Abundance and activity of nitrate reducers in an arable soil are more affected by temporal variation and soil depth than by elevated atmospheric [CO₂]

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Abstract

Elevated atmospheric carbon dioxide concentrations ([CO₂]) might change the abundance and the function of soil microorganisms in the depth profile of agricultural soils by plant-mediated reactions. The seasonal pattern of abundance and activity of nitrate-reducing bacteria was studied in a Mini-FACE experiment planted with oilseed rape (Brassica napus). Three depths (0–10, 10–20 and 20–30 cm) were sampled. Analyses of the abundances of total (16S rRNA gene) and nitrate-reducing bacteria (narG, napA) revealed strong influences of sampling date and depth, but no [CO₂] effects. Abundance and activity of nitrate reducers were higher in the top soil layer and decreased with depth but were not related to extractable amounts of nitrogen and carbon in soil. Dry periods reduced abundances of total and nitrate-reducing bacteria, whereas the potential activity of the nitrate reductase enzyme was not affected. Enzyme activity was only weakly correlated to the abundance of nitrate-reducing bacteria but was related to NH₄⁺ and NO₃⁻ concentrations. Our results suggest that in contrast to the observed pronounced seasonal changes, the elevation of atmospheric [CO₂] has only a marginal impact on nitrate reducers in the investigated arable ecosystem.

Introduction

The progressive increase in carbon dioxide (CO₂) in the atmosphere is estimated to result in CO₂ concentrations ([CO₂]) > 500 μmol mol⁻¹, within the 21st century (IPCC, 2007). Elevated [CO₂] can directly influence plant performance, which in turn can influence soil microorganisms (Jossi et al., 2006; Drigo et al., 2009) through, for example, changes in the quality and the quantity of plant exudates, increased soil moisture due to higher plant water use efficiency (Ainsworth & Long, 2005; Drigo et al., 2008), or enhanced plant growth. Enhanced growth results in greater demand by plants for nutrients, leading to increased competition with soil microorganisms for nutrient resources such as nitrogen (N) (Hu et al., 1999). Contradictory results have been reported on the impact of elevated [CO₂] on soil microbial communities involved in N-cycling (e.g. review by O’Neill, 1994; Freeman et al., 2004; Drigo et al., 2008; French et al., 2009). N₂O production and activity of denitrifying microorganisms in soil were shown to be stimulated by increased belowground allocation by Lolium perenne under elevated [CO₂] (Ineson et al., 1998; Baggs et al., 2003; Baggs & Blum, 2004). Under conditions favourable to denitrification, i.e. high soil moisture and available N, N₂O efflux was increased in a mixed stand of Trifolium and Phleum by elevated [CO₂] (Kettunen et al., 2007). Denitrifier enzyme activity (DEA) was also higher under elevated [CO₂] in the wheat rhizosphere (Smart et al., 1997). Contrary to this, DEA was lower under elevated [CO₂] in microcosms planted with Holcus lanatus (Barnard et al., 2005a). Barnard et al. (2004), however, found no effects of elevated [CO₂] on DEA or on nitrifier enzyme activity (NEA) in three of four different grassland ecosystems, whereas DEA was decreased by elevated [CO₂] at the fourth site. These discrepancies in the effects of elevated [CO₂] on N₂O fluxes, NEA, and DEA are assumed to derive from different N availabilities in the...
different studies, and Barnard et al. (2005b) suggested that the effects of N fertilization and deposition on N cycling were stronger than that of elevated \([\text{CO}_2]\). A few studies also investigated the effect of elevated \([\text{CO}_2]\) on the abundances or the structure of N-cycling bacterial communities. In one of the studies, the number of cultivable root-associated nitrate-dissimilating *Pseudomonas* species was increased by elevated \([\text{CO}_2]\) both in high and low N-fertilized swards (Fromin et al., 2005). However, neither the community structure of nitrate-reducing bacteria in the rhizosphere of *L. perenne* and *Trifolium repens* (Deiglmayr et al., 2004) nor the diversity of nitrate-reducing *Pseudomonas* strains has been found to be affected by elevated \([\text{CO}_2]\) (Roussel-Delf et al., 2005). Similarly, the abundance of denitrifying bacteria was not affected by elevated \([\text{CO}_2]\) in the rhizosphere of *Phaseolus vulgaris* L. (Haase et al., 2008).

Because the elevation of \([\text{CO}_2]\) changes the spatial and temporal patterns of water uptake by roots, it is likely that the \([\text{CO}_2]\) effect on soil microorganisms varies according to sampling time and soil depth. Moore and Field (2006) reported that a *Hemizonia congesta* monoculture grown under elevated \([\text{CO}_2]\) used water from deeper soil layers and major water uptake was recorded 2 weeks earlier in the season than under ambient \([\text{CO}_2]\). Billings et al. (2002) also highlighted the seasonal variation of the elevated \([\text{CO}_2]\) effect on microbial activity in soil. Also, the influence of elevated \([\text{CO}_2]\) on nitrate reductase activity was found to vary between seasons (Deiglmayr et al., 2004). However, knowledge of the effects of elevated \([\text{CO}_2]\) on N-cycling microorganisms over the plant growing season or at different soil depths is still limited despite its potential importance for future management of fertilization and irrigation of arable soils.

