The ISME Journal (2010) 4, 711–715

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SHORT COMMUNICATION

Microbial secondary succession in a chronosequence of chalk grasslands

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Although secondary succession has been studied extensively, we have little knowledge of the succession of soil-borne microbial communities. In this study, we therefore examined the structures of the microbial communities across two separate chronosequences of chalk grasslands in Limburg, the Netherlands, which are at different stages of secondary succession after being abandoned for between 17 and >66 years. Arable fields were also included in the investigation as non-abandoned references. Changes in the soil-borne microbial communities, as determined by phylogenetic microarray and quantitative PCR methodologies, were correlated with the prevailing environmental conditions related to vegetation and soil biochemistry. We observed clear patterns of microbial secondary succession related to soil age, pH and phosphate status, as exemplified by the overrepresentation of *Verrucomicrobia*, *Acidobacteria*, *Gemmatimonadetes*, and α-, δ- and ε-*Proteobacteria* at late successional stages. Moreover, effects of secondary succession versus changes in soil pH could be resolved, with pH significantly altering the trajectory of microbial succession.

The ISME Journal (2010) **4,** 711–715; doi:10.1038/ismej.2010.11; published online 18 February 2010
Subject Category: microbial population and community ecology
Keywords: phylogenetic microarray; secondary succession; microbial community; soil biochemistry; pH

In efforts to foster biodiversity and more natural ecosystem development, it has become common practice across Europe to take fields out of production to be set aside for nature development, and species-rich chalk grasslands are an important target community in such efforts. Several studies have examined secondary plant succession after field abandonment and discontinuation of fertilizer use, revealing patterns of plant species turnover, increases in plant diversity and decreases in total productivity (Willems, 1980; Bobbink and Willems, 1987; Olff and Bakker, 1991). Belowground, decreases in nitrogen turnover and shifts in ammonia-oxidizing bacterial communities have also been observed in the advanced stages of secondary succession (Kowalchuk *et al.*, 2000).

Microbial community dynamics in soils are closely linked to vegetation and soil characteristics (Marschner *et al.*, 2001), yet, although plant succession is well documented, for instance in the re-establishment of chalk grasslands (Kiehl and Pfadenhauer, 2007), little is known about accompanying responses in microbial community structure. We therefore studied two seminatural Dutch chalk grassland sites (Gerendal nature reserve and Wrakelberg), which each contain several stages of secondary vegetation succession due to the abandonment of intensive agriculture at different times in the past (17 to >66 years) (Supplementary material and methods), thereby representing ideal field experiments to study soil-borne microbial community shifts. Adjacent fields still in production were also included in the analyses of each successional series as references. The secondary successions exhibited clear patterns of increased diversity and richness of aboveground vegetation (Supplementary Table S1).

Soil NO_3^-, NH_4^+ and available P concentrations were significantly different across the various succession stages of the Gerendal and Wrakelberg sites, whereas organic matter and total N contents were not significantly different (Table 1). In general, soil NO_3^- and P decreased with time since the last

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Received 27 October 2009; revised 15 January 2010; accepted 18 January 2010; published online 18 February 2010

intensive agricultural use, except for the 22-year-old Gerendal site, which had a higher P concentration than the 17-year-old field. Soil pH in Wrakelberg was almost constant for the different succession stages and the adjacent agricultural fields. However, the intermediate grassland stages at Gerendal showed significantly lower soil pH than the adjacent fields and older successional stages. Correlation analyses between soil chemical properties and time since last intensive agricultural use revealed that P and NO_3^- were negatively correlated with time since abandonment ($r_s = -0.758$, $P < 0.0001$; $r_s = -0.622$, $P = 0.00002$, respectively, $N = 39$ for both).

To follow microbial community succession, we subjected total soil DNA extracts to analysis with 16S rRNA gene-based microarrays, PhyloChips (DeSantis *et al*., 2007), and phylum-specific quantitative (Q)-PCR (Supplementary material and methods) and compared resulting patterns with changes in environmental conditions during secondary vegetation succession. The high-throughput PhyloChip technique has proven to be a useful tool in the comparison of communities in diverse environmental samples (Brodie *et al*., 2007; Yergeau *et al*., 2009).

