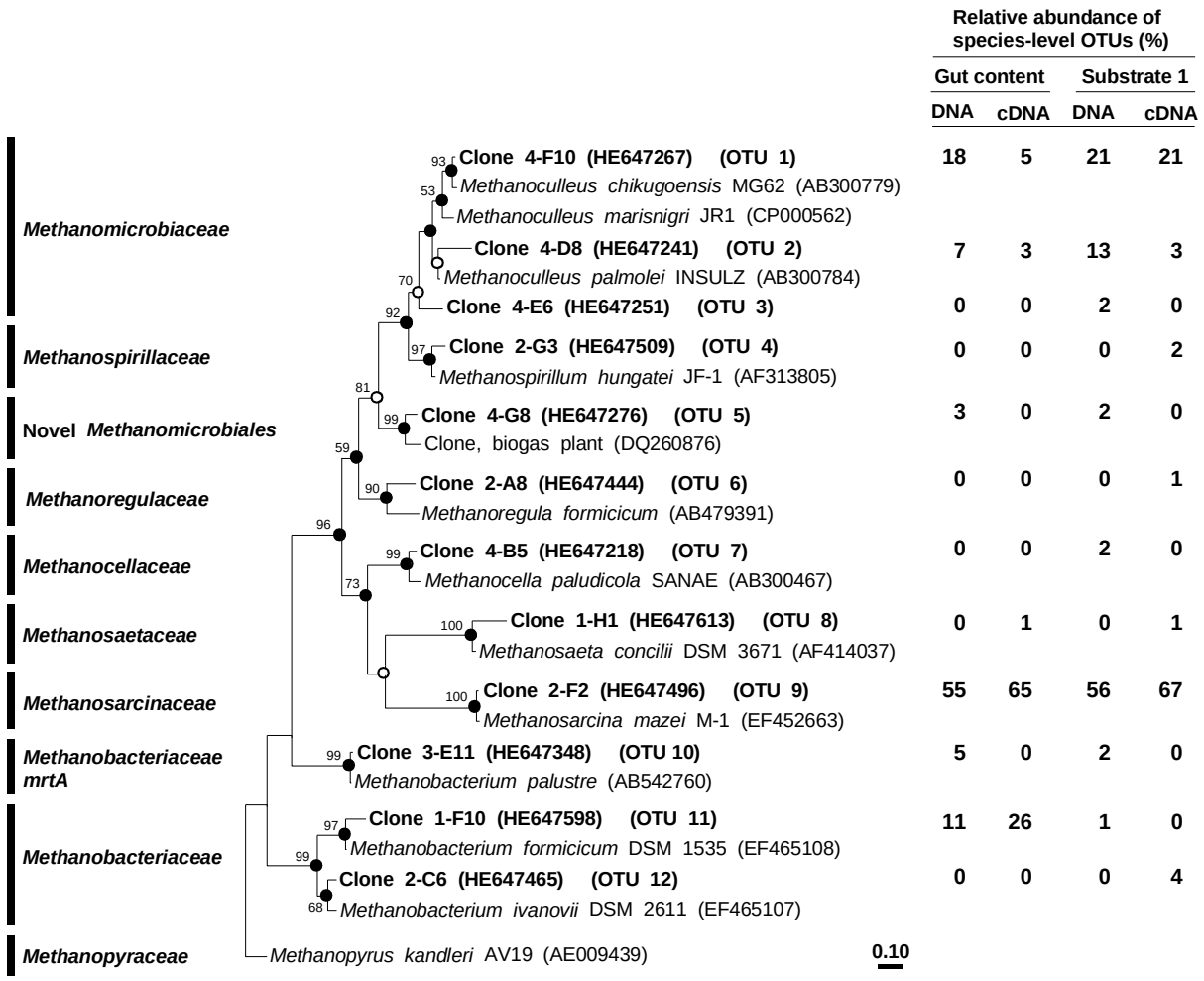


Figure 40: Phylogenic neighbor-joining tree of *in silico* translated gene and transcript sequences of *mcrA* and *mrtA* retrieved from gut contents of *E. eugeniae*, from Substrate 1, and affiliated reference sequences.

Tree is based on *in silico* translated amino acid sequences. Values next to the branches show the percentages of replicate trees in the bootstrap test (10,000 bootstraps) in which the associated taxa clustered together (values below 50 % are masked). Dots at nodes show the confirmation of tree the topology by all maximum-likelihood and maximum-parsimony calculations with the same data set as the displayed neighbor-joining tree. Empty circles indicate the confirmation of the tree topology by 3 of 4 calculations. Sequences displayed in the tree are *mcrA* sequences, if not otherwise indicated. The bar indicates a 0.1 estimated change per amino acid. Modified from Depkat-Jakob *et al.* (2012).

Detected *mcrA* gene and transcript sequences were affiliated with *Methanomicrobiaceae* (OTUs 1 to 3), *Methanospirillaceae* (OTU 4), *Methanoregulaceae* (OTU 6), *Methanocellaceae* (OTU 7), *Methanosaetaceae* (OTU 8), *Methanosarcinaceae* (OTU 9), *Methanobacteriaceae* (OTUs 11 and 12), with sequences of OTU 5 being distantly related to *Methanomicrobiales*. There was no OTU that was detected in the gut of *E. eugeniae* only but lacked in Substrate 1. Sequences of *mrtA* were detected on gene level only, and were affiliated with *Methanobacteriaceae* (OTU 10) (Figure 40).

Most *mcrA* gene sequences were affiliated with *Methanosarcina mazei* M-1 (*Methanosarcinaceae*, OTU 9), i.e., 55 % and 65 % for gut contents of *E. eugeniae* and Substrate 1, respectively. Other abundant taxa of *mcrA* gene sequences were *Methanomicrobiaceae* (OTUs 1 to 3) and *Methanobacteriaceae* (OTU 11) (Figure 40).

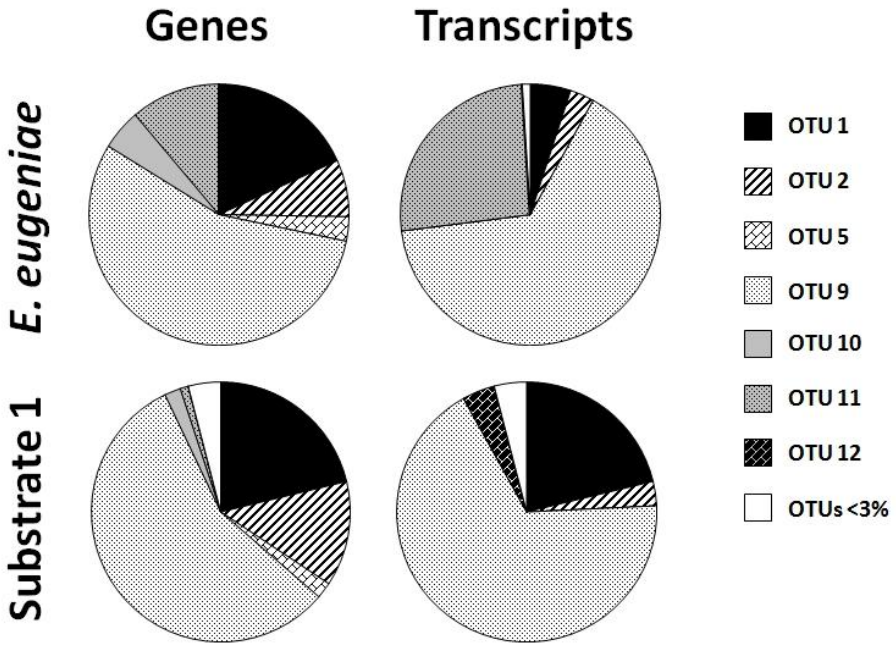


Figure 41: Relative distribution of *in silico* translated *mcrA* and *mrtA* gene and transcript OTUs derived from gut contents of *E. eugeniae* and from Substrate 1.

OTUs were calculated from in silico translated amino acid sequences of *mcrA* (OTUs 1 to 9, and 11 to 12) and *mrtA* (OTU 10) sequences. Sequences retrieved from DNA and cDNA (transcripts) are displayed. Numbering of OTUs at the right side correspond with that in the phylogenetic tree of *in silico* translated *mcrA* and *mrtA* sequences; OTUs below 3 % relative abundance were combined and are displayed as white OTUs (i.e., non-shaded wedges) in the pies.

Some sequences were detected on *mcrA* transcript level only and were affiliated with *Methanospirillaceae* (OTU 4), *Methanoregulaceae* (OTU 6), *Methanosaetaceae* (OTU 8),

and *Methanobacteriaceae* (OTUs 12), but were of minor abundance (Figure 40, Figure 41). For Substrate 1, *mcrA* gene and transcript sequences were rather similar. However, for the gut contents of *E. eugeniae*, transcripts differed especially in respect of OTU 10 with *Methanobacterium formicicum* DSM 1535 (*Methanobacteriaceae*) as its closest relative (Figure 40, Figure 41), and with 26 % and 11 % relative abundance on transcript and gene level, respectively. In Substrate 1, only one *mcrA* gene sequence was affiliated with OTU 10, and this OTU was absent in transcript analysis (Figure 40).

3.2.4. Enrichment of methanogens from the gut of *E. eugeniae*

Next to the genetic characterization of methanogens in the gut of *E. eugeniae* raised and maintained on Substrate 1, an isolation approach was started to receive methanogenic *Archaea* (2.3.2.3). Homogenized gut contents, coelom fluid and gut sections from the anterior part of the digestive system were used as inoculum for an anoxic, reduced mineral medium containing additional yeast extract, an autoclaved extract of Substrate 1, and H_2 and CO_2 in the headspace (2.3.2.3). This enrichment produced up to 5 % CH_4 in the headspace after 28 days of incubation (2.3.2.3). Thereafter, aliquots of different dilution steps were transferred into new medium as used before but with an additional extract of *L. terrestris* earthworms (2.3.1.1.17). After two additional transfer steps lasting approximately 8 weeks each, gases were measured after 50 days of incubation of the last enrichment step.

After 50 days of incubation, CH_4 was produced in all dilutions up to a concentration of 10.9 mM (i.e., 64.2 µmol CH_4) in the 10^{-1} dilution, i.e., supplemental H_2 (44.3 mM) was completely consumed yielding a ration of 4.1 of consumed H_2 divided by the CH_4 produced (Table 28). This consumption of H_2 did not include H_2 that might be produced by fermentation processes of microorganisms other than methanogens. With higher dilution, less H_2 was consumed within 50 days resulting in higher H_2/CH_4-ratios (Table 28). Altogether, active methanogens from the earthworm *E. eugeniae* have been successfully enriched.

An aliquot of the 10^{-5} dilution was used after 50 days for T-RFLP analysis (2.5.8) with amplified *mcrA*/*mrtA* gene fragments (2.5.7.2.1) to check purity and phylogeny of enriched methanogens. The main T-RF with a length of 157 bp accounted for 59 % of relative fluorescence and could *in silico* be affiliated with *Methanomicrobiaceae* (2.5.8.5). The T-RF with 260 bp length (34 % relative fluorescence) was affiliated with both *Methanomicrobiaceae* and *Methanospirillaceae*. One unaffiliated T-RF with 75 bp length accounted for 7 % relative fluoresecence. Thus, enriched methanogens derived from *E. eugeniae* were affiliated with *Methanomicrobiaceae* and maybe also *Methanospirillaceae* according to their detected *mcrA*/*mrtA* gene fragments.

Table 28: Production of CH_4 and consumption of H_2 of an enrichment culture of diluted gut contents of *E. eugeniae*.[a]

	Dilution[b]				
Gases after 50 days	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5} [c]
CH_4 produced (mM)	10.9	9.6	8.1	3.3	0.6
H_2 consumed (mM)[d]	44.3	40.6	35.1	18.7	10.5
consumed H_2 / produced CH_4	4.1	4.2	4.3	5.7	17.2

[a] An enrichment of gut content microorganisms of *E. eugeniae* pruducing CH_4 in a previous enrichment step was used in differnt dilutions for the displayed subsequent enrichment step. Incubation was in an anoxix, reduced mineral medium medium supplemented with yeast extract, earthworm extract and an extract of composted cow manure (i.e., Substrate 1). Headspace contained H_2 and CO_2. See methods psrt (2.3.2.3) for detailed information.

[b] The dilutions 10^{-1} and 10^{-2} were conducted in duplicates, and the mean values are displayed. The other dilution steps were were unique copies.

[c] This enrichment dilution was used for further enrichments and for T-RFLP analysis of *mcrA* genes.

[d] Possible production of H_2 from fermentations during incubation is disregarded.

3.2.5. Emission of CH_4 by the millipede *Gymnostreptus olivaceus*

Millipedes (Diplopoda) of the species *Gymnostreptus olivaceus* (approximately 1 g and 5 cm) belonging to the family Spirostreptidae (Fontanetti CS *pers. comm.*) were detected in the litter layer during the sampling of grassland soil (Substrate 4) in September 2011 (2.1.1.2), and were also tested for their ability to emit CH_4.

The two living specimens of *G. olivaceus* incubated under ambient air emitted 16 and 146 nmol CH_4 (g fw)$^{-1}$ after 6 h, respectively. Supplemental H_2/CO_2 in the headspace of four additional specimens (two specimens per incubation bottle) yielded 115 and 152 nmol CH_4 (g fw)$^{-1}$ after 6 h, respectively.

Although the results demonstrated that *G. olivaceus* emitted CH_4 *in vivo*, the low number of replicates does not provide a solid basis for making a conclusion on the influence of supplemental H_2/CO_2. However, the maximum amounts of CH_4 emitted by *G. olivaceus* after 6 h of incubation exceeded those of *E. eugeniae* on a per g fresh weight basis by a factor of up to approximately five (146 nmol CH_4 (g fw)$^{-1}$ for *G. olivaceus* versus 30 nmol CH_4 (g fw)$^{-1}$ for *E. eugeniae*). Emissions of CH_4 by *G. olivaceus* are higher than those of temperate millipedes (Šustr & Šimek 2009) but in the range of those reported from other tropical millipedes (Hackstein & Stumm 1994). Thus, these results will not be further discussed in this

study. However, a molecular analysis of methanogens from this species lacks in literature and might be a promising approach for future research as endogenous methanogens are known from the gut of other millipedes (Paul *et al.* 2012).

4. DISCUSSION

4.1. Denitrification and the emission of nitrogenous gases

Earthworms are known as an anoxic microzone in aerated soils (Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a) promoting anaerobic processes as fermentations and denitrification for ingested soil microorganisms. As a result of that, earthworms of the family Lumbricidae from Germany and New Zealand harboring rather small species are all emitting denitrification-derived nitrogenous gases, i.e., N_2O and N_2 (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Ihssen *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b). These emissions appear within earthworm species of all three main feeding guilds, i.e., endogeic (e.g., *A. caliginosa*), epigeic (e.g., *L. rubellus*), and anecic (e.g., *L. terrestris*) (Bouché 1977, Barois *et al.* 1999). The large *O. multiporus* from New Zealand was the first und so far only analyzed species from an alternative earthworm family, i.e., Megascolecidae and emitted no N_2O *in vivo* although its gut displayed a high denitrification potential (Wüst *et al.* 2009b). This raised the questions if (i) earthworm species belonging to families other than Lumbricidae are able to emit nitrogenous gases *in vivo*, if (ii) the earthworm size is a determinative factor, and if (iii) the earthworm feeding guild affects these emissions. Thus, ten earthworm species from Brazil affiliated with five different families and of different sizes and feeding guilds (Table 14) were analyzed for their *in vivo* emission of nitrogenous gases.

4.1.1. The emission of nitrogenous gases is a widespread feature of earthworms of different families, sizes, and feeding guilds

Earthworm species belonging to four families emitted *in vivo* N_2O, i.e., the families Megascolecidae (*A. gracilis* and *P. excavatus*), Glossoscolecidae (*P. corethrurus* and *R. alatus*), Eudrilidae (*E. eugeniae*), and Acanthodrilidae (*D. annae* and *Dichogaster* sp.) (Table 14). Three earthworm species emitted no N_2O *in vivo*, i.e., *E. andrei* (Lumbricidae), and *G. paulistus* and *Glossoscolex* sp. (both Glossoscolecidae) (Table 14). Of those earthworms emitting N_2O, all also emitted N_2, and *G. paulistus* emitted minor amounts of N_2 but no N_2O (Table 14). Earthworm species belonging to the family Lumbricidae from Germany and New Zealand emit *in vivo* both denitrification-derived N_2O and N_2 with an average of 1.5 N_2O nmol (g fw)$^{-1}$ h^{-1} and a maximum of 11 nmol N_2O (g fw)$^{-1}$ h^{-1} (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007,

Wüst *et al.* 2009b). The amounts of N_2O and N_2 emitted by earthworm species from Brazil were highly similar to those reported for Lumbricidae (Table 14, Figure 9, Figure 10). This demonstrates that the *in vivo* emission of nitrogenous gases is a widespread feature that can be attributed to every earthworm family analyzed so far, i.e., with representatives of Lumbricidae, Megascolecidae, Glossoscolecidae, Eudrilidae, and Acanthodrilidae (Hypothesis 1; 1.5). Most recently, casts from *P. corethrurus* (Glossoscolecidae), *D. annae* (Acanthodrilidae), *E. andrei* (Lumbricidae), and *Amynthas corticis* (Megascolecidae) were shown to emit N_2O and N_2 (Majeed *et al.* 2013) strongly confirming the above mentioned conclusions from the current study. As earthworms are widespread over the planet and often account for the dominant macrofauna in soils (Lee 1985), these invertebrates seem to significantly contribute to the global cycling of nitrogen including the potent greenhouse gas N_2O (Drake & Horn 2007, Lubbers *et al.* 2013). About 40 % of global emissions of N_2O from soils that are inhabited by earthworms are estimated to be derived from earthworms; either directly, or indirectly by their ecological lifestyle, i.e., the restructuring of soils (Drake & Horn 2007, Lubber *et al.* 2013).

Earthworm species of epigeic and endogeic feeding guilds emitted nitrogenous gases *in vivo* (Table 14). This is also documented for species of the family Lumbricidae and, in addition, is also valid for anecic species of this family (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b). However, *G. paulistus* (Glossoscolecidae) belonging to the endo-anecic feeding guild (Table 14) and the endogeic *O. multiporus* (Wüst *et al.* 2009a) emitted no N_2O whereas the anecic *L. terrestris* (Lumbricidae) and the endogeic *A. caligionsa* did (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2006b). Emissions of N_2O from casts of epigeic tropical earthworms were about three orders of magnitude higher than those of earthworm species belonging to endogeic feeding guilds (Majeed *et al.* 2013). Also for Lumbricidae, earthworms of different feeding guilds emitted different amounts of N_2O (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b). Thus, the feeding guild alone does not seem to be the determinative factor for the emission of nitrogenous gases, although it seems to influence these emissions (Hypothesis 1; 1.5). This conclusion is well supported for both the epigeic and endogeic feeding guild but lacks significance for the anecic feeding guild being represented by only one examined endo-anecic species in Brazil (Table 14).

E. eugeniae, *P. excavatus* (both emitting nitrogenous gases), and *E. andrei* (emits no N_2O) (Table 14) were purchased from an earthworm distributor (Table 26) as these species are commercially used, especially for vermicomposting (Gajalakshmi & Abbasi 2004). All other species were sampled from their natural habitat (Table 26), including species that emit nitrogenous gases and those that do not (Table 14). Thus, the emission of nitrogenous gases

by earthworms is not dependent on the appearance of a species, i.e., if it is commercially raised or living in its natural habitat. This observation was also made for specimens of both commercially raised and sampled specimens of *L. terrestris* (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a).

As an additional factor, the earthworm size was assumed to influence the emission of nitrogenous gases as the large *O. multiporus* was the first analyzed earthworm species that did not emit N_2O *in vivo* (Wüst *et al.* 2009b) (Hypothesis 1; 1.5). In Brazil, *G. paulistus*, *Glossoscolex* sp. (both Megascolecidae) and *E. andrei* (Lumbricidae) lacked the *in vivo* emission of N_2O and showed no to minor emission of N_2 (Table 14, Figure 9, Figure 10). *E. andrei* specimens were rather small (up to 7 cm and 0.7 g), but all other small species emitted N_2O (Table 14). *Glossoscolex* sp. specimens were larger with up to 29 cm and 4 g, but *A. gracilis* emitting significant amounts of N_2O was also up to 5 g with up to 12 cm (Table 14). *G. paulistus* specimens were large with up to 34 cm and 27 g, but *R. alatus* was even larger (up to 63 cm and 44 g) and emitted N_2O (Table 14). In addition, the whole worm, gut, and gut content of *G. paulistus* were able to emit N_2O when nitrite was added, i.e., displayed an, albeit small denitrification potential. Combined, two of three large earthworm species emitted no N_2O whereas only one of seven small species did not emit N_2O (Figure 9, Figure 10). Apart from *O. multiporus* mentioned above, former studies focussed on species of the family Lumbricidae displaying only relatively small inter-species differences in size. However, both small and larger species of the family Lumbricidae emitted nitrogenous gases (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a). Thus, if the emission of nitrogenous gases might be size dependent, the differences in size within the Lumbricidae seem to be insufficient to impact the detected gas emissions. Data from the current study with more pronounced differences in size between analyzed earthworm species suggest that the size of an earthworm is not the main determinative factor if N_2O is emitted *in vivo* (Hypothesis 1; 1.5). However, a huge size seems to negatively influence the emission of N_2O (Table 14, Figure 9, Figure 10; Wüst *et al.* 2009b).

Concerning this matter, it was speculated that a huge size of the earthworm and therefore of its gut might favor the release of N_2 instead of N_2O (Wüst *et al.* 2009b). Indeed, *R. alatus*, the largest species sampled emitted 67.2 nmol N_2 (g fw)$^{-1}$ in contrast to 1.9 nmol N_2O (g fw)$^{-1}$ after 6 h (Table 14). Also for the large *G. paulistus*, the relation of N_2/N_2O was significantly higher than that for the small *A. gracilis* when nitrite was applied (Figure 9, Figure 10). In addition, *G. paulistus* emitted no N_2O *in vivo* but N_2, albeit in minor amounts (Table 14). A long gut is indicative of a long gut passage time (Parlé 1963). Due to a long exposure time in the gut, denitrifiers might be able to conduct the complete denitrification pathway, i.e., from nitrate to nitrite, NO, N_2O and finally N_2 resulting in a preferred release of

N_2 instead of N_2O (Zumft 1997). In contrast, earthworms with a shorter gut and therefore shorter gut passage time might release more N_2O than N_2 as time is insufficient for the final conversion of N_2O to N_2. Next to the length of the gut, its enlarged diameter might also lead to a preferred release of N_2 instead of N_2O due to complete instead of incomplete denitrification. Indeed, the center of the gut of *L. terrestris* displays highest concentrations of N_2O whereas concentrations decline toward the gut wall (Horn *et al.* 2003, Wüst *et al.* 2009a). However, also some small earthworm species emitted more N_2 than N_2O (Table 14). Thus, the mechanism that determines if preferentially N_2O or N_2 is released from the earthworm gut, cannot exclusively be explained by the size of the gut and the retention time of denitrifiers in the gut lumen (Hypothesis 1; 1.5). Additional research with large earthworms at different stages of life, i.e., with different sizes of one species could elucidate this unresolved question.

If earthworm family, feeding guild, and size are not supposed to be the determinative factors for the *in vivo* emission of N_2O, the physical and chemical parameters of the soil or substrate the earthworm lives in and on might be. *G. paulistus* emitted no N_2O *in vivo* and was sampled from a pasture soil (Table 14). *A. gracilis* and *P. corethrurus* were sampled from the same grassland soil (Table 14). Although the grassland soil contained higher concentrations of ammonia, total organic carbon, total organic material, and total nitrogen than the pasture soil, both soils contained nitrate, the electron acceptor for denitrification (Zumft 1997) in comparable concentrations (Table 15). In addition, the soil of *G. paulistus* contained nitrate in amounts similar to those of N_2O-emitting earthworms of the family Lumbricidae (Matthies *et al.* 1999, Wüst *et al.* 2009b). Both soils emitted nitrogenous gases, predominantly N_2, with 3.2 and 1.4 nmol N_2 $(g\ fw)^{-1}$ after 5 h for pasture soil and grassland soil, respectively (Table 14) indicating that the soil itself had the pre-condition for denitrification processes. However, *A. gracilis* and *P. corethrurus* emitted N_2O *in vivo* whereas *G. paulistus* did not (Table 14, Figure 9, Figure 10). Furthermore it needs to be taken into consideration that the amount of *de facto* ingested soil highly differs between earthworms of different feeding guilds (Bouché 1977, Barois *et al.* 1999), i.e., *G. paulistus* and *P. corethrurus* are supposed to ingest larger amounts of soil than *A. gracilis* does (Barois *et al.* 1999, James & Guimarães 2010, GG Brown *pers. obs*.). In addition, the concentration of nitrate in non-sampled and -analyzed organic material and detritus is unknown although these materials also comprise the earthworm´s diet (1.4.3). However, the physical and chemical substrate parameters alone do not seem to be the determinative factor for the emission of nitrogenous gases by earthworms. The substrate as source of denitrifiers and dissimilatory nitrate reducers is analyzed later on (4.1.3, 4.1.4, 4.1.5).

4.1.2. The emission of nitrogenous gases is predominantly associated with denitrifiers in the earthworm gut

For species of the family Lumbricidae from Germany and New Zealand, denitrifiers in the earthworm gut are supposed to be affiliated with the emission of nitrogenous gases (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b). For earthworms of this family, this hypothesis was emphasized by the detection of transcripts of *nosZ* in the earthworm gut (3.1.2.1.1.2) and the isolation approach of the current study. Here, most *Bacteria* isolated with nitrite or N_2O as electron acceptor comprised the genera *Ensifer* (*Alphaproteobacteria*), *Pseudomonas* (*Gammaproteobacteria*), *Bacillus*, and *Paenibacillus* (both *Bacilli;* Ash *et al.* 1993) (Table 23, Table A 2). Although these isolates were not tested sufficiently for denitrification yet (3.1.2.2.2), all isolates were retrieved with nitrite or N_2O as sole electron acceptor and the detected genera harbor several species capable of denitrification (Zumft 1997, Shoun *et al.* 1998, Shapleigh 2006, Behrendt *et al.* 2010, Verbaendert *et al.* 2011a, Zhang *et al.* 2012) and were already detected in earthworm gut contents and casts (Furlong *et al.* 2002, Ihssen *et al.* 2003, Horn *et al.* 2005, Byzov *et al.* 2009, Knapp *et al.* 2009, Thakuria *et al.* 2010).

