RESEARCH ARTICLE

Soil and plant factors driving the community of soil-borne microorganisms across chronosequences of secondary succession of chalk grasslands with a neutral pH

Eiko Kuramae[1,2], Hannes Gamper[3], Johannes van Veen[1,4] & George Kowalchuk[1,2]

[1]Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands; [2]Institute of Ecological Science, Free University Amsterdam, Amsterdam, The Netherlands; [3]Institute of Botany, University Basel, Basel, Switzerland; and [4]Institute of Biology, Leiden University, Leiden, The Netherlands

Correspondence: Eiko Kuramae, Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, The Netherlands. Tel.: +31 317 473502; fax: +31 317 473675; e-mail: e.kuramae@nioo.knaw.nl

Received 3 November 2010; revised 1 April 2011; accepted 5 April 2011.
Final version published online 9 May 2011.

DOI:10.1111/j.1574-6941.2011.01110.x

Editor: Christoph Tebbe

Keywords
phylochips; phosphorus; pH; plant diversity.

Abstract

Although soil pH has been shown to be an important factor driving microbial communities, relatively little is known about the other potentially important factors that shape soil-borne microbial community structure. This study examined plant and microbial communities across a series of neutral pH fields (pH = 7.0–7.5) representing a chronosequence of secondary succession after former arable fields were taken out of production. These fields ranged from 17 to > 66 years since the time of abandonment, and an adjacent arable field was included as a reference. Hierarchical clustering analysis, nonmetric multidimensional scaling and analysis of similarity of 52 different plant species showed that the plant community composition was significantly different in the different chronosequences, and that plant species richness and diversity increased with time since abandonment. The microbial community structure, as analyzed by phylogenetic microarrays (PhyloChips), was significantly different in arable field and the early succession stage, but no distinct microbial communities were observed for the intermediate and the late succession stages. The most determinant factors in shaping the soil-borne microbial communities were phosphorous and NH_4^+. Plant community composition and diversity did not have a significant effect on the belowground microbial community structure or diversity.

Introduction

The conversion from agricultural fields to more natural grasslands has been a common practice in Europe in the recent decades. During this conversion process, soil nutrient levels decline, and plant diversity and composition change, with shifts towards a greater abundance of slow-growing plant species. Furthermore, plant species composition and secondary succession development has been observed to vary on a regional scale (Van der Putten *et al.*, 2000; Van der Wal *et al.*, 2006). This secondary succession, which results from the termination of intensive agricultural practices such as tillage and fertilizer application, results in increases in plant diversity and concomitant decreases in plant productivity (Willems, 1980; Bobbink & Willems, 1987; Olff & Bakker, 1991) and nitrogen turnover (Kowalchuk *et al.*, 2000) in the advanced stages of secondary succession.

Soil microorganisms are the main players in the nutrient cycling in soil systems, and soil microbial community structure has been shown to be influenced by both abiotic and biotic factors. With respect to abiotic factors, soil pH has been shown to be a key driver of microbial community structure (Enwall *et al.*, 2005; Wakelin *et al.*, 2008; Rousk, 2010; Smith *et al.*, 2010). Soil pH has been demonstrated to explain a significant degree of microbial community variation from local to continental scales (Lauber *et al.*, 2009) and has been strongly correlated with bacterial OTU richness, with neutral pH soil generally fostering greater bacterial richness than acidic conditions (Fierer & Jackson, 2006).

Plants are also known to influence microbial community structure and diversity, with the most pronounced effects observed in the zone directly adjacent to active plant roots, the rhizosphere (Kowalchuk *et al.*, 2002; Berg & Smalla, 2009). Specific plant species, plant diversity and plant community composition have all been shown to influence soil-borne microbial communities (Wardle *et al.*, 2004; Viketoft, 2008). However, despite the impact of vegetation on microbial communities (Bezemer *et al.*, 2006), the

impact of agricultural practices has been shown to persist in former agricultural fields after abandonment (Buckley & Schmidt, 2003). Secondary plant succession is often associated with a lowering of soil pH, and a lower pH in the rhizosphere has been shown to alter the environmental availability of various substrates (Harmsen & Eijsackers, 2005). Soil pH also influences the amounts of phosphate available to plants, as the negative charge of the ionic form of phosphate makes it more easily sequestered by cations such as Fe, Al and Ca (Vance *et al.*, 2003). Recently, Kuramae *et al.* (2010) have shown that soil pH can significantly alter the trajectory of microbial succession in the secondary succession of grasslands. Thus, a number of factors appear to influence soil-borne microbial community structure and diversity, but the dominance of pH in many systems makes it difficult to determine the importance of other underlying factors. In the current study, we therefore sought to examine the changes in the microbial communities in fields with similar pH, but taken out of production at different points in time. To address the potential impact of vegetation patterns on soil-borne microbial communities, we also undertook a detailed examination of vegetation composition across neutral fields at different stages of secondary succession. Thus, where Kuramae *et al.* (2010) described the general patterns of microbial succession with the predominance of pH as a driving soil factor, the analyses described here allowed for a focus on other key soil and vegetation factors in shaping microbial community composition and diversity.