The aim of the present study was to determine the effect of elevated \([\text{CO}_2]\) on the abundance and activity of an N-cycling community at different soil depths during the vegetation period of spring oilseed rape (*Brassica napus*). For this purpose, we investigated nitrate reducers as a model community. Nitrate reducers perform the first step of denitrification and of the dissimilatory reduction of nitrate to ammonium (DNRA) (Moreno-Vivián et al., 1999; Philippot & Højberg, 1999). Nitrate reduction is a step within the N-cycle that has economic and environmental consequences. The resulting nitrite can be further reduced by denitrification to N\(_2\)O or N\(_2\) gases, leading to losses of soluble N, a nutrient often limiting for plant growth in agriculture and under elevated \([\text{CO}_2]\). On the other hand, in the process of DNRA, nitrate is reduced to ammonium, a relatively immobile N form less sensitive to leaching and still assimilable by plants.

Here, we monitored the abundance and the activity of nitrate-reducing bacteria at three different soil depths (0–10, 10–20 and 20–30 cm) under ambient and elevated \([\text{CO}_2]\) between June and August 2007. Nitrate reducer abundance was estimated by quantification of the functional genes *narG* and *napA* and nitrate reductor activity by colorimetric measurements of nitrate reductase activity. By concomitant determination of NO\(_3^–\) concentrations, carbon (C) availability and soil moisture, we also examined the relationships between the measured environmental variables and nitrate reducer abundance and activity.

**Materials and methods**

**Study site**

The field experiment was performed in a Mini-FACE system located on an arable field near Hohenheim (Baden-Württemberg, Germany) (Erbs & Fangmeier, 2006). The soil is a slightly stagnic luvisol (9% sand, 69% silt, 22% clay; pH 6.8; C\(_{\text{org}}\) 15.5 g kg\(^{-1}\) soil dry weight in the top 10 cm of the soil). The Mini-FACE was established in spring 2002 and consists of 2-m-diameter plots with elevated atmospheric \([\text{CO}_2]\) (~540 µmol mol\(^{-1}\) CO\(_2\)) and ambient \([\text{CO}_2]\). Each treatment was replicated five times. The soil was tilled in spring 2007 before oil seed rape (*B. napus* L. cv. Campino) was seeded on 17 April (for details, see Franzaring et al., 2008). Plants were thinned to a final density of 70 plants m\(^{-2}\) on 31 May [45 days after sowing (DAS)]. Plots were fertilized at three application times before flowering (22 May, 6 June and 13 June) with a total of 130, 60, 120, 18 and 30 kg ha\(^{-1}\) of N, P, K, Mg and S applied, respectively. The plots were watered manually on demand, using the same volume for each of the plots. Mature plants were harvested on 21 August (127 DAS). Soil moisture and temperature were continuously recorded at 15 cm soil depth in the Mini-FACE experiment (Franzaring et al., 2008, Fig. 1).

**Soil sampling**

Soil samples were collected from each of the five replicates for each treatment every second week during the oil seed rape growing season on six days: June 4, June 18, July 2, July 16, July 30 and August 13. Samples were taken from three soil depths (0–10, 10–20 and 20–30 cm) per plot by soil auger. Resulting holes in the soil were filled by fitting polyvinyl chloride sticks to avoid preferential water flow and aeration of deeper soil layers. Due to an underlying stone layer, no samples could be taken in some plots for the 20–30 cm soil layer. Soil samples were cooled immediately after sampling and stored at −25 °C. Before further analyses, soil samples were thawed at 4 °C and sieved at 2 mm to exclude roots, stones and larger soil animals. Gravimetric soil water content was determined by drying aliquots at 105 °C for 24 h.
Soil extractable organic carbon (EOC), extractable nitrogen (EN), ammonium and nitrate

The fraction of soil organic carbon (EOC) and nitrogen (EN) extractable with 0.5 M K2SO4 was quantified in soil suspensions (soil fresh weight to extractant ratio of 1 : 4 w/v). Suspensions were shaken on a horizontal shaker for 30 min at 250 r.p.m. Soil particles < 0.1 mm, including the clay fraction, were separated by centrifugation (30 min at 4420 g). The clear supernatant was diluted 1 : 3 with distilled water, and EOC and EN concentrations were measured on a Dimatoc 100 DOC/TN-analyzer (Dimatec, Essen, Germany).

Nitrate (NO3−) and ammonium (NH4+) were extracted with 1 M KCl from the soil samples (soil to extractant ratio of 1 : 4 w/v). Soil suspensions were shaken on a horizontal shaker for 30 min at 250 r.p.m. and centrifuged afterwards (30 min at 4420 g). Concentrations of NO3− and NH4+ in extracts were measured with an autoanalyzer (Bran & Luebbe, Norderstedt, Germany).