In the current study, the emission of nitrogenous gases was tested for earthworms from Brazil only (3.1.1.2.1). Here, supplemental acetylene strongly enhanced the emission of N_2O for most species analyzed (Table 14, Figure 9, Figure 10) what relates to an emission of N_2 as this compound inhibits the N_2O reductase and therefore the final reduction of N_2O to N_2 (Yoshinari & Knowles 1976). For those earthworm species applied to, nitrite as an electron acceptor for denitrification (Zumft 1997) significantly increased emissions of both N_2O an N_2 (Figure 9, Figure 10). In a most recent study, casts from the same or similar earthworm species than in this study, i.e., *P. corethrurus*, *D. annae*, *E. andrei*, and *Amynthas corticis* emitted N_2O. These emissions were significantly increased with supplemented acetylene. In the same study, ammonium concentrations as indicator of occurring dissimilatory reduction of nitrate to ammonium (Tiedje 1988, Sudesh & Cole 2007) were not related to emissions of N_2O indicating that denitrification was the predominant process responsible for emissions of N_2O by earthworms (Majeed *et al.* 2013). Also N_2O emitted from marine invertebrates was identified as denitrification-derived (Stief *et al.* 2009, Heisterkamp *et al.* 2010). In gut homogenates of soil-feeding termites, dissimilatory nitrate reduction, i.e., the dissimilatory reduction of nitrate to ammonium appeared in higher rates than denitrification did. However, emitted N_2O from gut homogenates and living termites was predominantly affiliated with denitrification rather than dissimilatory nitrate reduction (Ngugi & Brune 2012). In addition, the dissimilatory nitrate reduction seems to be more important for oxic to mircoaerophilic conditions whereas denitrification is dominating in anoxic habitats (Baggs 2011). Thus, formation and emission of N_2O by earthworm families analyzed in Brazil is likely

predominantly due to highly active denitrifiers in the earthworm gut, similar to what is known for species of the family Lumbrcidae from Germany and New Zealand (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b).

However, to a certain extent, processes other than denitrification might also contribute to the emission of N_2O by earthworms. Concentrations of nitrite reach up to 4 mM in the alimentary canal of earthworms (Wüst *et al.* 2009a), and solutions 2 mM nitrite were applied in experiments with *G. paulistus* and *A. gracilis* (Figure 9, Figure 10). Nitrite can react unspecifically with nitrate reductases of dissimilatory nitrate reducers and thus be converted to NO that is further detoxified to N_2O (1.2.2; Smith 1983, Vine & Cole 2011). Indeed, non-denitrifying, dissimilatory nitrate reducers have been detected in earthworm gut contents and casts, often in high abundances (Furlong *et al.* 2002, Ihssen *et al.* 2003, Byzov *et al.* 2009, Knapp *et al.* 2009). In addition, a species of *Rhizobium* is known to produce N_2O from nitrite without the conservation of energy whereas classical denitrification implies the energy conservation upon the reduction of nitrogenous compounds (Casella *et al.* 1986, Zumft 1997). A *nirK*-encoded nitrite-reductase catalyzes this formation of N_2O in the *Rhizobium* species (Toffanin *et al.* 1996). Most *nirK* genes in *G. paulistus* and *A. gracilis* were affiliated with *Rhizobiales*, albeit mostly with taxa known to denitrify (Figure 18, 3.1.1.3.3.2). The majority of transcripts of *narG* in the gut of earthworm from Germany were affiliated with those of the genus *Mycobacterium* (Figure 27) where representatives are known for dissimilatory reduction of nitrate to nitrite with lacking information about further reduction to ammonium (Weber *et al.* 2000, Sohaskey & Wayne 2003, Hartmans *et al.* 2006, Giffin *et al.* 2012). It is unknown to which extent these processes other than denitrification contribute to the emission of N_2O by earthworms *in vivo*. However, based on studies with earthworms of the family Lumbricidae (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b; Table 23), and due to the strongly enhanced emission of N_2O detected via the inhibition of the N_2O reductase (Table 14, Figure 9, Figure 10), denitrification is supposed to be the main source of both N_2O and N_2 emitted by earthworms (Hypothesis 1; 1.5).

4.1.3. Denitrifiers and dissimilatory nitrate reducers in the earthworm gut are soil-derived

Denitrifiers are supposed to be the main source for nitrogenous gases released by the gut of earthworms from Brazil (4.1.2). Thus, gut contents and corresponding soils of *G. paulistus* and *A. gracilis*, two species with contrasting emissions of nitrogenous gases, families, sizes, and feeding guilds were analyzed for the appearance and composition of

genes indicative of denitrification (*nirK*, *nirS*, and *nosZ*) and of both denitrification and dissimilatory nitrate reduction (*narG*) (1.2.3, 3.1.1.3). The analyzed genes encode for the enzyme or a subunit of a dissimilatory nitrate reductase (*narG*), nitrite reductases (*nirK* and *nirS*), and N_2O reductase (*nosZ*) (1.2.1.1, 1.2.3). In addition, three earthworms of different feeding guilds of the family Lumbricidae from Germany (*A. caliginosa*, *L. terrestris*, and *L. rubellus*) and two soils were analyzed for genes and transcripts of *narG* and *nosZ* (3.1.2.1.1). For *L. terrestris* and its soil, *nirS* gene sequences were analyzed (3.1.2.1.2). With a *nosZ* primer system (Scala & Kerkhof 1998) distinct from that used for earthworm from Germany and Brazil (Rich *et al.* 2003; Table 6), *nosZ* gene sequences were analyzed from gut contents and soil of the large *O. multiporus* (Megascolecidae) from New Zealand (3.1.3.1).

The vast majority of detected *narG*, *nirK*, *nirS*, and *nosZ* OTUs from gut contents and soils from Brazil, Germany, and New Zealand harbored both gut- and soil-derived sequences (Figure 15, Figure 18, Figure 21, Figure 23, Figure 26, Figure 30, Figure 34, Figure 36). Only quantitatively very minor OTUs were detected exclusively in the earthworm gut, i.e., for all *narG* and *nosZ* libraries (Figure 15, Figure 23, Figure 26, Figure 30, Figure 36), and for *nirS* libraries of *L. terrestris* (Figure 34). In addition, most OTUs were closely related to sequences derived from uncultured soil *Bacteria* (Figure 15, Figure 18, Figure 21, Figure 23, Figure 26, Figure 30, Figure 34, Figure 36). This is congruent with former studies analyzing *nosZ* sequences in the earthworm gut (Horn *et al.* 2006a, Wüst *et al.* 2009b). The majority of the species and genera in this study to which most sequences were most closely related to, were originally isolated from or are frequently detected in various soils, e.g., *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, *Methylobacterium nodulans*, *Mycobacterium gilvum*, and *Pseudomonas fluorescens* (Gamble *et al.* 1977, Ramos *et al.* 2000, Furlong *et al.* 2002, Hartmans *et al.* 2006, Heylen *et al.* 2006, Moore *et al.* 2006, Sadowsky & Graham 2006, Falk *et al.* 2010). This is valid for all genes (i.e., *narG*, *nirK*, *nirS*, *nosZ*) (Figure 15, Figure 18, Figure 21, Figure 23, Figure 26, Figure 30, Figure 34, Figure 36) and all gene transcripts analyzed (i.e., *narG* and *nosZ*) (Figure 26, Figure 30).

Most *Bacteria* isolated from gut contents of species of the family Lumbricidae from Germany with nitrite as electron acceptor belonged to the genera *Ensifer* (*Alphaproteobacteria*), *Bacillus*, *Paenibacillus* (both *Bacilli*), and *Pseudomonas* (*Gammaproteobacteria*) (Table 23, Table A 2). Although these *Bacteria* were also detected in earthworm gut contents and casts (Furlong *et al.* 2002, Ihssen *et al.* 2003, Horn *et al.* 2005, Byzov *et al.* 2009, Knapp *et al.* 2009, Thakuria *et al.* 2010), they comprise species originally and frequently detected in soils (Gamble *et al.* 1977, Shirey & Sextone 1989, Ramos *et al.* 2000, Moore *et al.* 2006, Sadowsky & Graham 2006, Slepecky & Hemphill 2006, Behrendt *et al.* 2010). Only Isolate 201 with 97.7 % 16S rRNA gene similarity to its next related organism (*Mycoplana ramosa* DMS7292; Table A 2) might comprise a no novel species as the

similarity cutoff-value of the 16S rRNA gene fragments of 97 % is a very conservative one and is now assumed to be higher (Stackebrandt 2006, Stackebrandt & Ebers 2006) (Table 24, Table A 2). However, the combined data demonstrate that there is no cultivable, relevant number of endogenous *Bacteria* in the earthworm gut, if at all.

To sum it up, denitrifiers and dissimilatory nitrate reducers in the gut of earthworms are predominantly derived from ingested material and do not represent an endogenous microbiota in the earthworm gut (Hypothesis 2; 1.5). This was also postulated in former studies that used cultivation- and cultivation-independent methods to assess the microbial diversity in the earthworm gut and its casts in comparison to the surrounding soil (Furlong *et al.* 2002, Horn *et al.* 2003, Singleton *et al.* 2003, Egert *et al.* 2004, Horn *et al.* 2006a, Wüst *et al.* 2009b).

4.1.4. *Rhizobiales* are abundant and active denitrifiers in the earthworm gut

Denitrifiers were detected via *nirK*, *nirS*, and *nosZ* sequences in gut contents of earthworms from Brazil (3.1.1.3.3, 3.1.1.3.4, 3.1.1.3.5), Germany (3.1.2.1.1.2, 3.1.2.1.2.2), and New Zealand (3.1.3.1); analyzed *narG* sequences (3.1.1.3.2, 3.1.2.1.1.1) can detect both denitrifiers and dissimilatory nitrate reducers (1.2.3). Most detected sequences of *nirK* and *nirS* were most closely related to those of organisms that displayed a good correlation of their 16S rRNA gene to the corresponding *nirK* or *nirS* gene (Figure 12, Figure 14, 3.1.1.3.3.2, 3.1.1.3.4.2, 3.1.2.1.2.2.2). Thus, the calculated species-level cutoff values for *nirK* and *nirS* were estimated to be valid and highly applicable to detect and analyze organisms carrying one of these nitrite reductase genes (3.1.1.3.1.1, 3.1.1.3.1.2). However, the number of species-level *nirK* and *nirS* OTUs indicated only a minimum number of species in a library whereas the real number might be significantly higher (Palmer *et al.* 2009).

For *nirK*, *nirS*, and *nosZ* gene sequences from all earthworms analyzed, *Rhizobiales* were always detected in highest abundances (Figure 18, Figure 21, Figure 23, Figure 30, Figure 34), also within *nosZ* transcripts (Figure 30). This is also valid for *nosZ* sequences amplified with a different primer system (Scala & Kerkhof 1998), i.e., for gut contents of *O. multiporus* from New Zealand (Figure 36). Also of those *narG* sequences indicative of denitrifiers, i.e., whose next related cultured species is known to denitrify, *Rhizobiales* represented the vast majority (Figure 15, Figure 26). Within the *Rhizobiales*, the genera *Bradyrhizobium* (within sequences of *nirK*, *nirS*, and *nosZ*), *Rhodopseudomonas* (*nirK* and *nosZ*), *Methylobacterium* (*narG* and *nosZ*), and *Oligotropha* (*narG* and *nosZ*) displayed highest similarity to the sequences detected in all earthworm guts (Figure 15, Figure 18, Figure 21, Figure 23, Figure 26, Figure 30, Figure 34, Figure 36). The genera

Bradyrhizobium, Rhodopseudomonas, and *Oligotropha* are members of the family *Bradyrhizobiaceae* (Garrity *et al.* 2005, Sadowsky & Graham 2006). Related sequences of the structural genes *narG*, *nirK*, *nirS*, and *nosZ* of *Rhizobiales* were frequently and abundantly detected in various soils (Philippot *et al.* 2002, Priemé *et al.* 2002, Rich *et al.* 2003, Stres *et al.* 2004, Enwall *et al.* 2005, Henry *et al.* 2006, Horn *et al.* 2006a, Wüst *et al.* 2009b), and related *nosZ* sequences also in the alimentary canal of earthworms from Germany (Horn *et al.* 2006a) and New Zealand (Wüst *et al.* 2009b). Next to molecular analyses, members of the *Rhizobiales*, i.e., species of the genus *Ensifer* were frequently isolated from the alimentary canal of earthworms of the family Lumbricidae (Table 23, Table A 2). Other cultivation-dependent and cultivation-independent (i.e., 16S rRNA gene) approaches detected also *Rhizobiales* in the earthworm gut and casts (Furlong *et al.* 2002, Ihssen *et al.* 2003, Byzov *et al.* 2009, Knapp *et al.* 2009, Thakuria *et al.* 2010).

Rhizobiales harbor several denitrifying and nitrate reducing species (Gamble *et al.* 1977, Zablotowics *et al.* 1978, Daniel *et al.* 1982, Shapleigh 2006, Delgado *et al.* 2007) and genes indicative of denitrification are present in the genome of *O. carboxidovorans* OM5 (Paul *et al.* 2010, Volland *et al.* 2011; NCBI search). *Rhizobiales* are heterotrophic and saprophytic *Bacteria* that are either free-living, or in symbiosis with legumes where they fix N_2 (Sadowsky & Graham 2006). *Rhizobiales* can utilize a wide range of sugars (Sadowsky & Graham 2006) like those the earthworm´s mucus consists of (Wüst *et al.* 2009a). Thus, it is very likely that *Rhizobiales* are active in the earthworm gut. *Bradyrhizobium japonicum*, to whom most denitrification genes in this study were closely related to (Figure 18, Figure 21, Figure 23, Figure 30, Figure 34, Figure 36), is a facultative soil denitrifier of the family *Bradyrhizobiaceae* that conducts the whole denitrification process, i.e., from nitrate to N_2 (Bedmar *et al.* 2005, Delgado *et al.* 2007). For the reduction of nitrate, it harbors a less oxygen-sensitive *nap*-encoded nitrate reductase instead of a *nar*-encoded nitrate reductase (Moreno-Vivián *et al.* 1999, Delgado *et al.* 2003, Bedmar *et al.* 2005, Delgado *et al.* 2007), and could therefore not be detected via the *narG* analyses in this study (Figure 15, Figure 27). Next to the emission of N_2O, *B. japonicum* is also known to utilize atmospheric concentrations of N_2O (i.e., 0.34 ppm) indicating that related species might be capable of both the production and the efficient consumption of N_2O (Sameshima-Saito *et al.* 2006). These combined data demonstrate that *Rhizobiales*, especially *Bradyrhizobiaceae* seem to be of major importance for denitrification and therefore the emission of nitrogenous gases in and from the earthworm gut, respectively. As these taxa were dominant in all earthworm guts analyzed, they seem to be no major factor to determine if an earthworm emits nitrogenous gases.

In the gut of *G. paulistus* and *A. gracilis* from Brazil, 10 and 7 % of *narG* gene sequences were distantly related to those of the genus *Anaeromyxobacter* (Figure 15). A recent study

demonstrated that members of this genus can harbor a *nosZ*-encoded N_2O reductase that effectively reduces N_2O (Sanford *et al.* 2012) but cannot be detected with the available primers for this gene like those primers used in the current study (Scala & Kerkhof 1998, Rich *et al.* 2003; Table 6). In contrast to classical denitrification, this atypical N_2O reductase does not conserve energy (Sanford *et al.* 2012). Thus, these *Bacteria* are no classical denitrifiers *sensu stricto*. *Bacteria* with this novel *nosZ*-like N_2O reductase could also contribute to the fate of N_2O in the gut of earthworms, i.e., by reducing N_2O to N_2. Interestingly, 53 % of *narG* sequences were distantly affiliated with *Anaeromyxobacter* in the soil of *A. gracilis* (Figure 15) that emitted virtually no N_2O but N_2 (Table 14). Here, an effective reduction of produced N_2O might also be linked to *Anaeromyxobacter*-like species harbouring an atypical N_2O reductase (Sanford *et al.* 2012).

The isolation approach yielded several Gram-positive *Bacteria* of the genera *Bacillus* and *Paenibacillus* (Table 23, Table A 2). Although these isolates were not tested sufficiently for denitrification yet (3.1.2.2.2), they were isolated with nitrite and N_2O as sole electron acceptor, and these two genera harbor several species capable of denitrification (Zumft 1997, Shapleigh 2006, Behrendt *et al.* 2010, Verbaendert *et al.* 2011a, Zhang *et al.* 2012) and were already detected in earthworm gut contents and casts (Furlong *et al.* 2002, Ihssen *et al.* 2003, Horn *et al.* 2005, Byzov *et al.* 2009, Knapp *et al.* 2009, Thakuria *et al.* 2010). Thus, Gram-positive denitrifiers might significantly contribute to denitrification processes in the earthworm gut but cannot be detected with primers targeting *nirK*, *nirS*, and *nosZ* up to now (Behrendt *et al.* 2010, Green *et al.* 2010, Verbaendert *et al.* 2011b; see 4.5).

4.1.5. Abundant and active dissimilatory nitrate reducers in the earthworm gut

Dissimilatory nitrate reducers were detected via *narG* sequences in gut contents of earthworms from Brazil (Figure 15) and Germany (Figure 27). For all earthworms analyzed, *narG* sequences related to *Proteobacteria* were always detected in highest abundances with *Rhizobiales* representing the most abundant phylum within *Proteobacteria* (Figure 15, Figure 27). Next to *Proteobacteria*, *Actinomycetales* were the second most abundant phylogenetic group in gut contents from earthworms from both Germany and Brazil (Figure 15, Figure 27). Species and *narG*-sequences related to taxa of *Proteobacteria* and *Actinomycetales* have been frequently detected in soil (Philippot *et al.* 2002, Chèneby *et al.* 2003, Enwall *et al.* 2005, Deiglmayr *et al.* 2006), but also from earthworm gut contents and casts (Furlong *et al.* 2002, Ihssen *et al.* 2003, Byzov *et al.* 2009, Knapp *et al.* 2009, Thakuria *et al.* 2010).

For earthworms from Germany only, *narG* transcripts were evaluated (Figure 27). Within *narG* transcripts, the vast majority of sequences was related to those affiliated with the genus

Mycobacterium (Figure 27) whereas *narG* sequences of *Proteobacteria* lacked nearly completely in the earthworm gut and were abundantly detected in the mineral soil only (Figure 27).

Mycobacterium tuberculosis is known as an obligate aerobe and a facultative human pathogen that can survive but not replicate under anoxic conditions (Hartmans *et al.* 2006, Giffin *et al.* 2012). *Mycobacterium*-affiliated isolates and *narG*-sequences are commonly found in soils (Philippot *et al.* 2002, Deiglmayr *et al.* 2006, Hartmans *et al.* 2006). Species of the genus *Mycobacterium* and other *Actinobacteria* occur in earthworm gut contents and casts might be associated with gut walls of earthworms of the family Lumbricidae (Furlong *et al.* 2002, Fischer *et al.* 2003, Byzov *et al.* 2009, Knapp *et al.* 2009, Thakuria *et al.* 2010). Also *Actinobacteria*-related 16S rRNA sequences are more abundant in the earthworm gut than in soil (Furlong *et al.* 2002, Knapp *et al.* 2009, Nechitaylo *et al.* 2010). Species of *Mycobacterium* are not known to denitrify but can reduce nitrate to nitrite (Weber *et al.* 2003, Hartmans *et al.* 2006). *M. tuberculosis* constitutively expresses a *narG*-containing nitrate reductase, i.e., also during aerobic growth without nitrate or nitrite, and with entering the hypoxic and anoxic state, the nitrate reductase activity is strongly enhanced (Weber *et al.* 2000, Sohaskey & Wayne 2003, Sohaskey 2008, Giffin *et al.* 2012) what might explain the high relative abundance of *Mycobacterium*-related *narG* transcripts in the earthworm gut (Figure 27). *M. tuberculosis* normally conducts the assimilation of nitrate via this nitrate reductase (Malm *et al.* 2009), but this enzyme is also supposed to be used for the dissimilation of nitrate even though information about further reduction of nitrite to ammonium lacks (Weber *et al.* 2000, Sohaskey & Wayne 2003, Sohaskey 2008). The different functions of non-redundant *narG* copies in *Mycobacterium* species are still largely unresolved (Sudesh & Cole 2007). *Mycobacterium*-related *narG* transcripts were also abundant in the uppermost soil (Figure 27) that was assumed to be rich in decaying plant material, i.e., organic carbon (2.1.2). *Mycobacterium* species were highly active in soil enriched with biochar (Anderson *et al.* 2011). The gut of earthworms contains also high amounts of carbon albeit as organic carbon (Barois & Lavelle 1986, Horn *et al.* 2003, Drake & Horn 2007, Wüst *et al.* 2009a) what might be a highly favourable precondition for a high activity of *Mycobacterium* species (Anderson *et al.* 2011). Thus, species of the genus *Mycobacterium* seem to be highly active and important for both the dissimilation and the assimilation of nitrate in the earthworm gut of species of the Family Lumbricidae. However, no isolates from the current study were affiliated with the genus *Mycobacterium* (Table 23, Table A 2). This might be due to the fact that these species are hard to isolate with common media but need a specific medium, e.g., the Löwenstein-Jensen medium (Portales *et al.* 1987, Juste *et al.* 1991, Hartmans *et al.* 2006).

Rhizobiales-related *narG* transcripts were of minor abundance in species of the family Lumbricidae (Figure 27). This might be du to the fact that *Bradyrhizobium*-species frequently detected via *nirK*, *nirS*, and *nosZ* analyses (4.1.4) use Nap instead of Nar to reduce nitrate to nitrite (Moreno-Vivián *et al.* 1999, Delgado *et al.* 2003, Bedmar *et al.* 2005). Thus, the real contribution of *Rhizobiales* to the reduction of nitrate in the earthworm gut might be neglected by the lack of information about *napA* sequences.

Non-denitrifying, dissimilatory nitrate reducers have been detected in earthworm gut contents and casts, often in higher abundances than denitrifiers (Furlong *et al.* 2002, Ihssen *et al.* 2003, Byzov *et al.* 2009, Knapp *et al.* 2009), but the dissimilatory nitrate reduction to ammonium seems to be of minor importance in the gut of soil-feeding earthworms (Ihssen *et al.* 2003) or termites (Ngugi & Brune 2012) (4.1.2). Thus, it is very likely that denitrifiers and dissimilatory nitrate reducers compete for the nitrate in the gut of earthworms whereas nitrite seems to be predominantly used by denitrifiers instead of ammonium producing nitrite reducers. Analyses of *narG* with earthworms from Brazil were restricted to gene sequences (Figure 15). However, on gene level, *narG* sequences of German and Brazilian earthworms were similar (Figure 15, Figure 27). Thus, it can be speculated that dissimilatory nitrate reducers as those of the highly active genus *Mycobacterium* might compete for nitrate with denitrifiers also in the gut of earthworm from Brazil, i.e., *G. paulistus* and *A. gracilis*.

4.1.6. Ingested denitrifiers and dissimilatory nitrate reducers are selectively activated during gut passage

Denitrifiers and dissimilatory nitrate reducers are supposed to be soil-derived and do not represent an endogenous microbiota in the earthworm gut (4.1.3). However, detected relative abundances of *narG*, *nirK, nirS* and *nosZ* sequences differed between soil- and gut-derived libraries (3.1.1.3.2, 3.1.1.3.3, 3.1.1.3.4, 3.1.1.3.5, 3.1.2.1.1.1, 3.1.2.1.1.2, 3.1.2.1.2.2, 3.1.3.1, Table A 1) indicating an activation that is not evenly distributed among all taxa but more pronounced for some *Bacteria*.

The most significant way to elucidate the activation of ingested *Bacteria* in the earthworm gut is to analyze transcript sequences, as conducted for *narG* and *nosZ* from German earthworms and their soils (3.1.2.1.1). Here, gut- and mineral soil-derived *nosZ* transcripts strongly differed on transcript level (Figure 30, Figure 32, Figure 33). In addition, *nosZ* transcripts displayed lower diversity in the gut than in the soil (Table 21). For *narG* transcripts, differences between gut- and mineral soil-derived sequences were even more pronounced (Figure 26, Figure 28, Figure 29). Gut-derived *narG* transcripts were also less diverse than those from soil (Table 20). However, these differences within active denitrifiers and dissimilatory nitrate reducers could not be attributed to certain taxa as changes mostly

occurred within cultured and uncultured members of the *Rhizobiales* (*nosZ*; Figure 30, Figure 32) or *Actinomycetales* (*narG*; Figure 26, Figure 28). These combined data indicate that different and less diverse denitrifiers and dissimilatory nitrate reducers are active in the earthworm gut compared to the mineral soil to be a synonym for a selective activation.

Indeed, it is known that detected bacterial communities can differ significantly between the earthworm gut and pre-ingested soil (Egert *et al.* 2004, Knapp *et al.* 2008) concomitant with an elevated relative abundance of *Bacteria* capable of nitrate reduction in earthworm casts compared to bulk soil (Furlong *et al.* 2002, Chapuis-Lardy *et al.* 2010). *Bacteria* (Furlong *et al.* 2002, Horn *et al.* 2003, Singleton *et al.* 2003, Egert *et al.* 2004, Horn *et al.* 2006a, Wüst *et al.* 2009b), and *narG* and *nosZ* sequences in the earthworm gut (4.1.3; Horn *et al.* 2006a, Wüst *et al.* 2009b) are predominantly soil-derived. The activity and cultivability of most physiological groups, also denitrifiers and dissimilatory nitrate reducers is up to three orders of magnitude higher in the gut compared to pre-ingested soil (Daniel & Anderson 1992, Karsten & Drake 1995, Karsten & Drake 1997, Ihssen *et al.* 2003, Drake & Horn 2007). In contrast, total cell counts increase only marginally if at all during gut passage (Krištůfek *et al.* 1992, Krištůfek *et al.* 1995, Schönholzer *et al.* 1999, Wolter & Scheu 1999, Schönholzer *et al.* 2002). With a gut passage time of no longer than 20 h for *A. caliginosa* and *L. terrestris* (Barley 1961, Wüst *et al.* 2009a), a significant growth of ingested Bacteria seems unlikely. However, certain bacterial taxa get disrupted or killed during gut passage by grinding effects in the crop/gizzard and by chemicals secreted into the lumen of the digestive system (Schönholzer *et al.* 1999, Wolter & Scheu 1999, Schönholzer *et al.* 2002, Khomyakov *et al*. 2007, Oleynik & Byzov 2008). Thus, at least a few *Bacteria* seem to replicate during gut passage to end up with no lower total cell numbers in the gut compared to the pre-ingested soil. A strongly enhanced metabolism of denitrifiers and dissimilatory nitrate reducers in the earthworm gut seems very likely as the earthworm gut provides highly favorable conditions for both denitrifiers and dissimilatory nitrate reducers, i.e., the key factors anoxia and nitrate (Sudesh & Cole 2007, van Spanning *et al.* 2007). In addition to nitrite, a high moisture content, a nearly neutral pH, a low redox potential, and a high amount of organic carbon such as sugars and amino acids are prevalent (Barois & Lavelle 1986, Daniel & Anderson 1992, Lattaud *et al*. 1997, Trigo & Lavelle 1999, Horn *et al*. 2003, Horn *et al*. 2006b, Drake & Horn 2007, Wüst *et al*. 2009a, Schmidt *et al*. 2011). These combined findings strongly support the hypothesis of selectively activated denitrifiers and dissimilatory nitrate reducers in the earthworm gut (Hypothesis 2; 1.5).

Interestingly, *narG* and *nosZ* transcripts, i.e., active denitrifiers and dissimilatory nitrate reducers in the uppermost soil were highly similar to those in the earthworm gut but different from those of the mineral soil (Figure 26, Figure 29B, Figure 30, Figure 33B). Conditions in the uppermost soil overlay were not analyzed but texture and visual evaluation indicated a

high water content and a high content of organic carbon due to decaying plant material. A high water content can rapidly lead to anoxia due to the strongly reduced diffusion efficiency of O_2 in water. Experiments with soil incubated under conditions prevailing in the earthworm gut demonstrated that this activation of soil microbes leads to processes highly comparable to those prevailing earthworm gut content (Horn *et al*. 2003, Ihssen *et al*. 2003). Thus, conditions in the uppermost soil layer might have been quite similar to those in the earthworm gut upon sampling date. In summary, the detected differences between denitrifiers and dissimilatory nitrate reducers in the earthworm gut compared to mineral soil are very likely due to a selective activation of taxa that can adapt more quickly and efficiently to the conditions in the gut than others (Hypothesis 2; 1.5).