The south-eastern portion of the Netherlands contains several chalk-rich grasslands that allow for the examination of biotic and abiotic drivers of soil-borne microbial community under buffered, pH-neutral, soil conditions. Furthermore, this region has grassland fields that have been taken out of production at various times in the past, thereby creating a semi-natural experiment to test the effects of changing vegetation and soil nutrient status on the development of soil-borne microbial communities. We hypothesized that both plant species composition and soil nutritional factors would have a major influence on the soil-borne microbial community composition and diversity. In order to address this issue, we examined the microbial communities inhabiting neutral soils across secondary succession chalk grasslands using high-density phylogenetic microarrays (PhyloChips) and related observed patterns of microbial community structure and diversity to vegetation and soil properties.

Materials and methods

Site description, soil sampling and chemical analyses

Fields from four succession stages (stages: W_1–W_4) of chalk grassland along a slope (15–20° inclination) exposed to the SW were selected in a nature reserve at Wrakelberg, in the Limburg province of the Netherlands. An adjacent conventional arable field with winter wheat in grasslands represented the reference field (W_0). The three grasslands that had last been ploughed about 17, 22 and 36 years ago represented the intermediate transitional restoration stages. The soils of the oldest restoration stage were most probably never ploughed, and a lack of tillage can be verified for at least the last 66 years. Bulk soil samples were taken at each succession stage in late February 2007, along linear transects of 20 m for all five fields. At each of the five locations along each 20-m transect (5 m apart), five cores (10 cm depth, 2 cm diameter) were taken < 10 cm apart and combined, yielding a total of 25 composite soil samples (Supporting information, Fig. S1). The soils were sieved to < 3 mm to exclude larger root fragments and pieces of chalk. Chemical soil analyses were performed according to Novozamsky *et al.* (1984). Available soil phosphorus was measured calorimetrically in 0.01 M $CaCl_2$ suspensions using the Molybdenum blue method and mineral N as the NH_4^+ and NO_3^- concentrations in KCl extracts, using a Traacs 800 autoanalyzer.

Plant species

In the 1 m^2 surrounding each of the spots from which soil samples were taken, the vegetation composition was determined, and a plant species presence (0)/absence (1) matrix was constructed. A total of 52 different plant species were observed: these were *Arhenatherum elatius*, *Avenula* sp., *Avenula* sp. (hard), *Brachypodium pinatum*, *Briza media*, *Campanula* sp., *Carex flacca*, *Carex cariophyllaceae*, *Cirsium* sp., *Centaurea scabiosa*, *Convolvulus arvensis*, *Crataegus* sp., *Dactylis glomerata*, *Daucus carota*, *Euphrasia stricta*, *Festuca ovina*, *Galium mollugo*, *Genista tinctoria*, *Gymnadenia conopsea*, *Hieracium pilosella*, *Hypericum perforatum*, *Inula conyza*, *Knautia arvense*, *Leontodon hispidus*, *Leucanthemum vulgare*, *Linum catharticum*, *Lolium perenne*, *Lotus corniculatus*, *Medicago lupulina*, *Ononis repens*, *Oreganum vulgare*, *Pimpinella saxifraga*, *Plantago lanceolata*, *Poa* sp., *Poligola comosa*, *Potentilla* sp., *Prunella vulagris*, *Prunus avium*, *Ranunculus* sp., *Reseda lutea*, *Rhinanthus minor*, *Rosa* sp., *Rubus* sp., *Sanguisorba minor*, *Scabiosa columbaria*, *Senecio* sp., *Thymus pulegioides*, *Trifolium pratense*, *Trifolium* sp., *Triticun aestivum*, *Vicia* sp. and *Viola* sp. Open space was also scored as present (1) and absent (0) in the different chronosequence stages.

Nucleic acid extractions and 16S rRNA gene amplification

A total of 25 samples, representing each soil spot, were subjected to DNA isolation. DNA from each sample was extracted from 0.25 g of soil, using the Power Soil kit (MoBio, Carlsbad, CA), with bead-beating at 5.5 m s^{-1} for 10 min. The total DNA concentration was quantified on an ND-1000 spectrophotometer (Nanodrop Technology,

Wilmington, DE). 16S rRNA gene amplification was performed using the bacterial-specific primers, 27F (5′-AGAGT TTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTT GTTACGACTT-3′), and the archaeal-specific primers, 4fa (5′-TCCGGTTGATCCTGCCRG-3′) and 1492R (5′-AGAG TTTGATCCTGGCTCAG-3′). PCR amplifications were carried out with 1 × *Ex Taq* buffer (Takara Bio Inc., Japan), 0.8 mM dNTP, 0.02 U mL^{-1} *Ex Taq* polymerase, 0.4 mg mL^{-1} bovine serum albumin and 1.0 mM of each primer. Three independent PCR amplifications were performed with the annealing temperatures 48, 51.9 and 58 °C for both bacteria and archaea, with an initial denaturation at 95 °C (3 min), 25 amplification cycles with denaturation at 95 °C (30 s), annealing (30 s) and extension at 72 °C (60 s), followed by a final extension at 72 °C (7 min). PCR products of bacteria and archaea were each pooled separately, and a 2 μL subsample of each was quantified on 2% agarose gels. The volume of pooled PCR products was reduced to < 40 μL using micrometer YM100 spin filters (Millipore, Billerica, MA) before application to hybridization analyses. Regression analysis confirmed that the quantity of PCR amplicon applied to the array was not correlated with any organism abundances as estimated by the fluorescence intensity of hybridization (data not shown).

