

## SPECIAL FEATURE

## PLANT–SOIL FEEDBACKS IN A CHANGING WORLD

# Independent variations of plant and soil mixtures reveal soil feedback effects on plant community overyielding

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## Summary

**1.** Recent studies have shown that the positive relationship between plant diversity and plant biomass ('overyielding') can be explained by soil pathogens depressing productivity more in low than in high diverse plant communities. However, tests of such soil effects in field studies were constrained by experimental limitations to manipulate soil community composition independent of plant community composition. Here, we report of an experiment where feedback effects to plants were tested for both plant and soil monocultures and mixtures.

**2.** Our results demonstrate that overyielding is the result of plant species in mixture being more growth-limited by 'own' soil biota than by soil biota of other plant species. This effect disappeared when the soils had been sterilized by gamma-irradiation. Mixing plants themselves did not result in overyielding except when grown in the soil of one of the species (*Leucanthemum vulgare*), where growth of one species disproportionately increased in mixture compared to monoculture.

**3.** Soil nutrient availability could not explain differences in growth between the non-sterilized soils. Therefore, our results suggest that plant species-specific soil biota rather than the plants have contributed to the plant community overyielding.

**4.** Species biomass ranking in mixtures highly differed between non-sterilized soils of different histories of soil conditioning, whilst the ranking was more consistent in sterilized soil. Sterilized soils of different origin differed significantly in nutrient availability. These results suggest that shifts in competitive hierarchies depend on plant species-specific interactions influenced by soil biota and cannot be induced by mineral nitrogen.

**5. Synthesis.** Our results show that overyielding in four plant species mixtures can be due to species-specific interactions between plants and their specific soil biota. Neither mixing the plant species alone nor the differential responses of species to mineral nitrogen influenced community productivity, but mixing soil biota did.

**Key-words:** *Anthoxanthum odoratum*, biodiversity experiment, biodiversity–productivity relationship, competitive hierarchy, determinants of plant community diversity and structure, grasslands, *Leucanthemum vulgare*, micro-organisms, pathogens, plant–soil feedback

## Introduction

Productivity in experimental grassland communities is often positively correlated with plant species richness (Tilman *et al.* 2001; Cardinale *et al.* 2007; Marquard *et al.* 2009), and this so-called overyielding has been explained by a more complete use of niche space (Berendse 1982; Fargione & Tilman 2005;

Hooper *et al.* 2005; Levine & HilleRisLambers 2009). The niche complementarity hypothesis has recently been challenged by putting forward an alternative 'pathogen niche' hypothesis, suggesting that build-up of specific soil biota decreases plant productivity in species-poor grasslands (Westover & Bever 2001; Bever 2003; Reynolds *et al.* 2003; Petermann *et al.* 2008). Results from two recent biodiversity experiments indeed suggest that soil pathogens affect the relationship between plant species diversity and biomass

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production. Maron *et al.* (2011) and Schnitzer *et al.* (2011) showed that the positive relationship between biodiversity and productivity largely disappeared, as removing soil biota by sterilization or fungicide application increased the productivity of the low-diversity treatments more than productivity of the high-diversity treatments. Overyielding in biodiversity experiments, thus, appears to be due to the release from pathogens in mixtures compared to monocultures (Kulmatiski, Beard & Heavilin 2012).

Soil biota have very specific effects on plant growth and species competitive performance, and in general, plants suffer more from their own soil biota than from the soil biota of other plant species (e.g. van der Putten, Van Dijk & Peters 1993; Bever 1994; Kardol *et al.* 2007; Kulmatiski *et al.* 2008; Harrison & Bardgett 2010). However, we do not know how these species-specific interactions between plant community and soil community affect overyielding. The experimental treatments of soils as performed by Maron *et al.* (2011) and Schnitzer *et al.* (2011) revealed the effects of soil biota on plant diversity–functioning relationships by altering soil communities irrespective of plant species identity and thus left open the question whether these effects were due to a dilution of the species-specific infective potential of the soil.

To answer this question, we combined a biodiversity experiment with a plant–soil feedback experiment and investigated the interactions between soil communities and plant species on overyielding. A plant–soil feedback approach makes use of soil conditioning by plant species affecting the composition and proportional contribution of soil biota in the soil community and testing these effects on plant biomass production in a follow-up experiment (Bever, Westover & Antonovics 1997). We tested the hypothesis that plants growing in soil that was preconditioned by conspecifics produce less biomass than plants growing in a mixture of soils conditioned by hetero-specific plant species, thus leading to overyielding in plant species mixtures. To test this hypothesis, we performed the plant–soil feedback experiment with monocultures and mixtures of four plant species that were grown in soil conditioned by single plant species (soil monocultures) or in soil composed of a 1 : 1 : 1 : 1 mixture of those soils (soil mixtures). This factorial design enabled us to study the performance of plant species in monocultures and mixtures as a function of monoculture soils and a mixture of these soils and to quantify any additional effects of mixing plant species on top of effects of mixing species-specific soil biota.

We analysed biomass of plants in all combinations in both non-sterilized and sterilized soils in which all soil biota were eliminated (Brinkman *et al.* 2010). A limitation of plant–soil feedback experiments is that the results may be influenced by nutrient flushes due to soil sterilization (Kulmatiski *et al.* 2008) or due to different nutrient uptake during the soil conditioning phase (Kardol, Bezemer & van der Putten 2006). Therefore, we quantified the effects of sterilization on nutrient availability and carried out an additional nutrient experiment to test whether growth limitations under non-sterilized conditions were due to low nutrient availability (Troelstra *et al.* 2001). Comparing the effects of non-sterilized and sterilized

soils on plant biomass gives the opportunity to compare soils that differ in soil biota (and possibly nutrients) with soils that differ in nutrients only. We hypothesized that only the species-specific effects of soil biota (in non-sterilized soil) will result in overyielding, rather than the non-specific effects of differences in mineral nitrogen amongst the sterilized soils, and analysed how the underlying biomass hierarchies of the different species resulted in overyielding.