On gene level, differences between gut- and soil-derived *narG*, *nirK*, *nirS*, and *nosZ* sequences were smaller than detected for transcripts although some significant differences occurred on gene level (3.1.1.3.2, 3.1.1.3.3, 3.1.1.3.4, 3.1.1.3.5, 3.1.2.1.1.1, 3.1.2.1.1.2, 3.1.2.1.2.2, 3.1.3.1). These less pronounced differences on gene level can be explained by the fact that prokaryotes are assumed to replicate only marginally in the earthworm gut if at all (Krištůfek *et al.* 1992, Krištůfek *et al.* 1995, Schönholzer *et al.* 1999, Wolter & Scheu 1999, Schönholzer *et al.* 2002). However, diversity of *narG*, nirK, *nirS*, and *nosZ* gene sequences was sometimes higher in the gut than in soil (3.1.1.3.2, 3.1.1.3.3, 3.1.1.3.4, 3.1.1.3.5, 3.1.2.1.1.1). As gene sequences were analyzed instead of transcript sequences, these higher detected diversities could be due to an enhanced cell disruption during DNA extraction. Compared to the earthworm gut, the soil is a relatively dry habitat with few nutrients, resulting in a highly reduced metabolism and Bacteria display a low metabolic activity. Microorganisms in this stage of life are assumed to get less effectively disrupted than cells with a high metabolism. Thus, the beneficial conditions in the gut might lead to an enhanced detectability of *Bacteria* as gut conditions stimulate the cultivability of *Bacteria* (Karsten & Drake 1995, Karsten & Drake 1997, Ihssen *et al.* 2003, Drake & Horn 2007) and the germination of spores (Johnstone 1994, Fischer *et al.* 1997). However, this assumed higher detectability of microorganisms in the earthworm gut due to a higher metabolism also correlates with an activation of ingested organisms.

Most earthworms from Brazil emitted nitrogenous gases, especially N_2O in significantly higher amounts than their corresponding soil (Table 14), although the gut content comprises only about one fourth of the earthworm´s fresh weight (data not shown). Thus, even without the analysis of gene transcripts, a strong selective activation of ingested denitrifiers and dissimilatory nitrate reducers also for earthworms analyzed in Brazil can be assumed (Hypothesis 2; 1.5).

4.1.7. The earthworm feeding guild affects the diversity and activity of ingested denitrifiers and dissimilatory nitrate reducers

The emission of nitrogenous gases was reported for species of all three feeding guilds, i.e., endogeic, anecic, and epigeic, for earthworms from Germany, Brazil, and New Zealand (Table 14; Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b) and sequences indicative of denitrifiers were predominantly affiliated with *Rhizobiales* in the gut of all earthworms analyzed so far (4.1.4; Horn *et al.* 2006b, Wüst *et al.* 2009b). However, diversity of gut denitrifiers of those earthworms representing the endogeic (*A. caliginosa* and *O. multiporus*) or endo-anecic (*G. paulistus*) feeding guild was highly similar to that of the corresponding mineral soil. In contrast, denitrifiers in the gut of epigeic (*L. rubellus*) and epi-endogeic (*A. gracilis*) species were distinct from those of the mineral soil (Figure 20, Figure 22, Figure 25, Figure 33). Thus, there is a direct correlation between the amount of mineral soil that is normally ingested by an earthworm (Bouché 1977, Barois *et al.* 1999) and the similarity of gut denitrifiers to this soil.

It was not possible to determine the exact pre-ingested material of an earthworm upon sampling. Thus, the actual diet of an earthworm other than soil was not analyzed for denitrifiers and dissimilatory nitrate reducers. In addition, the two soils of *G. paulistus* and *A. gracilis* were sampled from distinct sampling locations (Table 14). However, the three German earthworms species analyzed were sampled from the same sampling area with the same mineral soil, and both gene and transcript sequences of *narG* and *nosZ* were evaluated (3.1.2.1). Thus, denitrifiers and dissimilatory nitrate reducers detected in their guts and their selective activation could be directly compared. Here, detected *nosZ* sequences indicative of denitrifiers differed in the gut of *A. caliginosa*, *L. terrestris*, and *L. rubellus*. This trend was detectable on gene level but was more pronounced on transcript level (Figure 32, Figure 33). For *narG* sequences indicative of both denitrifiers and dissimilatory nitrate reducers, differences were also detectable on gene level and more pronounced on transcript level (Figure 28, Figure 29). However, differences for nosZ were more pronounced than for *narG*. That is indicative of an activation of denitrifiers and dissimilarity nitrate reducers influenced by the feeding guild. In addition, this influence of the feeding guild on denitrifiers is stronger than on dissimilatory nitrate reducers in common.

For both *narG* and *nosZ*, sequences derived from gut content of the endogeic *A. caliginosa* were most similar to those from mineral soil whereas sequences of the epigeic *L. rubellus* were most dissimilar to those from mineral soil; the anecic *L. terrestris* was displaying a position in between (Figure 28, Figure 29, Figure 32, Figure 33). That means,

the more soil an earthworm was expected to normally ingest (Bouché 1977, Barois *et al.* 1999), the more similar were the denitrifiers and dissimilatory nitrate reducers detected in its gut compared to mineral soil. For the epigeic *L. rubellus*, the amount of isolated *Gammaproteobacteria* was higher than for the other earthworm species, whereas *Bacilli* were rare to absent within isolates derived with nitrite and N_2O as nitrogenous electron acceptor, respectively (Table 23). Thus, the isolation of assumed denitrifiers and dissimilatory nitrate reducers revealed also an influence of the earthworm feeding guild on the detected diversity.

In addition to the differences of genes indicative for denitrification and dissimilatory nitrate reduction, and of isolates detected in this study, *A. caliginosa*, *L. terrestris*, *and L. rubellus* all emit nitrogenous gases but different amounts (Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009b). Also for earthworms of different feeding guilds from Brazil (Table 14) and other tropical regions (Majeed *et al.* 2013), earthworms and their casts emitted different amounts of nitrogenous gases, even when sampled from the same soil.

It is known that the feeding guild influences the composition of bacterial taxa in the earthworm gut (Krištůfek *et al.* 1992, Aira *et al.* 2009, Knapp *et al.* 2009). This can be explained by the different feeding behaviors of the different feeding guilds. Anecic and epigeic earthworm ingest more organic materials and less mineral soil than endogeic species (Lee 1985, Barois *et al.* 1999, Brown & Doube 2004, Curry & Schmidt 2007). Although analyses are lacking in this study, the amount and composition of *Bacteria* in mineral soil and organic material is assumed to be different. Also nitrate concentrations might be different for mineral soil and organic material influencing the activity of denitrifiers and dissimilatory nitrate reducers in the earthworm gut. Before entering the gut lumen, some microorganisms are disrupted in the gizzard (Piearce & Philips 1980, Reddell & Spain 1991, Schönholzer *et al*. 1999, Wolter & Scheu 1999, Brown *et al*. 2000). It is likely that this grinding effect is stronger for endogeic species than for epigeic species due to more mineral particles as sand in the diet of endogeic earthworms.

Substances secreted by the earthworm into the lumen of the digestive system can inhibit, kill or digest microorganisms, sometimes specifically some taxonomical groups (Khomyakov *et al*. 2007, Oleynik & Byzov 2008). These fluids might be dependent on the feeding guild in respect of amount and composition. Actimomycetes whose activity might be also feeding guild-dependent are known to produce antibiotics that might selectively influence the activity of other microbes in the earthworm gut (Ravasz *et al.* 1986, Krištůfek *et al.* 1993, Brown 1995, Masignani *et al.* 2006). The amount of produced mucus is highest for endogeic earthworms and least for epigeic earthworms with anecic species displaying an intermediate production of mucus (Edwards & Bohlen 1996, Trigo *et al.* 1999, Brown *et al.*

2000). It is conceivable that these different amounts of mucus influence the activity of ingested *Bacteria* that entered the gut lumen unharmed. However, experiments with gut contents of *G. paulistus* demonstrated that additional organic carbon did not stimulate the emission of nitrogenous gases indicating that mucus-derived organic carbon was not limited, at least in the endo-anecic *G. paulistus* (Figure 10E).

To sum it up, the earthworm feeding guild affects the diversity and activity of ingested denitrifiers and dissimilatory nitrate reducers (Hypothesis 2; 1.5). However, the distinct factors that lead to the detected differences are still unresolved.

4.2. Methanogenesis and the emission of CH_4

Earthworms are known to emit nitrogenous gases and H_2 via ingested nitrate reducers and fermenters, respectively, that get both activated in the anoxic earthworm gut (4.1.2; Horn *et al.* 2003, Drake & Horn 2007, Wüst *et al.* 2009a, Wüst *et al.* 2009a). Methanogenesis is so far exclusively known from strictly anaerobic *Archaea* (Hedderich & Whitman 2006, Liu & Whitman 2008). The digestive tract of vertebrates, e.g., humans and cows and also of invertebrates, e.g., termites and cockroaches is known to harbor methanogens emitting high amounts of the greenhouse gas CH_4 (Miller & Wolin 1986, Brusa *et al.* 1993, Hackstein & Stumm 1994, Brune 2006, Alley *et al.* 2007, Forster *et al.* 2007, Denman *et al.* 2007, EPA 2010, Schauer *et al*. 2012). However, attempts restricted to species of the family Lumbricidae failed to demonstrate the emission of CH_4 by earthworms (Karsten & Drake 1995, Šustr & Šimek 2009). Thus, eight earthworm species from Brazil affiliated with five different families and of different sizes and feeding guilds were tested for their ability to emit CH_4, in addition to their substrates (3.2.2).

4.2.1. The earthworms *E. eugeniae*, *P. corethrurus*, and *R. alatus* emit CH_4 *in vivo*

Three earthworm species emitted *in vivo* CH_4, i.e., *E. eugeniae*, *P. corethrurus* and *R. alatus*. All other species emitted no CH_4 (Figure 38A) (Hypothesis 3; 1.5). *E. eugeniae* displayed the highest emissions with up to 41 nmol CH_4 (g fw)$^{-1}$ after a 5 h incubation, whereas highest emission for *P. corethrurus* was nmol CH_4 (g fw)$^{-1}$ after a 5 h incubation (Figure 38A). *R. alatus* emitted CH_4 when sampled from its natural substrate (March 2011), and subjected to Substrate 1 when sampled in diapause (September 2011) (Figure 38A). Emissions of CH_4 from *E. eugeniae* were relatively linear (Figure 39) and specimens raised on Substrate 1 emitted CH_4 at a rate of approximately 5 nmol CH_4 (g fw * h)$^{-1}$ (Figure 38A, Figure 39). This is in the range of emissions reported for those of N_2O and H_2 (Table 14;

Karsten & Drake 1997, Matthies *et al.* 1999, Horn *et al.* 2003, Horn *et al.* 2006b, Drake & Horn 2007, Wüst *et al.* 2009b). Studies with the epigeic *Eisenia andrei* indicated earthworms might alter the CH_4 production and oxidation rates whereas the net CH_4 flux seems be unaffected by the presence of earthworms in cattle-impacted soils (Bradley *et al.* 2012, Koubova *et al.* 2012). However, this study is the first report of emission of CH_4 by earthworms.

Other invertebrates as termites, cockroaches, beetle larvae, and millipedes from tropical regions are known to emit about one order of magnitude more CH_4 than *E. eugeniae* (Hackstein & Stumm 1994, Brune 2006, Schauer *et al*. 2012) whereas emission rates of temperate millipedes are in the range of the maximum rates determined for earthworms in the current study (Šustr & Šimek 2009). Most specimens of *E. eugeniae* emitted CH_4 but some did not (Figure 38A) although earthworms were of comparable weight and displayed all a healthy behavior (data not shown). This phenomenon is also known from earthworms in respect of the emission of N_2O (Matthies *et al.* 1999).

Earthworms of the family Eudrilidae (*E. eugeniae*) and Glossoscolecidae (*P. corethrurus* and *R. alatus*) emitted CH_4 *in vivo* (Figure 38A, Figure 39). In contrast, *G. paulistus* and *Glossoscolex* sp. of the family Glossoscolecidae emitted no CH_4 (Figure 38A). *R. alatus* was the largest earthworm studied whereas *P. corethrurus* and *E. eugeniae* were rather small (Table 14). All other big and small species emitted no CH_4. In respect of the feeding guild, *E. eugeniae* is epigeic whereas *P. corethrurus* and *R. alatus* are endogeic (Table 14). All other species are also categorized into these feeding guilds (Table 14) but emitted no CH_4 (Figure 38A). Thus, the earthworm family, size, and feeding guild alone are no determinative factor for the emission of CH_4.

The earthworm substrate might be another factor influencing the emission of CH_4. Specimens of *E. eugeniae* raised on Substrate 1 emitted the highest amounts of CH_4 (Figure 38A, Figure 39). Substrate 1 consisted of composted cow manure rich in organic material (Table 27). Although aerobically composted for several weeks, these residues of cow manure are supposed to be a source of methanogens (Flint 1997, Janssen & Kirs 2008). Indeed, sequences indicative of methanogenic *Archaea* were detected in Substrate 1 and this substrate was the only substrate that emitted small amounts of CH_4 under the aerated conditions used to assess the *in vivo* emission of CH_4 by earthworms (Figure 38B). Thus, the methanogenic activity of *E. eugeniae* might be associated with Substrate 1. In addition, *R. alatus* sampled in diapause, i.e., without gut content in September 2011 emitted no CH_4 whereas CH_4 was emitted after a 60 h incubation with Substrate 1 (Figure 38A). However, *E. eudrilus* specimens raised on Substrate 2 and Substrate 3 emitted similar amounts of CH_4 than on Substrate 1 (Figure 38A) although these substrates emitted no CH_4 and consisted of processed sugarcane residues and no mammalian feces potentially rich in methanogens

(Miller & Wolin 1986, Brusa *et al.* 1993, Hedderich & Whitman 2006) as Substrate 1 (Table 27, Figure 38B). In addition, the amount of organic matter in the substrate was not strictly correlated with the amount of CH_4 emitted, as *E.* eugeniae raised on Substrate 3 displayed a higher capacity to emit CH_4 than specimens raised on Substrate 2, which displayed o lower amount of organic material than Substrate 3 (Figure 38A, Table 27). Also substrates for *P. corethrurus* (Substrate 4, i.e., grassland soil) and *R. alatus* (Substrate 7, i.e., uncharacterized soil) emitted no CH_4 and contained no mammalian feces (Figure 38A). *P. excavatus* and *E. andrei* raised on Substrate 1 and Substrate 3, respectively emitted no CH_4 although *E. eugeniae* specimens raised on these substrates did (Figure 38A). Thus, the substrate alone does not seem to determine if an earthworm emits CH_4. However, Substrate 1, i.e., composted cow manure seems to favor methanogenesis best.

In respect of the earthworm appearance, *P. corethrurus* and *R. alatus* were sampled from their natural habitat (Table 26). In contrast, *E. eugeniae* was purchased (Table 26) as this species is commercially used, especially for vermicomposting (Gajalakshmi & Abbasi 2004). *E. eugeniae,* originally domiciled in Central and Western Africa, tolerates only high temperatures of 25 to 28°C and is also naturally occurring in many tropical and subtropical habitats as pastures and tree plantations, also in Brazil where it comprises an exotic earthworm species (Fragoso *et al.* 1999, James & Brown 2006). *P. excavatus* and *E. andrei* were also purchased whereas all other species were sampled from their natural habitat (Table 26). Thus, the emission of CH_4 by earthworms is not restricted to antropogenically used species as *E. eugeniae*, but is also present in earthworms in their natural habitat, i.e., *P. corethrurus* and *R. alatus*. The earthworm *P. corethrurus* tolerates temperatures of 14 to 28°C (Fragoso *et al.* 1999) and is abundant in many tropical and subtropical regions (Marichal *et al.* 2010), especially in Brazil (James & Brown 2006) and preferentially invades deforested, cropland, and pasture areas where it comprises the dominant earthworm species (James & Brown 2006, Nunes *et al.* 2006, Marichal *et al.* 2010, Rossi *et al.* 2010). In contrast, *R. alatus* occurs only in restricted areas in Brazil (James & Brown 2006). Although other invertebrates emit significantly more CH_4 than earthworms from this study (Hackstein & Stumm 1994, Brune 2006, EPA 2010, Schauer *et al*. 2012) (Figure 38A, Figure 39), the contribution of earthworms to the biogenic emission of CH_4 should not be overlooked. This is even more relevant as in the current study, only eight earthworm species from tropical regions have been analyzed for their capacity to emit CH_4 indicating that research in this direction might reveal an even more pronounced contribution of tropical earthworms to the emission of the potent greenhouse gas CH_4.

4.2.2. Methanogens associated with the *in vivo* emission of CH_4 by *E. eugeniae*

The only biogenic process to produce CH_4 is methanogenesis conducted by methanogenic *Archaea*, strict anaerobes (Hedderich & Whitman 2006, Liu & Whitman 2008). BES is an inhibitor of methanogenesis (Gunsalus *et al.* 1978). For *E. eugeniae* raised on Substrate 1, gut contents incubated under anoxic conditions produced CH_4 when no BES was applied whereas no CH_4 was emitted when BES was added (Figure 38A). Thus, as expected, methanogenesis is responsible for the emission of CH_4 detected for *E. eugeniae*, and is also assumed for *P. corethrurus* and *R. alatus* (Figure 38A). The emission of CH_4 from gut contents of *E. eugeniae* was lower than that for living specimens on a per g fresh weight basis (Figure 38A). This reduced emission might have been due to a short exposure to oxygen during preparation what could have severly inhibited the strict anaerobic methanogenic *Archaea* (Hedderich & Whitman 2006, Liu & Whitman 2008) whereas the earthworm gut itself is strictly anoxic (Horn *et al.* 2003, Wüst *et al.* 2009a).

Gene and transcript analyses of *mcrA* and *mrtA* (encoding for the alpha subunit of methyl-CoM reductase and its isoenzyme, respectively [Springer *et al.* 1995]) were conducted with gut contents of *E. eugeniae* raised on Substrate 1, and with Substrate 1 (3.2.3) to identify methanogenic taxa. On transcript level, only *mcrA* sequences were detected (Figure 40). Those transcripts derived from the gut were affiliated with several methanogenic taxa but predominantly with *Methanosarcinaceae* and *Methanobacteriaceae* (Figure 40, Figure 41). Transcripts derived from Substrate 1 were distributed more evenly and lacked sequences affiliated with *Methanobacteriaceae* (Figure 40). On a per g fresh weight basis, *E. eugeniae* emitted up to 90-fold more CH_4 than Substrate 1 (Figure 38A, Figure 38B). In addition, only about on fourth of the earthworm´s fresh weight is assumed to consist of gut content (data not shown). This leads to an up to 360-fold higher emission of CH_4 by *E. eugeniae* compared to the emission by Substrate 1. In the gut of *L. terrestris*, an average redox potential of 150 mV was measured (Schmidt *et al.* 2011), a value that seems inadequate to favor methanogenesis that usually occurs at an in any case negative redox potential (Thauer *et al.* 2008). Thus, the redox potential of *E. eugeniae* might be more negative than in *L. terrestris* to facilitate the detected methanogenesis.

The isolation of methanogens from gut contents of *E. eugenae* raised and maintained on Substrate 1 that still stands on the level of enrichment cultures yielded an enrichment of *Methanomicrobiales*, predominantly *Methanomicrobiaceae* via T-RFLP analysis of *mcrA*/*mrtA* genes (3.2.4). These methanogens are predominantly capable of hydrogenotrophic methanogenesis (Garcia *et al.* 2006, Liu & Whitman 2008). This fits well with the data obtained from the enrichment cultures of low dilution steps that utilized H_2 and produced CH_4 in a ratio of 4.1 to 1, a ratio that is near the ideal utilization of H_2 for

hydrogenotrophic methanogenesis, disregarded of CO_2 and an increase in biomass (Liu & Whitman 2008; Equation 2a). However, at the time of sampling, this ratio was higher (about 17) for the highest enrichment step of which the T-RFLP analysis was conducted from (Table 28), and hydrogenotrophic methanogens might have been extremely favored by the enrichment conditions applied, i.e., high concentrations of H_2/CO_2 and no supplemental alternative substrates for methanogenesis as acetate (1.3). Thus, the data directly retrieved from gut contents of *E. eugeniae*, i.e., without a pre-enrichment appear to be more reliable in respect of *in situ* conditions in the living earthworm.

These combined data indicate a strong activation of ingested methanogens in the earthworm gut (Hypothesis 3; 1.5). There, *Methanosarcinaceae* and *Methanobacteriaceae* seem to be the the most active methanogens under *in situ* conditions.

Methanosarcinaceae are capable of hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis whereas *Methanobacteriaceae* are predominantly hydrogenotrophic methanogenes (Bonin & Boone 2006, Kendall & Boone 2006). In the gut of *L. terrestris*, fermentations occur, concomitant with the production of H_2 and acetate (Wüst *et al.* 2009a; Figure 6) and the assumed production of CO_2 from earthworm respiration and microbial fermentation processes. The substrate of *E. eugeniae* is rich in organic material and nutrients (Table 27). Thus, similar fermentation processes are very likely to occur also in the gut of *E. eugeniea* yielding the substrates H_2/CO_2, and acetate for methanogenesis (Hedderich & Whitman 2006, Liu & Whitman 2008). For *E. eugeniae* raised on Substrate 1 or 3, supplemented H_2/CO_2 did not stimulate the *in vivo* emission of CH_4 (Figure 38A). This indicates (i) that the hydrogenotrophic methanogenesis is not the main source of CH_4 or (ii) that hydrogenotrophic methanogens in the gut of *E. eugeniae* are already saturated with H_2/CO_2 resulting in no enhanced production of CH_4 with supplemented H_2/CO_2. However, the emissions of CH_4 by *E. eugeniae* raised on Substrate 2, and of *R. alatus* incubated with Substrate 1 after diapause were slightly stimulated with H_2/CO_2 (Figure 38A) indicating that methanogenesis is not at its maximum rate in some earthworms analyzed or under certain conditions.

4.2.3. Origin of methanogens in earthworms from Brazil

The majority of methanogenic taxa detected in the gut of *E. eugeniae* raised on Substrate 1 was also detected in Substrate 1 although differences in diversity were detected (Figure 40, Figure 41). In addition, Substrate 1 emitted small amounts of CH_4 demonstrating that methanogens not inhibited by oxygen were present in Substrate 1 (Figure 38B). Thus, the emission of CH_4 by *E. eugeniae* is assumed to be due to an activation of ingested methanogens rather than endogenous *Archaea* reported for other invertebrates as termites,

cockroaches, and millipedes (Brune 2006, Paul *et al.* 2012, Schauer *et al*. 2012). This is congruent with findings concerning denitrifiers and dissimilatory nitrate reducers in the gut of earthworms (4.1.3; Horn *et al.* 2003, Horn *et al.* 2006a, Drake & Horn 2007, Wüst *et al.* 2009b). However, as knowledge about methanogens in earthworms does not exist in literature up to now, scenarios other than a diet-derived microbiota that gets activated and subsequently decasted will be discussed in the following.

Genes of *mcrA* affiliated with *Methanobacteriaceae* were abundantly detected in the gut content of *E. eugeniae* whereas only one sequence was affiliated with *Methanobacteriaceae* in Substrate 1. On transcript level, sequences affiliated with *Methanobacteriaceae* were abundantly (i.e., 26 % relative abundance) and exclusively detected in the gut of *E. eugeniae* (Figure 40, Figure 41). This imbalance is less pronounced for most analyses of denitrifiers and dissimilatory nitrate reducers in the earthworm gut (Figure 15, Figure 18, Figure 21, Figure 23, Figure 26, Figure 30, Figure 34, Figure 36; Horn *et al.* 2006a, Wüst *et al.* 2009b) and might indicate that methanogens are not just activated upon their gut transit.

E. eugeniae specimens raised on Substrate 1, Substrate 2, and Substrate 3 emitted CH_4 and maintained a reduced ability to emit CH_4 after a 60 h incubation on Substrate 4 (Figure 38A), i.e., on dry, reddish mineral soil poor in organic material (Table 27). During this pre-incubation, the original gut content of *E. eugeniae* specimens (i.e., Substrate 1, Substrate 2, and Substrate 3) was completely replaced by Substrate 4. This was verified by the reddish instead of darkish casts already after a few hours of incubation and is in accordance with the mean gut passage time of *E. eugeniae* of 2 to 6 h (Mba 1982). Molecular information about methanogens in Substrate 4 lacks and the CH_4-emitting *P. corethrurus* was also sampled from Substrate 4 (Figure 38A, Table 26). However, it seems unlikely that this dry and nutrient-poor soil harbors enough methanogens that could be activated within the 2 to 6 h of gut passage through *E. eugeniae* and *P. corethrurus* (Mba 1982, Mba 1989) and produce the detected amounts of emitted CH_4 (Figure 38A). Thus, it is disputable if an, even sufficient but oxygen-exposed amount of methanogens can be activated fast enough to produce up to approximately 5 nmol CH_4 $(g\ fw * h)^{-1}$ as detected for *E. eugeniae* (Figure 38A, Figure 39) even though microsites with permanent anoxia might exist in Substrate 4. It appears also conceivable that diet-derived methanogens are at least retained in the digestive system of the earthworm and therefore stay longer in the gut than 6 h, the maximum gut passage time for *E. eugeniae* and *P. corethrurus* (Mba 1982, Mba 1989). In these earthworms, *Archaea* could be retained in foldings of the inner gut wall (Breidenbach 2002), attach to the gut tissue itself as reported for some prokaryotes in Lumbricidae (Jolly *et al.* 1993) or inhabit specialized bacterisomes as known from symbionts of other invertebrates (Baumann *et al.* 2006).

P. excavatus raised or pre-incubated on Substrate 1, Substrate 2, and Substrate 4 emitted no CH_4, with no single specimen although *E. eugeniae* specimens emitted CH_4 when raised or pre-incubated on these substrates (Figure 38A). This indicates that conditions in the earthworm alimentary canal seem to strictly determine if methanogenesis occurs, and that the substrate is not the only determinant factor. In summary, it remains still unresolved if the gut anatomy (Breidenbach 2002), the redox potential, substances secreted by the earthworm (Khomyakov *et al*. 2007, Oleynik & Byzov 2008), compartments filled with retained or endogenous *Archaea*, or a combination of these factors are determinative for the emission of CH_4.

In a highly speculative scenario of *Archaea* tightly affiliated with the digestive system of the earthworm, and also for only transient methanogens, the question about the advantage of the earthworm from this symbiosis arises. The earthworm is assumed to be interested in the effective digestion of ingested material and in fermentations in the gut resulting in the production of organic acids and alcohols which the worm can assimilate (Trigo *et al.* 1999, Brown *et al.* 2000, Wüst *et al.* 2009a). During fermentation processes in the gut of *L. terrestris*, H_2, acetate (Wüst *et al.* 2009a), and most likely CO_2 are produced. Fermentations are also supposed to occur in the gut of *E. eugeniae* and *P. corethrurus* as for *L. terrestris* fed on bulk soil and decaying plant material, fermentations were detected right from the beginning of the alimentary canal, i.e., after a short period of time inside the gut (Wüst *et al.* 2009a; Figure 6). *Archaea* producing CH_4 from H_2/CO_2 or acetate could keep the partial pressure or concentration of these fermentation products low. These low concentrations of end products of fermentation processes would result in enhanced and more effective fermentations (Schink & Stams 2006, Liu & Whitman 2008) resulting in more organic carbon to be assimilated by the earthworm. This symbiosis could then be termed a 'mutualism', a term that is already used to describe the relationship between the digestive system of earthworms and ingested microorganisms as a whole (Barois & Lavelle 1986, Lavelle *et al.* 1995, Trigo *et al.* 1999, Brown *et al.* 2000). However, further research is needed to confirm or disprove these speculations.

