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LETTER

Reduction of rare soil microbes modifies plant–herbivore interactions

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Abstract

Rare species are assumed to have little impact on community interactions and ecosystem processes. However, very few studies have actually attempted to quantify the role of rare species in ecosystems. Here we compare effects of soil community assemblages on plant-herbivore interactions and show that reduction of rare soil microbes increases both plant biomass and plant nutritional quality. Two crop plant species growing in soil where rare microbes were reduced, had tissues of higher nutritional quality, which theoretically makes them more susceptible to pest organisms such as shoot-feeding aphids and root-feeding nematodes. Reduction of rare microbes increased aphid body size in the absence of nematodes; nematodes always reduced aphid body size independent of the soil microbial community. This study is the first to show that rare soil microbes are not redundant but may play a role in crop protection by enhancing aboveground and belowground plant defence. It remains to be tested whether these are direct effects of rare soil microbes on plants and herbivores, or indirect effects via shifts in the microbial soil community assemblages.

Keywords

Biodiversity, dominant, species interactions.

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INTRODUCTION

Dominant species are assumed to have more impact on ecosystem functioning than subordinate or rare species. Here, we use the term 'rare' to indicate low abundance in the soils under study, which is not necessarily 'rare' in a global population perspective. The mass ratio hypothesis postulates that effects of species on ecosystems are in proportion to their contribution to primary production (Grime 1998). However, very few studies have examined the influence of rare species empirically. Rare species can play major roles in ecosystem processes when they have particular functional characteristics (Hooper *et al.* 2005). Nevertheless, relatively few of such rare but important species have been recognized (Clay & Holah 1999; Peltzer *et al.* 2009). Especially in the soil, where microbial communities are extremely species rich (Torsvik *et al.* 2002) and functional redundancy is supposed to be high (Nannipieri *et al.* 2003), rare species would be predicted to have little significance for community interactions and ecosystem processes. Here, we address the question whether rare microbes in soil may influence plant production and plant–herbivore interactions both aboveground and belowground.

Communities of soil organisms are extremely diverse and there are numerous interactions within and between species from most levels of the soil food web (Hunt *et al.* 1987;

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De Ruiter *et al.* 1995). Selective removal studies point at high redundancy within the microbial community for a range of functions, such as decomposition, degradation and ammonification (Degens 1998; Griffiths *et al.* 2001; Wertz *et al.* 2006). Yet, biodiversity loss can impair resistance or resilience to abiotic stress (Griffiths *et al.* 2000). However, we are not aware of any study examining the effects of rare soil microorganisms on aboveground–belowground plant–herbivore interactions.

We tested the hypothesis that loss of rare microbial species in soil affects primary production (plant biomass and quality) and herbivore performance, as well as the effects of herbivores on plants. To test our hypothesis, we used two crop plants (*Beta vulgaris* and *Brassica oleracea*) and determined their biomass and nutritional quality after exposure to soil communities with and without rare soil microbes. The rare microbes were reduced by diluting filtrates from field soil and re-inoculating these filtrates into sterilized soil (Wertz *et al.* 2006). After a period of establishment enabling similar soil microbial biomass production, plants were grown in these soils and exposed to factorial combinations of aboveground and belowground invertebrate herbivores. Primary production depends on aboveground and belowground mutualists and herbivores, as well as on decomposer organisms in the soil (Scheu 2001; Wardle *et al.* 2004). In addition, nutritional quality plays an important role in plant suitability for aboveground (van der Meijden *et al.* 1988) and belowground herbivores (Van Dam 2009). Therefore, we determined both plant biomass and quality indices.

In our predictions, we considered plant-associated organisms and higher trophic-level organisms, such as specialized predators and microbial parasites to be influenced most by dilution, because these organisms are often less abundant in soil food webs (Hunt & Wall 2002). Moreover, plant-associated soil organisms are supposed to have less functional redundancy (van der Heijden *et al.* 1998; Brinkman *et al.* 2005) than decomposer organisms (Degens 1998; Liiri *et al.* 2002; Wertz *et al.* 2006). Therefore, we predicted that reduction of rare microbes will affect aboveground and belowground herbivores and, therefore, plant biomass and quality. The outcome of this study will be of importance for both fundamental and applied ecology, because it elucidates the role of rare species in community organization and ecosystem functioning.

MATERIAL AND METHODS

We removed all species from the soil microbial community by sterilization with gamma radiation. Then, microbial suspensions were obtained from unsterilized soil, diluted to reduce rare species (Wertz *et al.* 2006) and inoculated to the sterilized soil. To allow the total microbial biomass to recover, we incubated the soils without plants for 30 weeks in 10-L microcosms.