## Materials and methods

### SPECIES SELECTION

Two grasses, *Anthoxanthum odoratum* L. and *Festuca rubra* L., and two forbs, *Leucanthemum vulgare* L. and *Plantago lanceolata* L., were used, because they are known for above-ground (van Ruijven & Berendse 2005) and below-ground overyielding (Mommer *et al.* 2010). Seeds of the four plant species were surface-sterilized for 3 h in a desiccator of 3 L containing two beakers of 50 mL sodium hypochlorite and 1.5 mL HCl each. Seeds were germinated in small containers covered with sterile glass lids on sterilized riverine sand moistened with sterilized 0.25-strength Hoagland's solution (Arnon 1950; Johnson 1953). Fourteen days after starting germination, seedlings were transferred to the experimental units.

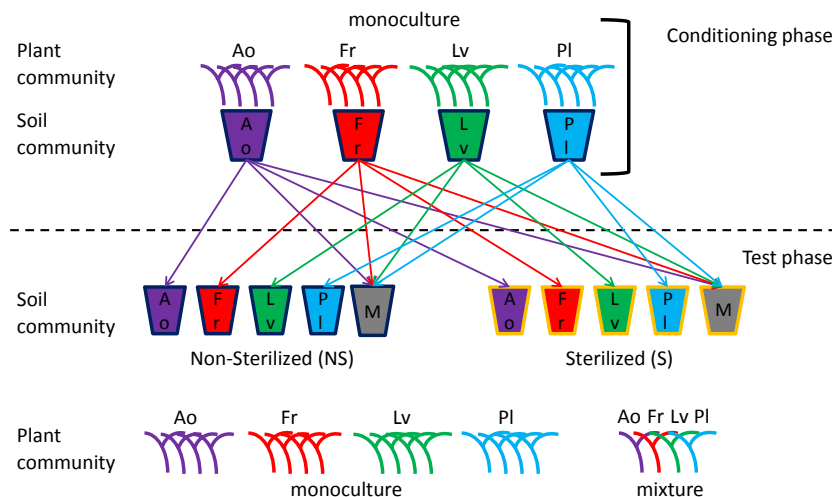
### EXPERIMENTAL SET-UP

Plant–soil feedback experiments consist of two phases, a conditioning phase and a feedback phase (Brinkman *et al.* 2010). In the conditioning phase, we used four monocultures to condition the soil to create a legacy effect. In the feedback phase of this study, we used two sterilization treatments, five different conditioned soil types and five different planting schemes (Fig. 1). Each different treatment and planting scheme was replicated five times. During the experiment, plants were watered three times a week, and every week, we reset each pot to the initial soil moisture content of 15% (w/w). Plants were grown in a climate chamber (day/night regime: 16 h light/8 h dark, temp 20/16 °C; 236  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

#### Phase 1: soil conditioning

Soil from a previous outdoor biodiversity experiment in Nijmegen, installed to investigate rooting patterns and biomass production of monocultures and mixed plant communities under near ambient conditions (Mommer *et al.* 2010), was used as inoculum in this experiment. We choose to use the conditioning step as the outdoor experiment was not large enough to collect soil immediately for the feedback phase without destroying the experiment. To collect soil for inoculation, six root cores of 7.2 cm diameter and 24 cm depth of each of the four original monocultures were taken in winter 2009, which was 4 years after the start of the outdoor biodiversity experiment, and those root cores were stored at 4 °C prior to inoculation. Rhizomes were removed from these root cores, and roots were cut into pieces of 1–2 cm. This inoculum was added to a mixture of riverine sand and loamy sand (v/v = 2 : 1) that had been sterilized by 25 kGy at Isotron Ede, The Netherlands, prior to inoculation. We added 12% inoculum to the sterilized soil (w/w).

We do not report results on soil of the mixed communities from the experimental units previously used by Mommer *et al.* (2010) in the main text, because after four growing seasons, they were domi-



**Fig. 1.** Experimental design of conditioning and test phase. In the conditioning phase, four different plant communities (monocultures) *Ao*: *Anthoxanthum odoratum*, *Fr*: *Festuca rubra*, *Lv*: *Leucanthemum vulgare*, *Pl*: *Plantago lanceolata* were planted in soil from the experimental units of these four species previously used by Mommer *et al.* (2010). In the test phase, five different preconditioned soil types were used; four were conditioned with a monoculture of each of the four species, and the fifth soil type (M) was created by mixing 25% of each of the monospecifically conditioned soils. There were two treatments: conditioned soils that were sterilized (S) and conditioned soils that remained non-sterilized (NS). In these non-sterilized or sterilized preconditioned soils, five different plant communities (four monocultures and a mixture of all four plant species) were planted. Average monoculture and mixture biomasses were calculated.

nated by one of the species (*F. rubra*) and were therefore no longer a good representation of the four-species mixtures. Additionally, during the conditioning phase, in which these soils were trained by a mixture of the four plant species, all four species produced a different amount of biomass in the plant mixture (See Fig. S2 in Supporting Information), and therefore, the four species did not have an equal contribution to the microbial community of the soil. As our aim was to disentangle the specific effects of plant and soil communities, we had to control the contribution of each species to soil conditioning. Therefore, our 'real mix conditioning' was unsuitable for answering our question because the four plant species had very different relative growth rates, and the nutrient conditions of the 'real mix' were similar to *P. lanceolata*. For completeness, data of growth on the real mix are provided in the Fig. S3.

Monocultures were created by planting four seedlings of each plant species (except for *L. vulgare*, of which six seedlings were planted because of their small size) in pots of 13 × 13 × 13 cm filled with 2.1 kg of the inoculated soil mixture. After 2 months of growth, shoots were clipped off, dried and weighed, and roots were cut into pieces of 1–2 cm, and the soil of 20 pots of each treatment was homogenized, as we were not interested in variation that developed during the conditioning phase. The soils were stored at 4 °C until the feedback phase started.