DNA extraction

DNA was extracted from 0.25 g of soil (fresh weight) with the MoBIO PowerSoil DNA Kit (MoBIO Laboratories, Carlsbad, CA), according to the protocol of the manufacturer and redissolved in 100 μL H2O. The quantity of the DNA extracted was measured with a Nanodrop ND1000 (NanoDrop Technologies Inc., Wilmington).

Quantitative PCR assay

Quantification of the nitrate reducers was performed using the narG and napA genes encoding the catalytic subunits of the membrane-bound and periplasmic nitrate reductases, respectively, as molecular markers (Philippot, 2002). Amplification of quantitative PCR products was carried out with an ABI Prism 7900 (Applied Biosystems) using SYBR green as the detection system. The real-time PCR assay was performed in a 20 μL reaction volume containing the SYBR green PCR mastermix (Absolute QPCR SYBR Green Rox, Thermo, France), 1 μM of each primer, 500 ng of T4gp32 (Q-BIOgene, France) and 20 ng of soil DNA. Primer sequences and thermal-cycling conditions for the 16S rRNA gene [total bacteria (López-Gutiérrez et al., 2004)] and the narG and napA functional genes are listed in Table 1 (Bru et al., 2007). Standard curves were obtained with serial plasmid dilutions of a known amount of plasmid DNA containing a fragment of the 16S rRNA gene or the narG and napA genes (López-Gutiérrez et al., 2004; Henry et al., 2006; Bru et al., 2007). PCR efficiency for the different assays ranged between 82% and 98%. Tests for the potential presence of PCR inhibitors in DNA extracted from soil were performed by mixing soil DNA extracts with a known amount of standard DNA before qPCR. In all cases, no inhibition was detected. Methodological evaluation of the qPCR assays showed a good reproducibility of 95.6%.

Potential nitrate reductase activity

The potential nitrate reductase activity was determined by anaerobic incubation of soil following a modified protocol.
(Abdelmagid & Tabatabai, 1987; Kandeler, 1996). Briefly, 0.2 g of soil was weighed in five replicates into 2.0 mL reaction tubes. To inhibit nitrite reduction, 2,4-dinitrophenol was added in a concentration of 167 μg g⁻¹ soil (fresh weight). After 24 h incubation in 1 mM KNO₃ in a total volume of 1 mL at 25 °C in the dark, the soil mixture was extracted with 4 M KCl and centrifuged for 1 min at 1400 g. The accumulated nitrite in the supernatant was determined by colorimetric reaction.

**Statistical analysis**

Soil water content, the concentrations of NH₄⁺, NO₃⁻, EOC, EN, total DNA yield and copy numbers of 16S rRNA gene, narG and napA genes were calculated on an oven-dry soil weight (g⁻¹ soil DW) basis. Soil samples were taken with soil corers, and samples from deeper layers were therefore considered to be influenced by the upper soil layers. To account for this dependence, one-way ANOVA with soil samples of the different depths (depths: 0–10, 10–20 and 20–30 cm) treated as repeated measures were performed. With this approach, differences according to the factor CO₂ treatment ([CO₂], ambient and elevated) were tested separately for each sampling date. To evaluate the effect of sampling date, an additional one-way ANOVA with the factor CO₂ treatment was performed for every soil depth, and the CO₂ treatment ([CO₂], ambient and elevated) were tested separately for each sampling date. To evaluate the effect of sampling date, an additional one-way ANOVA with the factor CO₂ treatment was performed for every soil depth, and the six sampling dates (Date) were now treated as repeated measures. Before analysis, data were checked for homogeneity of variance (Levene’s test) and log-transformed if required. Correlation analyses were performed to determine inter-relationships between measured parameters. Multiple regression analysis was applied to evaluate the relationship between soil physicochemical factors (soil water content, NH₄⁺, NO₃⁻, EOC, EN) and DNA yield, copy numbers of 16S rRNA gene, narG and napA genes and nitrate reductase activity. All statistical analyses were performed using the STATISTICA 6.0 software package (Statsoft, Tulsa, OK).

**Results**

**Soil temperature and moisture**

On the sampling dates, soil temperature at 15 cm depth varied between 14.0 and 21.8 °C with the lowest temperatures on July 2 and the highest at the July 16 sampling (Fig. 1). Average soil temperatures were found to be slightly lower under elevated [CO₂], but this was not significant because of high variation between plots. The specific temporal pattern of volumetric soil moisture measured by TDR probes at 15 cm depth in the field experiment (Fig. 1) was well reflected by gravimetric soil water content measurements in the collected soil samples (Table 2). Between the end of June and the beginning of August soil moisture tended to be higher under elevated [CO₂] (Fig. 1), and the gravimetric soil water content was significantly greater on August 13 (Table 2). With the exception of the July 16 sampling, soil samples from the top layers showed on average higher water content than samples from the lower soil layers (Table 2). The water content of the soil samples differed significantly between the different sampling dates for all three depths (Date; \( P < 0.001 \)), with samples from June 4 and 18 and again those from the last sampling (August 13) showing higher water content than samples from the July date (Table 2).