Two non-mycorrhizal crop plant species (*Beta vulgaris* L. and *Brassica oleracea* L.) were seeded in the soils. Plants were exposed to shoot-feeding aphids (*Brevicoryne brassicae* on *B. oleracea* and *Myzus persicae* on *B. vulgaris*) and root-feeding nematodes (*Heterodera schachtii* on both plant species). The microcosms were kept under controlled conditions in the greenhouse. Plant biomass, primary and secondary metabolites and the numbers of herbivores per plant were determined at the end of the experiment when the plants were 13 (*B. vulgaris*) and 16 (*B. oleracea*) weeks old.

Soil collection and treatment

Soil was collected from a field on sandy loam soil in Hoeven (Brabant, the Netherlands; 51°35′13 N, 4°34′13 E). The current crop was *Zea mays* L. (maize), while the previous crops were *Solanum tuberosum* L. (potato; 2005) and *Beta vulgaris* L. (sugar beet; 2004). *Brassica oleracea* had never been grown on this soil. At distances of more than 1 m, seven soil samples of 1 kg were collected at a depth of 0–20 cm below the soil surface, to be used as inoculum. The soil was stored in plastic bags at 4 °C until inoculation. At the same day, an additional amount of 300 kg soil was collected from the same field. The soil was sieved and homogenized before γ-sterilization (> 25 kGray, Isotron, Ede, the Netherlands) and divided over plastic bags. After sterilization, the pH_{CaCl2} was 5.7 and the main extractable nutrient concentrations were: ammonium (0.001 M $CaCl_2$) 3.8 mg kg^{-1}; nitrate (0.001 M $CaCl_2$) 84 mg kg^{-1}; phosphorus (0.001 M $CaCl_2$) 8 mg kg^{-1}; potassium (0.001 M $CaCl_2$) 92 mg kg^{-1}.

Seven suspensions were made, using the seven soil samples described above. Soil suspensions were made by mixing 44 g fresh weight (fw) soil with 400 mL sterile distilled water using a Waring blender. After 1 min at maximum speed, the blender with content was cooled on ice for 2 min and this procedure was repeated four times. The suspensions were decanted in centrifuge tubes and centrifuged at 4 °C 1000 ***g*** to remove soil particles. Supernatant was sieved over 45 μm to remove soil fauna. Then, the supernatant was diluted to reduce rare soil microbes (see below), added to bags with sterilized soil and homogenized thoroughly. Every bag contained 6 kg of soil and was inoculated with 270 mL suspension. For the undiluted suspensions, the amount of soil that forms the basis of this suspension equals (270/400) × 44 g = 30 g fw soil. Thirty grams soil in 6 kg means it is diluted 200 times. Seven bags were inoculated with the undiluted supernatant, seven bags received a 10^2 dilution and another seven bags were inoculated with a 10^4 dilution resulting in final dilutions, compared with the field situation, of 2×10^{-2}, 2×10^{-4} and 2×10^{-6}. In the least diluted soils, the suspension equalled

30 g soil added to 6 kg of sterilized soil, so in theory, assuming 100% extraction efficiency, all species with > 1 individuals per 30 g field soil should have been present. The medium diluted soils were inoculated with 10^{-2} of the previous suspension and should therefore theoretically have contained abundant and subordinate species with > 1 individuals per 0.3 g field soil, while in the most diluted soils only abundant species with > 1 individuals per 0.003 g field soil will have been present.

After addition of the suspensions to the sterilized soil, additional sterile distilled water was added to reach 20% moisture (w/w). Then, all bags were closed with a cotton wool plug to avoid contamination by air-borne microbes, but at the same time allowing exchange of gasses. The bags were kept in a greenhouse under controlled conditions (ambient light, 21 °C/16 °C) and the contents were regularly homogenized by turning the bags over. Moisture content was slowly declining to 18%, with no significant difference between the bags. No water was added during the incubation, because we were aiming for 14% moisture when the plants would be growing in the soil. After 30 weeks, soil samples were taken from the 21 bags for assessing microbial biomass and composition and nutrient content. The intention was to reach similar microbial biomass, but reduced species richness. A side consequence may have been increased species dissimilarity in the soils with the most diluted suspensions. Total N and organic C in soil were measured with a Flash EA 1112 NC analyzer (Interscience, Breda, the Netherlands).