### Phase 2: soil feedback

Half the soil of the conditioning phase (averaged pH 7.8; 1.2–1.4% organic material, 0.036% N, 0.84% C; Table S3) was sterilized as explained above, after which subsamples for nutrient analysis were taken from each of the soil types. All soil types were stored at 4 °C until used; total storage time was <4 weeks. Pots were filled with the differently conditioned soils and planted with 2-week-old seedlings obtained from surface-sterilized seeds. We used ten soil treatments: both non-sterilized and sterilized versions of four soils originated

from each of the four monocultures and of one soil that was a 1 : 1 : 1 : 1 mixture of the four soils conditioned by the monocultures. Every soil was tested by five different plant communities: monocultures of four individuals of *A. odoratum*, *F. rubra*, *L. vulgare* or *P. lanceolata*, and a mixture containing one individual of every plant species (Fig. 1). Each treatment was carried out in five replicates.

In this feedback phase, plants were harvested 6 weeks after planting, which is a short duration, but not uncommon in plant–soil feedback studies (Bonanomi, Giannino & Mazzoleni 2005; Dostál & Palečková 2011; van de Vooorde, van der Putten & Martijn Bezemer 2011). The rationale for this short duration, however, was the need for disentangling the individual plants below-ground. The roots of all individuals were washed from the soil, and all four individuals – being the same in monocultures or four different species in the mixtures – were separated. Root and shoot biomass were dried for > 48 h at 70 °C and weighed.

### NUTRIENT EXPERIMENT

As elimination of soil biota by soil sterilization may cause nutrient flushes, with release of  $\text{NH}_4^+$  in particular (Troelstra *et al.* 2001), an experiment was performed to check whether growth limitation in non-sterilized soil would disappear after nutrient addition. To perform this experiment, we used a second set of monocultures of the same four plant species growing in soil conditioned by conspecifics. Every week, half the pots received 20-ml demineralized water, and the other half received 20-ml 0.50-strength Hoagland's nutrient solution (Hoagland & Arnon 1950; Johnson *et al.* 1957). The total amount of nutrients added was at least double the amount of nutrients required to compensate for the increased nutrient availability in sterilized soils (see Table S4 in Supporting Information and Appendix S1 for calculation). After 6 weeks of growth, we harvested the experiment and made analyses similar to those applied to the results from the general feedback experiment.

## NUTRIENT ANALYSES

The amounts of extractable nitrogen (N) ( $\text{mg kg}^{-1}$  dry soil) were determined by diluting soil samples (20 g) in 50 mL of KCl solution (0.2 M), gently shaking them for 1 h to dissolve the nutrients in the solution and analysing them with an Auto Analyser 3 system (Bran + Luebbe, Norderstedt, Germany) (Table S3).

## CALCULATIONS AND STATISTICAL ANALYSES

Root biomass was on average  $45 \pm 0.33\%$  of total biomass. As the patterns of root and shoot biomass were similar (results not shown), only analyses of total community biomass are presented. Disentangling effects of plant and soil communities on plant community biomass (Fig. 2) were based on the following calculations. For every monoculture replicate (on mono and mixed soil), we first calculated the average weight of a plant in a pot and then summed all species for one replicate (average  $A_{\text{rep-x}}$  + average  $F_{\text{rep-x}}$  + average  $L_{\text{rep-x}}$  + average  $P_{\text{rep-x}}$ ) in a given soil type and calculated the average of the replicates. For every mixed plant community in mono soil, we first calculated the average weight of a plant in a pot, then summed these average values for one replicate on the different monoculture soils (average weight in  $A_{\text{rep-x}}$  + average weight in  $F_{\text{rep-x}}$  + average weight in  $L_{\text{rep-x}}$  + average weight in  $P_{\text{rep-x}}$ ) and calculated the average of the replicates. For every monoculture replicate on foreign soil, we first calculated the average weight of a plant in a pot and then averaged this average plant weight per species for the three different 'foreign' soil types. As an example, for  $A_{\text{foreign}}$ , we calculated  $((\text{average } F_{\text{rep-x}} + \text{average } L_{\text{rep-x}} + \text{average } P_{\text{rep-x}})/3)$  and then summed these average values of foreign soil for one replicate ( $A_{\text{foreign\_rep-x}} + F_{\text{foreign\_rep-x}} + L_{\text{foreign\_rep-x}} + P_{\text{foreign\_rep-x}}$ ) and then calculated the average of the replicates. Like this, we always used an equal number of replicates ( $N = 5$ ) for the statistical analyses of the calculated community biomass values. For the mixture on mixed soil, we just calculated the average of the five replicates of this treatment.

To decipher what responses of what plant species to what soil types were responsible for the biomass at the community level, we further analysed the biomass of plant individuals in the mixtures. The biomass of individuals in the monoculture was calculated by dividing the total biomass per pot by four. This resulted in the data used for Fig. 3 (panel b–e). Panel 3a shows the sum of all individuals of each plant species on each soil type.

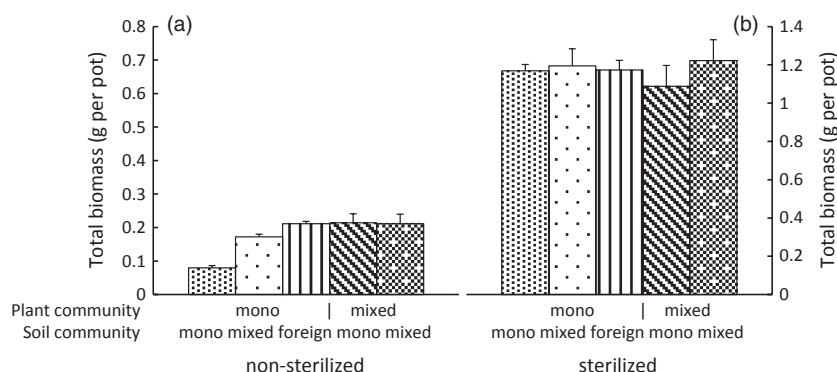
Statistical analyses were performed using full factorial univariate ANOVAS, with plant community (monoculture/mixture), soil community (mono/foreign/mixed) and sterilization as fixed factors, and total biomass as the dependent variable (Tables S1 and S2). We analysed plant biomass of plant individuals under all four conditions in both non-sterilized and sterilized soils. Soil sterilization is known to cause much stronger growth effects than comparing own vs. foreign (both non-sterilized) soils (Kulmatiski *et al.* 2008) so that we focused our interpretations on comparisons within non-sterilized and within sterilized soils. So, data of non-sterilized and sterilized treatments have been split due to many interactions in the full model (Table S1). Thus, we used two separate two-way ANOVAS (General Linear Model, SPSS 17.0; IBM, Armonk, NY, USA) to test the hypothesis that overyielding in diverse plant communities is the result of diluting soil conditioning effects. In these ANOVAS, we compared monospecific vs. mixed plant communities in monospecifically conditioned soils vs. a 1 : 1 : 1 : 1 mixture of these soils. To meet assumptions of ANOVA, variables were  $\ln$ -transformed.