**EOC, EN, ammonium and nitrate**

EOC was not affected by elevated [CO₂] and was not significantly different between the three soil layers (Table 2). In the 0–10 cm depth, EOC was significantly higher on the first and last sampling dates (Date; \( P = 0.02 \)). EOC in the 10–20 cm depth was also affected by sampling date (Date; \( P = 0.004 \)), but no clear trend was observed. EN was affected neither by an elevated [CO₂] nor by the sampling date (Table 2). EN amounts decreased significantly with soil depth on June 4 and August 13.

At the beginning of June, NO₃⁻ concentration in soil tended to be reduced under elevated [CO₂] (\( P = 0.078 \); Table 2).

**Table 1.** Primer sequences and thermal profiles for quantification of the 16S rRNA gene, narG and napA genes

<table>
<thead>
<tr>
<th>Primer target</th>
<th>Sequence (5'-3')</th>
<th>Thermal conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S 341F</td>
<td>CCT ACG GGA GGC AGC AG</td>
<td>1 cycle, 15 min at 95 °C</td>
</tr>
<tr>
<td>16S 534R</td>
<td>ATT ACC GCG GCT GCT GGC A</td>
<td>40 cycles, 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, 30 s at 80 °C</td>
</tr>
<tr>
<td>(López-Gutiérrez et al., 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>narG G-F</td>
<td>TCG CCS ATY CCG GCS ATG TC</td>
<td>1 cycle, 15 min at 95 °C</td>
</tr>
<tr>
<td>narG G-R</td>
<td>GAG TTG TAC CAG TCR GCS GAY TCS G</td>
<td>6 cycles, 15 s at 95 °C, 30 s at 63 °C, touchdown of –1 °C by cycle</td>
</tr>
<tr>
<td>(Bru et al., 2007)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>napA 3F</td>
<td>TGG ACV ATG GGY TTY AAY C</td>
<td>1 cycle, 15 min at 95 °C</td>
</tr>
<tr>
<td>napA 4R</td>
<td>ACY TCR CGH GCV GTR CCR CA</td>
<td>6 cycles, 15 s at 95 °C, 30 s at 63 °C, touchdown of –1 °C by cycle</td>
</tr>
<tr>
<td>(Bru et al., 2007)</td>
<td></td>
<td></td>
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</tbody>
</table>

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Table 2. Soil water content, EOC and EN, soil ammonium and nitrate concentrations, nitrate reductase activity and total extractable DNA (DNA yield) on six sampling dates during the growing season of spring oilseed rape in the Mini-FACE experiment under ambient (Amb) and elevated (Elev) atmospheric CO₂ concentrations at three different soil depths (0–10, 10–20 and 20–30 cm).