Experimental setup and maintenance

After the 30 weeks incubation, each bag with inoculated sterilized soil was used to fill eight pots with 550 g soil. These eight pots were used for setting up one replicate of a multi-factorial experiment with treatment combinations: plant species (two species), aphids (absent or present) and nematodes (absent or present). This resulted into 3 dilutions × 7 replicates × 8 treatment combinations, equalling 168 pots in total. In half the pots, two seeds were sown from sugar beet 'Shakira' (KWS Saat A.G., Einbeck, Germany), which is one of the main current cultivars used by sugar beet growers and this race is sensitive to *Heterodera schachtii*. As these were commercial seeds, they had been pelleted with fungicides: hymexazole against the soil oomycete *Aphanomyces euteiches* and TMTD against seed fungi. The remaining 84 pots were sown with two seeds of Brussels sprouts *Brassica oleracea* L. var. gemmifera cv. 'Maximus' (Syngenta Seeds, Enkhuizen, the Netherlands). Pots were placed randomly on a table in a greenhouse with controlled conditions (60% RH; 16 h L, 8 h D, 21 °C/16 °C). Additional illumination was provided by 400 W growing bulbs (Philips SON-T Agro, Philips, Eindhoven, The Netherlands). Light intensity at plant level was 225 μmol PAR. Germination of the seeds was > 95% and after 1 week seedlings were thinned randomly, so that every pot then contained one seedling. Water was provided regularly to keep moisture levels at 14% w/w. To compensate for possible side effects of rare microbes on nutrient availability, we supplied Hoagland solution as 0.5 double phosphorus Hoagland (van Dam *et al.* 2004) 40 mL per week.

When the seedlings were 6 weeks old, each pot of the nematode treatment was inoculated with *H. schachtii*. A population of *Heterodera schachtii* was collected from a sugar beet field in the Netherlands and then kept in culture on sugar beet in the greenhouse. Per pot 500 juveniles were suspended in 5 mL water. This initial density of nematodes is low compared with the advised threshold in the field for growing resistant cultivars (Heijbroek *et al.* 2002). Non-nematode treatments received 5 mL water only, without nematodes.

Ten weeks after sowing the seeds, aphids were placed on the plants allocated to the aphid treatments. *Brevicoryne brassicae* were collected from *B. oleracea* and cultured in a growth cabinet on *B. oleracea* 'Maximus'. Five individuals (4th instar) were placed on the *B. oleracea* plants allocated to the aphid treatment. *Myzus persicae* were cultured in a growth cabinet on sugar beet 'Shakira' and five individuals (4th instar) were placed per plant on the *B. vulgaris* plants allocated to the aphid treatment. Every pot, so also the pots without aphids, was placed individually inside a fine-maze net that was closed with a zipper. These nets are effective in keeping in the aphids, while still allowing observations and counting at regular intervals. The number of aphids per plant was estimated every 2–3 days. Winged adult aphids were rare and therefore not scored separately.

When the seedlings were 13 weeks old, all sugar beet plants were harvested. First, *Myzus persicae* were collected from the plants using a fine brush. The aphids were stored in 70% ethanol for image analysis with the software WINSEEDLE PRO v.7 (Regent Instruments, Ottawa, Canada) to determine their body length as a fitness-related parameter; aphid size is positively related with reproductive output (Vorburger 2005). The aim was to scan at least 100 individuals per plant, but six of the 42 plants had less than 100 individuals; in those cases all aphids were scanned. The plant shoot, beet and roots were weighed to determine fresh weight, and after freeze-drying weighed again to determine dry weight.

The *B. oleracea* plants were only harvested when the seedlings were 16 weeks old, as *B. brassicae* populations developed slower than *M. persicae*. *Brevicoryne brassicae* were not collected for size measurements, because they were very difficult to remove from the plants without damaging them. Two young fully expanded leaves were flash frozen in liquid nitrogen for glucosinolate analysis on HPLC (van Dam *et al.*

2004). The remaining part of the plant was weighed to determine fresh weight, freeze-dried and weighed again for dry weight.

Plant and soil analyses

The freeze-dried plant material was ground in a Retsch Mill MM301 (RETSCH, Haan, Germany) for 2 times 1 min at 30 rpm. Three milligrams of the leaves and the sugar beet were used for measuring total N and organic C with a Flash EA 1112 NC analyzer (Interscience, Breda, the Netherlands). Methanol extracts were made with 100 mg ground material to determine sugar content, amino acids (both plant species) and glucosinolates (for *B. oleracea* only) with HPLC (van Dam *et al.* 2004).

Total number of nematode cysts per pot was determined by adding 5 L water to the soil from every single pot, stirring the suspension and decanting it over 180 μm mesh. This decantation procedure was repeated three times for every pot. The material that remained on the surface of the sieve was collected on a paper filter and cysts were separated from the organic debris using the aceton method (Den Ouden 1954). Number and size of the cysts was determined using the image analysis software WINSEEDLE PRO v.7 (Regent Instruments).

Soil from 21 pots of each plant species that had not been inoculated with *H. schachtii* was checked for nematode contamination by extracting 75 g soil with the Oostenbrink elutriator (Oostenbrink 1960). No nematodes were found, indicating that the microbial suspensions were free of nematodes or nematode eggs and that no contamination had occurred during the experiment.