PhyloChip processing, scanning and normalization

The pooled PCR products described above were spiked with known concentrations of amplicons derived from yeast and prokaryotic metabolic genes. This amplicon mix was fragmented to 50–200 bp using DNase I (0.02 U ug^{-1} DNA; Invitrogen) and One-Phor-All buffer following Affymetrix's protocol. The mixture was then incubating at 25 °C for 20 min and 98 °C for 10 min before labeling. Biotin labeling was carried out using the GeneChip DNA labeling reagent following the manufacturer's instructions (Affymetrix). Next, the labeled DNA was denatured at 99 °C for 5 min and hybridized to custom-made Affymetrix GeneChips (16S rRNA gene PhyloChips G2) (DeSantis *et al.*, 2007) at 48 °C and 60 r.p.m. for 16 h in a hybridization chamber. PhyloChip washing and staining were performed according to standard Affymetrix protocols (Masuda & Church, 2002). Each PhyloChip was scanned and recorded as a pixel image, and initial data acquisition and intensity determination were performed using standard Affymetrix software (Genechip Microarray Analysis Suite, version 5.1).

To account for scanning intensity variation from array to array, the intensities resulting from the internal standard probe sets were natural-log-transformed. Adjustment factors for each PhyloChip were calculated by fitting a linear model with the least-squares method. A PhyloChip adjustment factor was subtracted from each probe set's natural log of intensity. Background subtraction, noise calculation, and detection and quantification were essentially as reported previously (Brodie *et al.*, 2006).

Statistical analyses

The hierarchical clustering analysis of plant presence and absence was performed using the R (R statistical programming environment) 'Stats Package' applying the 'Ward' agglomeration method. The clustering analysis of normalized intensities of OTUs derived from the PhyloChips was performed by hierarchical clustering and the average linkage method. The heatplot of normalized intensities in the PhyloChips was obtained using the 'MADE4' package in R.

Pearson correlations were calculated between soil characteristics (NH_4^+, NO_3^-, P, pH), environmental variables (succession stage) and normalized OTU hybridization intensities using the 'MULTTEST' package in R. *P*-values were corrected for multiple testing, using the false discovery rate controlling procedure (Benjamini & Hochberg, 1995).

In order to visualize the changes in the microbial community structure, plant community structure and soil factors with respect to time since abandonment, nonmetric multidimensional scaling (NMDS) was applied. This analysis was used because it avoids the assumption of linear relationships among variables, and it is reported to be the most generally effective ordination method for ecological community data (Clarke, 1993). Normalized intensities of OTUs given by PhyloChips were used as data for the microbial communities, and plant species data were converted to presence or absence in order to generate a binary data set. For each data set, a Bray–Curtis distance measure matrix was calculated. NMDS representations were assessed using the Analysis of Similarity (ANOSIM). The significance was examined using a permutation test, where *R* was recomputed for 10 000 permutations. ANOSIM was used to assess significant differences in the profile composition characteristics for the different succession stages. This is a nonparametric permutation procedure that uses the rank similarity matrix underlying an ordination plot to calculate an *R* test statistic, which can range from 0 to 1. *R*-values near 0 indicate a true null hypothesis of no difference between groups, whereas those > 0 indicate a discrimination between groups. ANOSIM statistics reach a maximum of 1 when the similarities within groups are greater than any similarity between groups. For each ANOSIM test, the null hypothesis that there were no significant differences between groups was rejected when the significance level, *P*, was < 0.05.

The results from pair-wise ANOSIM were analyzed to infer where the main differences occurred between microbial community and plant composition in soils with different times since abandonment. We interpreted *R*-values > 0.75 as well-separated bacterial/vegetation compositions;

$R > 0.5$ as overlapping, but clearly different; and $R < 0.25$ as practically not separable. Calculations of similarity coefficients, NMDS and ANOSIM analyses were carried out using the PAST program (Hammer *et al.*, 2001).

The normalized intensities of OTUs were correlated with soil factors and plant species composition by multivariate regression tree (MRT) analysis using the 'MVPART' package in R, and the distance matrix was based on Bray–Curtis reconstructed by the function 'gdist'.

Results

Plant communities

The hierarchical clustering analysis of the presence and absence of 52 different plant species grouped the samples of chronosequences W_1–W_4 and arable field (W_0) into two main clusters: A.I and A.II (Fig. 1a). Cluster A.I comprised samples representing young field at succession stage W_1 and arable field (W_0), while cluster A.II contained samples of succession stages W_2, W_3 and W_4. Cluster A.I could be further subdivided into clusters A.Ia and A.Ib, comprising young field and arable field, respectively. Cluster A.II subdivided into clusters A.IIa, A.IIb and A.IIc, corresponding to succession stages W_2, W_3 and W_4, respectively (Fig. 1a). The young field of succession stage W_1 had lower plant species richness and plant diversity as compared with the intermediate and late succession stages (Table 1). The field older than 66 years (W_4) had higher plant species richness and higher plant diversity than the intermediate (W_2 and W_3) and young stages (W_1) (Table 1). Thus, the analysis of the presence and absence of 52 different plant species grouped the plant communities according to field age (Fig. 1a).