<table>
<thead>
<tr>
<th>Soil water content (%)DW</th>
<th>June 4</th>
<th>June 18</th>
<th>July 2</th>
<th>July 16</th>
<th>July 30</th>
<th>August 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elev 0–10 cm</td>
<td>26.46 (0.48)</td>
<td>26.66 (1.26)</td>
<td>16.36 (1.08)</td>
<td>12.67 (0.24)</td>
<td>17.76 (1.57)</td>
<td>24.75 (0.93)</td>
</tr>
<tr>
<td>Elev 10–20 cm</td>
<td>26.35 (0.74)</td>
<td>24.08 (1.29)</td>
<td>15.09 (0.51)</td>
<td>14.10 (0.68)</td>
<td>13.02 (0.95)</td>
<td>20.69 (1.32)</td>
</tr>
<tr>
<td>Elev 20–30 cm</td>
<td>23.68 (0.48)</td>
<td>19.64 (0.87)</td>
<td>15.40 (1.15)</td>
<td>14.26 (0.20)</td>
<td>12.34 (0.88)</td>
<td>14.50 (0.36)</td>
</tr>
<tr>
<td>Elev 0–10 cm</td>
<td>26.32 (1.09)</td>
<td>26.14 (0.97)</td>
<td>17.05 (0.83)</td>
<td>12.61 (1.18)</td>
<td>18.21 (0.44)</td>
<td>24.26 (0.81)</td>
</tr>
<tr>
<td>Elev 10–20 cm</td>
<td>25.33 (0.82)</td>
<td>22.87 (0.66)</td>
<td>15.68 (0.59)</td>
<td>15.15 (1.26)</td>
<td>13.23 (0.81)</td>
<td>23.14 (0.36)</td>
</tr>
<tr>
<td>Elev 20–30 cm</td>
<td>23.25 (1.02)</td>
<td>21.25 (0.30)</td>
<td>14.62 (1.33)</td>
<td>15.51 (0.69)</td>
<td>12.18 (1.79)</td>
<td>18.80 (1.59)</td>
</tr>
<tr>
<td>Elev 0–10 cm</td>
<td>95.16 (7.51)</td>
<td>76.81 (7.38)</td>
<td>86.59 (6.10)</td>
<td>78.24 (3.51)</td>
<td>87.79 (4.46)</td>
<td>110.05 (12.57)</td>
</tr>
<tr>
<td>Elev 10–20 cm</td>
<td>103.01 (9.74)</td>
<td>74.01 (6.06)</td>
<td>91.51 (5.93)</td>
<td>67.86 (1.96)</td>
<td>78.24 (3.51)</td>
<td>94.85 (4.88)</td>
</tr>
<tr>
<td>Elev 20–30 cm</td>
<td>89.36 (13.63)</td>
<td>60.66 (15.58)</td>
<td>90.80 (6.77)</td>
<td>73.18 (7.95)</td>
<td>74.02 (3.38)</td>
<td>78.29 (5.17)</td>
</tr>
<tr>
<td>Amb 0–10 cm</td>
<td>106.11 (6.72)</td>
<td>86.83 (3.59)</td>
<td>95.80 (4.90)</td>
<td>91.87 (17.01)</td>
<td>88.55 (8.34)</td>
<td>89.58 (7.04)</td>
</tr>
<tr>
<td>Amb 10–20 cm</td>
<td>121.06 (12.72)</td>
<td>77.66 (20.57)</td>
<td>90.24 (12.72)</td>
<td>82.75 (17.04)</td>
<td>78.92 (13.72)</td>
<td>82.75 (17.04)</td>
</tr>
<tr>
<td>Amb 20–30 cm</td>
<td>7.57 (1.12)</td>
<td>7.62 (2.15)</td>
<td>9.12 (2.20)</td>
<td>9.25 (1.00)</td>
<td>12.52 (2.20)</td>
<td>30.96 (13.56)</td>
</tr>
<tr>
<td>Nitrate-N (µmol-N g⁻¹ soil)</td>
<td>5.63 (0.68)</td>
<td>6.23 (1.86)</td>
<td>3.26 (1.05)</td>
<td>6.84 (0.78)</td>
<td>5.94 (0.72)</td>
<td>9.17 (1.15)</td>
</tr>
<tr>
<td>Nitrate red. activity (µg N g⁻¹ soil Day⁻¹)</td>
<td>4.40 (0.44)</td>
<td>3.21 (0.46)</td>
<td>1.46 (0.13)</td>
<td>4.89 (0.50)</td>
<td>4.14 (0.86)</td>
<td>6.97 (1.06)</td>
</tr>
<tr>
<td>DNA yield (µg g⁻¹ soil)</td>
<td>1.27 (0.18)</td>
<td>1.09 (0.75)</td>
<td>3.80 (0.26)</td>
<td>1.38 (0.45)</td>
<td>1.88 (0.30)</td>
<td>1.06 (0.46)</td>
</tr>
<tr>
<td>Nitrate-N (µmol-N g⁻¹ soil)</td>
<td>2.26 (0.44)</td>
<td>3.34 (1.72)</td>
<td>1.75 (0.04)</td>
<td>3.01 (0.39)</td>
<td>1.71 (0.49)</td>
<td>4.61 (0.03)</td>
</tr>
<tr>
<td>Nitrate red. activity (µg N g⁻¹ soil Day⁻¹)</td>
<td>3.98 (0.94)</td>
<td>2.88 (0.52)</td>
<td>2.31 (0.78)</td>
<td>6.76 (0.66)</td>
<td>7.76 (1.37)</td>
<td>8.71 (1.84)</td>
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<td>Nitrate red. activity (µg N g⁻¹ soil Day⁻¹)</td>
<td>2.43 (1.13)</td>
<td>0.54 (0.31)</td>
<td>3.58 (0.24)</td>
<td>2.60 (1.51)</td>
<td>2.72 (0.29)</td>
<td>1.11 (0.43)</td>
</tr>
<tr>
<td>Nitrate-N (µmol-N g⁻¹ soil)</td>
<td>1.12 (0.45)</td>
<td>0.09 (0.08)</td>
<td>3.15 (0.28)</td>
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<td>3.15 (0.52)</td>
<td>2.25 (0.55)</td>
<td>3.26 (1.53)</td>
</tr>
</tbody>
</table>

Significance (ANOVA) of the fixed factors [CO₂] and soil depth (Depth) as repeated measures are indicated in the headline of each parameter separately for each sampling date. Means of three to five replicates with (SE).

*P < 0.1
*P < 0.05
**P < 0.01
***P < 0.001
NS, not significant.
During the following sampling dates, no [CO₂] effect was detectable. On June 2 and from July 2 on, nitrate concentrations were significantly lower in deeper soil layers. The date of sampling significantly influenced the nitrate concentration, especially in the top 10 cm of the soil, with values increasing over the last two sampling dates. On most of the dates, ammonium concentrations in soil were lower than nitrate concentrations but were affected neither by soil depth nor by elevated [CO₂] on June 4 and 18 (Table 2). On July 16 elevated [CO₂] tended to decrease ammonium concentration (P = 0.06). Soil depth differentially influenced NH₄⁺ in soil. On July 2 and 30 concentrations were higher in the upper soil, whereas on July 16 and August 13 they were higher in the deeper layers.