Microbial assessments

Ergosterol, as indicator of fungal biomass, was extracted (De Ridder-Duine *et al.* 2006) from soils after 30 weeks incubation in the greenhouse, before seeding. Total DNA was extracted from soil samples after 30 weeks incubation in the greenhouse to determine microbial composition by PCR-DGGE and bacterial abundance by real-time PCR. DNA extraction was performed on 0.25 g soil with the MoBio PowerSoil™ DNA Isolation kit according to the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA, USA). PCR amplifications were performed for general bacteria (968-gc/1378; Heuer *et al.* 1997), for actinomycetes (Acti243/1378; Heuer *et al.* 1997) and for *Bacillus* (BacF/1378; Garbeva *et al.* 2003). Fungal biomass was low in all treatments and therefore no further molecular analyses of community composition were done. Yet, this does not mean that all observed effects are due to bacteria and hence we speak of 'microbes' throughout the manuscript. DGGE analysis was performed using the method of Muyzer *et al.* (1993), incorporating modifications described in Kowalchuk *et al.* (2006). Members of the actinomycetes and *Bacillus* are very common soil organisms; DGGE is showing dominant bands only. Moderately abundant species and rare species are not detected, so that DGGE shows a subset of the microbial soil biodiversity, mainly the dominant taxa. Although according to our theoretical calculation the dominant microorganisms should not be affected by dilution, we performed DGGE to provide information on variation in the dominant microorganisms within and among treatments. Then, we correlated this variation with that in plant biomass as to test whether variation in dominant microbes could have been the cause of the observed variation in plant biomass. DGGE gels can be problematic to align; therefore we choose to only compare samples within the same gel. This limited the sample size to 15 of the 21 samples in total (three dilutions and seven replicates). DGGE banding profiles were examined with the ImageMaster Elite Database program (Amersham Biosciences, Roosendaal, the Netherlands), binary coded and band matching data were exported via Excel for statistical analysis. To determine the similarity in relative abundance of bacterial DNA between soils, quantitative PCR with general bacteria primers Ba519f and Ba907r was performed according to Lueders *et al.* (2004a,b).

Statistical analysis

Microbial abundance ($n = 21$), the number of DGGE bands ($n = 15$) and dissimilarity of DGGE pattern ($n = 15$) were analysed with a linear mixed model with block as random factor and dilution as fixed continuous explanatory variable. Dissimilarity within treatment was calculated as the average Jaccard distances between replicates within a treatment based on absence/presence of DGGE bands (Dissimilarity = 1 − (number of bands present in both samples x and y/(number of bands present only in sample x + number of bands present only in sample y + number of bands present in both samples x and y). To assess whether changes in abundant microorganisms could explain changes in total plant biomass, the correlations between Jaccard distances and variation in total plant biomass was calculated. Variation in plant biomass was defined as the difference in plant biomass between two samples divided by the average plant biomass of those two samples. In total, 24 correlations were calculated, based on three DGGEs (for total bacteria, actinomycetes and Bacillus) and eight plant biomass groups (two plant species, absence/presence aphids, absence/presence nematodes). To retain power despite the multiple testing, we applied control of false discovery rate (Verhoeven *et al.* 2005) with $\alpha = 0.10$.

For analysis of plant parameters ($n = 84$), a linear mixed model was used with block as random factor, nematode and

aphids as categorical factors and dilution as continuous explanatory variable. Analyses were done separately for each plant species since their experimental period differed as well as the aphid species on the plant species. Stepwise model simplification was used to obtain the minimum model. This includes the removal of non-significant (interaction-)terms. When dilution interacted with nematode or aphid effect, the percentage change due to nematodes or aphids was calculated separately for each dilution level. Normality of errors was assessed by visual inspection of q–q plots. Glucosinolate and amino acid concentrations were log-transformed to obtain normality of errors. Nitrogen concentrations in the shoot were included as covariable when analysing the effect of nematodes on aphid body size. Exponential growth curves were fitted for the numbers of aphids plotted against days since introduction ($n = 42$ for *M. persicae*). There is some density dependence but this did not affect the treatment effects. Analysis of part of the data set, omitting the last data points that were most likely density dependent, gave the same result as analysis of the full data set (data not shown). For estimation of aphid growth rate on *B. oleracea*, we excluded nine plants from the analyses where aphid populations did not develop well and had < 10 individuals ($n = 33$ for *B. brassicae*). Since there was some mortality under the initial 5 aphids per plants, both initial number and growth rate was estimated and tested for treatment effects using a linear mixed model with block as random factor, nematode as categorical fixed factor and dilution as fixed continuous explanatory variable. An alpha level of 0.05 was used to determine significant effects. All analyses were performed with R Statistical package version 2.3.1. (Team 2008).