NMDS plots for plant species suggested that the plant composition was different in each succession stage (Fig. 2a). The final stress for plant composition was 0.05059, which indicated a good representation of the plant data in a 2D space. Furthermore, the ANOSIM test corroborated the NMDS plot data, showing significantly different ($R = 0.8644$; $P < 0.0001$) plant composition in fields with different

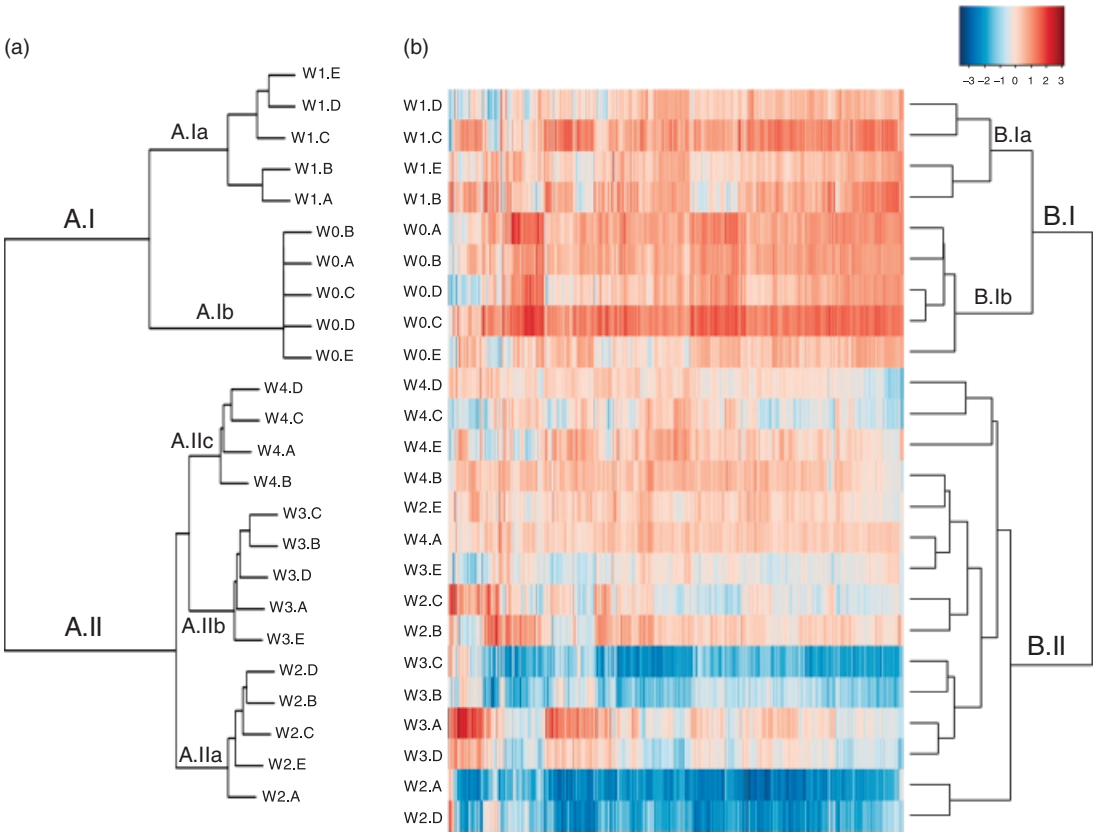

Fig. 1. (a) Hierarchical clustering analyses of presence and absence of 52 different plant species in different succession stages of chalk grasslands. (b) Heat-plot of normalized intensities (blue, low intensity; red, high intensity) of 2222 OTUs given in the PhyloChips. A.I and B.I represent the reference and the early stage fields and A.II and B.II represent the intermediate and late stage fields. Samples are coded as W (Wrakelberg), followed by a number indicating the succession stage (0, arable fields; 1–4, fields abandoned for 17, 22, 36 and > 66 years, respectively). A, B, C, D and E represent the subplots within each succession stage.

Table 1. Plant species richness, plant Shannon–Weaver index, numbers of OTUs, soil NH_4^+, NO_3^-, available P, pH and percentage of organic matter of arable fields and different succession stages of samples from Wrakelberg