Firmicutes was the most dominant bacterial group (25.64%) in the Gerendal fields, and the abundance of this phylum in Wrakleberg fields (20.19%) was similar to that observed for *Actinobacteria* (20%) (Supplementary Figure S1). The main difference was that intermediate succession stages of Gerendal (G1, G2, G3) had a higher proportion of *Firmicutes* as compared with intermediate aged fields of Wrakelberg (Supplemmentary Table S2). Members of this phylum have been reported to be abundant in grassland soils in the Netherlands (Felske *et al*., 1998, 2000) and relatively low in several other locations, such as Canada, Brazil, United States, United Kingdom, Switzerland, Austria, Australia and Germany (Janssen, 2006). The second most abundant phylum was *Actinobacteria* representing 17.65% in Gerendal and 20.00% in Wrakelberg followed by *α-Proteobacteria* (13.53% G, 16.25% W), *β-Proteobacteria* (13.11% G, 12.36% W) and *γ-Proteobacteria* (8.18% G, 10.40% W) (Supplementary Figure S1). The relative dominance of the phyla *Firmicutes* and *Actinobacteria* in these Dutch chalk grassland soils was distinct from soils from Florida, Brazil, Canada and Illinois (Roesch *et al*., 2007), where the most abundant groups were *Bacteroidetes* and *Acidobacteria* (Fulthorpe *et al*., 2008).

We detected clear patterns of microbial secondary succession with time since abandonment of intensive agricultural practices (Figure 1). Fields still subjected to intensive agriculture (G0 and W0) contained higher P than intermediate and old restoration stages, and all these samples had highly similar microbial community structures. These arable fields, in general, had relatively high representations of *Bacteroidetes*, *Cyanobacteria* and *Euryarchaeota*, and relatively low representations of *Acidobacteria*, *Actinobacteria*, *Crenarchaeota*, *δ-Proteobacteria* and *Verrucomicrobia* (Supplementary Table S2).

Soils of the latest succession stages (G4 and W4) at both locations had similar microbial community structures, probably because of similar pH and low levels of available nutrients (NO_3^- and P). These sites had high representations of *Actinobacteria*, *α-Proteobacteria*, *β-Proteobacteria* and *Planctomycetes*,

Table 1 Soil organic matter, NH_4^+, NO_3^-, available P, pH and total *N* and associated Kruskal–Wallis analysis of variance tests for fields abandoned for different time located at Gerendal and Wrakelberg

Location	*Time since abandonment (years)*	*Succession stage*	*OM (%)*	*NH_4^+ (mg kg^{-1} soil DW)*	*NO_3^- (mg kg^{-1} soil DW)*	*P (mg kg^{-1} soil DW)*	*pH*	*Total* N *(%)*
Gerendal	0	G0	4.03 ab	6.05 a	15.30 a	92.94 a	7.07 ab	21.34 a
	17	G1	11.08 ab	3.59 a	2.37 a	17.50 ab	5.73 ab	5.96 a
	22	G2	10.30 a	3.65 a	1.34 a	51.13 ab	5.37 ab	4.99 a
	36	G3	5.51 ab	5.07 a	0.64 a	10.19 ab	5.14 a	5.70 a
	66	G4	9.44 b	9.93 a	1.16 a	8.55 b	7.45 b	11.09 a
Stage effect			**	NS	†	*	*	NS
Wrakelberg	0	W0	3.40 x	2.00 x	1.55 xy	115.35 x	7.85 x	3.55 x
	17	W1	10.22 x	10.09 y	7.24 x	15.61 xy	7.48 y	17.33 x
	22	W2	9.20 x	8.56 xy	0.51 xy	6.76 y	7.54 xy	9.07 x
	36	W3	9.16 x	8.68 xy	0.50 xy	5.85 y	7.53 xy	9.07 x
	66	W4	9.10 x	8.84 xy	0.63 y	7.80 xy	7.62 xy	9.47 x
Stage effect			†	**	**	**	**	NS
Location			NS	NS	NS	*	***	NS

Abbreviation: OM, organic matter; NS, not significant.
†$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
Samples are coded as W (Wrakelberg) and G (Gerendal) followed by a number indicating the succession stage (0: fields in production, 1–4: fields abandoned for 17, 22, 36 and >66 years, respectively).
Calculations were based on three replicates per succession stage of Gerendal and five replicates per succession stage of Wrakelberg. The letters next to the numbers are Tukey-HSD (Honestly Significant Difference) letters, showing the difference between the stages.