## Results

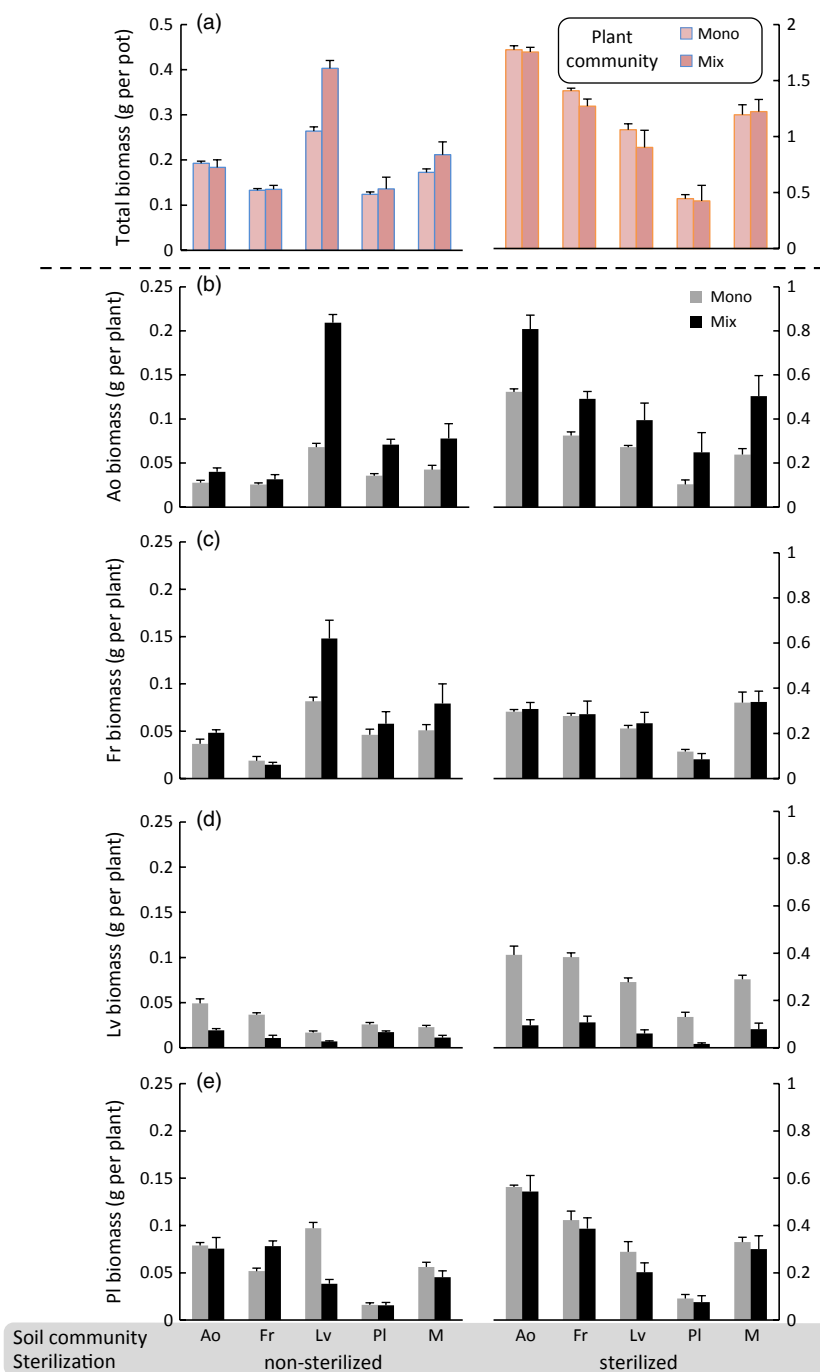
## EFFECTS OF PLANT COMMUNITY AND SOIL COMMUNITY ON OVERYIELDING

As in most biodiversity experiments, the four-species plant community in non-sterilized mixed soil produced 2.7 times more biomass than the average monocultures in soils conditioned by conspecifics (bar 5 vs. bar 1 in Fig. 2a). The observed overyielding was highly significant ( $F_{1,8} = 36.6$ ;  $P < 0.001$ ) in non-sterilized soil, whilst in the sterilized soils, there was no overyielding ( $F_{1,8} = 0.100$ ,  $P = 0.760$ ), as plant mixtures did not produce more biomass than monocultures. As expected, total biomass in sterilized soils was significantly higher than in non-sterilized soils (Fig. 2a vs. b and Table S1).

Plant community as well as soil community significantly affected the total amount of biomass produced (Table 1) in non-sterilized soil. Changing the plant community from monocultures to mixture resulted in higher biomass production, irrespective of soil type. Changing the soil community from mono to mixed resulted in higher biomass for the plant



**Fig. 2.** Disentangling effects of plant and soil community on overyielding. Total biomass (shoot + root; g dry weight per pot) of monocultures and mixtures of four plant species on non-sterilized (a) and sterilized (b) soils. 'Mono' soils have been conditioned by a conspecific monoculture 'foreign' soils are soils of the three other species, whilst mixed soils have been created by a 1 : 1 : 1 : 1 mixture of all four monoculture soils. The mixed plant community on 'mono soil' is based on the average biomass of the plant mixture on all mono soils. Note that y-axis scales are different for (a) and (b). Data are means  $\pm$  SE,  $N = 5$ .