4.3. Concomitant denitrification and methanogenesis in the earthworm gut

For most earthworm species and substrates analyzed in Brazil, the *in vivo* emissions of nitrogenous gases and CH_4 were analyzed concomitantly (Table 3). Except for *Dichogaster* sp., all earthworm species emitting N_2O also emitted N_2, whereas exclusively all species that emitted no N_2O also emitted no N_2 (Figure 42). All analyzed earthworm substrates emitted N_2 whereas only the substrate of *R. alatus* emitted N_2O under oxic conditions. This

demonstrates that the earthworm strongly shifts the emission of nitrogenous gases toward the potent greenhouse gas N_2O. *G. paulistus*, *Glossoscolex* sp., and *E. andrei* emitted neither nitrogenous gases nor CH_4. Thus, if conditions in the gut do not favor denitrification, also methanogenesis does not occur. *A. gracilis* and *P. excavatus* emitted nitrogenous gases but no CH_4 (Figure 42). Methanogenesis occurs at a lower redox potential than denitrification (Thauer *et al.* 2008, Schmidt *et al.* 2011) and might therefore not be present in earthworm species with a higher redox potential whereas denitrification can occur there. In addition, NO and N_2O as intermediates of denitrification are assumed to inhibit methanogenesis (Klüber & Conrad 1998, Roy & Conrad 1999, Choi *et al.* 2006). These combined factors might explain the lacking methanogenesis in earthworms that emit nitrogenous gases, especially for *P. excavatus* that was fed on Substrate 1, i.e., composed cow manure that displayed albeit minor emission of CH_4 (Figure 38B). In addition, denitrifiers are assumed to compete with methanogens for H_2 and therefore additionally inhibit methanogenesis (Klüber & Conrad 1998). However, supplemented H_2 did not result in an emission of CH_4 by *P. excavatus* (Figure 38A), and all three species emitting CH_4 also emitted nitrogenous gases, i.e., *E. eugeniae*, *P. corethrurus*, and *R. alatus* (Figure 42). Thus, other factors than NO and N_2O as inhibitors of methanogenesis and a low concentration of H_2 have to contribute to the contrasting emission features of earthworms *E. eugeniae* and *P. excavatus* fed on the same substrate. Thus, acetoclastic methanogens not dependent on a high concentration of H_2 (Hedderich & Whitman 2006, Liu & Whitman 2008) might be the dominant methanogens in these earthworms.

To sum it up, denitrification seems to occur more easily in the earthworm gut than methanogenesis. To what extent the substrate, the earthworm gut anatomy, the redox potential, concentration of substrates for methanogenesis, the composition of methanogens in the gut, a tight association of methanogens with the earthworm, inhibitory intermediates of denitrification, or other factors determine if methanogenesis occurs next to denitrification remains unresolved and needs further research (Figure 42, 4.1.1, 4.2.1, 4.2.3).

However, the earthworm substrate seems to influence but not determine if an earthworm emits nitrogenous gases and CH_4 (Figure 42, 4.1.1, 4.2.1). The earthworm size might influence which of the nitrogenous gases, i.e., N_2O or N_2 is emitted predominantly, whereas the earthworm family seems to be no influencing factor (Figure 42, 4.1.1). The feeding guild strongly influences what substrate the earthworm ingests and might therefore also influence the emission of nitrogenous gases and CH_4 (Figure 42, 4.1.1, 4.2.1).

Size	Family	Feeding guild		N_2O Worm	N_2O Substrate	N_2 Worm	N_2 Substrate	CH_4 Worm	CH_4 Substrate
	GLOSSO	an	***Rhinodrilus alatus***	+	+	+++	++	+	-
	GLOSSO	endo/an	***Glossoscolex paulistus***	-	-	-	+	-	-
	GLOSSO	endo	***Glossoscolex* sp.**	-	-	-	+	-	-
	GLOSSO	endo	***Pontoscolex corethrurus***	++	-	+	+	+	-
	MEGA	epi/endo	***Amynthas gracilis***	++	-	++	+	-	-
	MEGA	epi	***Perionyx excavatus***	+	-	+	++	-	+
	EUD	epi	***Eudrilus eugeniae***	++	-	++	++	++	+/-
	LUM	epi	***Eisenia andrei***	-	-	-	n.d.	-	-
	ACAN	epi	***Dichogaster annae***	+	-	+	++	n.d.	+
	ACAN	epi	***Dichogaster* sp.**	+	-	-	++	n.d.	+

Figure 42: Emission of N_2O, N_2, and CH_4 by earthworms and their substrates from Brazil.
Emission of N_2O, N_2, and CH_4 by earthworm species (black symbols) of different size, family, and feeding guild, and emission by the corresponding substrate an earthworm was associated with (gray symbols). Gray bar at the left indicates the relative size of an earthworm. Abbreviations: GLOSSO, Glossoscolecidae; MEGA, Megascolecidae; EUD, Eudrilidae; LUM, Lumbricidae; ACAN, Acanthodrilidae. an, anecic; endo, endogeic; epi, epigeic; -, < 0.02 nmol (g fw h)$^{-1}$; +, < 1 nmol (g fw h)$^{-1}$; ++, < 3 nmol (g fw h)$^{-1}$; +++, > 10 nmol (g fw h)$^{-1}$; n.d., not determined. Emissions were calculated from a 5, 6, and 9 h incubation under oxic conditions in March 2011, September 2011, and November 2010, respectively (Table 14, Figure 38). All symbols are based on gas emissions in nmol (g fw h)$^{-1}$ although most emissions were calculated from two points of measurement only. For species analyzed at different dates, the highest mean emission was used for this table. Only one of the three substrates of *E. eugeniae* emitted CH_4 whereas *E. eugeniae* specimens raised on any of the three substrates emitted CH_4.

4.4. Concluding model for the emission of nitrogenous gases and CH_4 by earthworms

Although the emission of nitrogenous gases and CH_4 was analyzed concomitantly for most earthworm species from Brazil, the analyses of genes indicative of these two processes were not conducted concomitantly for the same gut content of a species. However, several species emitted both nitrogenous gases and CH_4, and genes indicative of denitrifiers were affiliated with *Rhizobiales* for all species analyzed, i.e., for those collected in Germany, Brazil, and New Zealand (4.1.4). Thus, a model (Figure 43) was drawn to combine the results of the current study together with preceding knowledge about anoxic processes in the earthworm gut.

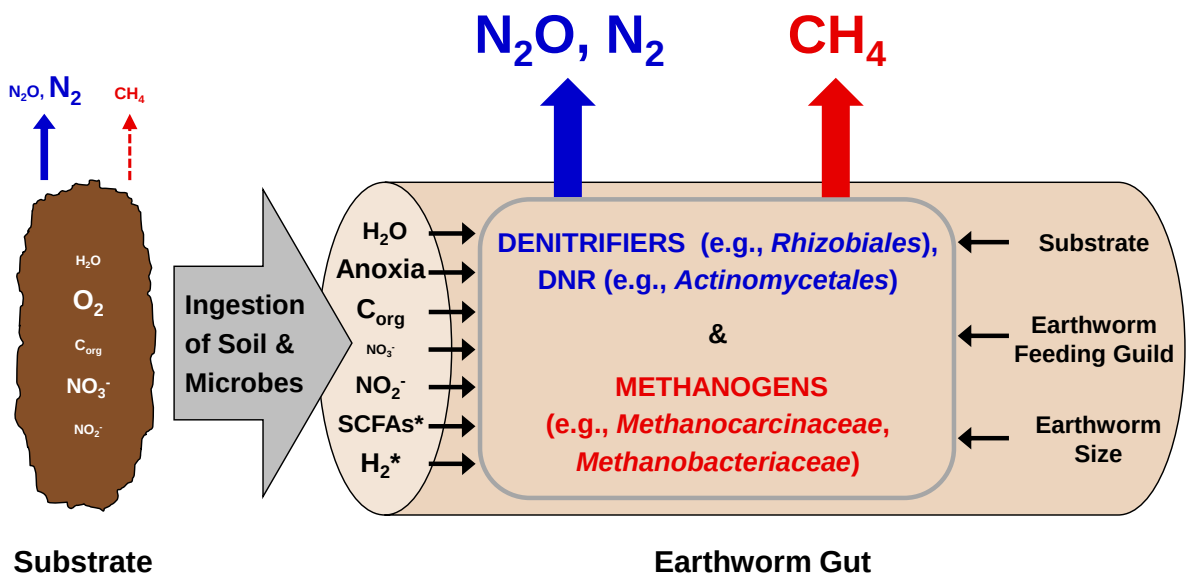


Figure 43: Model for the activation of ingested denitrifiers, dissimilatory nitrate reducers, and methanogens in the earthworm gut, and the associated emission of N_2O, N_2, and CH_4.
Substrate-derived microbes, i.e., *Bacteria* and *Archaea* get activated in the earthworm gut. Conditions in the substrate and the earthworm gut are displayed on the left and on the right side of the gray arrow, with relative concentrations indicated by the font size (modified from Horn *et al.* [2003] and Drake & Horn [2007]); compounds marked with an asterisk, i.e., SCFAs (short chain fatty acids) and H_2 are produced by fermenting *Bacteria* in the alimentary canal (Wüst *et al.* 2009a). All factors in the earthworm gut that impact on ingested denitrifiers, dissimilatory nitrate reducers (DNR), and methanogens (the analyzed ingested microorganisms are indicated by the gray box inside of the earthworm) are indicated by black arrows. In addition to the conditions mentioned above, the diversity and activity of denitrifiers, DNR (both processes and associated organisms are indicated by blue color), and methanogens (red color) is influenced by the ingested substrate and the earthworm feeding guild whereas the earthworm size might influence which nitrogenous gas in produces predominantly. The amount of N_2O, N_2, and CH_4 released from the substrate and the living earthworm is indicated by the arrow and font size; the dashed arrow indicates that some earthworms emitted CH_4 whereas their substrate did not. Denitrifiers (i.e., predominantly *Bradyrhizobiaceae* within the *Rhizobiales*) and, to a smaller extent dissimilatory nitrate reducers (i.e., predominantly *Mycobacterium* within the *Actinomycetales*) are assumed to release the detected N_2O and N_2. Methanogens (i.e., predominantly *Methanosarcinaceae* and *Methanobacteriaceae*) are assumed to release the detected CH_4. Not all earthworm species emitting nitrogenous gases also emitted CH_4, whereas all species emitting CH_4 also emitted nitrogenous gases (Figure 42). Abbreviations: H_2O, water content; C_{org}, organic carbon (predominantly earthworm-derived mucus).

In aerated soils, microbes face predominantly oxic conditions, a low water content, a low amount of easily available organic carbon, high nitrate and low nitrite concentrations (Figure 43). Anaerobic processes as denitrification and methanogenesis are occurring marginally only, if at all. Thus, the earthworm substrate, i.e., soil, microbes, and an earthworm feeding guild-dependent amount of decaying organic material emits minor amounts of N_2O and CH_4 if at all, with N_2 being emitted in higher amounts than N_2O. The earthworm ingests the substrate, and microbes reaching the gut lumen unharmed face conditions as permanent anoxia, a high water content, a high amount of easily available organic carbon (predominantly earthworm mucus), low nitrate and high nitrite concentrations. Fermenting *Bacteria* highly activated by these conditions produce high amounts of fermentation products as short chain fatty acids, alcohols, H_2, and CO_2 (Figure 6, Figure 43). All these conditions impact on the diversity and activity of denitrifiers, dissimilatory nitrate reducers and methanogens. In addition, the diversity and activity of these selectively activated physiological groups in the earthworm gut is influenced by the ingested substrate itself, whose composition is strongly influenced by the feeding behaviors of an earthworm as an equivalent to the earthworm feeding guild. The feeding guild might also influence microbes in the gut via a different earthworm gut anatomy and different amounts and compositions of secreted substances as mucus and inhibitory fluids, or by selectively activated microorganisms producing toxins that affect other microbes. The earthworm size might influence the relative emission of the nitrogenous gases N_2O and N_2, i.e., a large earthworm tends to result in a higher relative emission of N_2 than a small worm although this rule is not valid *sensu stricto*.

Denitrifiers (e.g., *Bradyrhizobiaceae* within the *Rhizobiales*) and dissimilatory nitrate reducers (e.g., *Mycobacterium* within the *Actinomycetales*) are active in the earthworm gut, and compete for nitrate. N_2O is assumed to be emitted predominantly by denitrifiers and to a small extent maybe also by dissimilatory nitrate reducers, whereas N_2 is the end product of denitrifiers only. Active methanogenic *Archaea* are affiliated with *Methanosarcinaceae* and *Methanobacteriaceae*, and may produce CH_4 predominantly via hydrogenotrophic and also acetoclastic methanogenesis. Influencing factors of methanogenesis are still largely unresolved. However, the earthworm emits nitrogenous gases and CH_4 in significantly higher amounts than the pre-ingested substrate. This turns the earthworm into a contributor of worldwide emission and turnover of the potent greenhouse gases N_2O and CH_4.

4.5. Outlook for future research

This study aimed to analyze different earthworm species for their capacity to emit nitrogenous gases and CH_4, and to link these emissions to microbial taxa, i.e., to denitrifiers, dissimilatory nitrate reducers, and methanogens. In addition, factors as the earthworm substrate, family, size, and feeding guild were analyzed for their impact on the diversity and activity of microbes in the gut.

Bradyrhizobiaceae within the *Rhizobiales* were identified as abundant and active denitrifiers in the earthworm gut (4.1.4). The molecular analysis of Gram-positive denitrifiers via *nirK*, *nirS*, and *nosZ* is not able with the primers available up to date (Behrendt *et al.* 2010, Green *et al.* 2010, Verbaendert *et al.* 2011b). However, active Gram-positive *Bacteria* were detected via *narG* (4.1.5), and potential Gram-positive denitrifiers were isolated from gut contents (4.1.4). Gram-positive denitrifiers might therefore significantly contribute to emission of nitrogenous gases. With knowledge about N_2O reductases of Gram-positive *Bacteria* beginning to get elucidated most recently (Sanford *et al.* 2012), new primers could be generated to detect Gram-positive denitrifiers in the earthworm gut. The evaluation of denitrification-linked nitrite reductases in Gram-positive *Bacteria* would also significantly help to understand denitrification in the earthworm gut, and at all. In addition, analysis of *napA*-encoded nitrate reductases could confirm the assumed dominant contribution of *Bradyrhizobium*-related, Gram-negative denitrifiers to the emission of nitrogenous gases as revealed by *nirK*, *nirS*, and *nosZ* analyses (4.1.4). Quantitative PCR on gene and transcript level could yield a direct comparison of denitrifiers and methanogens in the earthworm gut compared to its diet.

The earthworm size was assumed to influence the emission of nitrogenous gases (4.1.1). In this respect, specimens of large earthworm species could be analyzed for their capacity to emit nitrogenous gases and also CH_4 at different stages of life, i.e., at different sizes. The large *R. alatus* emitted both nitrogenous gases and CH_4, and would be an ideal study object in direct comparison to the assumed negative control, i.e., *G. paulistus* that emitted virtually no nitrogenous gases and CH_4 (Table 14, Figure 38A). Similar to *L. terrestris* (Wüst *et al.* 2009b), these two large species should also be analyzed for fermentation processes, as their long guts and long gut passage times should facilitate fermentations. In addition, gut contents of *E. eugeniae* should also be tested for fermentations, particularly for the occurrence of H_2 that is, next to CO_2 the substrate for hydrogenotrophic methanogenesis (Liu & Whitman 2008). Here, experiments with stable isotope probing (SIP) of carbon sources (e.g., sugar monomers as glucose, mannose or fucose as components of the earthworm´s mucus) (Wüst *et al.* 2011) would elucidate the food chain in the earthworm gut and lead to a better understanding of both, fermentations and methanogenesis.

To better understand the role of the earthworm substrate on the diversity and activity of gut microbes, the exact diet of an earthworm instead of the surrounding soil or substrate could be analyzed with molecular tools and directly compared to the earthworm´s gut content. In this respect, the impact of artificially defined diet on the *in situ* emission of gases could also be evaluated. Such experiments should be conducted in cooperation with earthworm ecologists with the appropriate knowledge about the exact feeding habits of earthworms and how to maintain the species in the laboratory for a longer period of time.

E. eugeniae raised on Substrate 1 (i.e., composted cow manure) maintained its ability to emit CH_4 when incubated on Substrate 4 (i.e., grassland soil) for 60 h (Figure 38A) but a molecular detection and quantification of methanogens in Substrate 4 lacks. An incubation *of E. eugeniae* with a substrate that is definitely free of methanogens could elucidate if CH_4-emitting methanogens are retained in *E. eugeniae*. A possible association of such methanogens with the earthworm gut tissue could be elucidated with gut slices analyzed with fluorescence *in situ* hybridization (FISH) that specifically detects taxa of methanogens abundantly detected in this study. Also the emission of CH_4 by the abundantly occurring species *P. corethrurus* needs further research and could also be elucidated with the means supposed for *E. eugeniae*. In addition, the inchoate isolation and characterization of methanogens from *E. eugeniae* that was started during the current study should be completed and also analyzed for the archaeal 16S rRNA gene next to the *mcrA*/*mrtA* gene.

In the current study, the emission of CH_4 was reported from three out of eight analyzed tropical earthworm species (Figure 38A) whereas more than 1,000 species are assumed to exist, in Brazil only (Brown & James 2007). This demonstrates the huge potential for hitherto undiscovered species that might also emit N_2O, CH_4, and H_2. Thus, an extended analysis of gas emissions from other tropical earthworm species is mandatory for a more global understanding of the contribution of earthworms to the global turnover of greenhouse gases.

5. SUMMARY

The earthworm gut is an anoxic microzone in aerated soils and is further characterized by a high water content and high amounts of nitrite and organic carbon. These conditions are in marked contrast to those in the pre-ingested earthworm substrate and ideal for microorganisms, especially for those capable of fermentations, for denitrifiers, and for dissimilatory nitrate reducers (DNR). Thus, denitrifiers derived from the ingested material (substrate) in the gut of species of the family Lumbricidae emit the greenhouse gas N_2O as well as N_2. Only one, large species of another earthworm family was tested so far but emitted no N_2O, leading to the hypothesis that large earthworms cannot emit nitrogenous gases, i.e., N_2O and N_2. In addition, there was no emission of the greenhouse gas CH_4 reported for all earthworm species tested so far.

Thus, the current study analyzed ten earthworm species of different families, sizes, and feeding guilds (i.e., burrow and feeding habits) from Brazil for the emission of nitrogenous gases and CH_4. The effect of nitrite and acetylene (an inhibitor of the N_2O reductase) on N_2O emissions and of H_2/CO_2 on CH_4 emissions was determined. Taxa affiliated with these emissions, i.e., denitrifiers, DNR, and methanogens were analyzed with cloning and pyrosequencing of marker genes, from gut contents and substrates of representative earthworm species from Brazil (*Amynthas gracilis, Glossoscolex paulistus, Eudrilus eugeniae*), Germany (*Aporrectodea caliginosa*, *Lumbricus terrestris*, *Lumbricus rubellus*), and New Zealand (*Octochaetus multiporus*) on gene and partly on transcript levels. Potential denitrifiers and methanogens were isolated and enriched from earthworm gut contents, respectively. Sequences of *narG* (encoding for a nitrate reductase; targets denitrifiers and DNR), *nirK*, *nirS* (both encoding for a nitrite reductase; target denitrifiers), *nosZ* (encoding for a N_2O reductase; targets denitrifiers), and *mcrA*/*mrtA* (encoding for the methyl-CoM reductase and its isoenzyme; target methanogenic *Archaea*) were analyzed. For *nirK* and *nirS*, cutoff values were calculated to define species-level affiliations from gene and amino acid sequences according to their sequence similarities.

Perionyx excavatus, A. gracilis (both Megascolecidae), *Pontoscolex corethrurus*, *Rhinodrilus alatus* (both Glossoscolecidae), *Dichogaster annae*, *Dichogaster* sp. (both Acanthodrilidae), and *E. eugeniae* (Eudrilidae) emitted nitrogenous gases *in vivo* whereas *G. paulistus*, *Glossoscolex* sp. (both Glossoscolecidae), and *Eisenia andrei* (Lumbricidae) did not. In contrast, earthworm substrates emitted smaller amounts of nitrogenous gases, predominantly N_2. *G. paulistus* emitted nitrogenous gases when provided with nitrite; however, total emissions of nitrogenous gases and the ratio of N_2O to N_2 were higher for *A. gracilis* when treated the same way. It was demonstrated that earthworms of all families, sizes, and feeding guilds can emit nitrogenous gases, and that the earthworm substrate, size, and feeding guild were influencing but not determinative factors taken alone.

For earthworms gut contents from Brazil, Germany, and New Zealand, gene sequences and transcripts indicative of denitrifiers were predominantly affiliated with *Bradyrhizobiaceae* (*Rhizobiales*), indicating that these *Bacteria* are responsible for the emission of nitrogenous gases from these earthworms. Active DNR were predominatly affiliated with *Mycobacterium* (*Actinomycetales*), and it is anticipated that these *Bacteria* compete with denitrifiers for nitrate. In contrast, denitrifiers are assumed to be the main utilizers of nitrite and producers of N_2O, and the only producers of N_2. Gene analyses and isolation approaches demonstrated that (i) both denitrifiers and DNR in the earthworm gut were derived from ingested material and (ii) detected diversity in the gut was influenced by the earthworm feeding guild. Detailed analyses of genes and transcripts from earthworms from Germany demonstrated that there was a selective activation of substrate-derived denitrifiers and DNR in the earthworm gut.

E. eugeniae emitted the highest amounts of CH_4. *P. corethrurus* and *R. alatus* emitted lower amounts of CH_4. All other tested species did not emit CH_4. Only one substrate emitted minor amounts of CH_4, all others did not. Certain substrates appeared to influence the emission of CH_4 by earthworms. However, the substrate taken alone was not determinative in respect of the emission of CH_4 by an earthworm as different earthworm species maintained on the same substrate either emitted CH_4 or did not. The capacity to emit CH_4 by *E. eugeniae* was not significantly affected by supplemental H_2/CO_2 and was at least partly retained when maintained on diverse alternative substrates.

Analysis of *mcrA*/*mrtA* genes and transcripts revealed that selectively activated hydrogenotrophic and acetoclastic methanogens of the *Methanosarcinaceae* and *Methanobacteriacea* in the gut were the source of the CH_4 emitted by *E. eugeniae*. These methanogens were assumed to be derived from the substrate (i.e., composted cow manure) although a symbiotic affiliation of methanogens with the earthworm digestive system cannot be excluded. Certain but not all earthworms emitted both CH_4 and nitrogenous gases, suggesting that methanogenesis and denitrification can be concomitant processes in the earthworm gut.

In conclusion, this study demonstrated that (i) earthworms from all families, sizes, and feeding guilds can emit N_2O and N_2, (ii) substrate-derived and selectively activated denitrifiers within the *Rhizobiales* are the main source of N_2O and N_2 whereas *Actinomycetales* are the main active DNR, (iii) the earthworm feeding guild affects the selective activation of ingested denitrifiers and DNR, (iv) certain earthworms emit CH_4, and *Methanosarcinaceae* and *Methanobacteriaceae* appear to be the main source of this CH_4, and (v) certain earthworms can concomitantly emit N_2O, N_2, and CH_4.

6. ZUSAMMENFASSUNG

Der Regenwurmdarm ist eine anoxische Mikrozone in belüfteten Böden, und ist weiterhin durch einen hohen Wassergehalt und große Mengen an Nitrit und organischem Kohlenstoff charakterisiert. Diese Bedingungen stehen im starken Kontrast zu jenen im Regenwurmsubstrat und sind zudem ideal für Mikroorganismen wie Gärer, Denitrifikanten und dissimilatorische Nitratreduzierer (DNR). Deshalb emittieren aus dem aufgenommenen Material (Substrat) stammende Denitrifikanten im Darm von Regenwurmarten der Familie Lumbricidae das Treibhausgas N_2O, wie auch N_2. Lediglich eine, große Regenwurmart aus einer anderen Familie wurde bisher untersucht, emittierte jedoch kein N_2O. Dies führte zu der Hypothese, dass große Regenwurmarten kein N_2O emittieren können. Keine der bisher getesteten Regenwurmarten emittierte zudem das Treibhausgas CH_4.

Deshalb untersuchte die vorliegende Studie zehn Regenwurmarten aus unterschiedlichen Familien mit unterschiedlichen Größen und mit unterschiedlichen Lebens- und Ernährungsweisen (Ökotypen) aus Brasilien auf die Emission von Stickstoffgasen (d.h. N_2O und N_2; N-Gase) und CH_4 hin. Es wurden die Auswirkungen von Nitrit und Acetylen (einem Inhibitor der N_2O-Reduktase) auf die Emission von N-Gasen, und von H_2/CO_2 auf die Emission von CH_4 untersucht. Taxa die mit diesen Emissionen in Verbindung gebracht werden, d.h. Denitrifikanten, DNR und Methanogene wurden mittels Klonierung und Pyrosequenzierung von Markergenen in Darminhalt und Substraten repräsentativer Regenwurmarten aus Brasilien (*Amynthas gracilis, Glossoscolex paulistus, Eudrilus eugeniae*), Deutschland (*Aporrectodea caliginosa*, *Lumbricus terrestris*, *Lumbricus rubellus*) und Neuseeland (*Octochaetus multiporus*) auf Gen- und teilweise Transkriptionsebene untersucht. Potenzielle Denitrifikanten und Methanogene wurden aus Regenwurmdarminhalten isoliert bzw. angereichert. Sequenzen von *narG* (codiert für eine Nitratreduktase; erfasst Denitrifikanten und DNR), *nirK* und *nirS* (codieren jeweils für eine Nitritreduktase; erfassten Denitrifikanten), *nosZ* (codiert für eine N_2O-Reduktase; erfasst Denitrifikanten) und *mcrA*/*mrtA* (codieren für eine Methyl-CoM-Reduktase und deren Isoenzym; erfassen methanogene Archaeen) wurden analysiert. Für *nirK* und *nirS* wurden Grenzwerte errechnet, um auf Gen- und Proteinsequenzebene Arten auf Basis von Sequenzunterschieden zu definieren.

Perionyx excavatus, *A. gracilis*, (beide Megascolecidae), *Pontoscolex corethrurus*, *Rhinodrilus alatus* (beide Glossoscolecidae), *Dichogaster annae*, *Dichogaster* sp. (beide Acanthodrilidae) und *E. eugeniae* (Eudrilidae) emittierten N-Gase *in vivo*, während *G. paulistus*, *Glossoscolex* sp. (beide Glossoscolecidae) und *Eisenia andrei* (Lumbricidae) keine N-Gase emittierten. Dagegen emittierten die Regenwurmsubstrate geringe Mengen an N-Gasen, hauptsächlich N_2. *G. paulistus* emittierte N-Gase, wenn Nitrit zugegeben wurde. Jedoch waren unter gleichen Bedingungen die Gesamtmenge an N-Gasen und das

Verhältnis von N_2O zu N_2 bei *A. gracilis* höher. Es wurde gezeigt, dass Regenwürmer jeder untersuchten Familie, jeder Größe und jedes Ökotyps N-Gase emittieren können. Hierbei zeigten sich Regenwurmsubstrat, -größe, und -ökotyp als Hauptfaktoren für diese Emissionen, wobei jedoch ein Faktor für sich allein nicht ausschlaggebend war.