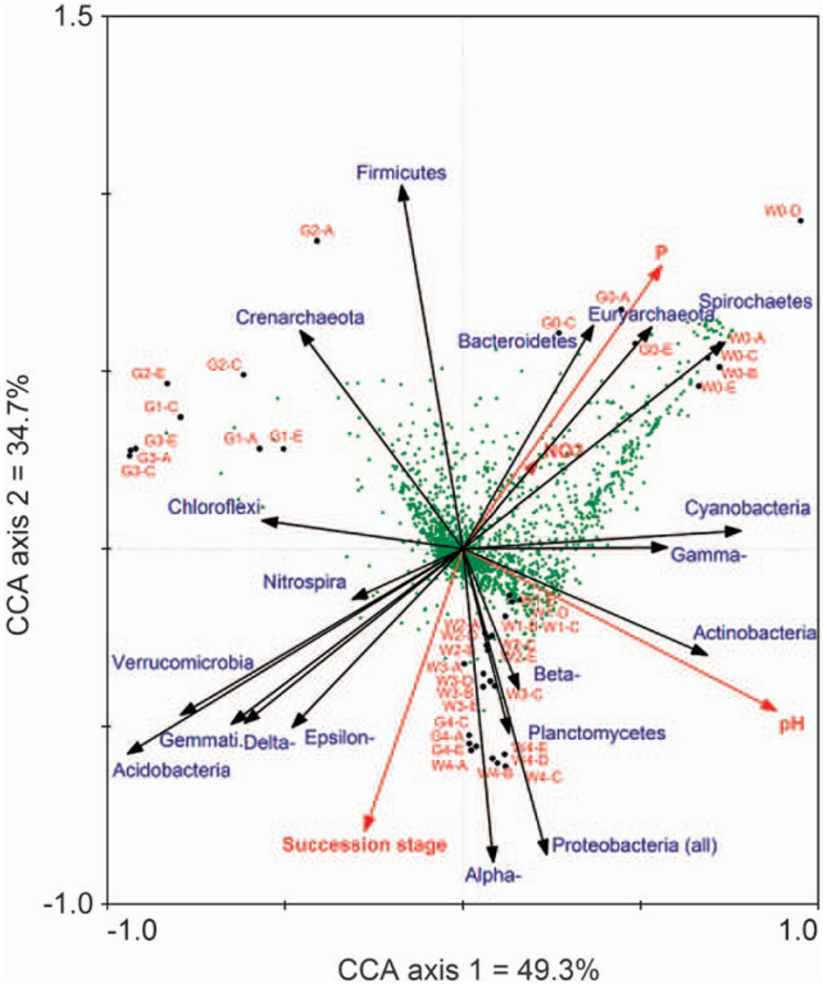

Figure 1 Triplot of canonical correspondence analysis, relating samples (black dots), microbial operational taxonomic units (OTUs; green dots), environmental variables (red arrows) and microbial groups (black arrows). Vectors for nonsignificant soil parameters (that is, organic matter content and total soil N) are not shown. Samples are coded as W (Wrakelberg) and G (Gerendal) followed by a number indicating the succession stage (0: fields in production, 1–4: fields abandoned for 17, 22, 36 and >66 years, respectively). A, B, C, D, E represent biological replicates within fields (Supplementary material and methods).

and low representations of *Firmicutes* and *Crenarchaeota* (Supplementary Table S2).

A major reason for choosing chalk grassland systems for our study was their buffering capacity, which typically results in slightly alkaline pH across fields of different ages. However, three of our study fields, the intermediately aged fields at the Gerendal location (G1, G2, G3), exhibited acidic soil pH. Thus, although the Wrakelberg site displayed a steady succession of soil-borne microbial communities, corresponding to changes in nutrient status, microbial secondary succession at the Gerendal site was also affected by soil pH (Figure 1), as shown by canonical correspondence analysis. Acidic soils at intermediate ages of Gerendal harbored relatively high representations of *Crenarchaeota*, *Firmicutes* and *Verrucomicrobia*, and low representations of *Bacterioidetes*, *α-Proteobacteria* and *γ-Proteobacteria* (Supplementary Table S2). Mantel tests were also used to highlight correlations between the similarities of samples due to soil, microbial community or vegetation properties. There was a significant microbial community–soil correlation ($r_m = 0.268$, $P = 0.001$), but no microbial community–plant community correlation ($r_m = -0.087$, $P = 0.222$) (for Wrakelberg fields where plant data were available). These tests indicate that similarly vegetated fields do not necessarily have similar microbial communities, but that fields with similar soil properties (including nutrients and pH) generally harbor more similar microbial communities. Although these factors were shown to be strong drivers of microbial community structure, the role of other soil factors that were not examined in this study cannot be ruled out.

Quantitative PCR targeting several important bacterial phyla and classes was used to confirm phylogenetic microarray results (Supplementary Table S3), showing strong correlations between the two independent