**Fig. 3.** Separation of effects of plant species and soil types on plant growth in monocultures and mixtures. Total community biomass (a) and individual plant biomass of all species (b–e) on differently conditioned soils, both non-sterilized and sterilized. The last two bars in panel a of both non-sterilized and sterilized soils are identical to the bars mono/mixed and mixed/mixed in Fig. 2. Ao, Fr, Lv, Pl represent *Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare* and *Plantago lanceolata*, respectively. Note: scales of y-axes of non-sterilized and sterilized soils are different. Scales of panel a differ from panel b–e. Data are means  $\pm$  SE,  $N = 5$ .

community, but this effect only occurred for monocultures, not for mixtures. There were no significant biomass differences between plant monocultures in foreign soil, plant mixtures in mono soils and plant mixtures in mixed soils (Fig. 2a). Apparently, both plant community and soil community had an effect on community biomass, as well as their interaction (Table 1).

How can these main and interaction effects be understood? Figure 3 disentangles these effects into the effects of specific soil types and individual plant species. In non-sterilized soils, all plant species generally produced more biomass when the soils had been conditioned by the other species compared to

growing in 'own' soil, varying on average from 1.6 to 4.5 times more than a species' own monoculture biomass (Fig. 3b–e). Consequently any combination of plant species and soil type in which plants were on average more exposed to foreign soil than to own soil yielded higher community biomass (Fig. 2). This explains why monocultures exposed to a mixture of soils develop a higher biomass (bar 2 vs. 1 in Fig. 2), and monoculture biomass is the highest on foreign soils (bar 3). It also explains why plant mixtures on mono soils are more productive than monocultures (bar 4 vs. 1 in Fig. 2), because three of four species within the mixtures are exposed to soil conditioned by other species. As plant individuals in mixtures are on average



**Table 1.** anova results of effects of plant community (averaged monoculture vs. mixture) and soil community (monoculture conditioned soil vs. 'foreign' conditioned soil vs. a mixture of conditioned soils) on total biomass production (shoots + roots) per pot, for both sterilization treatments separately. See Table S1 for full factorial statistical model. Analyses are performed on ln-transformed data.

	Total biomass		
	d.f.	F-value	P-value
Non-sterilized soil			
Plant community	1	40.01	0.000
Soil community	2	17.55	0.000
Plant × soil	1	12.35	0.002
Error (MS)	20	0.001	
Sterilized soil treatment			
Plant community	1	0.162	0.692
Soil community	2	0.738	0.491
Plant × soil	1	0.669	0.423
Error (MS)	20	0.022	

d.f., degrees of freedom; MS, Mean Square,  $N = 5$ .

equally exposed to foreign soil when grown on mono soils than on mixed soil, the average biomass of these mixtures is the same (bar 5 vs. 4 in Fig. 2).

These results show that the apparent plant community effect (Table 1 and Fig. 2) on community biomass was in fact largely a soil effect, that is, that responses of plant species growing in mixture were predominantly affected by the different soil biota, rather than by direct plant–plant interactions. Figure 3a corroborates this notion: biomass of both monocultures and mixtures differed strongly depending on the soil they were grown. However, a small plant community effect irrespective of soil was apparent. Mixing plants resulted in an increase in community biomass (bar 4 vs. 1 in Fig. 2) that was slightly larger than when mixing soils (bar 2 vs. 1 in Fig. 2), although plants were exposed to foreign vs. own soils in similar proportions. Figure 3a shows where this effect comes from by comparing the average biomass of monocultures and mixtures on a given soil type. In three of four soils conditioned by mono soils, the average biomass of all monocultures was similar to the biomass of plant mixtures (Fig. 3a). The only exception was *L. vulgare* soil: not only biomass production of the averaged monocultures was highest in this treatment, but biomass production in the mixture was even higher ( $F_{1,8} = 57.4$ ,  $P < 0.001$ ). On mixed soil, this effect of *L. vulgare* soil was diluted leading to a small (non-significant) increase in community biomass of plant mixtures vs. monocultures (bar 5 vs. 2 in Fig. 2; the two bars indicating mixed soil in Fig. 3a).

#### PLANT SPECIES-SPECIFIC EFFECTS ON OVERYIELDING

Although community biomass was similar between mixtures and average monocultures on four of the five soil types (Fig. 3a), relative species performances in mixtures were very different. Significant interactions occurred between plant com-

munity and soil community at the individual plant species level (Fig. 3, Table 2), as the magnitude of the plant–soil feedback effect significantly depended on the plant species – soil type combination involved (Fig. 3b–d, Table S5). Interaction effects between plant species and soil community in plant mixtures were such that some species increased and others decreased in the mixture, where generally, the lower biomass of some species on a given soil was compensated by the higher biomass of other species. The two grasses performed better in soils conditioned by forbs, and the two forbs performed better in grass-conditioned soils. The *L. vulgare* soil was exceptional in which the smaller biomass of *L. vulgare* on its own soil was overcompensated by a disproportionately high production of the grasses and particularly *A. odoratum*. This was only in part a grass–forb effect: *A. odoratum* produced almost twice as much biomass on *L. vulgare* soil than on *P. lanceolata* soil (Fig. 3b).

#### EFFECTS OF NUTRIENTS VS. SOIL BIOTA

There was a strongly significant effect of soil type on the biomass of the individual plant species (Table 2). Differences in community biomass between the sterilized soil types were large (up to fourfold; Fig. 3) and positively correlated with the amount of mineral nitrogen ( $\text{NO}_3^- + \text{NH}_4^+$ ) present in the soil at the start of the feedback phase (Fig. 4). In non-sterilized soil, however, there was no such correlation between mineral nitrogen and biomass (Fig. 4 insert) suggesting that in these soils, nutrient differences were overwhelmed by the effects of soil biota. This suggestion was further tested in a nutrient addition experiment. Adding nutrients to non-sterilized soils higher than the levels observed in the sterilized soils (Appendix S1) did not eliminate the soil sterilization effect, as plant biomass in sterilized soils without nutrient addition remained 2.1–9.1 times higher than plant biomass in non-sterilized soils with nutrient addition (Fig. S1). Thus, in the non-sterilized soils, other factors were more limiting to plant growth than nutrient availability.