Time since abandonment (years)	Succession stage	Block	Species richness ($S\ m^{-2}$)	Shannon–Weaver index ($H\ m^{-2}$)	#OTU	NH_4^+ ($mg\ kg^{-1}$ soil DW)	NO_3^- ($mg\ kg^{-1}$ soil DW)	P ($mg\ kg^{-1}$ soil DW)	pH (KCl)	OM (%)
0	W_0	A	–	–	1922	2.38	1.75	111.21	7.58	3.59
0	W_0	B	–	–	1725	1.95	1.11	104.88	7.68	2.91
0	W_0	C	–	–	1948	2.03	1.47	105.42	7.54	3.53
0	W_0	D	–	–	1952	2.03	2.03	159.14	7.51	3.86
0	W_0	E	–	–	1535	1.61	1.40	96.08	7.54	3.10
17	W_1	A	6	1.61	1553	8.15	7.67	17.34	6.91	8.48
17	W_1	B	6	1.56	1589	7.84	5.63	11.33	7.02	10.41
17	W_1	C	5	0.99	1440	11.68	7.87	17.83	6.98	9.80
17	W_1	D	6	1.43	1581	10.18	8.37	15.83	6.92	10.30
17	W_1	E	5	1.15	1559	10.68	7.08	17.47	6.96	10.36
22	W_2	A	11	1.97	953	9.60	1.12	8.04	7.10	9.22
22	W_2	B	14	2.04	1670	8.13	0.38	5.08	7.10	9.38
22	W_2	C	11	2.09	1126	8.88	0.66	8.64	7.08	9.26
22	W_2	D	14	1.63	1104	8.82	0.27	8.08	7.12	9.08
22	W_2	E	14	2.05	1234	7.39	0.11	3.95	7.15	9.09
36	W_3	A	10	2.17	1059	8.42	0.45	6.74	7.04	10.16
36	W_3	B	7	1.17	977	9.94	1.41	5.99	7.19	9.25
36	W_3	C	10	1.63	773	10.06	0.38	5.71	7.27	8.84
36	W_3	D	15	2.44	1017	7.63	0.03	7.25	7.14	9.21
36	W_3	E	15	2.33	1420	7.34	0.24	3.56	7.23	8.34
> 66	W_4	A	23	2.67	1421	7.39	0.14	6.35	7.11	9.06
> 66	W_4	B	26	2.48	1455	8.97	0.18	7.16	7.41	9.15
> 66	W_4	C	22	2.74	1337	8.93	0.00	7.66	7.51	8.54
> 66	W_4	D	22	2.52	1240	7.35	0.00	7.70	7.31	9.77
> 66	W_4	E	ND	ND	1127	11.56	2.82	10.14	7.37	8.98

OM, organic matter; ND, not determined.

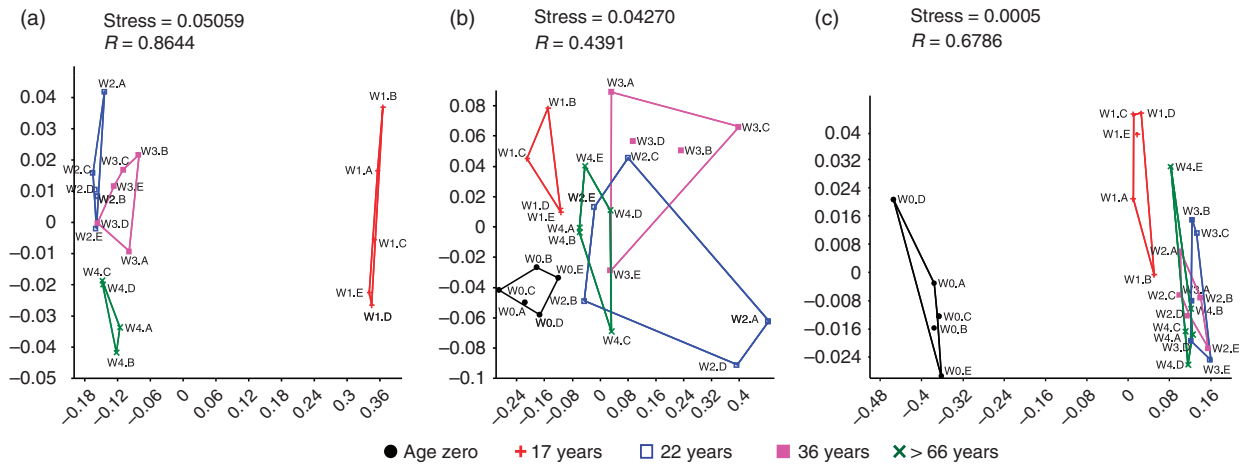

Fig. 2. Two-dimensional NMDS plots of (a) plant community, (b) microbial community and (c) soil factors in soil at different times since abandonment. The letters A, B, C, D and E indicate the subplots within each succession stage (W_0–W_4). The *R*-values provide a measure of (a) plant community, (b) microbial community and (c) soil factors significantly different in different succession stages.

times since abandonment. NMDS analysis and the ANOSIM test for plant community composition also showed that plant community was significantly different in the different succession stages.

Microbial communities

Of the 8741 OTUs represented on the PhyloChip, a total of 2222 (25.4%) were detected among the 25 samples, with 607

being detected in all 25 analyzed samples. From the 62 bacterial and archaeal phyla represented on the PhyloChip, 44 were detected.

In general, the numbers of OTUs detected by the PhyloChips were higher in arable field soil (W_0) than in any succession stage (W_1, W_2, W_3 and W_4) (Table 1). Younger field (W_1), abandoned for 17 years, had more detected OTUs than intermediate (W_2, W_3) and old fields (W_4). The numbers of detected OTUs decreased in the intermediate-stage fields, but showed a small increase in the oldest stage ($>$ 66 years) (Table 1).

The clustering analysis of the normalized intensities of 2222 different OTUs detected by the PhyloChips showed two main clusters: B.I and B.II (Fig. 1b). Cluster B.I subdivided into B.Ia and B.Ib, corresponding to samples from the arable field (W_0) and early succession stage (W_1), respectively. Cluster B.II corresponded to the samples representing intermediate (W_2, W_3) and late succession stages (W_4). The OTU clustering analysis did not cluster the soil samples according to their succession stage (Fig. 1b) or plant species richness and diversity, except for arable field and young field of 17 years. The fields of intermediate (W_2, W_3) and old succession stages (W_4) formed a single cluster. No specific microbial groups could be identified that distinguished the successional stages W_2, W_3 and W_4 from one another.