RESULTS

After 30 weeks of incubation, all three soils had similar fungal and bacterial biomass, but differed in species richness and dissimilarity as intended (Table 1). We confirmed that the soils were not significantly different in terms of concentrations of ammonium, nitrate, phosphorus and potassium (Table 2). Nitrate showed a marginally significant increase with dilution, therefore it was tested whether including this factor in the models would improve the models but this was not the case. pH showed a statistically significant but small decline with dilution. Thus, dilution followed by incubation yielded soils that did not differ in microbial biomass and nutrient availability, but that had different microbial communities.

Rare species reduction affects plant biomass and nutritional quality

Rare species reduction significantly influenced plant biomass production as well as plant quality. Belowground and total

Table 1 Microbial abundance, species richness and dissimilarities in diluted soils after 30 weeks incubation

	Mean values ± SEM for three microbial manipulation treatments					
Variable	(a) Abundant, subordinate and rare*	(b) Abundant and subordinate*	(c) Abundant*	*t*-value	d.f.	*P*
Microbial abundance						
Fungal biomass† (μg C g^{-1} soil)	34 ± 19	28 ± 10	25 ± 7	−1.44	13	0.17
Bacterial community size‡ (10^6 DNA copies g^{-1} soil)	2.6 ± 0.6	2.3 ± 0.2	2.1 ± 0.4	−0.72	13	0.48
Species richness§						
Actinomycetes	14.8 ± 1.9	15.4 ± 0.5	11.2 ± 0.8	−1.97	9	0.08
Bacillus	19.2 ± 0.6	16.8 ± 0.5	15.4 ± 1.4	−3.01	9	0.02
Total bacteria	15 ± 0.7	12.4 ± 1.3	10.2 ± 0.9	−3.21	9	0.01
Dissimilarity¶						
Actinomycetes	0.55 ± 0.05	0.44 ± 0.02	0.75 ± 0.04	2.61	9	0.028
Bacillus	0.40 ± 0.03	0.39 ± 0.03	0.48 ± 0.02	3.03	9	0.014
Total bacteria	0.45 ± 0.03	0.46 ± 0.04	0.61 ± 0.04	4.22	9	0.002

*In the least diluted soils (a), theoretically all species with > 1 individuals per 30 g field soil were present. The medium diluted soils (b) contained abundant and subordinate species with > 1 individual per 0.3 g field soil, while in the most diluted soils (c) only abundant species with > 1 individual per 0.003 g field soil were retained.

†Based on ergosterol measurements, $n = 7$.

‡Based on real-time PCR with general bacteria primers, $n = 7$.

§NO. DGGE bands, $n = 5$.

¶Jaccard distances within treatments, $n = 5$.

Table 2 Nutrients in soil after 30 weeks incubation

Variable	Mean values (± SEM) for three microbial manipulation treatments					
Nutrients*	(a) Abundant, subordinate and rare†	(b) Abundant and subordinate†	(c) Abundant†	*t*-value	d.f.	*P*
NH_4^+-N (mg kg^{-1})	1.6 ± 0.3	1.5 ± 0.3	1.3 ± 0.3	−1.00	13	0.34
NO_3^--N (mg kg^{-1})	61 ± 3	65 ± 4	71 ± 3	2.10	13	0.06
Phosphorus (mg kg^{-1})	9.7 ± 0.5	10.1 ± 0.2	10.1 ± 0.2	0.89	13	0.38
Potassium (mg kg^{-1})	85 ± 3	91 ± 1	92 ± 5	1.48	13	0.16
pH (0.01 M $CaCl_2$)	5.54 ± 0.01	5.50 ± 0.01	5.48 ± 0.01	−6.34	13	0.00

*For all parameters: $n = 7$.
†Headings are equivalent to those in Table 1.

biomass production of both plant species was enhanced when rare species were reduced; on average there was an increase of 8% biomass per dilution step (Fig. 1). The reduction of rare species also goes along with shifts in the abundant species, as shown by changes in DGGE patterns (Table 1). Yet, variability in abundant species does not explain the variability in plant biomass, as indicated by the low, mostly non-significant correlations between Jaccard distances and variation in total plant biomass (Table S1). Herbivore addition did not alter the effects of reducing rare microbes on plant biomass production (soil microbial community × herbivores $t = 0.13$, d.f. = 19, $P = 0.90$ for *B. vulgaris* and $t = -0.71$, d.f. = 19, $P = 0.47$ for *B. oleracea*). More biomass production per unit time may lead to a dilution of tissue nutrient concentration. However, rare species reduction enhanced carbon and nitrogen concentrations in *B. vulgaris* shoots (Table S2), whereas the shoot tissue composition of *B. oleracea* was not significantly affected. These changes in resource quantity and quality did not affect the numbers of nematode cysts per plant, the average cyst diameter, or the number of aphids per plant significantly ($P > 0.05$, Table S2).