#### BIOMASS HIERARCHIES IN NON-STERILIZED AND STERILIZED SOIL

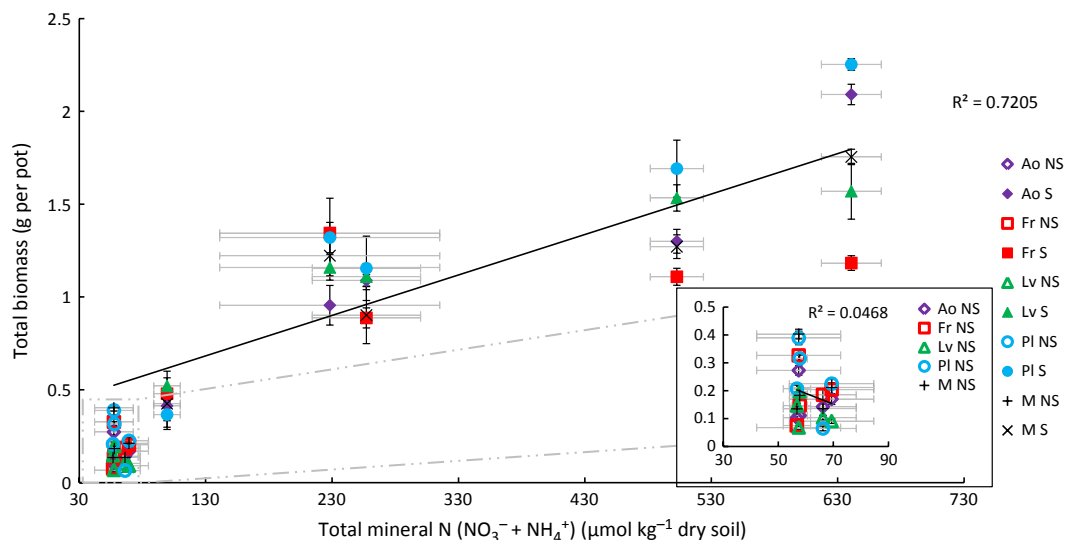
The overall consequence of the species-specific effects of soil biota was that competitive relationships were profoundly different amongst non-sterilized soils of different species origin (Fig. 5a). For example, the dicot *P. lanceolata* outperformed the other species in grass-trained soil; the two grasses outperformed the dicots in dicot-trained soil. The relatively high production of *A. odoratum* in *L. vulgare* soil (five times more biomass than in *A. odoratum* soil) was particularly striking. Overall, the competitive hierarchies were very different in non-sterilized soils with different histories of soil conditioning, with a significant interaction ( $F_{9,62} = 29.749$ ;  $P < 0.001$ ) between plant species and soil community in a two-way ANOVA. Overyielding in each of these soils was thus caused by other combinations of species, that is, the species that were less limited by the specific soil community present in a particular soil.

**Table 2.** anova results of effects of the plant community (monoculture vs. mixture) and soil community (soil of the four monocultures and the mixture of those soils) on total biomass per pot and per species, for both sterilization treatments separately. See Table S2 for full factorial statistical model. Data have been ln-transformed prior to analysis.

	Total biomass								
	d.f.	<i>Anthoxanthum odoratum</i>		<i>Festuca rubra</i>		<i>Leucanthemum vulgare</i>			<i>Plantago lanceolata</i>
		<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Non-sterilized soil									
Plant community	1	45.093	0.000	3.431	0.072	97.763	0.000	6.237	0.017
Soil community	4	37.031	0.000	29.423	0.000	15.389	0.000	56.009	0.000
Plant × Soil	4	3.887	0.009	1.219	0.319	3.249	0.022	8.005	0.000
Error (MS)	39*	0.087		0.170		0.094			0.074
Sterilized soil									
Plant community	1	23.443	0.000	1.233	0.273	125.723	0.000	3.315	0.076
Soil community	4	22.299	0.000	12.546	0.000	13.592	0.000	32.419	0.000
Plant × Soil	4	0.760	0.557	0.840	0.508	0.959	0.441	0.376	0.825
Error (MS)	40	0.141		0.229		0.286		0.188	

d.f., degrees of freedom; MS, mean square.  $N = 5$ .

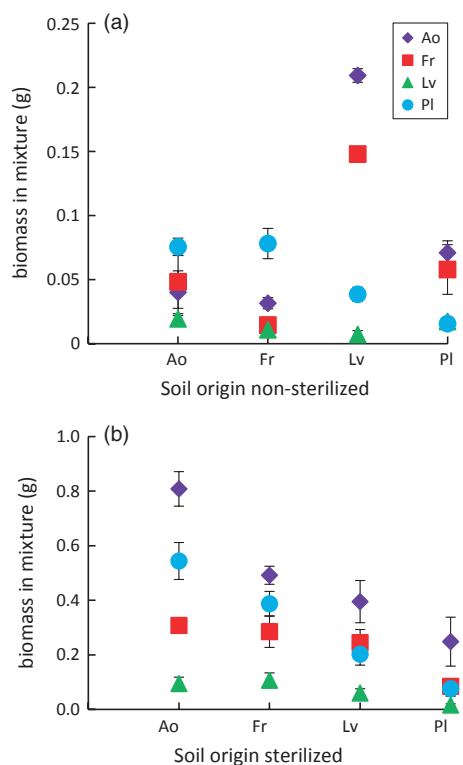
\*All d.f. = 39 except *P. lanceolata* with d.f. = 40.