On the NMDS plots, the points corresponding to samples from arable fields (W_0) and the early succession stage (W_1) formed a separate cluster (Fig. 2b), whereas samples of intermediate and late succession stages (22, 36 and 66 years since abandonment) did not show distinct clusters based on microbial community structure. Nevertheless, significantly different microbial communities were detected for the 36-year-old and $>$ 66-year-old samples, but with a high degree of overlap ($R = 0.35$; $P = 0.0168$). This suggests that changes in the microbial structure were relatively minor in the older stages (W_3 and W_4). The final stress value for PhyloChip data was 0.04270. Stress values < 0.2 typically indicate a good representation of the data in a 2D space. The ANOSIM showed significantly different microbial community structures, with some community overlap in all four ages (global $R = 0.44$). Statistically, the arable field and the 17-year-old field had distinct microbial communities, whereas the intermediate and older fields (ages 22, 36 and $>$ 66) had similar communities by ANOSIM pairwise comparison. Pairwise tests showed significant differences in the microbial community structure pairwise comparisons of soils: age 0:36 years ($R = 0.78$), age 0:66 years ($R = 0.85$), age 17:36 years ($R = 0.66$), age 17:66 years ($R = 0.66$).

Soil factors

In general, soil NH_4^+, NO_3^-, P concentrations and organic matter were different between arable field and all succession stages (Table 1). In the arable field, soil NH_4^+ and NO_3^- were lower, P was higher than the field of 17 years, whereas organic matter was lower than any succession stage (Table 1). The soil pH of succession stages fields ranged between 7.0 and 7.3, with a slightly higher pH of 7.5 in the arable field.

The NMDS plot based on soil factors revealed that soil samples did not group accordingly to the succession stage, except for arable fields (W_0) and samples from the early succession stage (W_1). It appeared that each succession stage separated into two different clusters, and the fields of intermediate and late stages overlapped (Fig. 2c), and this was supported by ANOSIM analysis ($R = 0.6786$; $P < 0.0001$). In fact, soil factors showed significant differences between the arable field and the remaining succession stages (W_0:W_1/W_2/W_3/W_4, $R = 1$) and between early succession samples and intermediate/late succession samples (W_1:W_2, $R = 1$; W_1:W_3/W_4, $R = 1$).

In order to find the main factors that drive the microbial community structure in soils of various stages of secondary succession in calcareous grasslands of neutral pH, we performed MRT analysis. The OTU intensities given by the PhyloChips were correlated with NH_4^+, NO_3^-, percentage organic matter, soil available phosphorus and plant species composition. The MRT analysis showed that the microbial communities of different succession stages and arable field were separated mainly by soil available P (Fig. 3). The microbial communities of arable field soil samples (W_0) and of early succession stage samples (W_1) formed a separate cluster (Fig. 3: CI, CII) from the one containing samples from the intermediate (W_2 and W_3) and late (W_4) succession stages (Fig. 3: CIII, CIV), where the soil P was $\geq 9.4\ mg\ kg^{-1}$ (Fig. 3). The exception was sample W_4.E, which grouped into cluster CI. This is probably explained by the higher P content in this particular sample as compared with the other four samples from this succession stage (Table 1).

A total of 386 of the 2222 detected OTUs were significantly correlated with the soil chemical properties. Samples with soil P $\geq 9.4\ mg\ kg^{-1}$ had a relative over-representation of OTUs belonging to *Actinobacteria*, *Bacterioidetes*, *Firmicutes*, *Planctomycetes* and *Nitrospira* and a relative under-representation of OTUs belonging to *Crenarchaeota*, *Verrucomicrobia*, *Deltaproteobacteria* and *Cyanobacteria* (Fig. 3). These differences were even more clear for samples with the highest amounts of available P ($>$ = 100.5), i.e. as present in arable fields (Fig. 3; Table 1). The combination of low soil available P ($< 9.4\ mg\ kg^{-1}$) content and a low soil NH_4^+ ($< 9.3\ mg\ kg^{-1}$) content, typical of the fields of succession stages clustered in CIII, seems to have a positive effect on a number of OTUs belonging to *Gammaproteobacteria*, *Betaproteobacteria*, *Planctomycetes*, *Firmicutes*, *Bacterioidetes* and *Actinobacteria*. On the other hand, under soil conditions with $P < 9.4$ and $NH_4^+ \geq 9.3$, several OTUs belonging to *Alphaproteobacteria*, *Cyanobacteria*, *Euryarcheota* and *Crenarcheota* seemed to be favored (Fig. 3;CIV).