**DNA yield and abundance of total bacteria**

Total amounts of DNA extractable per gram soil (DNA yield) were not affected by [CO₂] but decreased with soil depth, being on average 17.5% and 22.3% lower at 10–20 and 20–30 cm depth than in the upper 10 cm, respectively (Table 2). DNA yield was also significantly affected by the sampling date; amounts were highest early in June, decreased during the following three dates and slightly increased again on July 30 and August 13.

Copy numbers of the 16S rRNA gene per gram soil decreased with depth and were significantly affected by sampling date similar to total DNA (Fig. 2a, Table 2). No effects of [CO₂] on 16S rRNA gene copy numbers were observed. The relative abundance of the 16S rRNA gene (related to total DNA) also decreased with soil depth, on average 13.4% and 15.7% lower in the 10–20 and 20–30 cm depth than in the upper 10 cm (Supporting Information, Table S1). Over the sampling period, the decrease in the 16S rRNA gene copy numbers, which was observed in July, exceeded that of total DNA, indicating a stronger effect of the sampling date on 16S RNA gene copy numbers than on total DNA (Fig. 2a; Table 2 and Table S1).

**Abundance of nitrate reducers**

The copy numbers of *narG* and *napA* genes per gram soil were lower than those of the 16S rRNA gene (Fig. 2a–c). Similar to total bacteria, the abundance of nitrate reducers estimated using both *narG* and *napA* gene copy numbers did not differ between ambient and elevated [CO₂]. Nitrate reducers were significantly affected by soil depth and followed a depth gradient with highest *narG* and *napA* gene copy numbers in the top 10 cm and decreasing numbers with increasing soil depth (Fig. 2b and c). During the period of investigation, the *narG* and *napA* gene copy numbers followed roughly the same temporal patterns as observed for the 16S rRNA gene with significant effects according to the day of sampling (Fig. 2b and c). However, on June 4, the decrease in gene copy numbers was more pronounced for *napA* than for *narG* on the sampling dates in July. In addition, the *napA/narG* ratio, used as an indicator for changes in composition of the nitrate reducer community, was also significantly lower during these samplings (Table S1) but again not affected by elevated [CO₂].

**Nitrate reductase activity**

Neither [CO₂] nor sampling date showed an influence on the potential nitrate reductase enzyme activity in soil. Nitrate reductase activity varied strongly between samples,
and this was most pronounced in the top 10 cm of the soil (Table 2). Enzyme activities significantly decreased with increasing soil depth on July 2, July 30 and on August 13. Compared with the upper 10 cm, average values for the 10–20 and 20–30 cm soil layers were lower by 46.0% and 64.5%, respectively.

**Inter-relations between measured parameters**

Soil temperature tended to be negatively correlated with soil moisture ($r = 0.238$, $P = 0.071$) and positively correlated with NO$_3$ ($r = 0.385$, $P = 0.002$). The correlations of 16S rRNA gene to narG ($r = 0.932$) and to napA ($r = 0.942$) as well as the relation between the two functional genes themselves ($r = 0.954$) were all found to be positive and highly significant ($P < 0.001$). These positive relationships also persisted when data were analysed separately for the different depths (data not shown). Because soil temperature was measured only at the 15 cm depth in the field, soil temperature data were not included in the multiple regression analysis. Results from multiple regression analysis of the environmental properties soil water content, NH$_4$$_4$, NO$_3$, EOC and EN concentrations as predictor variables showed positive and significant relations to the dependent variables DNA yield, copy numbers of 16S rRNA gene, narG and napA, but not to nitrate reductase activities (Table 3). The 16S rRNA gene was slightly positively correlated to NH$_4$$_4$ concentrations. Nitrate reductase activity was significantly and positively correlated with NH$_4$$_4$ and NO$_3$ concentrations in soil (Table 3). Nitrate reductase activity and both functional genes narG ($r = 0.245$, $P = 0.002$) and napA ($r = 0.302$, $P < 0.001$) were only weakly correlated.

**Table 3.** Results of multiple regression analyses, including soil moisture, NH$_4$, NO$_3$, TN and EOC as independent factors. All regression models were significant at $P < 0.0001$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Soil</th>
<th>Standardized coefficient ($\beta$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA yield</td>
<td></td>
<td>0.511*** -0.080 0.062 0.164 -0.005</td>
</tr>
<tr>
<td>16S rRNA gene</td>
<td></td>
<td>0.635*** 0.250* -0.037 0.080 -0.036</td>
</tr>
<tr>
<td>narG</td>
<td></td>
<td>0.640*** 0.126 0.011 0.110 -0.074</td>
</tr>
<tr>
<td>napA</td>
<td></td>
<td>0.666*** 0.210* 0.038 0.065 -0.062</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td></td>
<td>-0.008 0.412** 0.289** 0.048 0.063</td>
</tr>
</tbody>
</table>

Significance levels ($P$) of the multiple regression models are as follows: *$P < 0.1$, **$P < 0.05$, ***$P < 0.01$, ****$P < 0.001$.