**Fig. 4.** Plant growth in sterilized soils strongly correlates with mineral nitrogen. Biomass at the end of the feedback phase is correlated with the amount of total mineral nitrogen at the end of the conditioning phase. All treatments are shown in the main figure. The insert shows the results for the non-sterilized soils only. For sterilized soils, the sum of the amount of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  ( $\mu\text{mol kg}^{-1}$  dry soil) at start of feedback phase (i.e. after conditioning phase) was positively correlated with the total biomass (g) produced in monocultures and mixture during the feedback phase ( $F_{1,23} = 59.7$ ;  $P < 0.001$ ). This correlation was not significant in non-sterilized soil ( $F_{1,23} = 0.864$ ;  $P = 0.362$ ). NS represents non-sterilized soils, and S represents sterilized soils. Ao, Fr, Lv, PI codes are similar to those in Fig. 3, indicating the four different monoculture plantings. M represents the mixture planting. Data are mean  $\pm$  SE,  $N = 5$  for biomass and  $N = 4$  for total N.

In sterilized soils, *A. odoratum* was always the strongest competitor and produced 1.2–3.0 times more biomass in mixtures than in monocultures, independent of soil preconditioning history (Fig. 5b, Table 2). Here, *L. vulgare* was the least competitive as biomass in mixtures was 0.67–0.12 times the biomass in monocultures. *Festuca rubra* and *P. lanceolata* were intermediate competitors, and their biomass did not differ significantly between mixtures and monocultures. These effects were not dependent on soil preconditioning or soil mixing.

Whilst competitive hierarchies hardly differed amongst soil types, there was a strong effect of soil type on plant productivity (Fig. 5b), which was clearly related to differences in the nutrient content of the soil (Fig. 4). These nutrient differences had a similar effect on all plant species as no interactions ( $F_{9,64} = 0.810$ ;  $P = 0.609$ ) occurred between plant species and soil community when soils were sterilized. Thus, community biomass varied with nutrient availability, but because the competitively superior species gained in biomass in proportion to



**Fig. 5.** Competitive relationships among four grassland species are more hierarchical in sterilized soil than in non-sterilized soil. Four individuals of *Anthoxanthum odoratum* (Ao), *Festuca rubra* (Fr), *Leucanthemum vulgare* (Lv) and *Plantago lanceolata* (Pl) were grown in mixtures in soils in which one of each of the plant species was pre-grown (soil origin). Competitive relationships were highly variable among soil types when soils contained the living biota (a, non-sterilized). In the absence of living soil biota, relationships were more hierarchical, with *A. odoratum* always winning and *L. vulgare* always losing, and the other two species in between (b, sterilized). Values are means  $\pm$  SE,  $N = 5$ .

the loss in biomass of the inferior species, communities at sterilized soils did not show overyielding.

## Discussion

As in most biodiversity experiments (Tilman *et al.* 2001; Cardinale *et al.* 2007; Marquard *et al.* 2009), we observed community overyielding, when comparing monocultures in their conspecifically trained soils to mixed plant communities in mixed soils. Our results are consistent with Maron *et al.* (2011), Schnitzer *et al.* (2011) and Kulmatiski, Beard & Heavilin (2012) in that the overyielding is driven by soil biota. As in their experiments, biomass production was lower in non-sterilized soils than in sterilized soils, and overyielding only occurred in soils where the soil biota were present (non-sterilized soil). Moreover, our set-up allowed a further discrimination of the effects of the four different 'monoculture' soil biota communities and the mixed community, suggesting that overyielding was due to the release of the conspecific soil biota in the monocultures, which hamper growth most. Our results further showed that overyielding did not occur in sterilized soils differing widely in nutrient availability, further

confirming that overyielding was the result of species-specific soil biota operating, and could not be invoked by the non-specific effects of differences in nutrient availability.

## SPECIES-SPECIFIC SOIL BIOTA DRIVE OVERYIELDING IN DIVERSE PLANT COMMUNITIES

Soils trained by heterospecific plant species were less limiting to plant growth than conspecifically trained soils (Fig. 2), as has been found in many other plant–soil feedback studies (see Kulmatiski *et al.* 2008). As a consequence, overyielding must occur in species mixtures because mixing the four soil biota communities evenly implies that conspecific soil biota are diluted, and plants are confronted mainly with heterospecific soil biota (de Kroon *et al.* 2012), as shown in our experiment (Fig. 2).

The novelty of our experimental design is that we could disentangle the contribution of the four different 'monoculture' and its 1 : 1 : 1 : 1 mixture soil communities to overyielding in different monoculture and mixed plant communities. Grasses produced less biomass in conspecific conditioned soil, but also in soils previously conditioned by other grass species, suggesting the involvement of grass-specific pathogens (Bezemer *et al.* 2006; Kardol *et al.* 2007; Petermann *et al.* 2008; Harrison & Bardgett 2010). Similarly, forbs produced less biomass in forb soil than in grass soils. However, taxonomic group responses cannot completely explain overyielding as the two forb soils did not have similar effects on the performance of the grasses. Soil biota of *L. vulgare*, much more than of *P. lanceolata*, alleviated the growth reduction in the two grasses, whereas the *L. vulgare*-specific soil biota strongly inhibited biomass production of *L. vulgare* itself, more so than the self-inhibition by the other species.

In general, increased plant diversity alone (compare monoculture vs. mixture on the five soil communities in Fig. 3a) did not increase community biomass, but there was one exception. In non-sterilized *L. vulgare* soil, the four-species plant mixture produced significantly more biomass than the average of the monocultures, which was mainly caused by a threefold biomass increase of *A. odoratum* (Fig. 3). The competitive advantage of *A. odoratum* in mixture was boosted in the *L. vulgare* soil and more than offset the growth reduction of the forb species. These specific effects of *L. vulgare* soil on *A. odoratum* biomass in mixtures may also have occurred in mixed soil community and probably resulted in a (non-significant) trend of higher biomass of plant mixtures compared to monocultures in the mixed soil community (Fig. 2a). Such non-additive effects from highly specific combinations of plant species and soil community composition are suggestive of indirect plant–plant facilitation through plant–soil biota interaction effects (van der Putten 2009). Exploring the below-ground interactions of these four very common grassland species (Roscher *et al.* 2004; van Ruijven & Berendse 2005; Marquard *et al.* 2009; Mommer *et al.* 2010) and their soil biota is an important new step towards understanding the below-ground mechanisms that may contribute to plant biodiversity effects on productivity.