Denitrifikanten aus Darminhalten von Regenwürmern aus Brasilien, Deutschland und Neuseeland waren hauptsächlich *Bradyrhizobiaceae* (*Rhizobiales*) zuordenbar, sowohl auf Gen- als auch auf Transkriptionsebene. Dies lässt darauf schließen, dass bei diesen Regenwürmern derartige Bakterien für die Emission von N-Gasen verantwortlich sind. Aktive DNR waren hauptsächlich *Mycobacterium* (*Actinomycetales*) zuordenbar, und es wird angenommen, dass diese Bakterien mit Denitrifikanten um das vorhandene Nitrat konkurrieren. Dagegen gelten Denitrifikanten als Hauptkonsumenten des Nitrits, Hauptproduzenten von N_2O und alleinige Produzenten von N_2. Genanalysen und Isolierungsansätze zeigten, dass sowohl Denitrifikaten als auch DNR im Regenwurmdarm aus dem Substrat stammten. Die im Darm detektierte Diversität von Denitrifikaten und DNR wurde hierbei vom Regenwurm-Ökotyp beeinflusst. Detaillierte Gen- und Transkriptionsanalysen mit Regenwürmern aus Deutschland zeigten, dass im Regenwurmdarm eine selektive Aktivierung von über das Substrat aufgenommenen Denitrifikanten und DNR stattfand.

E. eugeniae emittierte die größten Mengen an CH_4, *P. corethrurus* und *R. alatus* weniger, und alle anderen untersuchten Regenwurmarten gar kein CH_4. Nur ein Substrat emittierte geringe Mengen an CH_4, alle anderen emittierten kein CH_4. Einige Regenwurmsubstrate schienen hierbei Einfluss auf die Emission von CH_4 seitens des Regenwurms zu nehmen. Das Substrat allein war jedoch nicht der alleinige Faktor für die Emission von CH_4, da verschiedene Regenwurmarten auf dem gleichen Substrat CH_4 emittierten oder auch nicht. Die Emission von CH_4 seitens *E. eugeniae* wurde nur geringfügig von zugegebenem H_2/CO_2 beeinflusst und blieb auch nach einer Vorinkubation auf verschiedenen alternativen Substraten zumindest teilweise erhalten.

Die Analyse von *mcrA*/*mrtA* auf Gen- und Transkriptionsebene zeigte, dass aktivierte hydrogenotrophe und acetoklastische Methanogene der *Methanosarcinaceae* und *Methanobacteriaceae* die Quelle des von *E. eugeniae* emittierten CH_4 waren. Diese Methanogenen schienen aus dem Substrat (d.h. kompostierter Kuhdung) zu stammen, auch wenn eine symbiotische Beziehung mit dem Verdauungssystem des Regenwurms nicht ausgeschlossen werden kann. Einige, wenn auch nicht alle Regenwürmer emittierten sowohl CH_4 als auch N-Gase. Dies lässt darauf schließen, dass Methanogenese und Denitrifikation gleichzeitig im Regenwurmdarm ablaufen können.

Zusammengefasst zeigen die Daten der vorliegenden Studie, dass (i) Regenwürmer jeder Familie, jeder Größe und jedes Ökotyps N_2O und N_2 emittieren können, dass (ii) aus

dem Substrat stammende und selektiv aktivierte Denitrifikanten innerhalb der *Rhizobiales* die Hauptquelle der N-Gase zu sein scheinen während *Actinomycetales* die aktivsten DNR sind, dass (iii) der Regenwurm-Ökotyp diese selektive Aktivierung von Denitrifikanten und DNR beeinflusst, dass (iv) einige Regenwürmer CH_4 emittieren können und *Methanosarcinaceae* und *Methanobacteriaceae* die Quelle des emittierten CH_4 zu sein scheinen, und dass (v) bestimmte Regenwürmer N_2O, N_2 und CH_4 gleichzeitig emittieren können.

7. REFERENCES

Aaij C & Boerst P (1972) The gel electrophoresis of DNA. *Biochim Biophys Acta* **269:** 192-200.

Abdi H & Williams LJ (2010) Principal component analysis. *Wiley Interdispl Rev: Computat Stat* **2:** 433-459.

Adman ET S & Murphy MEP (2001) Copper nitrite reductses. In *Handbook of metalloproteins* (Messerschmidt A, Huber R, Poulos T & Wieghardt K, eds), pp. 1381-1389, Wiley, New York, NY, USA.

Aira M, Monroy F & Dominguez J (2009) Changes in bacterial numbers and microbial activity of pig slurry during transit of epigeic and anecic earthworms. *J Hazard Mater* **162:** 1404-1407.

Alley RB, Berntsen T, Bindoff NL, Chen Z, Chidthaisong A, Friedlingstein P, Gregory JM, Hegerl GC, Heimann M, Hewitson B, Hoskins BJ, Joos F, Jouzel J, Kattsov V, Lohmann U, Manning M, Matsuno T, Molina M, Nicholls N, Overpeck J, Qin D, Raga G, Ramaswamy V, Ren J, Rusticucci M, Solomon S, Somerville R, Stocker TF, Stott PA, Stouffer RJ, Whetton P, Wood RA & Wratt D (2007) Summary for policymakers. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M & Miller HL, eds), Cambridge University Press, Cambridge, UK & New York, NY, USA.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403-410.

Anderson CR, Condron LM, Clough TJ, Fiers M, Stewart A, Hill RA & Sherlock RR (2011) Biochar induced soil microbial community change: implications for biogeochemical cycling of carbon, nitrogen and phosphorus. *Pedobiologia* **54:** 309-320.

Anderson IC & Levine JS (1986) Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. *Appl Environ Microbiol* **51:** 938-945.

Ash C, Priest FG & Collins MD (1993) Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Ant Leeuwen* **64:** 253-260.

Augustenborg CA, Hepp S, Kammann C, Hagan D, Schmidt O & Muller C (2012) Biochar and earthworm effects on soil nitrous oxide and carbon dioxide emissions. *J Environ Qual* **41:** 1203-1209.

Baggs EM (2011) Soil microbial sources of nitrous oxide: recent advances in knowledge, emerging challenges and future direction. *Curr Opin Environ Sustain* **3:** 321-327.

Baker SC, Ferguson SJ, Ludwig B, Page MD, Richter OMH & van Spanning RJM (1998) Molecular genetics of the genus *Paracoccus*: metabolically versatile bacteria with bioenergetic flexibility. *Microbiol Mol Biol Rev* **62:** 1046-1078.

Balch WE, Fox GE, Magrum LJ, Woese CR & Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43:** 260-296.

Bapteste E, Brochier C & Boucher Y (2005) Higher-level classification of the Archaea: evolution of methanogenesis and methanogens. *Archaea* **1:** 353-363.

Barley KP (1961) The abundance of earthworms in agricultural land and theis possible significance in agriculture. *Adv Agron* **13:** 249-268.

Barois I & Lavelle P (1986) Changes in respiration rate and some physiochemical properties of a tropical soil during transit through *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta). *Soil Biol Biochem* **18:** 539-541.

Barois I, Brossard M, Lavelle P, Tondoh J, Kanyonyo J, Martínez A, Jiménez J, Rossi J, Angeles A, Lattaud C, Senapati B, Fragoso C, Blanchart E, Brown G & Moreno A (1999) Ecology of earthworm species with large environmental tolerance and/or extended distributions. In *Earthworm management in tropical agroecosystems* (Lavelle P, Hendrix P & Brussard L, eds), pp. 57-86, CAB International, London, UK.

Baumann B, Snozzi M, Zehnder AJB & van der Meer JR (1996) Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. *J Bacteriol* **178:** 4367-4374.

Baumann P, Moran NA & Baumann L (2006) Bacteriocyte-associated endosymbionts of insects. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 1, pp. 403-438, Springer-Verlag, New York, NY, USA.

Bazylinksi DA & Blakemore RP (1983) Denitrification and assimilatory nitrate reduction in *Aquaspirillum magnetotacticum*. *Appl Environ Mircobiol* **46:** 1118-1124.

Bedmar fEJ, Robles EF & Delgado MJ (2005) The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. *Biochem Soc Trans* **33:** 141-144.

Behrendt U, Schumann P, Stieglmeier M, Pukall R, Augustin J, Spröer C, Schwendner P, Moissl-Eichinger C & Ulrich A (2010) Characterization of heterotrophic nitrifying bacteria with respiratory ammonification and denitrification activity - description of *Paenibacillus uliginis* sp. nov., an inhabitant of fen peat soil and *Paenibacillus purispatii* sp. nov., isolated from a spacecraft assembly clean room. *Syt Appl Microbiol* **33:** 328-336.

Bell LC, Richardson DJ & Ferguson SJ (1990) Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*: the periplasmic enzyme catalyzes the first step in aerobic denitrification. *FEBS Lett* **265:** 85-87.

Bertora C, van Vliet PCJ, Hummelink EWJ & van Groenigen JW (2007) Do earthworms increase N_2O emissions in ploughed grassland? *Soil Biol Biochem* **39:** 632-640.

Bityutskii NP, Kaidun PI & Yakkonen KL (2012) The earthworm (*Aporrectodea caliginosa*) primes the release of mobile and available micronutrients in soil. *Pedobiologia* **55:** 93-99.

Blachnik R (1998) D´Ans Lax Taschenbuch für Chemiker und Physiker. 4th ed, vol 3, Springer Verlag, Berlin, Germany.

Blakemore RJ, Ito MT & Kaneko N (2007) Alien earthworms in the Asia/Pacific region with a checklist of species and the first records of *Eukerria saltensis* (Oligochaeta: Ocnerodrilidae) and *Eisenia tetraedra* (Lumbricidae) from Japan, and *Pontoscolex corethrurus* (Glossoscolecidae) from Okinawa. In *Assessment and control of biological*

invasion risks (Koike F. Clout MN, Kawamichi M, de Poorter M & Iwatsuki K, eds), pp. 173-181, Shoukadoh Book Sellers/IUCN, Kyoto, Japan.

Bleakley BH & Tiedje JM (1982) Nitrous oxide production by organisms other than nitrifiers or denitrifiers. *Appl Environ Microbiol* **44:** 1342-1348.

Bollag J-M & Tung G (1972) Nitrous oxide release by soil fungi. *Soil Biol Biochem* **4:** 271-276.

Bollmann A & Conrad R (1998) Influence of O_2 availability on NO and N_2O release by nitrification and denitrification in soils. *Global Change Biol* **4:** 387-396.

Bonin AS & Boone DR (2006) The order Methanobacteriales. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 3, pp. 231-243, Springer-Verlag, New York, NY, USA.

Bonkowski M & Schäfer M (1997) Interactions between earthworms and soil protozoa: a trophic component of the soil food web. *Soil Biol Biochem* **29:** 142-148.

Borg I & Groenen P (2005) Modern multidimensional scaling: theory and applications. 2nd ed, Springer Verlag, New York, NY, USA.

Borken W, Grundel S & Beese F (2000) Potential contribution of *Lumbricus terrestris* L. to carbon dioxide, methane, and nitrous oxide fluxes from a forest soil. *Biol Fertil Soils* **32:** 142-148.

Bouché MB (1977) Strategies lombriciennes. In *Soil organisms as components of ecosystems* (Lohm U & Persson T, eds), vol 25, pp. 122-132, Ecological Bulletins, Stockholm, Sweden.

Bradley RL, Chroňáková A, Elhottová A & Šimek M (2012) Interactions between land-use history and earthworms control gross rates of soil methane production in an overwintering pasture. *Soil Biol Biochem* **53:** 64-71.

Braker G, Ayala-del-Rio HL, Devol AH Fesefeldt A & Tiedje JM (2001) Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in pacific northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. *Appl Environ Microbiol* **67:** 1893-1901.

Braker G, Fesefeldt A & Witzel KP (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl Environ Microbiol* **64:** 3769-3775.

Braker G & Tiedje JM (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Appl Environ Microbiol* **69:** 3476-3483.

Bräuer SL, Yashiro E, Ueno NG, Yavitt JB & Zinder SH (2006) Characterization of scid-tolerant H2/CO2-utilizing methanogenic enrichment cultures from an acidic peat bog in New York State. *FEMB Microb Ecol* **57:** 206-216.

Breidenbach J (2002) Normalanatomie und Histologie des Lumbriciden *Lumbricus terrestris* L. (Annelida, Oligochaeta. *PhD thesis*. University of Münster, Germany.

Bremner JM (1997) Sources of nitrous oxide in soils. *Nutri Cycl Agroecosys* **49:** 7-16.

Brito-Vega H & Espinosa-Victoria D (2009) Bacterial diversity in the digestive tract of earthworms (Oligochaeta). *J Biol Sci* **9:** 192-199.

Brown GG (1995) How do earthworms affect microfloral and faunal community diversity? *Plant Soil* **170:** 209-231.

Brown GG, Barois I & Lavelle P (2000) Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. *Eur J Soil Biol* **36:** 177-198.

Brown GG & Doube BM (2004) Functional interactions between earthworms, microorganisms, organic matter, and plants. In *Earthworm ecology* (Edwards CA, ed), 3rd edn., pp. 213-239, CRC Press, Boca Raton, FL, USA.

Brown GG, Edwards CA & Brussaard L (2004) How earthworms affect plant growth: burrowing into the mechanisms. In *Earthworm ecology* (Edwards CA, ed), 2nd ed, pp. 13-50, CRC Press, Boca Raton, FL, USA.

Brown GG & James SW (2007) Ecologia biodiversidae e biogeografia das minhocas no Brasil. In *Minhocas na América Latina* (Brown GG & Fragoso C, eds), pp. 47-75, EmbrapaSoja, Londrina, Brazil.

Brown GG, Pashanasi B, Villenave C, Patron JC, Senepati BK, Giri S, Barois I, Lavelle P, Blanchart E, Blakemore RJ, Spain AV & Boyer J (1999) Effects of earthworms on plant production in the tropics. In *Earthworm management in tropical agroecosystems* (Lavelle P, Hendrix P & Brussard L, eds), pp. 87-147, CAB International, London, UK.

Bru D, Sarr A & Philippot L (2007) Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. *Appl Environ Microbiol* **73:** 5971-5974.

Brune A (2006) Symbiotic associations between termites and prokaryotes. In *The Prokaryotes* (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), 3rd ed, vol 1, pp. 439-474, Springer, New York, NY, USA.

Brusa T, Canzi E, Allievi L, del Puppo E & Ferrari A (1993) Methanogens in the human intestinal tract and oral cavity. *Curr Microbiol* **27:** 261-265.

Burth I, Benckiser G & Ottow JCG (1982) N_2O-release from nitrite (denitrification) by common fungi under aerobic conditions. *Naturwissensch* **69:** 598-599.

Byzov BA, Nechtitaylo TY, Bumazhkin BK, Kurakov AV, Golyshin PN & Zvyagintsev DG (2009) Culturable microorganisms from the earthworm digestive tract. *Mikrobiologiya* **78:** 360-368.

Cabello P, Roldan MD & Moreno-Vivian C (2004) Nitrate reduction and the nitrogen cycle in archaea. *Microbiol* **150:** 3527-3546.

Casella S, Shapleigh JP & Payne WJ (1986) Nitrite reduction in *Rhizobium* '*hedysari*' strain HCNT 1. *Arch Microbiol* **146:** 233-238.

Castillo F, Dobao MM, Reyes F, Blasco R, Roldan MD, Gavira M, Caballero FJ, Moreno-Vivian C & Martinez-Luque M (1996) Molecular and regulatory properties of the nitrate reductase systems of *Rhodobacter*. *Curr Microbiol* **33:** 341-346.

Castro-Gonzáles M, Braker G, Farias L & Ulloa O (2005) Communities of *nirS*-type denitrifiers in the water column of the oxygen minimum zone in the eastern South Pacific to detect denitrifying bacteria in environmental samples. *Environ Microbiol* **7:** 1298-1306.

Chang C-H & James S (2011) A critique of earthworm molecular phylogenies. *Pedobiologia* **54S:** S3-S9.

Chao A (1984) Non-parametric estimation of the number of classes in a population. *Scan J Statist* **11:** 265-270.

Chao A & Lee S-M (1992) Estimating the number of classes via sample coverage. *J Amer Statist Assoc* **87:** 210-217.

Chao A, Ma M-C & Yang MCK (1993) Stopping rules and estimation for recapture debugging with unequal failure rates. *Biometrika* **80:** 193-201.

Chapuis-Lardy L, Brauman A, Bernard L, Pablo AL, Toucet J, Mano MJ, Weber L, Brunet D, Razafimbelo T, Chotte JL & Blanchart E (2010) Effect of the endogeic earthworm Pontoscolex corethrurus on the microbial structure and activity related to CO_2 and N_2O fluxes from a tropical soil (Madagascar). *Appl Soil Ecol* **45:** 201-208.

Chèneby D, Hallet S, Mondon M, Martin-Laurent F, Germon JC & Philippot L (2003) Genetic characterization of the citrate reducing community based on *narG* nucleotide sequence analysis. *Microb Ecol* **46:** 113-121.

Choi PS, Naal Z, Moore C, Casado-Rivera E, Abruña HD, Helmann JD & Shapleigh JP (2006) Assessing the impact of denitrifier-produced nitric oxide on other bacteria. *Appl Environ Microbiol* **72:** 2200-2205.

Cicerone RJ (1987) Changes in atmospheric ozone. *Science* **237:** 35-42.

Citernesi U, Neglia R, Seritti A, Lepidi AA, Filippi C, Bagnoli G, Nuti MP & Galluzzi R (1977) Nitrogen-fixation in gastro-enteric cavity of soil animals. *Soil Biol Biochem* **9:** 71-72.

Cole AJ (1996) Nitrite reduction to ammonia by enteric: redundancy, or a strategy for survival during oxygen starvation? *FEMS MIcrobiol Lett* **136:** 1-11.

Cole AJ & Brown CM (1980) Nitrite reduction to ammonia by fermentative bacteria - short circuit in the biological nitrogen cycle. *FEMS MIcrobiol Lett* **7:** 65-72.

Colliver BB & Stephenson T (2000) Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnol Adv* **18:** 219-232.

Conrad R (1995) Soil microbial processes involved in production and consumption of atmospheric trace gases. In *Advances in Microbial Ecology* (Jones JG, ed), vol 14, pp. 207-250, Plenum Press, New York, NY, USA.

Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiol Rev* **60:** 609-640.

Cooke A (1983) The effects of fungi on food selection by *Lumbricus terrestris* L. In *Earthworm ecology: from Darwin to vermiculture* (Satchell JE, ed), pp. 365-373, Chapman & Hall, London, UK.

Cooke A & Luxton M (1980) Effect of microbes on food selection by *Lumbricus terrestris*. *Rev Ecol Biol Sol* **17:** 365-370.

Costa E, Pérez J & Kreft JU (2006) Why is metabolic labour divided in nitrification? *Trends Microbiol* **14:** 213-219.

Curry JP & Schmidt O (2007) The feeding ecology of earthworms - a review. *Pedobiologia* **50:** 463-477.

Cutruzzolà F, Rinaldo S, Centola F & Brunori M (2003) NO production by *Pseudomonas aeruginosa* cd_1 nitrite reductase. *IUBMB Life* **55:** 617-621.

Daniel O & Anderson JM (1992) Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus* Hoffmeister. *Soil Biol Biochem* **24:** 465-470.

Daniel SL & Drake HL (1993) Oxalate-dependent and glyoxylate-dependent growth and acetogenesis by *Clostridium thermoaceticum*. *Appl Environ Microbiol* **59:** 3062-3069.

Daniel R M, Limmer AW, Steele KW & Smith IM (1982) Anaerobic growth, nitrate reduction and denitrification of in 46 *Rhizobium* strains. *J Genl Microbiol* **128:** 1811-1815.

Darwin C (1881) The formation of vegetable mould through the action of worms, with observations of their habits. Murray, London, UK.

Davidson EA (1991) Fluxes of nitrous oxide and nitric oxide from terrestrial ecosytems. American Society for Microbiology, Washington DC, USA.

Davidson SK & Stahl DA (2006) Transmission of nephridial bacteria of the earthworm *Eisenia fetida*. *Appl Environ Microbiol* **72:** 769-775.

Davidson SK & Stahl DA (2008) Selective recruitment of bacteria during embryogenesis of an earthworm. *ISME J* **2:** 510-518.

Davidson SK, Powell R & James S (2013) A global survey of the bacteria within earthworm nephridia. *Mol Phylogenet Evol* **67:** 188-200.

Davidson SK, Powell RJ & Stahl DA (2010) Transmission of a bacterial consortium in *Eisenia fetida* egg capsules. *Environ Microbiol* **12:** 2277-2288.

de Vries S & Schröder I (2002) Comparison between the nitric oxide reductase family and its aerobic relatives, the cytochrome oxidases. *Biochem Soc Transact* **30:** 662-667.

de Vries S, Suharti & Pouvreau LAM (2007) Nitric oxide reductases: structural variations and catalytic mechanism. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 57-66, Elsevier Academic Press, Amsterdam, The Netherlands.

Deiglmayr K, Philippot L, Tscherko D & Kandeler E (2006) Microbial succession of nitrate-reducing bacteria in the rhizosphere of *Poa alpina* across a glacier foreland in the Central Alps. *Environ Microbiol* **8:** 1600-1612.

Delgado MJ, Bonnard N, Tresierra-Ayala A, Bedmar EJ & Muller P (2003) The *Bradyrhizobium japonicum napEDABC* genes encoding the perplasmativ nitrate reductase are essential for nitrate respiration. *Microbiol* **149:** 3395-3403.

Delgado MJ, Casella S & Bedmar EJ (2007) Denitrification in rhizobia-legume symbiosis. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 83-91, Elsevier Academic Press, Amsterdam, The Netherlands.

Denman KL, Brasseur G, Chidthaisong A, Ciais P, Cox PM, Dickinson RE, Hauglustaine D, Heinze C, Holland E, Jacob D, Lohmann U, Ramachandran S, da Silva Dias PL, Wofsy SC & Zhang X (2007) Couplings between changes in the climate system and biogeochemistry. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M & Miller HL, eds), pp. 499-588, Cambridge University Press, Cambridge, UK & New York, NY, USA.

Depkat-Jakob PS (2009) Einfluss des Regenwurm-Ökotyps auf die Diversität und Aktivität von Nitratreduzierern im Regenwurmdarm: Funktionelle Genanalyse und gerichtete Isolierung. *Diploma thesis*. University of Bayreuth, Germany.

Depkat-Jakob PS, Brown GG, Tsai SM, Horn MA & Drake HL (2013) Emission of nitrous oxide and dinitrogen by diverse earthworm families from Brazil and resolution of associated denitrifying and nitrate-dissimilating taxa. *FEMS Microbiol Ecol* **83:** 375-391.

Depkat-Jakob PS, Hilgarth M, Horn MA & Drake HL (2010) Effect of earthworm feeding guilds on ingested dissimilatory nitrate reducers and denitrifiers in the alimentary canal of the earthworm. *Appl Environ Microbiol* **76:** 6205-6214.

Depkat-Jakob PS, Hunger S, Schulz K, Brown GG, Tsai SM & Drake HL (2012) Emission of methane by *Eudrilus eugeniae* and other earthworms from Brazil. *Appl Environ Microbiol* **78:** 3014-3019.

Drake HL & Horn MA (2007) As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. *Annu Rev Microbiol* **61:** 169-189.

Dunn GM, Herbert RA & Brown CM (1979) Influence of oxygen tension on nitrate reduction by a *Klebsiella* sp. growing in chemostat culture. *J Gen Microbiol* **112:** 379-383.

Edwards CA & Bohlen BJ (1996) Biology and ecology of earthworms, 3rd ed, Chapman & Hall, London, UK.

Edwards CE & Fletcher KE (1988) Interactions between earthworms and microorganisms in organic-matter breakdown. *Agricul Ecosys Environ* **24:** 235-247.

Egert M & Friedrich MW (2003) Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl Environ Microbiol* **69:** 2555-2562.

Egert M, Marhan S, Wagner B, Scheu S & Friedrich MW (2004) Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). *FEMS Microbiol Ecol* **48:** 187-197.

Eisenhauer N & Scheu S (2008) Earthworms as drivers of the competition between grasses and legumes. *Soil Biol Biochem* **40:** 2650-2659.

Eisenhauer N, Schuy M, Butenschoen O & Schau S (2009) Direct and indirect effects of endogeic earthworms on plant seeds. *Pedobiologia* **52:** 151-162.

Ellington MJK, Bhakoo KK, Sawers G, Richardson DJ & Ferguson SJ (2002). Hierarchy of carbon source selection in *Paracoccus pantotrophus*: Strict correlation between reduction state of the carbon substrate and aerobic expression of the nap operon. *J Bacteriol* **184:** 4767-4774.

Enwall K, Philippot L & Hallin S (2005) Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Appl Environ Microbiol* **71:** 8335-8343.

EPA (2010) Methane and nitrous oxide emissions from natural sources. In *Inventory of U.S. greenhouse gas emissions and sinks: 1990-2008*, pp. 1-194, U.S. Environmantal Ptotection Agency, Washington, DC, USA.

Falk S, Liu B & Braker G (2010) Isolation, genetic and functional characterization of novel soil *nirK*-type denitrifiers. *Syst Appl Microbiol* **33:** 337-347.

Feldhaar H & Gross R (2009) Insects as hosts for mutualistic bacteria. *Internat J Med Microbiol* **299:** 1-8.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783-791.

Ferguson SJ & Richardson DJ (2004) Electron transport systems for reduction of nitrogen oxides and nitrogen anions. In *Diversity of prokaryotic respiratory systems* (Zannoni D, ed), vol 2, pp. 169-206, Kluwer Academic Publishers, The Netherlands.

Ferguson SJ, Richardson DJ & van Spanning RJM (2007) Biochemistry and molecular biology of nitrification. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 209-222, Elsevier Academic Press, Amsterdam, The Netherlands.

Ferry JG (2010) How to make a living by exhaling methane. *Ann Rev Microbiol* **64:** 453-473.

Fischer K, Hahn D, Hönerlage W & Zeyer J (1997) Effect of the passage through the gut of the earthworm *Lumbricus terrestris* L. on *Bacillus megaterium* studies by a whole cell hybridization. *Soil Biol Biochem* **29:** 1149-1152.

Fischer M, Alderson J, van Keulen G, White J & Sawers RG (2010) The obligate aerobe *Streptomyces coelicolor* A3(2) synthesizes three active respiratory nitrate reductases. *Microbiol* **76:** 3244-3254.

Fischer OA, Matlova L, Bart J, Dvorska L, Svastova P, du Maine P, Melicharek I, Bartos M & Pavlik I (2003) Earthworms (*Oligochaeta*, *Lumbricidae*) and mycobacteria. *Vet Microbiol* **91:** 325-338.

Flanagan DA, Gregory LG, Carter JP, Karakas-Sen A, Richardson DJ & Spiro S (1999) Detection of genes for periplasmic nitrate reductase in nitrite respiring bacteria and in community DNA. *FEMS Microbiol Lett* **177:** 263-270.

Flint HJ (1997) The rumen microbial ecosystem - some recent developments. *Trends Microbiol* **5:** 483-488.

Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey D, Haywood J, Lean J, Lowe D, Myhre G, Nganga J, Prinn R, Raga G, Schulz M & van Dorland R (2007) Changes in atmospheric constituents and in radiative forcing. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (Solomon S, Qin D, Manning

M, Chen Z, Marquis M, Averyt KB, Tignor M & Miller HL, eds), pp. 129-234, Cambridge University Press, Cambridge, UK & New York, NY, USA.

Fragoso C, Kanyonyo J, Moreno A, Senepati BK, Blanchart E & Rodriguez C (1999) A survey of tropical earthworms: taxonomy, biogeography and environmental plasticity. In *Earthworm management in tropical agroecosystems* (Lavelle P, Hendrix P & Brussard L, eds), pp. 1-26, CAB International, London, UK.

Furlong MA, Singleton DR, Coleman DC & Whitman WB (2002) Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Appl Environ Microbiol* **68:** 1265-1279.

Gajalakshmi S & Abbasi SA (2004) Earthworms and vermicomposting. *Ind J Biotech* **3:** 486-494.

Galloway JN (1998) The global nitrogen cycle. *Environ Pollution* **102:** 15-24.

Gamble TN, Betlach MR & Tiedje JM (1977) Numerically dominant denitrifying bacteria from world soils. *Appl Environ Microbiol* **33:** 926-939.

Garcia J-L, Ollivier B & Whitman W (2006) The order Methanomicrobiales. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 3, pp. 208-230, Springer-Verlag, New York, NY, USA.

Garrity GM, Bell JA & Lilburn T (2005) Family VII. *Bradyrhizobiaceae* fam. nov. In *Bergey´s maual of systematic bacteriology* (Brenner DJ, Krieg NR, Staley JT & Garrity GM, eds), 2nd ed, vol 2, p. 438, Springer-Verlag, New York, NY, USA.

Gebelein J (2011) Bakterielle Nitritreduktase-Gene (*nirK* und *nirS*) im Regenwurmdarm. *Bachelor thesis*. University of Bayreuth, Germany.

Giannopoulos G, Pullemann MM & van Groenigen JWV (2010) Interactions between residue placement and earthworm ecological strategy affect aggregate turnover and N2O dynamics in agricultural soil. *Soil Biol Biochem* **42:** 618-625.

Giffin MM, Raab RW, Morganstern M & Sohaskey CD (2012) Mutational analysis of the respiratory nitrate transporter NarK2 of *Mycobacterium tuberculosis*. *PLOS ONE* **7:** e45459.

Gilles A, Meglécz E, Pech N, Ferreira S, Malausa T & Martin J-F (2011) Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequnencing. *BMC Gen* **12:** 245.

Gomes CM, Guiffre A, Forte E, Vicente JB, Saraiva LM, Brunori M & Teixeira M (2002) A novel type of nitric-oxide reductase *Escherichia coli* flavorubredoxin. *J Biol Biochem* **277:** 25273-25376.

Good IJ (1958) The population frequency of species and the estimation of the population parameters. *Biometrics* **40:** 237-246.

Graff O (1983) Unsere Regenwürmer. Lexikon für Freunde der Bodenbiologie. Vol 2, M & H Schaper, Hannover, Germany.

Graff O & Makeschin K (1980) Crop yield of rye grass influenced by the excretions of three earthworm species. *Pedobiologia* **20:** 176-180.

Green MR & Sambrook J (2012) Molecular cloning. A laboratory manual. 4th ed, Cold Spring Harbor Laboratory Press, New York, NY, USA.

Green SJ, Prakash O, Gihring TM, Akob DM, Jasrotia P, Jardine PM, Watson DB, Brown SD, Palumbo AV & Kostka LE (2010) Denitrifying bacteria isolated from terrestrial subsurface sediments exposed to mixed-waste contamination. *Appl Environ Microbiol* **76:** 3244-3254.

Gregory LG, Bond PL, Richardson DJ & Spiro S (2003) Characterization of a nitrate-respiring bacterial community using nitrate reductase gene (*narG*) as a functional marker. *Microbiol* **149:** 229-237.

Gregory LG, Karakas-Sen A, Richardson DJ & Spiro S (2000) Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. *FEMS Microbiol Lett* **185:** 275-279.

Griffiths RI, Whiteley AS, O'Donnell AG & Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. *Appl Environ Microbiol* **66:** 5488-5491.

Gunsalus RP, Romesser JA & Wolfe RS (1978) Preparation of coenzyme-M analogs and their activity in methyl coenzyme-M reductase system of *Methanobacterium thermoautotrophicum*. *Biochem* **17:** 2374-2377.

Hackstein JHP & Stumm CK (1994) Methane production in terrestrial arthropods. *Proc Natl Acad Sci USA* **91:** 5441-5445.

Hallin S & Lindgren PE (1999) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Appl Environ Microbiol* **65:** 1652-1657.

Hallin S, Throback IN, Dicksved J & Pell M (2006) Metabolic profiles and genetic diversity of denitrifying communities in activated sludge after addition of methanol or ethanol. *Appl Environ Microbiol* **72:** 5445-5452.

Hamady M, Lozupone C & Knight R (2010) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *ISME J* **4:** 17-27.

Hartmann P, Scheitler M & Fischer R (1989) Soil fauna comparisons in healthy and declining noeway spruce stands. In *Forest decline and air pollution* (Schulze E-D, Lange OL & Oren R, eds), vol 77, pp. 137-150, Springer Verlag, Berlin, Germany.

Hartmans S, de Bont JAM & Stackebrandt E (2006) The genus *Mycobacterium* - nonmedical. In *The Prokaryotes* (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), 3rd ed, vol 3, pp. 889-918, Springer, New York, NY, USA.

Hartsock A & Shapleigh JP (2011) Physiological roles for two periplamic nitrate reductases in *Rhodobacter sphaeroides* 2.4.3 (ATCC 17025). *J Bacteriol* **193:** 6483-6489.

Heck KL, Vanbelle G & Simberloff D (1975) Explicit calculation of rarefaction diversity measurement and determination of sufficient sample size. *Ecology* **56:** 1459-1461.

Hedderich R & Whitman WB (2006) Physiology and biochemistry of the methane-producing Archaea. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 2, pp. 1050-1079, Springer-Verlag, New York, NY, USA.

Heisterkamp IM, Schramm A, de Beer D & Stief P (2010) Nitrous oxide production associated with coastal marine invertebrates. *Mar Ecol Prog Ser* **415:** 1-9.

Heltshe J & Forrester NE (1983) Estimating species richness using the jackknife procedure. *Biometrics* **39:** 1-11.

Heylen K, Vanparys B, Wittebolle L, Verstraete W, Boon N & de Vos P (2006) Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. *Appl Environ Microbiol* **72:** 2637-2643.

Hilgarth M (2009) Aktive Nitratreduzierer im Regenwurmdarm. *Bachelor thesis*. University of Bayreuth, Germany.

Hill TCJ, Walsh KA, Harris JA & Moffett BF (2003) Using ecological diversity measures with bacterial communities. *FEMS Microbiol Ecol* **43:** 1-11.

Horn MA, Drake HL & Schramm A (2006a) Nitrous oxide reductase genes (*nosZ*) of denitrifying microbial populations in soil and the earthworm gut are phylogenetically similar. *Appl Environ Microbiol* **72:** 1019-1026.

Horn MA, Ihssen J, Matthies C, Schramm A, Acker G & Drake HL (2005) *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. *Int J Syst Evol Microbiol* **55:**1255-1265.

Horn MA, Mertel R, Kästner M, Gehre M & Drake HL (2006b) In vivo emission of dinitrogen by earthworms via denitrifying bacteria in the gut. *Appl Environ Microbiol* **72:** 1013-1018.

Horn MA, Schramm A & Drake HL (2003) The earthworm gut: an ideal habitat for ingested N_2O-producing microorganisms. *Appl Environ Microbiol* **69:** 1662-1669.

Huber JA, Mark Welch DB, Morrison HG, Huse SM, Neal PR, Butterfield DA & Sogin ML (2007) Microbial population structures in the deep marine biosphere. *Science* **318:** 97-100.

Hulbert SH (1971) Nonconcept of species diversity - critique and altenative parameters. *Ecology* **52:** 577-586.

Hungate RE (1969) A roll tube method for the cultivation of strict anaerobes. In *Methods in microbiology* (Norris JR & Ribbons DW, eds), pp. 117-132, Academic Press, New York, NY, USA.

Hunger S, Schmidt O, Hilgarth M, Horn MA, Kolb S, Conrad R & Drake HL (2011) Competing formate- and carbon dioxide-utilizing prokaryotes in an anoxic methane-emitting fen soil. *Appl Environ Microbiol* **77:** 3773-3785.

Huse SM, Welch DM, Morrison HG & Sogin ML (2010) Ironing out the wrinkles in the rare biosphere through improved OUT clustering. *Environ Microbiol* **12:** 1889-1898.

Hutchinson GL (1995) Biosphere-atmosphere exchange of gaseous N oxides, In *Soils and Global Change* (Lal R, Kimble J, Levine E & Stewart BA, eds), pp. 219-236, CRC Press Inc., Boca Raton, FL, USA.

Hutha V (1979) Effects of liming and deciduous litter on earthworm (Lumbricidae) populations of a spruce forest, with an inoculums experiment on *Allobophora caliginosa*. *Pedobiologia* **19:** 340-345.

Hymen ED (1988) A new method of sequencing DNA. *Anal Biochem* **174:** 423-436.

Ihssen J, Horn MA, Matthies C, Gößner A, Schramm A & Drake HL (2003) N_2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. *Appl Environ Microbiol* **69:** 1655-1661.

Iwai S, Chai B, Sul WJ, Cole JR, Hashsham SA & Tiedje JM (2010) Gene-targeted-metagenomics reveals extensive diversity of aromatic dioxygenase genes in the environment. *ISME J* **4:** 279-285.

James SW & Brown GG (2006) Earthworm ecology and diversity in Brazil. In *Soil biodiversity in Amazonian and other Brazilian ecosystems* (Moreira FMS, Siqueira JO & Brussaard L, eds), pp. 56-116, CAB International, Wallingford, UK.

James SW & Davidson SK (2012) Molecular phylogeny of earthworms (Annelida: Crassiclitellata) based on 28S, 18S, and 16S gene sequences. *Invert Syst* **26:** 213-229.

James SW & Guimarães A (2010) Discovery and development of new species for vermiculture. In *Vermiculture technology: earthworms, organic wastes and environmental management* (Edwards CA, Arancon NQ & Sherman RL, eds), pp. 41-52, CRC Press, Boca Raton, FL, USA.

Jamieson BGM, Tillier S, Tillier A, Justine JL, Ling E, James S, McDonald K & Hugall AF (2002) Phylogeny of the Megascolecidae and Crassiclitellata (Annelida, Oligochaeta): combined versus partitioned analysis using nuclear (28S) and mitochondrial (12S, 16S) rDNA. *Zoosystema* **24:** 707-734.

Janssen PH & Kirs M (2008) Structure of the archaeal community of the rumen. *Appl Environ Microbiol* **74:** 3619-3625.

Jetten MSM (2008) The microbial nitrogen cycle. *Environ Microbiol* **10:** 2903-2909.

John P (1977) Aerobic and anaerobic bacterial respiration monitored by electrodes. *J Gen Microbiol* **98:** 231-238.

Johnstone K (1994) The trigger mechanism of spore germination - current concepts. *J Appl Bacteriol* **76:** S17-S24.

Jolly JM, Lappinscott HM, Anderson JM & Clegg CD (1993) Scanning electron microscopy of the gut microflora of two earthworms: *Lumbricus terrestris* and *Octolasion cyaneum*. *Microb Ecol* **26:** 235-245.

Jones CG, Lawton JH & Shachak M (1994) Organisms as ecosystem engineers. *Oikos* **69:** 373-386.

Jouquet P, Dauber J, Lagerlöf J, Lavelle P & Lepage M (2006) Soil invertebrates as ecosystem engineers: intended and accidental effects on soil and feedback loops. *Appl Soil Ecol* **32:** 153-164.

Judas M (1992) Gut content-analysis of earthworms (Lumbricidae) in a beechwood. *Soil Biol Biochem* **24:** 1413-1417.

Juste RA, Marco JC, de Ocáriz CS & Adúriz JJ (1991) Comparison of different media for the isolation of small ruminant strains of *Mycobacterium tuberculosis*. *Veterin Microbiol* **28:** 385-390.

Kamman C, Hepp S, Lenhart K & Müller C (2009) Stimulation of methane consumption by endogenous CH_4 production in aerobic grassland soil. *Soil Biol Biochem* **41:** 622-629.

Karsten GR & Drake HL (1995) Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. *Appl Environ Microbiol* **61:** 1039-1044.

Karsten GR & Drake HL (1997) Denitrifying bacteria in the earthworm gastrointestinal tract and *in vivo* emission of nitrous oxide (N_2O) by earthworms. *Appl Environ Microbiol* **63:** 1878-1882.

Kaspar HF & Tiedje JM (1981) Dissimilatory reduction of nitrate and nitrite to ammonium: nitrous oxide production and effect of acetylene. *Appl Environ Microbiol* **41:** 705-709.

Kellner RLL (2002) Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaeus* (Coleoptera: Staphylinidae). *Insect Biochem Molec Biol* **32:** 389-365.

Kendall MM & Boone DR (2006) The order Methanosarcinales. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 3, pp. 244-256, Springer-Verlag, New York, NY, USA.

Keppler F, Hamilton JTG, Braß M & Röckmann T (2006) Methane emissions from terrestrial plants under aerobic conditions. *Nature* **439:** 187-191.

Kester RA, Meijer ME, Libochant JA, De Boer W & Laanbroek HJ (1997) Contribution of nitrification and denitrification to the NO and N_2O emissions of an acid forest soil, a river sediment and a fertilized grassland soil. *Soil Biol Biochem* **29:** 1655-1664.

Khomyakov NV, Kharin SA, Yu T, Nechitaylo TY, Golyshin PN, Kurakov AV, Byzov BA & Zvyagintsev DG (2007) Reaction of microorganisms to the digestive fluid of earthworms. *Microbiology* **76:** 45-54.

Kiehl JT & Trenberth KE (1997) Earth's annual global mean energy budget. *Bull Amer Meteor Soc* **78:** 197-208.

Klüber HD & Conrad R (1998) Effects of nitrate, nitrite, NO and N_2O on methanogenesis and other redox processes in anoxic rice field soil. *FEMS Microbiol Ecol* **25:** 301-318.

Knapp BA, Podmirseg SM, Seeber J, Meyer E & Insam H (2009) Diet-related composition of gut microbiota of Lumbricus rubellus as revealed by a molecular fingerprinting technique and cloning. *Soil Biol Biochem* **41:** 2299-2307.

Köhler T, Dietrich C, Scheffrahn RH & Brune A (2012) High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). *Appl Environ Microbiol* **78:** 4691-4701.

Kool DM, Dolfing J, Wrage N & van Groenigen JW (2011). Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. *Soil Biol Biochem* **43:** 174-178.

Koubová A, Goberna M, Šimek M, Chroňáková A, Pižl V, Insam H & Elhottová D (2012) Effects of the earthworm *Eisenia andrei* on methanogens in a cattle-impacted soil: a microcosm study. *Eur J Soil Biol* **48:** 32-40.

Kraft B, Strous M & Tegetmeyer HE (2011) Microbial nitrate respiration - genes, enzymes and environmental distribution. *J Biotech* **155:** 104-117.

Krištůfek V, Pizl V & Ravasz K (1995) Epifluorescent microscopy of earthworms intestinal bacteria. *Acta Microbiol Immunolog Hungar* **42:** 39-44.

Krištůfek V, Pizl V & Szabo IM (1990) Composition of the intestinal streptomycete community of earthworms (Lumbricidae). In *Microbiology in* Poecilotherms (Lesel R, ed.), pp. 137-140, Elsevier, Amsterdam, The Netherlands.

Krištůfek V, Ravasz K & Pizl V (1992) Changes in densities of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea caliginosa* (Oligochaeta, Lumbricidae). *Soil Biol Biochem* **24:** 1499-1500.

Krištůfek V, Ravasz K & Pizl V (1993) Actinomycete communities in earthworm guts and surrounding soil. *Pedobiologia* **37:** 379-384.

Kumar S, Nei M, Dudley J & Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9:** 299-306.

Kunin V, Engelbrektson A, Ochman H & Hugenholz P (2010) Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environ Microbiol* **12:** 118-123.

Küsel K & Drake HL (1995) Effects of environmental parameters on the formation and turnover of acetate by forest soils. *Appl Environ Microbiol* **61:** 3667-3675.

Laossi K-R, Noguera CD, Bartolomé-Lasa A, Mathieu J, Blouin M & Barot S (2009) Effects of an endogeic and an anecic earthworm on the competition between four annual plants and their relative fecundity. *Soil Biol Biochem* **41:** 1668-1673.

Lattaud C, Locati S, Mora P, Rouland C & Lavelle P (1998) The diversity of digestive systems in tropical geophageous earthworms. *Appl Soil Ecol* **9:** 189-195.

Lattaud C, Zhang BG, Locati S, Rouland C & Lavelle P (1997) Activities of the digestive enzymes in the gut and in tissue culture of a tropical geophagous earthworm, *Polypheretima elongate* (Megascolecidae). *Soil Biol Biochem* **29:** 335-339.

Lavelle P, Bignell D, Legape M, Wolters V, Roger P, Ineson P, Heal OW & Ghillion S (1997) Soil function in a changing world: the role of invertebrate ecosystem engineers. *Eur J Soil Biol* **33:** 159-193.

Lavelle P, Lattaud C, Trigo D & Barois I (1995) Mutualism and biodiversity in soils. *Plant Soils* **170:** 23-33.

le Treut H, Somerville R, Cubasch U, Ding Y, Mauritzen C, Mokssit A, Peterson T & Prather M (2007) Historical overview of climate change. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M & Miller HL, eds), pp. 93-128, Cambridge University Press, Cambridge, UK & New York, NY, USA.

Lee KE (1959a) The earthworm fauna of New Zealand, vol 130. Owen RE, Government Printer: Wellington, New Zealand.

Lee KE (1959b) A key for the identification of New Zealand earthworms. *Tuatara* **8:** 13-60.

Lee KE (1985) Earthworms. Their ecology and relationships with soil and land use. Academic Press, Sydney, Australia.

Liu WT, Marsh TL, Chenh H & Forney LJ (2010) Characterization of microbial diversity by tetermining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* **63:** 4516-4522.

Liu YC & Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann N Y Acad Sci* **1125:** 171-189.

Logsdon SD & Linden DR (1992) Interactions of earthworms with soil physical conditions influencing plant growth. *Soil Sci* **154:** 330-337.

Lozupone C & Knight R (2005) UniFrac a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71:** 8228-8235.

Lubbers IM, van Groenigen KJ, Fonte SJ, Six J, Brussaard L & von Groenigen JW (2013) Greenhouse gas emissions from soils increased by earthworms. *Nature Climate Change* **3:** 187-194.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann D, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A & Schleifer K-H (2004) ARB: a software environment for sequence data. *Nucl Ac Res* **32:**1363-1371.

Lueders T, Chin KJ, Conrad R & Friedrich M (2001) Molecular analyses of methyl-coenzme M reductase α-subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3:** 194-204.

Magurran AE (2004) Measuring biological diversity. Blackwell, Oxford, UK.

Majeed MZ, Miambi E, Barois I, Blanchart E & Brauman A (2013) Emission of nitrous oxide from casts of tropical earthworms belonging to different ecological categories. *Pedobiologia* **56:** 49-58.

Makeschin F (1997) Earthworms (Lumbricidae: Oligochaeta): important promoters of soil development and soil fertility. In *Fauna in soil ecosystems* (Benckiser G, ed), pp. 173-223, Marcel Dekker Inc., New York, NY, USA.

Malm S, Tiffert Y, Micklinghoff J, Schultze S, Joost I, Weber I, Horst S, Ackermann B, Schmidt M, Wohlleben W, Ehlers S, Geffers R, Reuther J & Bange F-C (2009) The roles of the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in nitrate assimilation of *Mycobacterium tuberculosis*. *Microbiol* **155:** 1332-1339.

Mansell GP, Syers JK & Gregg PEH (1981) Plant availability of phosphorus in dead herbage ingested by surface-casting earthworms. *Soil Biol Biochem* **13:** 163-167.

Margulies M, Egholm M, Altman W, Attiya S, Bader J, Bemben L, Berka J, Braverman M, Chen Y & Chen Z (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437:** 376-380.

Marichal R, Martinez AF, Praxedes C, Ruiz D, Carvajal AF, Oszwald J, del Pilar Hurtado M, Brown GG, Grimaldi M, Desjardins T, Sarrazin M, Decaëns T, Valasquez E & Lavelle P (2010) Invasion of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta) in landscapes of the Amazonian deforestation arc. *Appl Soil Ecol* **46:** 443-449.

Martin A, Cortez J, Barois I & Lavelle P (1987) Les mucus intestinaux de Ver de Terre, moteur de leurs interactions avec la microflore. *Rev Ecol Biol Sol* **24:** 549-558.

Martínez-Espinosa RM, Dridge RM, Bonete MJ, Butt JN, Butler CS, Sargent F & Richardson DJ (2007) Look at the positive side! The orientation, identification and bioenergetics of 'Archaeal' membrane-bound nitrate reductases. *FEMS Microbiol Lett* **276:** 129-139.

Masignani V, Pizza M & Rappuoli R (2006) Bacterial toxins. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 2, pp. 893-955, Springer-Verlag, New York, NY, USA.

Matthies C, Griesshammer A, Schmittroth M & Drake HL (1999) Evidence for involvement of gut-associated denitrifying bacteria in emission of nitrous oxide (N_2O) by earthworms obtained from garden and forest soils. *Appl Environ Microbiol* **65:** 3599-3604.

Maziarski S (1903) Recherches cytologiques sur les organs segmentaires des vers de terre. *Arch Polon Biol Med* **3:** 1-81.

Mba CC (1982) Dietary influence on the activity of two Nigeria oligochaetes: *Eudrilus eugeniae* and *Pontoscolex corethrurus*. *Pedobiologia* **23:** 244-250.

Mba CC (1989) Biomass and vermicompost production by the earthworm *Eudrilus eugeniae* (Kinberg). *Revist Biol Trop* **37:** 11-13.

McCune B & Grace JB (2002) Analysis of ecological communities. MjM Software Design, Gleneden Beach, OR, USA.

Merilä P, Galand PE, Fritze H, Tuittila ES, Kukko-Oja K, Laine J & Yrjälä K (2006) Methanogen communities along a primary succession transect of mire ecosystems. *FEMS Microbiol Ecol* **55:** 221-229.

Metzker ML (2005) Emerging technologies in DNA sequencing. *Genome Res* **15:** 1767-1776.

Mierswa I, Wurst M, Klinkenberg R, Scholz M & Euler T (2006) YALE: rapid prototyping for complex data mining tasks. In *Proceedings of the 12th ACM SIGKDD International Conference on Knowledge Discovery and Data Mining (KDD-06)*.

Miles HB (1963) Soil protozoa and earthworm nutrition. *Soil Sci* **95:** 407-409.

Miller TL & Wolin MJ (1986) Methanogens in human and animal intestinal trasts. *Syst Appl Microbiol* **7:** 223-229.

Mohan SB, Schmid M, Jetten MSM & Cole J (2004) Detection and widespread distribution of *nrfA* gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. *FEMS Microbiol Ecol* **49:** 433-443.

Moir JWB & Wood NJ (2001) Nitrate and nitrite transport in bacteria. *Cell Mol Life Sci* **58:** 215-224.

Moody SA, Briones MJI, Piearce TG & Dighton J (1995) Selective consumption of decomposing wheat-straw by earthworms. *Soil Biol Biochem* **27:** 1209-1213.

Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R & Castillo F (1999) Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. *J Bacteriol* **181:** 6573-6584.

Morley N & Baggs EM (2010) Carbon and oxygen controls on N_2O- and N_2-production during nitrate reduction. *Soil Biol Biochem* **42:** 1864-1871.

Mounier E, Hallet S, Cheneby D, Benizri E, Gruet Y, Nguyen C, Piutti S, Robin C, Slezack-Deschaumes S, Martin-Laurent F, Germon JC & Philippot J (2004) Influence of maize mucilage on the diversity and activity of the denitrifying community. *Environ Microbiol* **6:** 301-312.

Murai K, Miyake K, Andoh J & Iijima S (2000) Transcriptional regulation of *nir* and *nor* operons of *Paracoccus denitrificans*. *J Biosci Bioeng* **89:** 384-387.

Mülhardt C (2009) Die Polymerase-Kettenreaktion (PCR). In Der Experimentator: Molekularbiologie/Genomics. 6th ed, pp. 94 - 119, Spektrum Akademischer Verlag, Heidelberg, Germany.

Muszynski S (2012) Physiologische und molekularbiologische Charakterisierung von Bakterienisolaten und ihr potentialler Effekt aud fie N_2O-Produktion im Regenwurmdarm. *Bachelor thesis*. University of Bayreuth, Germany.

Nebert LD, Bloem J, Lubbers IM & van Groenigen JWV (2011) Association of earthworm-denitrifier interactions with increased emission of nitrous oxide from soil mesocosms amended with crop residue. *Appl Environ Microbiol* **77:** 4097-4104.

Nechitaylo TY, Timmis KN & Golyshin PN (2009) *'Candidatus* Lumbricincola', a novel lineage of uncultured *Mollicutes* from earthworms of family Lumbricidae. *Environ Microbiol* **11:** 1016-1026.

Nechitaylo TY, Yakimov MM, Godinho M, Timmis KN, Belogolova E, Byzov BA, Kurakov AV, Jones DL & Golyshin PN (2010) Effect of the earthworm *Lumbricus terrestris* and *Aporrectodea caliginosa* on bacterial diversity in soil. *Microb Ecol* **59:** 574-587.

Nebel ME, Wild S, Holzhauser M, Hüttenberger L, Reitzig R, Sperber M, Wild S & Stoeck T (2011) JAguc - a software package for environmental diversity analyses. *J Bioinform Comput Biol* **9:** 749-773.

Ngugi DK & Brune A (2012) Nitrate reduction, nitrous oxide formation, and anaerobic ammonia oxidation to nitrite in the gut of soil-feeding termites (*Cubitermes* and *Ophiotermes* spp.). *Environ Microbiol* **14:** 860-871.

Ngugi DK, Rong J & Brune A (2010) Nitrogen mineralization, denitrification, and nitrate ammonification by soil-feeding termites: a ^{15}N-based approach. *Biobeochem* **103:** 355-369.

Nozaki M, Miura C, Tozawa Y & Miura T (2009) The contribution of endogenous cellulose digestion in the gut of earthworm (*Pheretima hilgendorfi*: Megascolecidae). *Soil Biol Biochem* **41:** 762-769.

Nunes DH, Pasini A, Benito NP & Brown GG (2006) Earthworm diversity in four land use systems in the region of Jaguapitã, Paraná State, Brazil. *Carib J Sci* **3:** 331-338.

Oleynik AS & Byzov BA (2008) Response of bacteria to earthworm surface excreta. *Microbiology* **77:** 765-773.

Omodeo P (1998) History of *Clitellata*. *Ital J Zoo* **365:** 51-73.

Palmer K, Biasi C & Horn MA (2012) Contrasting denitrifier communities relate to contrasting N_2O emission patterns from acidic soils in arctic tundra. *ISME J* **6:** 1058-1077.

Palmer K, Drake HL & Horn MA (2009) Genome-derived criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units of nitrate reducers. *Appl Environ Microbiol* **75:** 5170-5174.