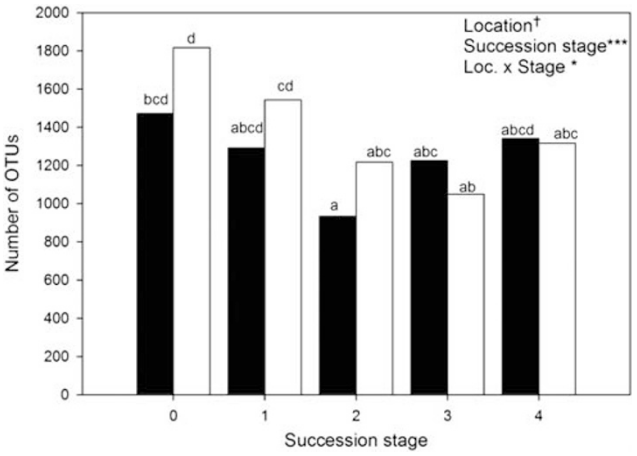

Figure 2 Richness determined as the number of operational taxonomic units (OTUs) scored as present (see Supplementary material and methods for more details) for fields at different succession stages (0: fields in production, 1–4: fields abandoned for 17, 22, 36 and >66 years, respectively) located in Gerendal (■) and Wrakelberg (□). Different letters mean significantly different averages at $P<0.05$ following a Tukey-HSD (Honestly Significant Difference) test. Effects of different factors following analysis of variance tests are presented in the top left corner of the figure with $^{\dagger}P<0.10$, $^{*}P<0.05$ and $^{***}P<0.001$.

analyses: *Actinobacteria* ($r_s=0.324$, $P=0.044$), *α-Proteobacteria* ($r_s=0.540$, $P<0.001$), *Bacteroidetes* ($r_s=0.649$, $P<0.001$) and *Firmicutes* ($r_s=0.867$, $P<0.001$). In contrast, real-time PCR quantifications for *Acidobacteria* and *β-Proteobacteria* did not correlate significantly with their corresponding relative intensity from the PhyloChip. This might be explained by the different primer sets used for PhyloChip and Q-PCR analyses or different levels of coverage of this phylum by the two platforms. It is known, for instances, that some groups of *Acidobacteria* are not targeted by bacterial universal primers (Jones *et al*., 2009; Kielak *et al*., 2009).

In contrast to plant diversity and richness, we observed a decrease in microbial richness with field age (Spearman's rank-order correlation: $r=-0.497$, $P=0.0013$), with the highest microbial richness observed in the arable fields (Figure 2). This result is in line with the results of Roesch *et al*. (2007) that agricultural soils harbored greater species richness than forest soils. The youngest fields (succession stage 1), abandoned for 17 years, had more operational taxonomic units (OTUs) than intermediate and old fields. The numbers of OTUs decreased in intermediate stage fields, but showed a slight increase in the oldest stage (>66 years) (Figure 2). This decrease in OTU richness with time since the last intensive agricultural use seems to be at least partly related to the changes in soil nutrient concentrations and in soil pH, as OTU richness was significantly correlated to soil NO_3^-, P and pH ($r_s=0.358$, $P=0.025$; $r_s=0.412$, $P=0.009$; $r_s=0.343$, $P=0.032$; respectively, $N=39$ for all), but not soil organic matter content ($r_s=-0.211$, $P=0.197$, $N=39$). Studying several soil types across South and North America though, Fierer and Jackson (2006) also found strong correlations between soil pH and bacterial OTU richness, with pH 6.8 soil having 60% more bacterial OTUs than pH 5.1 soil. These authors compared soil with similar vegetation and climate but different pH, and concluded that pH is an important factor determining soil-borne microbial community structure.

The importance of fungi in soil-borne communities generally tends to increase with ecosystem stability, given their susceptibility to tillage and adaptation to degradation of complex organic matter (van der Wal *et al*., 2006); however, we observed the highest fungal:bacteria ratio in the arable fields of Wrakelberg and the earliest succession stage (G1) of Gerendal (Supplementary Table S4). However, it should be noted that our calculations of fungal:bacterial ratios could be subjected to bias caused by the fungal and bacterial universal primers used for Q-PCR. For instance, the primers used for fungal PCR are known to be able to amplify some non-fungal eukaryotic sequences (Lueders *et al*., 2004).

Our results revealed clear patterns of microbial succession in secondary succession chalk grasslands. Fields at similar stages of succession were generally most similar, regardless of location, and this pattern seemed to be driven more by the changes in soil nutrient characteristics measured in this study that developed during secondary field succession as opposed to changes in vegetation. In addition, pH altered the trajectory of succession for fields differing in this key environmental factor. It is interesting that the patterns of microbial OTU richness were opposite to changes in plant diversity. One might expect increased plant diversity to provide a greater variety of substrates and niches in soil, thereby supporting greater microbial diversity. However, we observed no evidence for such a relationship, although it must be stressed that we examined bulk soil samples, where plant effects might be expected to be less pronounced. By integrating high-throughput microarray approaches into the study of ecosystem properties, we show that patterns of microbial community succession can be tracked in the field and related to some of the environmental factors driving soil-borne microbial communities. Coupling of such comprehensive phylogenetic data with functional studies should provide critical data toward understanding the role of microbial secondary succession in terrestrial ecosystem development.

Acknowledgements

We thank Lina C Wong and Agata S Pijl for laboratory assistance, and Wiecher Smant for soil chemical analysis. This work was supported by the Bsik program of 'Ecogenomics' (http://www.ecogenomics.nl/). Publication number 4732 of the NIOO-KNAW, Netherlands Institute of Ecology.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)