We did not analyse the soil community composition. It is known that plant species can support different amounts of microbial biomass and community diversity (Grayston *et al.* 1998; Kowalchuk *et al.* 2002; Bezemer *et al.* 2010); however, these assays do not reveal which species actually have been responsible for the observed phenomena. Such studies would call for extensive molecular community profiling, culturing species and reinoculations in monospecific and mixed species assemblages. Whilst our results strongly suggest that overyielding was the result of release from species-specific soil pathogens, effects of other soil biota may have occurred as well. The specific overyielding on *L. vulgare* soil (especially driven by *A. odoratum* biomass increase) could potentially be caused by AMF-mediated negative feedbacks [as suggested by Bever (2002) and Casper & Castelli (2007)], where *L. vulgare* is culturing AMF more beneficial to *A. odoratum* than to *L. vulgare* itself.

The growing time of plants in our experiment was rather short, which may have favoured direct interactions between plants and soil biota, rather than (decomposition-related) indirect interactions (Wardle *et al.* 2004). However, plant responses were similar to those in two outdoor experiments of Mommer *et al.* (2010) and van Ruijven & Berendse (2005, 2009), run over several years, in which similar growth-promoting effects have been found on (root) growth of *A. odoratum* in mixtures of the same four plant species. Also in these experiments, *A. odoratum* contributed most to overyielding in mixtures, which may have been due to the effect of *L. vulgare* soil biota as found in our experiment. *Leucanthemum vulgare* performance in the monocultures of these experiments crashed after a few years and recovered some years later (J. van Ruijven & F. Berendse, unpubl. data), suggesting an accumulation of highly pathogenic micro-organisms, as has been shown before in a mesocosm experiment (van Ruijven, De Deyn & Berendse 2003).

Because the patterns in biomass production in our pot experiment were consistent with those observed under more natural conditions, our results suggest that also in the field, direct interactions between plants and soil biota may play an important role in explaining the observed outcomes of plant community interactions, even though it is difficult to generalize across more diverse plant communities as we only compared monocultures with four-species mixtures. In plant communities in nature, legacy effects on soil biota composition will thus be determined by the number of species, species abundance and their (non)-additive interactions. These interactions were noticeable in the 'real mixed conditioning' (Fig. S3) in which an interplay occurred between the original soil community composition resulting from the previous experiment (Mommer *et al.* 2010) and the community that developed during the conditioning phase. The mixing of soil as was practiced in our experiment enabled us to create time-independent conditions in order to test our hypothesis. In natural plant communities, the relative abundance of plant species is varying dynamically over time with profound effects on the soil community composition feeding back on community productivity and composition in the future.

#### SOIL BIOTA VS. SOIL NUTRIENTS AFFECTING COMPETITIVE HIERARCHIES AMONGST PLANT SPECIES

Total biomass of mixtures was similar in the five different non-sterilized soils, but the composition of the plant community biomass depended on soil pretreatments (Fig. 5a). This result suggests that competitive relationships (in particular between *A. odoratum* and *P. lanceolata*) were strongly influenced by soil conditioning, as has been predicted (Bever, Westover & Antonovics 1997; Bever 2003) and shown in other studies (e.g. Kardol *et al.* 2007). Small differences in nutrient availability between these non-sterilized soils could not explain the community responses. Plant mixtures in all of the soils overyielded, but the higher biomass compared to the average monocultures was produced by the species that were less limited by the specific soil biota.

In soils from which the species-specific soil communities had been removed by soil sterilization, differences in biomass production between soil types correlated with differences in mineral nitrogen at the start of the feedback experiment. In contrast to the species-specific effects observed in non-sterilized soils, nutrient differences in sterilized soils affected the growth of all plant species in essentially the same way, without changing the competitive hierarchy (Fig. 5b) and without overyielding (Fig. 2b). Because all species produced more biomass in soils containing more nutrients, and competitive abilities between species remained essentially the same, overyielding was absent in soils that differed only in nutrient availability. Therefore, overyielding as well as changing competitive hierarchies were dependent on species-specific effects of soil biota and could not be invoked by non-specific effects of differences in nutrient availability between the sterilized soils. As shown here and observed earlier (Mommer *et al.* 2010), it is inconceivable that differences in nutrient availability can inverse competitive hierarchies (de Kroon *et al.* 2012).

#### Conclusions

Our results strongly suggest that species-specific pathogenic effects of the soil biota are driving overyielding. Complementing recent studies (Maron *et al.* 2011; Schnitzer *et al.* 2011), we have been able to unravel the effects of soil biota, soil nutrient availability and plant diversity, leaving an overwhelming effect of soil biota on community productivity. Our results underscore suggestions that plant species identity should obtain more attention in the biodiversity debate (Schmidtke *et al.* 2010) when considering plant–soil interaction effects on diversity–productivity relationships. Species-specific interactions mediated by soil biota may not only drive community productivity but, by changing competitive hierarchies, simultaneously play a crucial role in maintaining coexistence.

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## Supporting Information

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

**Figure S1.** Soil biota are more important than additional nutrients.

**Figure S2.** Uneven production of above-ground biomass during conditioning phase in ‘real mixture soil’.

**Figure S3.** Results on real mixed soil during test phase.

**Table S1.** Full model ANOVA results of effects of soil sterilization, plant and soil community on total biomass production.

**Table S2.** Full model ANOVA results of effects of soil sterilization, plant community, plant species and soil community (‘own’, ‘foreign’ and mixed) on total biomass production.

**Table S3.** Additional nutrient data.

**Table S4.** Amount of nutrients needed to compensate for the difference between non-sterilized and sterilized treatments.

**Table S5.** Plant-soil feedback coefficients for each species.

**Appendix S1.** Nutrient addition experiment.