Parlé JN (1963) Microorganisms in the intestine of earthworms. *J Gen Microbiol* **31:** 1-13.

Paul D, Bridges S, Burgess S, Dandass Y, & Lawrence M (2010) Complete genome and comparative analysis of the chemolithoautotrophic bacterium *Oligotropha carboxidovorans* OM5. *BMC Gen* **11:** 51.

Paul K, Nonoh JO, Mikulski L & Brune A (2012) "*Methanoplasmatales*", *Thermoplasmatales*-related Archaea in termite guts and other environments, are the seventh order of methanogens. *Appl Environ Microbiol* **78:** 8245-8253.

Pauleta SR, Dell´Acqua S & Moura I (2013) Nitrous oxide reductase. *Coord Chem Rev* **257:** 332-349.

Pearson IV, Page MD, van Spanning RJM & Ferguson SJ (2003) A mutant of *Paracoccus denitrificans* with disrupted genes coding for cytochrome c_{550} and pseudoazurin establishes these two proteins as the in vivo electron donors to cytochrome cd_1 nitrite reductase. *J Biol Biochem* **185:** 6308-6315.

Petri R & Imhoff JK (2000) The relationship of nitrate reducing baceteria on the basis of *narH* gene sequences and comparison of *narH* and 16S rDNA based phylogeny. *Syst Appl Microbiol* **23:** 47-57.

Philippot L (2005) Tracking nitrate reducers and denitrifiers in the environment. *Biochem Soc Transact* **33:** 200-204.

Philippot L & Hojberg O (1999) Dissimilatory nitrate reductases in bacteria. *Biochim Biophy Acta* **1446:** 1-23.

Philippot L, Mirleau P, Mazurier S, Siblot S, Hartmann A, Lemanceau P & Germon JC (2001) Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar, nir, nor* and *nos* genes. *Biochim Biophy Acta* **1517:** 436-440.

Philippot L, Piutti S, Martin-Laurent F, Hallet S & Germon JC (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. *Appl Environ Microbiol* **68:** 6121-6218.

Piearce TG & Philips MJ (1980) The fate of ciliates in the earthworm gut: an in vitro study. *Microb Ecol* **5:** 313-319.

Piearce TG, Roggero N & Tipping R (1994) Earthworms and seeds. *J Biol Edu* **28:** 195-202.

Pielou EC (1977) Mathematical ecology. Wiley Interscience, New York, NY, USA.

Pinel N, Davidson SK & Stahl DA (2008) *Verminephrobacter eiseniae* gen. nov., sp. nov., a nephridial symbiont of the earthworm *Eisenia foetida* (Savigny). *Int J Syst Evol Microbiol* **58:** 2147-2157.

Portales F, de Muynck A & Sylla MP (1987) Selective isolation of Mycobacteria from soil: a statistical analysis approach. *Microbiology* **134:** 849-855.

Prabha ML, Jayaraaj IA, Jeyaraaj R & Rao S (2007) Comparative studies on the digestive enzymes in the gut of earthworms, *Eudrilus eugeniae* and *Eisenia fetida*. *Ind J Biotech* **6:** 567-569.

Pratscher J, Dumont MG & Conrad R (2011) Ammonia oxidation coupled to CO_2 fixation by archaea and bacteria in an agricultural soil. *Proc Natl Acad Sci USA* **108:** 4170-4175.

Precht M, Kraft R & Bachmaier M (2005) Angewandte Statistik 1. 7th ed, Oldenburg Verlag, München, Germany.

Prieme A, Braker G & Tiedje JM (2002) Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Appl Environ Microbiol* **68:** 1893-1900.

Prosser JI (2007) The ecology of nitrifying bacteria. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 223-243, Elsevier Academic Press, Amsterdam, The Netherlands.

Purkhold U, Pommerening-Roser A, Juretschko S, Schmidt MC, Koops HP & Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Appl Environ Microbiol* **668:** 5368-5382.

Quince C, Lanzen A, Curtis TP, Davenport RJ, Hall N, Head IM, Read LF & Sloan WT (2009) Accurate determination of microbial diversity from 454 pyrosequencing data. *Nat Methods* **6:** 639-641.

Ramos C, Molbak L & Molin S (2000) Bacterial activity in the rhizosphere analyzed at a single-cell level by monitoring ribosome contents and synthesis rates. *Appl Environ Microbiol* **66:** 801-809.

Ravasz K, Zicsi A, Contreras E, Szell V & Szabo IM (1986) Über die Darmaktinomycetengemeinschaft einiger Regenwurmarten. *Opusc Zool Budapest* **22:** 85-102.

Ravishankara AR, Daniel JS & Portmann RW (2009) Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science* **326:** 123-125.

Reddell P & Spain AV (1991) Transmission of effective Frankia (Actinomycetales) propagules in casts of the endogeic earthworm *Pontoscolex corethrurus* (Oligochaeta, Glossoscolecidae). *Soil Biol Biochem* **23:** 775-778.

Reynolds JW & Cook DC (1993) Nomenclatura Oligochaetologica. Supplementum tertium. A catalogue of names, description and type specimens of the Oligochaeta. New Brunswick Museum Monographic Series, New Brunswick, Quebec, Canada.

Rich JJ, Heichen RS, Bottomley JP, Cromack K & Myrold DD (2003) Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Appl Environ Microbiol* **69:** 5974-5982.

Richardson DJ, Berks BC, Sussell DA, Spiro S & Taylor CJ (2001) Functional biochemical and genetic dicersity of prokaryotic nitrate reductases. *Cell Molec Life Sci* **298:** 165-178.

Richardson DJ, van Spanning RJM & Ferguson SJ (2007) The prokaryotic nitrate reductases. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 21-35, Elsevier Academic Press, Amsterdam, The Netherlands.

Righi G (1990) Minhocas de Mato Grosso e de Rondônia. In *Programa Polo Noroeste, Relatorio de Pesquisa No. 12. SCT/PR-CNPq.* Programa do Tropico Umido. CNPq/AED. Brasilia, Brazil.

Rinaldo S & Cutruzzolà F (2007) Nitrite reductases in denitrification.. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 37-55, Elsevier Academic Press, Amsterdam, The Netherlands.

Risgaard-Petersen N, Langezaal AM, Ingvardsen S, Schmid MC, Jetten MSM, Op den Camp HJM, Derksen JWM, Pina-Ochoa E, Eriksson SP, Nielsen LP, Revsbech NP, Cedhagen T & van der Zwaan GJ (2006) Evidence for complete denitrification in a benthic foraminifer. *Nature* **443:** 93-96.

Rizhiya E, Bertora C, van Vliet PCJ, Kuikman PJ, Faber JH & van Groenigen JW (2007) Earthworm activity as an determinant for N_2O emission from crop residue. *Soil Biol Biochem* **39:** 2058-2069.

Rodinov DA, Dubchak IL, Arkin AP, Alm EJ & Gelfand MS (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput Biol* **1:** e55.

Ronaghi M, Uhlén M & Nyrén P (1998) A sequencing method based on real-time pyrophosphate. *Science* **281:** 363-365.

Rösch C, Mergel A & Bothe H (2002) Biodiversity of denitrifying and nitrogen-fixing bacteria in an acidic forest soil. *Appl Environ Microbiol* **68:** 3818-3829.

Rossi J-P, Celini L, Mora P, Mathieu J, Lapied E, Nahmani J, Ponge JF & Lavelle P (2010) Decreasing fallow duration in tropical slash-and-burn agriculture alters soil macroinvertebrate diversity: a case study in southern French Guiana. *Agric Ecosyst Environ* **135:** 148-154.

Roy R & Conrad R (1999) Effect of methanogenic precursors (acetate, hydrogen, propionate) on the suppression of methane production by nitrate in anoxic rice field soil. *FEMS Microbiol Ecol* **28:** 49-61.

Rudolf M & Kroneck PM (2005) The nitrogen cycle: its biology. *Met Ions Biol Syst* **43:** 75-103.

Rütting T, Boeckx P, Müller C & Klemedtsson L (2011) Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. *Biogeosci* **8:** 1779-1791.

Russell JB & Moselio S (2009) Rumen. *Encyclopedia of Microbiology*, pp. 163-174, Academic Press, Oxford, UK.

Sachs L (1999) Angewandte Statistik, Springer Verlag, Berlin, Germany.

Sadowsky M & Graham P (2006) Root and stem nodule bacteria of legumes. In *The Prokaryotes* (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), 3rd ed, vol 2, pp. 818-841, Springer, New York, NY, USA.

Sahrawat KL & Keeney DR (1986) Nitrous oxide emission from soils. *Adv Soil Sci* **4:** 103-148.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB & Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487-491.

Saitou N & Nei M (1987) The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4:** 406-425.

Sakai S, Imachi H, Hanada S, Ohashi A, Harada H & Kamagata Y (2008) *Methanocella paludicola* gen. nov., sp nov., a methane-producing archaeon, the first isolate of the lineage 'Rice Cluster I', and proposal of the new archaeal order *Methanocellales* ord. nov. *Int J Syst Evolut Microbiol* **58:** 929-936.

Sameshima-Saito,R, Chiba K, Hirayama J, Itakura M, Mitsui H, Eda S & Minamisawa K (2006) Symbiotic *Bradyrhizobium japonicum* reduces N_2O surrounding the soybean root system via nitrous oxide reductase. *Appl Environ Microbiol* **72:** 2526-2532.

Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-Garcia C, Rodriguez G, Massol-Deyá A, Krishnani KK, Ritalathi KM, Nissen S, Konstandinidis KT & Löffler FE (2012) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc Natl Acad Sci USA* **48:** 19709-19714.

Sanger F, Nicklen S & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci* **74:** 5463-5467.

Scala DJ & Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiol Lett* **162:** 61-68.

Schaefer M (2000) Brohmer. Fauna von Deutschland. 20th ed, Quelle & Meyer, Wiebelsheim, Germany.

Schauer C, Thompson CL & Brune A (2012) The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. *Appl Environ Microbiol* **78:** 2758-2758.

Scheu S (1987) Microbial activity and nutrient dynamics in earthworm casts. *Biol Fertil Soils* **5:** 230-234.

Scheu S (2003) Effects of earthworms on plant growth: Patterns and perspectives: The 7th international symposium on earthworm ecology. *Pedobiologia* **47:** 846-856.

Schink B & Stams AJM (2006) Syntrophism among prokaryotes. In *The Prokaryotes* (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), 3rd ed, pp. 309-336, Springer, New York, NY, USA.

Schlesinger WH (2009) On the fate of anthropogenic nitrogen. *P Natl Acad Sci USA* **106:** 203-208.

Schloss PD & Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71:** 1501-1506.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, van Horn DJ & Weber CF (2009) Introducing mother: open-source, platform independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537-7541.

Schmidt I, van Spanning RJM & Jetten MSM (2004) Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild type and nirK- and norB-deficient mutants. *Microbiol* **150:** 4107-4114.

Schmidt O, Wüst PK, Hellmuth S, Borst K, Horn MA & Drake HL (2011) Novel [NiFe]- and [FeFe]-hydrogenase gene transcripts indicative of active facultative aerobes and obligate anaerobes in earthworm gut contents. *Appl Environ Microbiol* **77:** 5842-5850.

Schönholzer F, Hahn D & Zeyer J (1999) Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. *FEMS Microbiol Ecol* **28:** 235-248.

Schönholzer F, Hahn D, Zarda B & Zeyer J (2002) Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of *Lumbricus terrestris* L. *J Microbiol Methods* **48:** 53-68.

Schramm A (2003) In situ analysis of structure and activity of the nitrifying community in biofilms, aggregates, and sediments. *Geomicrobiol J* **20:** 313-333.

Schramm A, Davidson SK, Dodsworth JA, Drake HL, Stahl DA & Dubilier N (2003) *Acidovorax*-like symbionts in the nephridia of earthworms. *Environ Microbiol* **5:** 804-809.

Schultz JE & Breznak JA (1978) Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. *Appl Environ Microbiol* **35:** 930-936.

Schwartz E & Friedrich B (2006) The H_2-metabolizing prokaryotes. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 2, pp. 496-563, Springer-Verlag, New York, NY, USA.

Sears HJ, Little PJ, Richardson DJ, Berks BC, Spiro C & Ferguson SJ (1997) Identification of an assimilatory nitrate reductase in mutants of *Paracoccus denitrificans* GB17 deficient in nitrate respiration. *Arch Microbiol* **167:** 61-66.

Segers R (1998) Methane production and methane consumption: A review of processes underlying wetland methane fluxes. *Biogeochem* **41:** 23-51.

Selengut JD & Haft DH (2010) Unexpected abundance of coenzyme F420-dependent enzymes in Mycobacterium tuberculosis and other actinobacteria. *J Bacteriol* **192:** 5788-5798.

Shannon CE & Weaver W (1949) The mathematical theory of communication. University of Illinois Press, Urbana, IL, USA.

Shapleigh JP (2006) The denitrifying prokaryotes. In *The Prokaryotes* (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), 3rd ed, vol 2, pp. 769-792, Springer, New York, NY, USA.

Shirey JJ & Sextone AJ (1989) Denitrification and nitrate reducing bacterial populations. *FEMS Microbiol Ecol* **62:** 59-70.

Shoun H, Kano M, Baba I, Takaya N & Matsuo M (1998) Denitrification by actinomycetes and purification of dissimilatory nitrate reductase and azurin from *Streptomyces thioluteus*. *J Bacteriol* **180:** 4413-4415.

Shoun H, Kim DH, Uchiyama H & Sugiyama J (1992) Denitrification by fungi. *FEMS Microbiol Lett* **94:** 277-281.

Siddiqui RA, Warnecke-Eberz U, Hengsberger A, Schneider B, Kostka S & Friedrich B (1993) Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. *J Bacteriol* **175:** 5867-5876.

Simon J (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol Rev* **26:** 285-309.

Simpson EH (1949) Measurement of diversity. *Nature* **163:** 688.

Singleton DR, Hendrix PF, Coleman DC & Whitman WB (2003) Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). *Soil Biol Biochem* **35:** 1547-1555.

Slepecky RA & Hemphill HE (2006) The genus *Bacillus* - nonmedical. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 2, pp. 1050-1079, Springer-Verlag, New York, NY, USA.

Smith EP & van Belle G (1984) Nonparametric estimation of species richness. *Biometrics* **40:** 119-129.

Smith MS (1982) Dissimilatory reduction of nitrite to ammonium and nitrous oxide by a soil *Citrobacter* species. *Appl Environ Microbiol* **43:** 854-860.

Smith MS (1983) Nitrous oxide production by *Escherichia coli* is correlated with nitrate reductase activity. *Appl Environ Microbiol* **45:** 1545-1555.

Smith MS & Zimmerman K (1981) Nitrous oxide production by nondenitrifying soil nitrate reducers. *Soil Sci Soc Am J* **45:** 865-871.

Sohaskey CD (2008) Nitrate enhances the survival of *Mycobacterium tuberculosis* during inhibition of respiration. *J Bacteriol* **190:** 2981-2986.

Sohaskey CD & Wayne LG (2003) Role of *narK2X* and *narGHJI* in hypoxic upregulation of nitrate reduction by *Mycobacterium tuberculosis*. *J Bacteriol* **185:** 7247-7256.

Spain AV, Saffigna PG & Wood AW (1990) Tissue carbon sources of *Pontoscolex corethrurus* (Oligochaeta: Glossoscolecidae) in a sugarcane ecosystem. *Soil Biol Biochem* **29:** 703-706.

Springer E, Sachs MS, Woese CR & Boone DR (1995) Partial gene sequences for the -subunit of methyl-coenzyme M reductase (*mcrA*) as a phylogenetic tool for the family *Methanosarcinaceae*. *Int J Syst Bacteriol* **45:** 554-559.

Springett JA (1992) Distribution of lumbricid earthworms in New Zealand. *Soil Biol Biochem* **24:** 1377-1381.

Springett JA, Gray RAJ, Barker DJ, Lambert MG, Mackay AD & Thomas VJ (1998) Population density and distribution of the New Zealand indigenous earthworm *Octochaetus multiporus* (Megascolecidae: Oligochaeta) in hill pastures. *NZL J Ecol* **22:** 87-93.

Stackebrandt E (2006) Defining taxonomic ranks. In *The Prokaryotes* (Falkow S, Rosenberg E & Dworkin M, eds), 3rd ed, vol 1, pp. 29-57, Springer-Verlag, New York, NY, USA.

Stackebrandt E & Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33:** 152-155.

Stein LY (2011) Surveying N_2O-producing pathways in bacteria. In *Methods in Enzymology* (Klotz MG, ed), vol 486, pp. 131-152, Elsevier Academic Press, Amsterdam, The Netherlands.

Stevens RJ, Laughlin RJ & Malone JP (1998) Soil pH affects the processes reducing nitrate to nitrous oxide and dinitrogen. *Soil Biol Biochem* **30:** 1119- 1126.

Stief P, Poulsen M, Nielsen LP, Brix H & Schramm A (2009) Nitrous oxide emission by aquatic macrofauna. *P Natl Acad Sci USA* **106:** 4296-4300.

Stolz JF & Basu P (2002) Evolution of nitrate reductase: molecular and structural variations on a common function. *Chembiochem* **3:** 198-206.

Storch V & Welch U (1999) *Kükenthals Leitfaden für das zoologische Praktikum*, 24. Auflage, Spektrum Akademischer Verlag, Heidelberg, Germany.

Stres B, Mahne I, Avgustin G & Tiedje MJ (2004) Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. *Appl Environ Microbiol* **70:** 301-309.

Strohm TO, Griffin B, Zumft WG & Schink B (2007) Growth yields in bacterial denitrification and nitrate ammonification. *Appl Environ Microbiol* **73:** 1420-1424.

Sudesh BM & Cole JA (2007) The dissimilatory reduction of nitrate to ammonia by anaerobic bacteria. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 93-106, Elsevier Academic Press, Amsterdam, The Netherlands.

Suharti & de Vries S (2005) Membrane-bound denitrification in the Gram-positive bacterium *Bacillus azotoformans*. *Biochem Soc Transact* **33:** 130-133.

Sun YJ, Cai YP, Liu L, Yu FH, Farrell ML, McKendree W & Farmerie W (2009) ESPRIT: estimating species richness using large collections of 16S rRNA pyrosequences. *Nucleic Acids Res* **37:** e76.

Tavares P, Pereira AS, Moura JJG & Moura I (2006) Metalloenzymes of the denitrification pathway. *J Inorg Biochem* **100:** 2087-2100.

Thakuria D, Schmidt O, Finan D, Egan D & Doohan FM (2010) Gut wall bacteria of earthworms: a natural selection process. *ISME J* **4:** 357-366.

Thauer RK, Kaster AK, Seedorf H, Buckel W & Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nature *Rev Microbiol* **6:** 579-591.

Thies JE (2007) Soil microbial community analysis using terminal restriction fragment length polymorphisms. *Soil Sci Soc Amer J* **71:** 579-591.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Ac Res* **22:** 4673-4680.

Thomsen JK, Geest R & Cox RP (1994) Mass spectrometric studies of the effect of pH on the accumulation of intermediates in denitrification by *Paracoccus denitrificans*. *Appl Environ Microbiol* **60:** 536-541.

Tiedje JM (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium, In *Biology of Anaerobic Microorganisms* (Zehnder AJB, ed), pp. 179-243, John Wiley and Sons, New York, NY, USA.

Tillinghast EK, O'Donnell R, Eves D, Calvert E & Taylor J (2001) Water-soluble luminal contents of the gut of the earthworm *Lumbricus terrestris* L. and their physiological significance. *Compar Biol Physiol* **129:** 345-353.

Tiwari SC, Tiwar BK & Mishra RR (1989) Microbial populations, enzyme activities and nitrogen-phosphorus-potassium enrichment in earthworm casts and in the surrounding soil of a pineapple plantantion. *Biol Fertil Soils* **8:** 178-182.

Toffanin A, Wu QG, Maskus M, Casella S, Abruna HD & Shapleigh JP (1996) Characterization of the gene encoding nitrite reductase and the physiological consequences of its expression in the nondenitrifying *Rhizobium* "*hedsari*" strain HCNT1. *Appl Environ Microbiol* **62:** 4019-4025.

Toyota K & Kimura M (2000) Microbial community indigenous to the earthworm *Eisenia foetida*. *Biol Fertil Soils* **31:** 187-190.

Treusch AH, Leininger S, Schleper C, Kietzin A, Klenk HP & Schuster SC (2005) Novel genes for nitrite reductase and Amoa-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* **7:** 1985-1995.

Trigo D, Barois I, Garvin MH, Huerta E, Irisson S & Lavelle P (1999) Mutualism between earthworms and soil microflora. *Pedobiologia* **43:** 866-873.

Tsutsuki K & Kuwatsuka S (1979) Chemical studies on soil humic acids. *Soil Sci Plant Nutr* **25:** 373-384.

Unden G, Becker S, Bongaerts J, Holighaus G, Schirawski J & Six S (1995) O_2-sensing and O_2-dependent gene regulation in faculatvely anaerobic bacteria. *Arch MIcrobiol* **164:** 81-90.

van Breemen N & Feijtel TCJ (1990) Soil processes and properties involved in the production of greenhouse gases, with special relevance to soil taxonomic systems. In *Soils and the Greenhouse Effect* (Bouwman AF, ed), pp. 195- 223, John Wiley & Sons, Chichester, UK.

van Cleemput O (1998) Subsoils: Chemo- and biological denitrification, N_2O and N_2 emissions. *Nutr Cyc Agroecosys* **52:** 187-194.

Verbaendert I, Boon N, de Vos P & Heylen K (2011a) Denitrification is a common feature among members of the genus *Bacillus*. *Syt Appl Microbiol* **34:** 385-391.

Verbaendert I, de Vos P, Boon N & Heylen K (2011b) Denitrification in gram-positive bacteria: an underexplored trait. *Biochem Soc Transact* **39:** 254-258.

Vine CE & Cole JA (2011) Unresolved sources, sinks, and pathways for the recovery of enteric bacteria from nitrosative stress. *FEMS Microbiol Lett* **325:** 99-107.

Vilar-Sanz A, Piug S, García-Lledó A, Trias R, Balaguer MD, Colprim J & Bañeras L (2013) Denitrifying bacterial communities affect current production and nitrous oxide accumulation in a microbial fuel cell. *PLOS ONE* **8:** e63460.

Warnecke-Eberz U & Friedrich B (1993) Three nitrate reductase activities in *Alcaligenes eutrophus*. *Arch Microbiol* **159:** 405-409.

Wang J, Hersketh JD & Wooley JT (1986) Preexisting channels and soybean rooting patterns. *Soil Sci* **141:** 432-437.

Ward BB (2011) Measurement and distribution of nitrification rates in oceans. *FEMS Methods Enzymol* **486:** 307-323.

Volland S, Rachinger M, Strittmatter A, Daniel R, Gottschalk G & Meyer O (2011) Complete genome sequences of the chemolithoautotrophic *Oligotropha carboxidovorans* strains OM4 and OM5. *J Bacteriol* **193:** 5043.

Weber I, Fritz C, Ruttkowski S, Kreft A & Bange F-C (2000) Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virolence in immunedieficient mice. *Mol Microbiol* **35:** 1017-1025.

Webster EA & Hopkins DW (1996) Contribution of different microbial processes to N_2O emission from soil under different moisture regimes. *Biol Fertil Soils* **22:** 331-335.

Weinert LA, Tinsley MC, Temperley M & Jiggins FM (2007) Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles . *Biol Lett* **3:** 678-681.

Welander PV & Metcalf WM (2005) Loss of the mrt operon in Methanosarcina blocks growth on methanol, but not methanogenesis, and reveals an unknown methanogenic pathway. *Proc Natl Acad Sci USA* **102:** 10664-10669.

Westheide W & Rieger R (2007) Spezielle Zoologie Teil 1: Einzeller und Wirbellose Tiere. Spektrum Akademischer Verlag, Munich, Germany.

Will C, Thürmer A, Wollherr A, Nacke H, Herold N, Schrumpf M, Gutknecht J, Wubet T, Buscot F & Daniel R (2010) Horizon-specific bacterial community composition of German grassland soil, as revealed by pyrosequencing-based analysis of 16S rRNA genes. *Appl Environ Microbiol* **76:** 6751-6759.

Wolter C & Scheu S (1999) Changes in bacterial numbers and hyphal length during the gut passage through *Lumbricus terrestris* (Lumbricidae, Oligochaeta). *Pedobiologia* **43:** 891-900.

Wrage N, Velthof GL, van Beusichem ML & Oenema O (2001) Role of nitrifier-denitrification in the production of nitrous oxide. *Soil Biol Biochem* **33:** 1723-1732.

Wüst PK (2010) Mikrobielle Kohlenstoff- und Stickstofftransformationen im Verdauungstrakt von Regenwürmern (Oligochaeta). *PhD thesis*. University of Bayreuth, Germany.

Wüst PK, Horn MA & Drake HL (2009a) In situ hydrogen and nitrous oxide as indicators of concomitant fermentation and denitrification in the alimentary canal of the earthworm *Lumbricus terrestris*. *Appl Environ Microbiol* **75:** 1852-1859.

Wüst PK, Horn MA & Drake HL (2009c) Trophic links between fermenters and methanogens in a moderately acidic fen soil. *Environ Microbiol* **11:** 1395-1409.

Wüst PK, Horn MA & Drake HL (2011) *Clostridiaceae* and *Enterobacteriaceae* as active fermenters in earthworm gut content. *ISME J* **5:** 92-106.

Wüst PK, Horn MA, Henderson G, Janssen PH, Rehm BH & Drake HL (2009b) Gut-associated denitrification and in vivo emission of nitrous oxide by the earthworm families Megascolecidae and Lumbricidae in New Zealand. *Appl Environ Microbiol* **75:** 3430-3436.

Yoshinari T & Knowles R (1976) Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Bio*chem Biophys Res Commun* **69:** 705-710.

Zablotowics RM, Eskew DL & Focht DD (1978) Denitrification in *Rhizobium*. *Can J Microbiol* **24:** 757-760.

Zehnder AJB & Stumm W (1988) Geochemistry and Biogeochemistry of anaerobic habitats, In *Biology of Anaerobic Microorganisms* (Zehnder AJB, ed), pp. 1-38, John Wiley & Sons, New York, NY, USA.

Zhang QL, Liu Y, Ai GM, Miao LL, Zheng HY & Liu ZP (2012) The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. *Bioresource Techn* **76:** 3244-3254.

Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61:** 533-616.

Zumft WG (2002) Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. *J Molec Microbiol Biotech* **4:** 277-286.

Zumft WG (2005) Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J Inorg Biochem* **99:** 194-215.

Zumft WG & Körner H (2007) Nitrous oxide reductases. In *Biology of the nitrogen cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 67-81, Elsevier Academic Press, Amsterdam, The Netherlands.

Zumft WG & Kroneck PMH (2007) Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by bacteria and archaea. *Adv Microb Phys* **52:** 107-227.

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9. PUBLICATIONS AND PRESENTATIONS

To date, the results of the current work yielded three publications. In addition, results of this work have been presented at four national and three international conferences.

9.1. Publications in peer-reviewed journals

Depkat-Jakob PS, Hilgarth M, Horn MA & Drake HL (2010) Effect of earthworm feeding guilds on ingested dissimilatory nitrate reducers and denitrifiers in the alimentary canal of the earthworm. *Appl Environ Microbiol* **76:** 6205-6214.

Depkat-Jakob PS, Hunger S, Schulz K, Brown GG, Tsai SM & Drake HL (2012) Emission of methane by *Eudrilus eugeniae* and other earthworms from Brazil. *Appl Environ Microbiol* **78:** 3014-3019.