Interactions between rare species reduction and herbivore effects

Nematodes significantly reduced aphid numbers (Fig. S1) and aphid growth rates on both plant species (Table S3). Nematodes on *B. vulgaris* reduced aphid body size independent of the soil community composition. Such nematode effects on aphid reproduction emerged before (Bezemer *et al.* 2005), but here we show that rare soil microbes also reduced aphid body size in the absence of nematodes (Fig. 2). The presence of rare soil microbes influenced plant

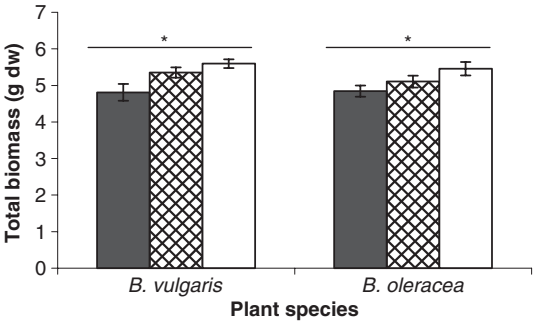

Figure 1 Effect of soil microbial community on total plant biomass of *B. vulgaris* and *B. oleracea* (mean dry weight ± SEM, $n = 28$). Black bars: least diluted soils, all species with > 1 individual per 30 g field soil were present. Hatched bars: medium diluted soils containing abundant and subordinate species with > 1 individual per 0.3 g field soil. White bars: most diluted soils where only abundant species with > 1 individual per 0.003 g field soil were retained. Significant effect of soil microbial community on total biomass (*$P < 0.05$).

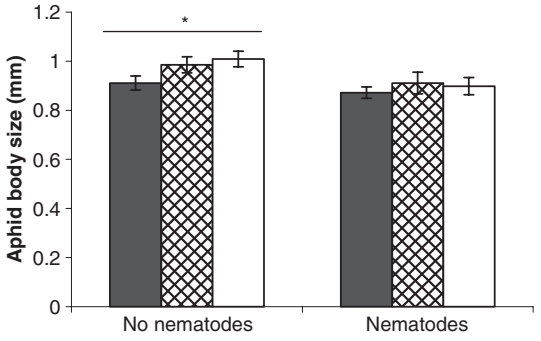

Figure 2 Effects of soil microbial community and nematodes on aphid body size on *B. vulgaris* (mean body length ± SEM, $n = 7$). Black bars: least diluted soils, all species with > 1 individual per 30 g field soil were present. Hatched bars: medium diluted soils containing abundant and subordinate species with > 1 individual per 0.3 g field soil. White bars: the most diluted soils where only abundant species with > 1 individual per 0.003 g field soil were retained. Significant effect of soil community on aphid body size (*$P < 0.05$).

tissue composition upon infestation with nematodes and aphids (Fig. 3). The effects varied, depending on the precise combination of interacting species. In *B. vulgaris*, the percentage of sugar in the beets depended on a combination of rare species reduction and nematode presence (soil microbial community × nematodes $t = 2.19$, d.f. = 19, $P = 0.041$). Nematodes reduced the percentage sugar and reduction was most severe in the soils in which rare microbes were present (Fig. 3a). In *B. oleracea*, the presence of rare species enhanced glucosinolate concentration in the shoots upon aphid infestation (soil microbial community × aphids $t = -3.01$, d.f. = 40, $P = 0.0015$; Fig. 3b). In *B. oleracea*, the presence of rare soil microbes also reduced the concentration of amino acids on aphid-infested plants (soil microbial community × aphid $t = 2.17$, d.f. = 40, $P = 0.036$) (Fig. 3c).