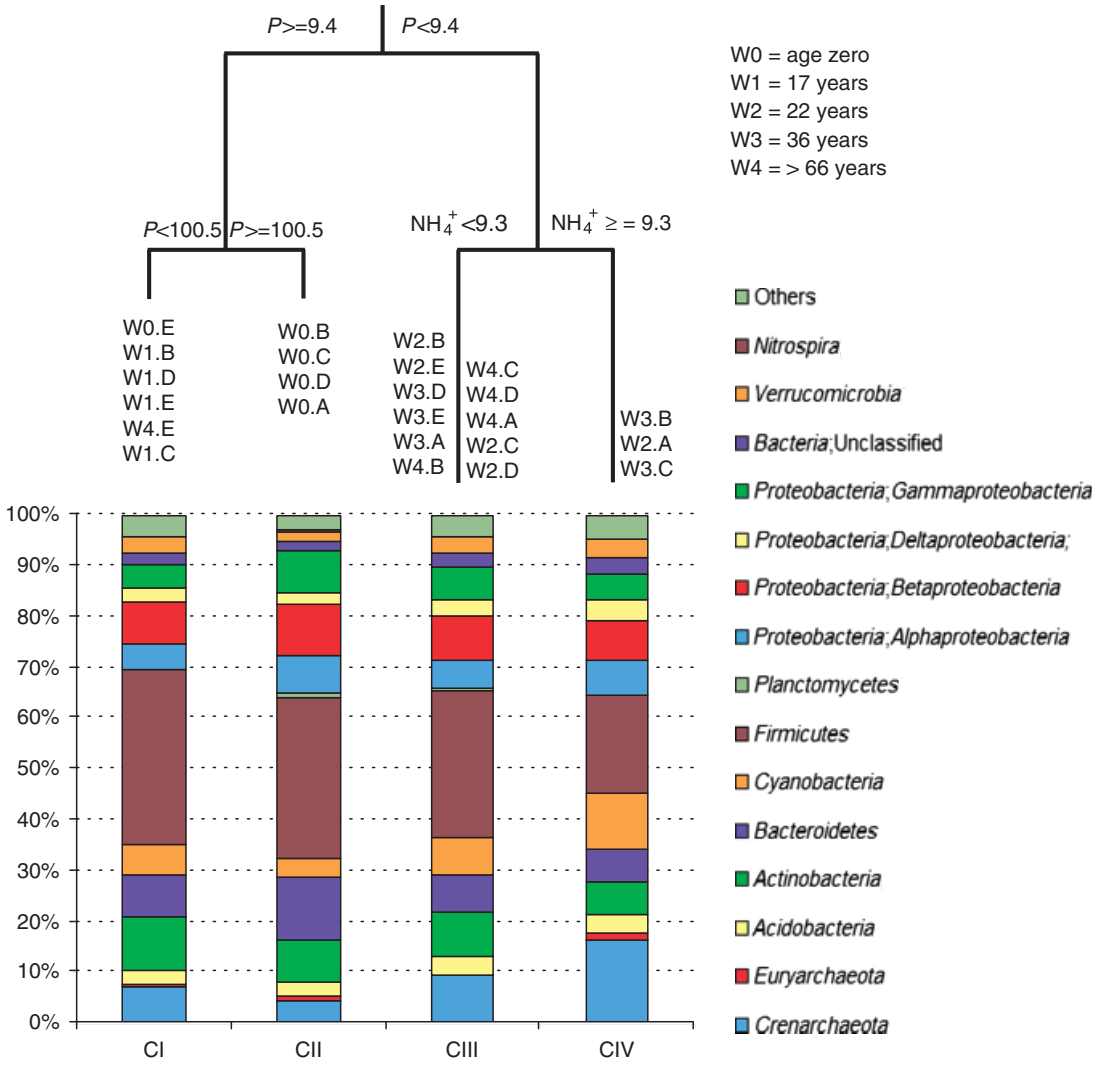

Fig. 3. MRT analysis of 2222 normalized OTUs intensities detected by PhyloChip analysis and soil factors (soil available P, NH_4^+). Bar-plots show the average relative abundance of the 386 OTUs that were significantly correlated with soil factors, % organic matter, soil P, NO_3^-, NH_4^+. The OTUs were classified into 15 major phyla and 'others', which included OTUs belonging to phyla *Aquificae*, *Chlamydiae*, SPAM, *Thermotogae*, TM7, OD1, OP8. The bar-plots represent the samples in clusters I (CI), II (CII), III (CIII) and IV (CIV).

Discussion

The increases in plant species richness and diversity with time since abandonment of intensive agricultural practices in the chalk grasslands of the Wrakelberg are typical of secondary grassland succession, and similar trends have been observed for other grasslands on similar soils in the Netherlands (Willems, 1980; Bobbink & Willems, 1987). However, contrary to our hypothesis, the aboveground plant species richness and community composition did not appear to have a significant effect on the belowground microbial communities. It should, however, be noted that our analyses involved bulk soil samples, and it could be that a focus on the rhizosphere may have yielded a greater impact of vegetation (Kowalchuk *et al.*, 2002). Given the extended length of time for plant-induced impacts on microbial communities to accumulate over the course of secondary succession, we anticipated that such effects would indeed become manifest throughout the bulk soil, but this was clearly not the case. NMDS and ANOSIM analyses confirmed that the microbial communities in the arable and young fields (17 years after abandonment) were different from the intermediate and the late succession stages. Also, the succession stages of 22, 36 and older than 66 years have a large overlap within their microbial communities, with no clear separation between them.