Depkat-Jakob PS, Brown GG, Tsai SM, Horn MA & Drake HL (2013) Emission of nitrous oxide and dinitrogen by diverse earthworm families from Brazil and resolution of associated denitrifying and nitrate-dissimilating taxa. *FEMS Microbiol Ecol* **83:** 375-391.

9.2. Presentations at national and international conferences

9.2.1. Oral presentation

Depkat-Jakob PS, Brown GG, Tsai SM, Horn MA & Drake HL (2012) Emission of denitrification-derived nitrogenous gases by Brazilian earthworms. Annual meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie. BIOspectrum Tagungsband, Abstract SMV004.

9.2.2. Poster presentations

Depkat-Jakob PS, Horn MA & Drake HL (2009) Activation of ingested dissimilatory nitrate reducers and denitrifiers in the earthworm gut is dependent on earthworm feeding guilds. Annual meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie. BIOspectrum Tagungsband, Abstract PJ03.

Depkat-Jakob PS, Wüst PK, Horn MA & Drake HL (2010) Denitrifiers differ in the alimentary canals of the earthworms *Octochaetus multiporus* (*Megascolecidae*) and *Lumbricus rubellus* (*Lumbricidae*). Annual meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie. BIOspectrum Tagungsband, Abstract HMP19.

Depkat-Jakob PS, Hilgarth M, Horn MA & Drake HL (2010) Effect of earthworm feeding guilds on selective activation of ingested dissimilatory nitrate reducers and denitrifiers during gut passage. 13th International Symposium on Microbial Ecology (abstracts on disc).

Depkat-Jakob PS, Hilgarth M, Gebelein J, Horn MA & Drake HL (2011) Activation of nitrous oxide-producing nitrate reducers during gut passage through earthworms of different feeding guilds. Annual meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie. BIOspectrum Tagungsband, Abstract EMP101.

Depkat-Jakob PS, Tsai SM, Brown GG, Horn MA & Drake HL (2011) Emission of denitrification derived nitrous oxide from Brazilian earthworms. Annual Meeting of the American Society for Microbiology (abstracts on disc).

Depkat-Jakob PS, Hunger S, Schulz K, Brown GG, Tsai SM, Horn MA & Drake HL (2012) Microbial symbioses yielding the emission of the greenhouse gases nitrous oxide and methane by earthworms in Brazil: functional links to taxa. 14th International Symposium on Microbial Ecology (abstracts on disc).

10. DECLARATION

I declare that this document has been composed by myself; it describes my own work, unless acknowledged otherwise in the text (see 2.7).

This dissertation has not been submitted for the award of any other degree or diploma in any other tertiary institution. Material from the published and unpublished work of others which is referred to in the dissertation, and all sources of information are acknowledged.

Peter S. Depkat-Jakob Bayreuth, 02. July 2013

11. APPENDICES

Table A 1. FastUnifrac significance test of gene libraries derived from gut contents and the corresponding soil of the earthworms *G. paulistus* and *A. gracilis*.

		Library			
Gene	Library	Gut *GP*	Gut *AG*	Soil *GP*	Soil *AG*
narG	Gut *GP*[a]	n.a.[c]	0.468	1.000	≤ 0.002
	Gut *AG*[b]	0.468[d]	n.a.	1.000	≤ 0.002
	Soil *GP*	1.000	1.000	n.a.	≤ 0.002
	Soil *AG*	≤ 0.002	≤ 0.002	≤ 0.002	n.a.
nirK	Gut *GP*	n.a.	≤ 0.002	1.000	≤ 0.002
	Gut *AG*	≤ 0.002	n.a.	≤ 0.002	≤ 0.002
	Soil *GP*	1.000	≤ 0.002	n.a.	≤ 0.002
	Soil *AG*	≤ 0.002	≤ 0.002	≤ 0.002	n.a.
nosZ	Gut *GP*	n.a.	≤ 0.002	1.000	≤ 0.002
	Gut *AG*	≤ 0.002	n.a.	≤ 0.002	≤ 0.002
	Soil *GP*	1.000	≤ 0.002	n.a.	≤ 0.002
	Soil *AG*	≤ 0.002	≤ 0.002	≤ 0.002	n.a.

[a] *GP*, *G. paulistus*.

[b] *AG*, *A. gracilis*.

[c] n.a., not applicable.

[d] p-values have been corrected for multiple comparisons using the Bonferroni correction; $p > 0.1$ indicates no significant difference; $0.001 < p < 0.1$ indicates a significant difference.

Table A 2. List of bacterial isolates retrieved from gut contents of *L. rubellus*, *A. caliginosa*, *L. terrestris*, and *O. lacteum* isolated under anoxia with nitrite and N_2O as electron acceptor.

e^--acceptor[a]	Number[b]	Source[c]	Next related species[d]	Accession number	Similarity (%)[e]
NO_2^-	101	*LR*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	103	*LR*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	105	*LR*	*Pseudomonas gessardii* P25	AY972182	99
NO_2^-	114	*LR*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	115	*LR*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	119	*LR*	*Aeromonas encheleia* E195	AJ458416	100
NO_2^-	120	*LR*	*Aeromonas veronii* 4pW23	FJ940810	99
NO_2^-	122	*LR*	*Oerskovia paurometabola* DSM 14281	AJ314851	100
NO_2^-	123	*LR*	*Oerskovia enterophila* CG30(2)-2	AB562466	100
NO_2^-	124	*LR*	*Bacillus drentensis* WN575	DQ275176	99
NO_2^-	201	*AC*	*Mycoplana ramosa* DMS7292	EU022308	97.7*
NO_2^-	202	*AC*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	203	*AC*	*Paenibacillus amylolyticus* KT5501	AB115960	99
NO_2^-	204	*AC*	*Bacillus circulans* WSBC 20030	Y13062	99
NO_2^-	206	*AC*	*Bacillus boroniphilus* PL68	GU001897	99
NO_2^-	207	*AC*	*Paenibacillus borealis* RFNB5	FJ266316	98
NO_2^-	208	*AC*	*Paenibacillus borealis* KK19	AJ011322	99.0*
NO_2^-	211	*AC*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	214	*AC*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	215	*AC*	*Paenibacillus xylanexedens* B22a	EU558281	100
NO_2^-	216	*AC*	*Paenibacillus xylanexedens* B22a	EU558281	100
NO_2^-	217	*AC*	*Paenibacillus pabuli* Gt-1	GU201854	100
NO_2^-	219	*AC*	*Paenibacillus xylanexedens* B22a	EU558281	100
NO_2^-	220	*AC*	*Paenibacillus xylanexedens* B22a	EU558281	100
NO_2^-	222	*AC*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	223	*AC*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	224	*AC*	*Paenibacillus wynnii* LMG 22176T	AJ633647	97
NO_2^-	302	*LT*	*Paenibacillus graminis* 801	FJ544322	99
NO_2^-	306	*LT*	*Bacillus drentensis* WN575	DQ275176	99
NO_2^-	307	*LT*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	308	*LT*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	309	*LT*	*Bacillus niacini* J2S5	EU221359	99
NO_2^-	311	*LT*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	316	*LT*	*Buttiauxella agrestis* HS-39	DQ440549	100
NO_2^-	318	*LT*	*Bacillus drentensis* WN575	DQ275176	99
NO_2^-	320	*LT*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	321	*LT*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	322	*LT*	*Bacillus niacini* J2S5	EU221359	99
NO_2^-	323	*LT*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	324	*LT*	*Cellulomonas humilata* NCTC 25174	NR_026226	99
NO_2^-	401	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	402	*OL*	*Bacillus niacini* J2S5	EU221359	99
NO_2^-	403	*OL*	*Paenibacillus borealis* 15	JX122146	99.8*
NO_2^-	404	*OL*	*Bacillus drentensis* WN575	DQ275176	100

Sequel to Table A 2.

e⁻-acceptor[a]	Number[b]	Source[c]	Next related species[d]	Accession number	Similarity (%)[e]
NO_2^-	405	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	407	*OL*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	408	*OL*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	411	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	412	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	413	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	415	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	416	*OL*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	418	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	420	*OL*	*Bacillus drentensis* WN575	DQ275176	100
NO_2^-	421	*OL*	*Paenibacillus graminis* 801	FJ544322	98
NO_2^-	422	*OL*	*Ensifer adhaerens* LC04	EU928872	100
NO_2^-	423	*OL*	*Ensifer adhaerens* LC04	EU928872	99
N_2O	502	*LR*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	503	*LR*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	504	*LR*	*Pseudomonas migulae* PD 17	DQ377758	100
N_2O	505	*LR*	*Aeromonas encheleia* E195	AJ458416	100
N_2O	507	*LR*	*Pseudomonas fluorescens* Mc07	EF672049	98
N_2O	508	*LR*	*Aeromonas hydrophila* DSM 30019	HM007582	99
N_2O	509	*LR*	*Pseudomonas fluorescens* Mc07	EF672049	99
N_2O	510	*LR*	*Pseudomonas fulva* 6	FJ418772	98
N_2O	511	*LR*	*Pseudomonas putida* KL3B4	DQ208660	99
N_2O	512	*LR*	*Pseudomonas fulva* 6	FJ418772	98
N_2O	513	*LR*	*Pseudomonas jessenii* PJM15	AM707022	99
N_2O	515	*LR*	*Erwinia billingiae* Eb661	FP236843	100
N_2O	516	*LR*	*Bosea thiooxidans* As5-4b	FN392632	99
N_2O	519	*LR*	*Aeromonas veronii* 4pW23	FJ940810	99
N_2O	521	*LR*	*Pseudomonas fluorescens* Mc07	EF672049	98
N_2O	522	*LR*	*Aeromonas encheleia* E193	AJ458414	100
N_2O	523	*LR*	*Pseudomonas fluorescens* Mc07	EF672049	98
N_2O	524	*LR*	*Pseudomonas putida* KL3B4	DQ208660	99
N_2O	601	*AC*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	602	*AC*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	603	*AC*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	604	*AC*	*Oerskovia enterophila* CG30(2)-2	AB562466	99
N_2O	605	*AC*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	606	*AC*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	608	*AC*	*Paenibacillus graminis* 801	FJ544322	98
N_2O	609	*AC*	*Bacillus cereus* 1TL12b	HM163559	100
N_2O	610	*AC*	*Bacillus boroniphilus* D7028	FJ161333	99
N_2O	614	*AC*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	615	*AC*	*Aeromonas hydrophila* DSM 30019	HM007582	99
N_2O	616	*AC*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	617	*AC*	*Bacillus boroniphilus* D7028	FJ161333	99
N_2O	618	*AC*	*Paenibacillus xylanexedens* B22a	EU558281	99
N_2O	619	*AC*	*Paenibacillus borealis* RFNB5	FJ266316	99
N_2O	620	*AC*	*Aeromonas hydrophila* DSM 30019	HM007582	99

Sequel to Table A 2.

e⁻-acceptor[a]	Number[b]	Source[c]	Next related species[d]	Accession number	Similarity (%)[e]
N_2O	621	*AC*	*Paenibacillus caespitis* LMG 23879T	AM745263	99
N_2O	623	*AC*	*Bacillus boroniphilus* PL68	GU001897	99
N_2O	ISO 4	*AC*	*Pantoea agglomerans* HDDMN03	EU879089	99.0*
N_2O	701	*LT*	*Bacillus drentensis* WN575	DQ275176	99
N_2O	702	*LT*	*Bacillus weihenstephanensis* HY3	FJ390462	99
N_2O	703	*LT*	*Bacillus drentensis* WN575	DQ275176	99
N_2O	704	*LT*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	705	*LT*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	706	*LT*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	707	*LT*	*Bacillus weihenstephanensis* HY3	FJ390462	99
N_2O	708	*LT*	*Bacillus drentensis* WN575	DQ275176	99
N_2O	709	*LT*	*Aeromonas encheleia* E195	AJ458416	99
N_2O	711	*LT*	*Bacillus boroniphilus* PL68	GU001897	99
N_2O	713	*LT*	*Erwinia billingiae* Eb661	FP236843	99
N_2O	715	*LT*	*Aeromonas molluscorum* 869N	AY532692	99
N_2O	716	*LT*	*Aeromonas hydrophila* DSM 30019	HM007582	99
N_2O	717	*LT*	*Bosea thiooxidans* As5-4b	FN392632	99
N_2O	718	*LT*	*Bacillus drentensis* WN575	DQ275176	99
N_2O	719	*LT*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	721	*LT*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	722	*LT*	*Flexibacter canadensis* IFO 15130	AB078046	100
N_2O	723	*LT*	*Pseudomonas fluorescens* PTA-268	AM293678	98
N_2O	725	*LT*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	726	*LT*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	A93	*LT*	*Paenibacillus borealis* RFNB5	FJ266316	98
N_2O	A94	*LT*	*Aminobacter aminovorans* A27	AM285009	100
N_2O	A105	*LT*	*Pseudomonas fluorescens* PTA-268	AM293678	100
N_2O	A110	*LT*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	A118	*LT*	*Bosea thiooxidans* As5-4b	FN392632	99
N_2O	A124	*LT*	*Pseudomonas migulae* PD 17	DQ377758	100
N_2O	801	*OL*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	802	*OL*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	803	*OL*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	804	*OL*	*Paenibacillus riograndensis* SBR5	EU257201	98
N_2O	806	*OL*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	807	*OL*	*Paenibacillus xylanexedens* B22a	EU558281	100
N_2O	808	*OL*	*Mesorhizobium chacoense* PR5	AJ278249	99
N_2O	809	*OL*	*Paenibacillus ginsengisoli* ES_MS40c	EU888522	99
N_2O	811	*OL*	*Paenibacillus xylanexedens* B22a	EU558281	100
N_2O	813	*OL*	*Aeromonas encheleia* E195	AJ458416	99
N_2O	814	*OL*	*Aeromonas encheleia* E195	AJ458416	100
N_2O	815	*OL*	*Paenibacillus graminis* 801	FJ544322	99
N_2O	816	*OL*	*Bacillus boroniphilus* D7028	FJ161333	99
N_2O	818	*OL*	*Paenibacillus graminis* 801	FJ544322	99
N_2O	819	*OL*	*Ensifer adhaerens* LC04	EU928872	100
N_2O	820	*OL*	*Bacillus drentensis* WN575	DQ275176	100
N_2O	821	*OL*	*Bacillus drentensis* WN575	DQ275176	100

Sequel to Table A 2.

e^--acceptor[a]	Number[b]	Source[c]	Next related species[d]	Accession number	Similarity (%)[e]
N_2O	822	*OL*	*Pseudomonas syringae* pv. *porri* P55	FN554248	99
N_2O	823	*OL*	*Bacillus drentensis* +Y73	JX067900	100*
N_2O	824	*OL*	*Flavobacterium frigidimaris* KUC-1	AB183888	98

[a] NO_2^- or N_2O was added as electron acceptor to isolate denitrifiers instead of dissimilatory nitrate reducers. Carbon sources consisted of fermentation products typically detectable in the earthworm gut. See methods part (2.3.2.1) for detailed information.

[b] isolate number as used during isolation process.

[c] Earthworm species of which the diluted gut content was used as inoculum for isolation. *LR*, *L. rubellus*; *AC*, *A. caliginosa*; *LT*, *L. terrestris*; *OL*, *O. lacteum*.

[d] next related species, its accession number, and similarity of 16S rRNA gene fragment as determined by BLAST search.

[e] similarity of the 16S rRNA gene fragment of an isolate to that of the next related species based on a fragment size of ca. 600 to 800 bp; *, results are based on a 16S rRNA gene fragment of ca. 1100 to 1400 bp.

Table A 3. Sequences of *nirK* and the corresponding 16S rRNA gene sequences derived from public databases.

	Accession numbers	
Species	*nirK*	16S rRNA gene
Acidovorax caeni R-24613	AM230881	AM084007
Acidovorax caeni R-24614	AM230882	AM084008
Acidovorax sp. R-25052	AM230883	AM084039
Acidovorax sp. R-25075	AM230843	AM084109
Acidovorax sp. R-25076	AM230844	AM084035
Afipia sp. 4AS1	GQ404514	FJ851428
Agrobacterium tumefaciens C58	NC_003063	NC_003063
Alcaligenes sp. CJANPY1 (A-II)	EF202175	EF205260
Alcaligenes sp. ESPY2 (A-III)	EF202174	EF205261
Alcanivorax dieselolei N1203	AB453733	AB453731
Blastobacter denitrificans IFAM 1005	AJ224906	AF338176
Bosea sp. MF18	EF363545	EF219051
Bradyrhizobium japonicum USDA 110	NC_004463	AF363150
Bradyrhizobium sp. BTAi1	NC_009485	NC_009485
Bradyrhizobium sp. ORS278	NC_009445	AF239255
Brucella abortus 2308 A	NZ_ACOR01000007	NZ_ACOR01000003
Brucella canis ATCC 23365	NC_010104	NC_010104
Brucella ceti Cudo	NZ_ACJD01000006	NZ_ACJD01000006
Brucella melitensis ATCC 23457	NC_012442	NC_012442
Brucella microti CCM 4915	NC_013118	NC_013118
Brucella ovis ATCC 25840	NC_009504	NC_009504
Brucella suis ATCC 23445	NC_010167	NC_010167
Cardiobacterium hominis ATCC 15826	NZ_ACKY01000036	NZ_ACKY01000036
Castellaniella sp. ROi28	EF363542	EF219044
Devosia sp. GSM-205	FN600574	FN600566
Enterococcus sp. R-25205	AM230873	AM084029
Mesorhizobium sp. 4FB11	AY078254	AF229877
Mesorhizobium sp. TSA37	AB542297	AB542413
Mesorhizobium sp. TSA41b	AB542300	AB542398
Mesorhizobium sp. TSA41s	AB542299	AB542414
Nitrosomonas sp. C-56	AF339044	M96400
Ochrobactrum anthropi ATCC 49188	CP000758	NC_009667
Ochrobactrum anthropi YX0703	GU207402	FJ873801
Ochrobactrum intermedium LMG 3301	NZ_ACQA01000001	NZ_ACQA01000001
Ochrobactrum sp. 2FB10	AY078249	AF229865
Ochrobactrum sp. 3CB4	AY078250	AF229883

Sequel to Table A 3.

Species	Accession numbers	
	nirK	16S rRNA gene
Ochrobactrum sp. 3CB5	AY078251	AF229884
Ochrobactrum sp. 4FB13	AY078252	AF229879
Ochrobactrum sp. R-24291	AM230826	AM231053
Ochrobactrum sp. R-24343	AM230828	AM231054
Ochrobactrum sp. R-24618	AM230812	AM084042
Ochrobactrum sp. R-24638	AM230816	AM084005
Ochrobactrum sp. R-24653	AM230815	AM084004
Ochrobactrum sp. R-25055	AM230884	AM084018
Ochrobactrum sp. R-26465	AM230839	AM231060
Paracoccus sp. R-24650	AM230830	AM084045
Paracoccus sp. R-24652	AM230818	AM083998
Paracoccus sp. R-25058	AM230885	AM084019
Pseudomonas chlororaphis subsp. *aureofaciens* ATCC 13985	Z21945	AF094722
Pseudomonas fluorescens Pf-5	NC_004129	NC_004129
Pseudomonas sp. R-24261	AM230874	AM231055
Pseudomonas sp. R-24609	AM230878	AM084013
Pseudomonas sp. R-25208	AM230838	AM084017
Pusillimonas sp. I-Bh37-12	FN555530	FN555411
Pusillimonas sp. I-Bh37-5	FN555529	FN555410
Rhizobium etli CFN 42	NC_007766	NC_007761
Rhizobium sp. NGR234	NC_012587	NC_012587
Rhizobium sp. PY13	DQ096645	DQ096643
Rhizobium sp. R-24658	AM230834	AM084043
Rhizobium sp. R-24663	AM230832	AM083999
Rhizobium sp. R-26467	AM230836	AM231056
Rhizobium sp. R-31549	AM403562	AM403621
Rhodopseudomonas palustris TIE-1[a]	NC_011004	NC_011004
Rhodopseudomonas palustris TIE-1	NC_011004	NC_011004
Rhodopseudomonas sp. 2-8	GU332847	GU332846
Silicibacter sp. TrichCH4B	NZ_GG703520	NZ_ACNZ01000059
Sinorhizobium medicae WSM419	NC_009621	CP000738
Sinorhizobium meliloti 1021	NC_003037	AL591688
Sinorhizobium sp. I-Bh25-4	FN555528	FN555404
Sinorhizobium sp. R-24605	AM230817	AM084000
Sinorhizobium sp. R-25067	AM230840	AM084031

Sequel to Table A 3.

	Accession numbers	
Species	*nirK*	16S rRNA gene
Sinorhizobium sp. R-25078	AM230841	AM084032
Staphylococcus sp. R-25050	AM230837	AM084016
Starkeya novella DSM 506	CP002026	CP002026

[a] this species possesses two distinct *nirK* sequences.

Table A 4. Sequences of *nirS* and the corresponding 16S rRNA gene sequences derived from public databases.

	Accession numbers	
Species	*nirS*	16S rRNA gene
Achromobacter sp. DBTN3	GU122964	GQ214399
Acidovorax sp. R-25212	AM230905	AM084022
Alicycliphilus sp. R-24604	AM230888	AM084015
Alicycliphilus sp. R-24611	AM230896	AM084014
Aromatoleum aromaticum EbN1	NC_006513	NC_006513
Arthrobacter sp. TSA68	AB542303	AB542420
Azoarcus tolulyticus 2FB6	AY078272	AF229861
Azospirillum sp. TSA19	AB542308	AB542385
Bacillus sp. TSA4w	AB542306	AB542372
Bordetella petrii DSM 12804	NC_010170	NC_010170
Bradyrhizobium sp. TSA1	AB542304	AB542368
Bradyrhizobium sp. TSA26	AB542313	AB542389
Bradyrhizobium sp. TSA27b	AB542314	AB542390
Bradyrhizobium sp. TSA27s	AB542315	AB542391
Bradyrhizobium sp. TSA43	AB542321	AB542399
Bradyrhizobium sp. TSA44	AB542322	AB542400
Comamonas sp. R-25066	AM230897	AM084024
Cupriavidus metallidurans CH34	CP000352	CP000352
Cupriavidus sp. TSA25	AB542312	AB542388
Cupriavidus sp. TSA35b	AB542319	AB542394
Cupriavidus sp. TSA49	AB542325	AB542403
Cupriavidus sp. TSA5	AB542307	AB542373
Dechloromonas sp. R-28400	AM230913	AM084133
Dechlorospirillum sp. I-Bh37-22	FN555562	FN555412
Dinoroseobacter shibae DFL 12	NC_009952	NC_009952
Halomonas campisalis ATCC 700597	FJ686151	NR_028702
Halomonas cerina 15CR	GQ384046	EF613111
Halomonas cerina R53	GQ384052	EF613110
Halomonas denitrificans Al13	GQ384047	EU541350
Halomonas desiderata DSM 9502	FJ686153	NR_026274
Halomonas sp. 4CR	GQ384045	GQ384061
Halomonas sp. C8	GQ384048	GQ384062
Halomonas sp. F15	GQ384053	GQ384063
Halomonas sp. HGD1	GQ384049	GQ384064
Halomonas sp. HGDK1	GQ384050	GQ384066
Halomonas sp. N64	GQ384051	GQ384065

Sequel to Table A 4.

	Accession numbers	
Species	*nirS*	16S rRNA gene
Herbaspirillum sp. I-Bh15-17	FN555558	FN555399
Herbaspirillum sp. TSA20w	AB542309	AB542410
Herbaspirillum sp. TSA21	AB542310	AB542386
Herbaspirillum sp. TSA29	AB542316	AB542392
Herbaspirillum sp. TSA31	AB542318	AB542393
Herbaspirillum sp. TSA46	AB542323	AB542401
Herbaspirillum sp. TSA50y	AB542326	AB542404
Herbaspirillum sp. TSA51	AB542327	AB542405
Herbaspirillum sp. TSA53b	AB542328	AB542406
Herbaspirillum sp. TSA54	AB542329	AB542407
Herbaspirillum sp. TSA57y	AB542330	AB542408
Herbaspirillum sp. TSA63y	AB542333	AB542412
Herbaspirillum sp. TSA65	AB542334	AB542417
Hydrogenobacter thermophilus TK-6	NC_013799	NC_013799
Paracoccus sp. I-Bh37-1	FN555561	FN555408
Paracoccus sp. R-24615	AM230906	AM084001
Paracoccus sp. R-24616	AM230901	AM084041
Paracoccus sp. R-24617	AM230900	AM084023
Paracoccus sp. R-24665	AM230903	AM084107
Paracoccus sp. R-26466	AM230902	AM231059
Pseudomonas aeruginosa ATCC 10145	DQ386157	AF094713
Pseudomonas aeruginosa LESB58	NC_011770	NC_011770
Pseudomonas aeruginosa PA7	NC_009656	NC_009656
Pseudomonas aeruginosa PAO1	NC_002516	NC_002516
Pseudomonas aeruginosa DBT1BNH3	FJ976652	FJ976651
Pseudomonas aeruginosa UCBPP-PA14	NC_008463	NC_008463
Pseudomonas grimontii PD 10	DQ518192	DQ377751
Pseudomonas grimontii PD 9	DQ518191	DQ377750
Pseudomonas lini PD 15	DQ518194	DQ377756
Pseudomonas lini PD 28	DQ518188	DQ377769
Pseudomonas mandelii PD 8	DQ518190	DQ377749
Pseudomonas migulae PD 1	DQ518189	DQ377742
Pseudomonas migulae PD 17	DQ518195	DQ377758
Pseudomonas qianpuensis C10-2	DQ088665	DQ088664
Pseudomonas sp. I-Bh25-14	FN555560	FN555406
Pseudomonas sp. I-Bh4-8	FN555557	FN555395
Pseudomonas sp. PD 13	DQ518193	DQ377754

Sequel to Table A 4.

	Accession numbers	
Species	*nirS*	16S rRNA gene
Pseudomonas sp. PD 21	DQ518196	DQ377762
Pseudomonas sp. PD 22	DQ518186	DQ377763
Pseudomonas sp. PD 26	DQ518187	DQ377767
Pseudomonas sp. PD 6	DQ518185	DQ377747
Pseudomonas stutzeri A1501	NC_009434	NC_009434
Pseudovibrio sp. JE062	NZ_DS996807	ABXL01000006
Ralstonia eutropha H16	NC_008314	NC_008314
Rhodothermus marinus DSM 4252	CP001807	CP001807
Roseobacter denitrificans OCh 114	NC_008209	NC_008209
Ruegeria pomeroyi DSS-3	NC_006569	NC_003911
Sideroxydans lithotrophicus ES-1	CP001965	DQ386264
Thauera aromatica 3CB3	AY078259	AF229882
Thauera aromatica T1	AY078257	U95176
Thauera chlorobenzoica 4FB1	AY078262	AF229867
Thauera chlorobenzoica 4FB2	AY078263	AF229868
Thauera selenatis AX	AY078264	X68491
Thauera sp. 27[a]	AY838762	AY838760
Thauera sp. 27	AY838759	AY838760
Thauera sp. Q20-C	GU566032	EU850614
Thauera sp. R-25071	AM230899	AM084033
Thauera sp. TGOPY13 (T-I)	EF204941	EF205255
Thauera terpenica 21Mol	AY078267	AJ005818
Thiohalomonas denitrificans HLD 2T	AM492191	EF117909

[a] this species possesses two distinct *nirS* sequences.