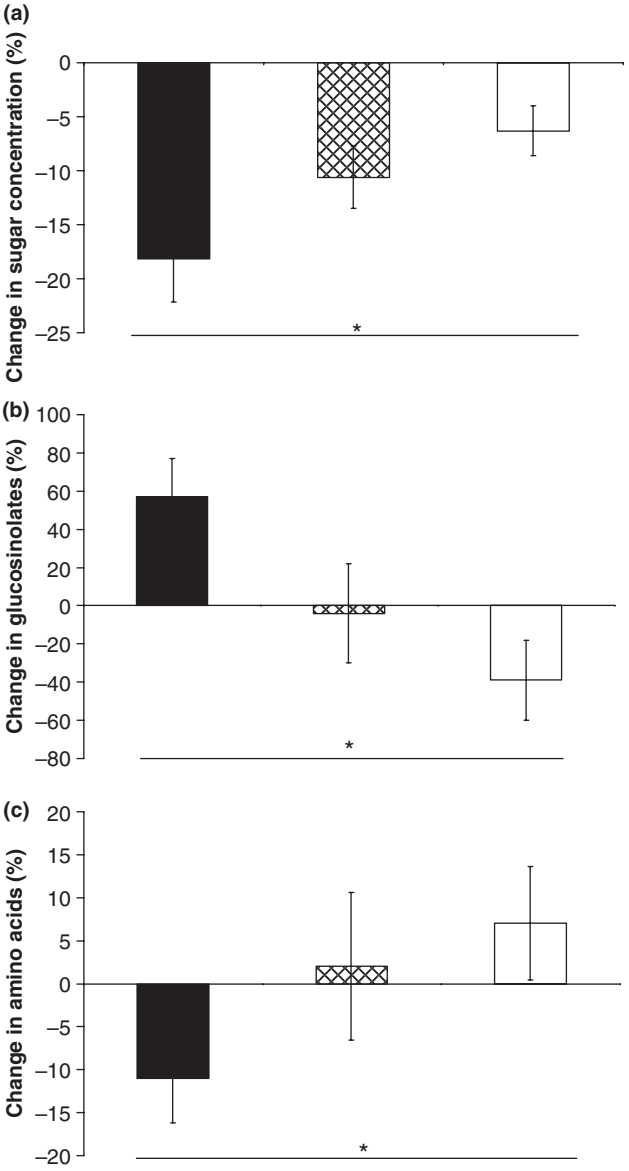

Figure 3 Soil dilution modifies effects of nematodes and aphids on plants (a–c). Mean ± SEM are given, $n = 14$. Effects of nematodes on sugar concentration in sugarbeet (a); effects of aphids on glucosinolates in *B. oleracea* (b) and effect of aphids on amino acids in *B. oleracea*. (c). Effects are calculated as 100 × (difference between plants with and without nematodes or aphids/plants without nematodes or aphids). Black bars: least diluted soils, all species with > 1 individual per 30 g field soil were present. Hatched bars: medium diluted soils containing abundant and subordinate species with > 1 individual per 0.3 g field soil. White: the most diluted soils where only abundant species with > 1 individual per 0.003 g field soil were retained. Significant effect of soil community (*$P < 0.05$).

DISCUSSION

We show that manipulation of the microbial community, most likely a major reduction of especially those species that were rare in the soil microbial community, influences plant biomass and plant quality. The biomass of both plant species responded positively to rare species reduction, suggesting that rare species may have a detrimental effect on plant production. A possible mechanism for the positive effect of rare species reduction on plant growth could be the decreasing number of microbial species interactions, which may lower the production of phytotoxic metabolites by the microbial communities (de Boer *et al.* 2007). Testing this or other possible mechanisms would require additional studies. Identification of the organisms responsible for plant growth reduction was not attempted because it was unknown whether to look for single species or combinations of species. Moreover, isolation of rare microorganisms is hampered by their low densities and the general low cultivability of more than 90% of all soil microorganisms (Riesenfeld *et al.* 2004). Therefore, we cannot exclude that this positive effect of rare species reduction on plant biomass might also be due to filtering out soil pathogens (Suslow & Schroth 2009); however, no visual disease symptoms were observed in plants on soils with rare microbial species still present. In addition, soil microbial communities were allowed to develop during 30 weeks after inoculation in the absence of plants. This is not a situation where plant-pathogenic microbes are expected to develop well as they are weak competitors for growth on soil organic compounds. Nutrient availability in the soil could be eliminated as a cause for the observed effects on plant biomass and quality, because there were no differences in soil nutrient parameters among dilution treatments at the start of the experiment and additional nutrients were given during the experiment.

Although rare microbes reduced plant biomass, their net effect is not necessarily negative, because plants exposed to all microbes were less suitable for herbivores; the levels of

defensive compounds were higher and concentrations of nitrogen, sugar and amino acids were lower. This may explain why aphid body size was reduced in the presence of rare soil microbes on *B. vulgaris* (we could not determine aphid body size on *B. oleracea*). The lower average aphid body size can result from a shift in stage distribution towards younger aphids, as well as from a smaller aphid body size within developmental stages. As aphid body size is related to fitness (Godfray 1994), we expect that these reductions of plant quality may depress the long-term aphid population development in the field when rare soil microbes are present in the soil. During the experiment, the number of aphids per plants was rather variable (Tables S2 and S3) and therefore there were no statistically significant effects on aphid population size by the soil microbial community even though average aphid body size was affected in the absence of nematodes. However, the effects of the nematodes, which were stronger than the effect of the dilution, did affect both aphid body size and aphid growth rate as expected (Bezemer *et al.* 2005). The experiment did not last long enough to show effects of aphids on plant biomass. Ultimately, to understand consequences of microbial alterations in the soil under field conditions, it needs to be assessed if the net effects of moderate plant biomass reduction due to the presence of rare microbes in the soil community may be outweighed by reduced exposure to shoot feeding by aphids.