**Discussion**

**Effects of elevated CO$_2$**

For the same Mini-FACE experiment and season (2007) plant biomass and environmental conditions were also determined, and significantly increased shoot biomass of *B. napus* as well as increased soil moisture were observed under elevated [CO$_2$] (Franzaring et al., 2008). We expected that the higher plant biomass under elevated [CO$_2$] was likely to result in higher C availability in soil due to increased rhizodeposition, which could provide additional electron donors here during the nitrate reduction process. Higher soil moisture due to higher water use efficiency of plants could therefore increase the number of microsites with low oxygen concentrations. Both are prerequisites for nitrate reduction and we therefore expected nitrate reducers to be favoured under elevated [CO$_2$]. However, in the present experiment, we found no effects of elevated [CO$_2$] on C availability in soil (EOC; Table 2) and no significant effect of [CO$_2$] on the abundance and activity of nitrate reducers during the six sampling dates. This contrasts with both the greater numbers of cultivable, root-associated nitrate-dissimilating bacteria belonging to the genus Pseudomonas (Fromin et al., 2005) and the enhanced frequencies of Pseudomonas species that were found in the rhizosphere of *L. perenne* in a grassland FACE experiment under elevated [CO$_2$] (Roussel-Delif et al., 2005).

In two related studies, Marhan et al. (2010) and Franzaring et al. (2010) reported that the increase in soil moisture in response to elevated CO$_2$ over several years at the same Mini-FACE experiment was detectable but small (+2.7 vol%). It is therefore likely that the effects of elevated [CO$_2$] on soil moisture in the investigated period (Table 2) were too small to result in differences in bacterial abundance and activity. In addition, the significant changes in soil moisture during the sampling period, which resulted in more pronounced changes in nitrate reducer abundances, might mask or change the effects of elevated [CO$_2$].

We hypothesized that the nitrate-reducing community would be affected by elevated [CO$_2$] due to a stronger competition for NO$_3$ between nitrate-reducing bacteria and faster-growing plants. However, the decrease in NO$_3$ availability under elevated [CO$_2$] was too small ($P < 0.1$; Table 2) to result in changes in activity or abundance of nitrate-reducing bacteria in soil.

In the present study, we also found that the napA to narG ratio was not different between ambient and elevated [CO$_2$]. Roussel-Delif et al. (2005) found contrasting results for Pseudomonas isolates, with isolates harbouring the narG gene being more abundant close to the root surface of plants under elevated [CO$_2$] than isolates with the napA gene. We did not analyse the narG- and napA-harbouring bacterial
community structure but because nitrate reducers possess either the membrane bound nitrate reductase encoded by narG or the periplasmic nitrate reductase encoded by napA or both types of nitrate reductases (Philippot & Højberg, 1999), our results suggest that, at least in the bulk soil, the effects of elevated \([\text{CO}_2]\) on the community structure of nitrate-reducing bacteria were insignificant.

**Effects of sampling time and soil depth**

The measured soil chemical, physical and biological properties (soil water content, \(\text{NO}_3^-\), \(\text{NH}_4^+\), EN, DNA yield, copy numbers of 16S rRNA gene, narG and napA) showed a decrease from the upper 10 cm to the 20–30 cm layer (Table 2, Fig. 2a–c). Depending on the sampling date, the abundance of total bacteria in the top layer of the arable soil ranged between \(1.7 \times 10^9\) and \(6.6 \times 10^9\) copies of 16S rRNA gene per gram soil (dry weight) (Fig. 2a). These numbers are within the range of those measured in soils of an arable long-term fertilization experiment (Hallin et al., 2009), and those of an arable soil treated with artificial root exudates (Henry et al., 2008), with both soils having similar C\(_{\text{org}}\) content as our soil. For spruce forest soils, total and nitrate-reducing bacteria were also found to be more abundant in the upper than in deeper soil layers (Mergel et al., 2001a; Kandeler et al., 2009). For the tilled arable soil horizon (0–30 cm) in the present study, the observed steep depth gradient of total bacteria was not expected but can be explained by more suitable living conditions in the upper soil layers; greater root biomass and exudation resulting in a higher supply of C resources and nutrients combined with better aeration.