Soil biochemistry, as opposed to plant species composition and richness, seemed to be the main driver of microbial

community structure. This is supported by the ANOSIM analysis, which showed that microbial communities were significantly different in soils with different amounts of NH_4^+ ($R = 0.50$, $P = 0.0079$) and available phosphorus ($R = 0.61$, $P = 0.0155$). Soil pH was previously shown to be the dominant factor steering microbial communities in chalk grasslands of this region (Kuramae *et al.*, 2010), but this overriding effect masks the importance of other drivers of microbial community structure. We, therefore, focused specifically on soils that have buffered conditions, thereby allowing a far more detailed analysis of nutritional and vegetation factors that may drive microbial community structure and diversity. This approach showed a clear discrimination between arable young and old fields, with soil available phosphorus being the most dominant factor in driving this distinction. It should, however, be mentioned that the importance of other factors, not assessed in this study, that might correlate with available phosphorus cannot be ruled out. It should also be noted that these results represent a single time point (February, 2007), and community dynamics may shift throughout the year. Although seasonal dynamics might affect the trends observed across the microbial taxa observed, previous studies have shown consistent impacts of changing nutrient conditions on microbial activities in such grassland systems (Kowalchuk *et al.*, 2000), and the long-term patterns within these microbial communities are expected to remain generally intact. Although the soil conditions at the Wrakelberg appear to be rather typical of such chalk grasslands (Kowalchuk *et al.*, 2000), it must be mentioned that the lack of a true field-level replication in this study makes it impossible to rule out that specific peculiarities of some of the fields studied here may have contributed to the observed result.

The higher nutrient conditions present in the arable field samples (e.g. $P > 100.5\ mg\ kg^{-1}$ soil) apparently selected for specific members of the *Bacterioidetes*, *Planctomycetes*, *Alpha-*, *Beta-* and *Gammaproteobacteria*, and it is reasonable to suppose that many of these populations represent soil-borne copiotrophs. In marine environments, the gammaproteobacterial order and the *Alteromonadales* clade have been put forth as containing a large proportion of copiotrophs (Lauro *et al.*, 2009). In addition to the *Alteromonadales*, we found that the orders *Pseudomonadales* and *Xanthomonadales* were positively correlated with available phosphorus, which is in agreement with the negative selection imposed by nutrient-limited bulk soil conditions on members of the genus *Pseudomonas* (Van Overbeek & Van Elsas, 1997). Extending these arguments to later succession stages, it is to be expected that the lower nutrient status of these soils should select for a greater proportion of oligotrophic microorganisms. Among the groups that were relatively over-represented in the late succession stage, *Verrucomicrobia*, *Crenarcheota* and *Cyanobacteria* are known to have a large proportion of slow-growing oligotrophic representatives (Lauro *et al.*, 2009).

During secondary succession, the total plant productivity is decreased, and it is presumed that there is a greater reliance on arbuscular mycorrhizal fungi (AMF) to facilitate P uptake. Thus, although plant community composition had no detectable direct effects on microbial communities, shifts in plant nutrient acquisition strategies and efficiencies may affect soil-borne microorganisms that compete for these nutrients, and it has been demonstrated that plant species can differentially affect soil microorganisms due to differences in their efficiency of nutrient acquisition (Bardgett *et al.*, 1999). Furthermore, as AMF become more abundant and diverse, their impact on soil-borne microbial communities is expected to increase, because AMF have been shown to influence microbial community structure in the rhizosphere (Drigo *et al.*, 2010). It may also be that the neutral soil conditions facilitate phosphate uptake by plants, as low (acid) soil pH has been shown to contribute to the immobilization of phosphate by bivalents such as Ca, Fe and Al (Vance *et al.*, 2003).

We also observed interesting and unexpected patterns with respect to microbial biodiversity, with the highest microbial diversity detected in the fields still under intensive management or only abandoned recently. Thus, microorganisms and plants showed opposite patterns of diversity. It is still a matter of speculation as to why microbial diversity should be the highest under the high nutrient and relatively disturbed conditions of arable or recently reclaimed fields. Also, potential biases of the PhyloChip platform should be kept in mind, as the OTUs detected on the PhyloChip are based on the sequence information that is presently known, thereby potentially being biased toward greater representation of OTUs typically found in agricultural vs. more natural settings. However, the microbial diversity detected did increase again slightly in the oldest fields, which may reflect the increased tropic complexity that develops after several decades of abandonment.

In total, the focus on fields with neutral pH across a land reclamation gradient allowed us to relate patterns of microbial diversity and community composition with detailed patterns of vegetation dynamics and chemical soil factors other than pH. In a previous study (Kuramae *et al.*, 2010), we showed that pH had an overriding effect on the composition of microbial communities in some of the field soils also included in the analyses described in the present study. In order to obtain a more detailed picture of the factors that drove microbial community structure in soils of similar (neutral) pH, we conducted this study including the same conditions as in Kuramae *et al.* (2010). The results of the present study suggest that soil nutrient conditions, especially available P levels, were among the most important drivers of microbial succession after field abandonment, with plant diversity and species composition playing an insignificant role.

Acknowledgements

We thank A.S. Pijl for laboratory assistance and W. Smant for soil chemical analysis. This work was supported by the Bsik program of 'Ecogenomics' (http://www.ecogenomics.nl/). Publication number 5023 of the NIOO-KNAW, Netherlands Institute of Ecology.

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Supporting Information

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

Fig. S1. Schematic representation of soil sampling of chalk grassland in Wrakelberg, the Netherlands.

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