Below ground, we did not observe such fitness effects on herbivores. The size of the nematode cysts, which is indicative of their egg production (Monroy and Van der Putten 2009), was not influenced, so that plants with nematodes may not have a direct net benefit from the presence of the rare soil microbes. Interestingly, in the absence of nematodes, the presence of rare microbes had the same negative effect on aphid body size as nematodes had, implying that rare soil microbes may have an important insurance value for the capacity of communities of soil organisms to reduce aboveground pest incidence. Nematodes are patchily distributed in the field (Robertson & Freckman 1995), so that their effect on aphids is expected to be spatially variable. While keeping in mind that extrapolation from a greenhouse to the field has its limitations and that this effect was found for one aphid species only, our results offer the tempting possibility that in fields with low nematode abundance rare microbial species could reduce aphid body size. Therefore, rare soil microbes may have a critical value for crop protection by providing a baseline in reducing aboveground aphid development.

Identification of the mechanism by which rare microbes or nematodes reduced aphid body size on *Beta vulgaris* was not possible with the plant parameters measured. The changes in plant biomass, nitrogen and carbon did not correlate with aphid body size. This is not surprising, because host quality is probably determined by a combination of factors, not by simple correlations with one or a few compounds (Karban & Baldwin 1997). In addition, host plant quality for aphids probably relates better to phloem content (Cole 1997) than the measured overall changes in foliar concentrations. We can only speculate about the mechanism causing the interaction between herbivores and rare microbes on plant parameters. In both plant species, we measured parameters that were initially not affected by rare microbes (sugar content in *B. vulgaris*, amino acids in *B. oleracea*), but showed differential responses to herbivores depending on the soil microbial community. In the presence of rare microbes, nematodes reduced sugar content more than when the rare microbes were absent. Aphids could reduce or increase foliar amino acid concentration depending on microbial soil community. Studies using *Arabidopsis thaliana* showed that rhizosphere bacteria can prime genes to respond faster upon pathogen attack via jasmonic acid or ethylene signalling (Verhagen *et al.* 2004). Perhaps, rare microbes could play a role in such priming processes.

Other studies that used dilution-to-extinction approaches to examine soil microbial communities and their influence on ecosystem processes showed high functional redundancy within microbial communities for general soil processes (Griffiths *et al.* 2001; Wertz *et al.* 2006). Our results, however, strongly suggest that rare soil microbial species play an important role in complex community interactions, namely by affecting plant productivity and priming defensive responses in the interactions between plants and herbivores. However, it will always remain extremely difficult to demonstrate the role of organisms that are so rare that their absence or presence is hard to prove. Although our dilution method was aiming at only changing rare species, there were also shifts in abundant species as shown by the DGGE results. Apparently, the whole soil community has been affected by the dilution and subsequent re-growth, including some changes in the proportional composition of the most abundant species, as was revealed by the DGGE analyses. This can be due to changing species interactions or due to the selectivity of the extraction and incubation process. The extraction procedure will have selected species that are resistant to the collection and inoculation method, while the incubation period probably favored r-strategists. Yet, variability in abundant species did not explain the variability in plant biomass.

In agricultural plant breeding, priority has been given to productivity over defence (Harlan 1992). Our results suggest that a similar trade-off exists in managing the microbial soil community: simplification of soil microbial communities by land use intensification (Gomez *et al.* 2004) could enhance productivity, but reduce the insurance value of the soil microbial community in controlling pests and pathogens.

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SUPPORTING INFORMATION

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

Figure S1 Aphid population development in time for *Myzus persicae* on *Beta vulgaris* (Fig S1a; $n = 42$) and *Brevicoryne brassicae* on *Brassica oleracea* (Fig S1b, $n = 33$). Number of aphids per plant (mean with standard errors) are shown.
Table S1 Correlations between Jaccard distances and variation in plant biomass. Significant Pearson correlations (FDR, $\alpha = 0.1$) are indicated in bold.
Table S2 Plant parameters (carbon, nitrogen and C/N ratio's) and herbivore parameters (cysts/plant, median cyst size, aphids/plant) at the end of the experiment.
Table S3 Effect of nematode (*Heterodera schachtii*) presence on aphid parameters on *Beta vulgaris* (*Myzus persicae*) and *Brassica oleracea* (*Brevicoryne brassicae*) at the end of the experiment.

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