Total bacteria as well as nitrate reducers followed similar temporal patterns with a maximum in the early phase of the vegetation period and a minimum during July (Fig. 2b and c). High temporal fluctuations in numbers of bacterial cells and of culturable bacteria harbouring the narG gene were also found in a spruce forest soil, showing lowest numbers during summer months (Mergel et al., 2001b). Whereas neither Mergel et al. (2001b) nor Kandeler et al. (2009) found significant correlations between total and nitrate-reducing bacteria numbers and soil water content, the gene copy numbers from the Mini-FACE, including extractable DNA yields, showed significant and positive correlations with soil water content on the respective sampling dates (Tables 2 and 3). This result was probably due to the higher temporal resolution of soil sampling in the present study as compared with the previous studies. This indicates that the total bacterial community and the nitrate-reducing community are strongly influenced by changing soil moisture conditions, exhibiting a decline when soil moisture is low during dry periods. In arable soils, hot and dry periods during summer months have been shown to strongly reduce microbial biomass C (Van Gestel et al., 1992) and 16S rRNA gene copy numbers (Dandie et al., 2008) in comparison with moist late autumn and winter months. In our study, the relatively higher rate of increase in both total and nitrate-reducing bacteria in the 0–10 cm soil layer as compared with the deeper layers between July 16 and August 13 indicates that environmental conditions became more favourable and bacterial populations recovered after rewetting of the surface soil by rainfall during this period (Figs 1 and 2a–c).

The narG copy numbers were on average twofold higher than copy numbers of napA, with greater differences between the two genes in the 20–30 cm layer (Fig. 2b and c; Table S1). Approximately twice as many copies of narG as compared with napA were also found in soils of spruce forest (Kandeler et al., 2009), pasture (Philippot et al., 2009) and glacier foreland (Bru et al., 2007), whereas Bru et al. (2007) observed similar narG and napA gene copy numbers in several arable soils. In comparison with narG, the decline between June 4 and July 16 was more pronounced for napA (Fig. 2b and c; also see napA/narG ratio in Table S1), indicating a stronger influence of environmental factors on napA-harbouroing bacteria than on those with narG. Correlations of both functional genes with chemical soil properties were weak. However, napA gene copy numbers were slightly more closely correlated with nitrate reductase activity \((r = 0.302, P < 0.001)\) than the narG copy numbers \((r = 0.245, P = 0.002)\), suggesting that napA nitrate reducers are more important contributors to nitrate reduction than narG nitrate reducers. However, the relationship between size and activity of the nitrate reducer community was generally weak, which is consistent with the facultative nature of respiratory nitrate reduction, which depends on three factors: the presence of nitrate, oxygen limitation and electron donor availability. However, when environmental conditions are not limiting for the expression of nitrate reductase, differences in the size of the nitrate-reducing community can be reflected in nitrate reduction rates (Chêneby et al., 2009).

In the present study, dissimilatory nitrate reductase enzyme activity using the method developed by Abdelmagid & Tabatabai (1987) and modified by Kandeler (1996) provided data about the potential activity of both periplasmic and membrane-bound nitrate reductases. Nitrate reductase activity was significantly affected by soil depth and showed a gradient with highest values in the upper (0–10 cm) and lowest values in the deepest layer (20–30 cm). We also found soil water content and \(\text{NO}_3^-\)concentration to increase in the top 10 cm (Fig. 1, Table 2). The dependence of nitrate reduction on the availability of an alternative electron acceptor, i.e. nitrate concentration and absence of oxygen, which is linked to soil moisture (Smith & Tiedje, 1979; Firestone et al., 1980), is well known and has been reported in several studies (Deiglmayr et al.,...
Effects of elevated CO₂ on nitrate reducers in arable soil

2006; Kandeler et al., 2009). The higher nitrate concentration in the upper layer was somewhat surprising because high nitrate uptake by plant roots was expected. However, similar results have previously been reported with higher concentrations of nitrate as well as nitrate reductase activities in the rhizosphere soil of barley than in the bulk soil without roots (Højberg et al., 1996). In our experiment, this increasing NO₃⁻ concentration in the soil during the last four sampling dates can be explained by the lower demands of the oilseed rape plants for nutrients during the ripening period.

In conclusion, neither the abundance nor the activity of the nitrate-reducing bacteria was significantly affected by elevated atmospheric [CO₂] with regard to soil depth or sampling time. However, we showed that medium-term changes (1–2 weeks) in soil moisture and mineral N availability during the main vegetation period of oil seed rape corresponded to shifts in the abundances of nitrate-reducing bacteria, whereas nitrate reductase activity was only depth dependent and showed no seasonal changes. Our results suggest that the elevation of atmospheric [CO₂] has only a marginal impact on nitrate reducers in the investigated arable ecosystem. However, the observed pronounced seasonal changes suggests that the predicted climate change with increased soil temperature, more frequent drought periods and heavy rainfall events could influence nitrate reducers, and likely N-cycling in arable soil.

Acknowledgements

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gene copy numbers of 16S rRNA gene, narG and napA per nanogram DNA, ratios of the two functional genes to 16S rRNA gene and the ratio of napA to narG in three different soil depths (0–10, 10–20 and 20–30 cm) on six sampling dates during the growing season of spring oilseed rape in a Mini-FACE experiment under ambient (Amb) and elevated (Elev) atmospheric CO₂ concentrations at three different soil depths (0–10, 10–20 and 20–30 cm).

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