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Increase of fast nutrient cycling in grassland microcosms through insect herbivory depends on plant functional composition and species diversity

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Nutrient cycling in terrestrial ecosystems is affected by various factors such as plant diversity and insect herbivory. While several studies suggest insect herbivory to depend on plant diversity, their interacting effect on nutrient cycling is unclear.

In a greenhouse experiment with grassland microcosms of one to six plant species of two functional groups (grasses and legumes), we tested the influence of plant species richness (diversity) and functional composition on plant community biomass production, insect foliar herbivory, soil microbial biomass, and nutrient concentrations in throughfall. To manipulate herbivory, zero, three or six generalist grasshoppers (*Chorthippus parallelus*) were added to the plant communities.

Increasing plant species richness increased shoot biomass and grasshopper performance, without significantly affecting root biomass or insect herbivory. Plant functional composition affected all of these parameters, e.g. legume communities showed the highest shoot biomass, the lowest grasshopper performance and suffered the least herbivory. Nutrient concentrations (dissolved mineral N, PO_4-P, SO_4-S) and pH in throughfall increased with herbivory. PO_4-P and pH increases were positively affected by plant diversity, especially under high herbivore pressure. Plant functional composition affected several throughfall variables, sometimes fully explaining diversity effects. Increasing plant diversity tended to increase soil microbial biomass, but only under high herbivore pressure. Faeces quantities strongly correlated with changes in pH and PO_4-P; frass may therefore be an important driver of throughfall pH and a main source of PO_4-P released from living plants. Our results indicate that insect herbivory may significantly influence fast nutrient cycling processes in natural communities, particularly so in managed grasslands.

In the past two decades, a growing number of studies have investigated the importance of biodiversity, in particular of plant species richness, for processes at the ecosystem level (Naeem et al. 2009). These studies have bridged the traditional division of ecology into community ecology and ecosystem ecology (Jones and Lawton 1995). Interestingly, while the question of how the diversity of a plant community affects N- and P-cycling in the ecosystem was raised early in the history of functional biodiversity research (Schulze and Mooney 1993), we are still far from understanding this relationship. A number of studies have shown that increasing plant diversity, in particular plant species richness, can affect particular components of the nutrient cycles, e.g. by decreasing nitrate (NO_3^-) leaching into groundwater (Tilman et al. 1996, Scherer-Lorenzen et al. 2003), decreasing extractable soil NO_3^-, and increasing N-pools in plant biomass (Oelmann et al. 2007b, 2011).

While plants are main drivers of nutrient cycling in terrestrial ecosystems, other organisms may modify the relationship between plant diversity and N- or P-cycling (Klironomos et al. 2000), or, as in the case of microbial communities, to a large extent drive these processes (Bardgett et al. 2003, Zak et al. 2003). One group of organisms that is a dominant component of overall biodiversity with potential effects on nutrient cycling is insect herbivores. A number of previous studies have reinforced the view that the consumption of living plant tissue by insect herbivores can exert measurable effects on nutrient cycling (Belovsky and Slade 2000, Frost and Hunter 2004, Weisser and Siemann 2004). Hunter (2001) distinguishes seven mechanisms by which insect herbivores can cause changes in nutrient cycles. Three of them, the inputs of frass, of cadavers, and of modified throughfall, i.e. precipitation that passes through plant canopy and thereby accumulates nutrients leaching from

damaged tissue as well as greenfall and faeces, are considered to be particularly important for short-term changes in the availability of nutrients for the soil microbial community and for plants themselves, because no decomposition of complex organic matter is required. Such insect herbivore effects on this 'fast cycle', a term originally coined by McNaughton et al. (1988) for vertebrate herbivory, have been shown repeatedly, in particular for N and in outbreak situations (Hunter 2001, Lovett et al. 2002). In their seminal review, Seastedt and Crossley (1984) argued that measurable effects on nutrient cycles can also be exerted at 'background' herbivory levels, and they proposed that leaching losses from foliage are more pronounced for mobile elements that are transported through plant tissues, such as potassium (K^+) or sulfate (SO_4^{2-}). They also argued that inorganic forms of N and P are less likely to be leached from foliage, possibly in part because of uptake by microbes in the phyllosphere. More than twenty years onwards their conclusions are still valid. However, results of subsequent studies have been equivocal with respect to the magnitude of effects observed, the resulting effect on soil nutrient status, and responses of the soil community to these inputs (Hunter 2001). In fact, there are examples of both positive and negative effects of herbivory on nutrient cycling (Hunter 2001), observed mainly in forest ecosystems, making further studies necessary.

The relationship between plant diversity and insect herbivory is complex. Root (1973) originally proposed that herbivore loads, in particular for specialist herbivores, should be higher in simple than in species-rich plant communities, and that the higher abundance of specialist herbivores in monocultures translates into greater herbivore damage of individual plants compared to polycultures. Subsequent studies have found mixed support for this prediction and more recent experiments where plant species richness was carefully manipulated showed little effects of plant diversity on rates of herbivory (Scherber et al. 2006) or even increasing herbivory levels with increasing plant species richness (Mulder et al. 1999, Loranger et al. 2014). Herbivore abundance has often been shown to increase with plant species richness (Scherber et al. 2010a). Thus, from these studies no clear prediction can be derived if and how insect herbivore effects on the nutrient cycle should depend on plant diversity. In addition, it is not clear how a gradient of herbivory should translate into effects on the nutrient cycle. It is likely that higher levels of herbivory result in increasing amounts of nutrients being made available through the fast cycle across a plant diversity gradient if plant nutrient concentrations from the differently diverse communities stay constant as observed by Oelmann et al. (2007a).

Plant diversity may also induce changes in herbivore behaviour with increasing plant species richness, in particular in generalist species (Unsicker et al. 2008). Moreover, if generalist herbivore feeding preferences result in the selection and consumption of more nutrient-rich or nutrient-poor plants, or if herbivores show differing consumption rates in more diverse than simple plant mixtures, nutrient concentrations in throughfall may also be affected. Preferential feeding on particular plant functional groups such as grasses, as expected in grasshoppers, additionally relates nutrient release from plants to plant community composition.

Insect herbivory results in a number of plant responses e.g. in increased or altered root exudation (Dyer et al. 1991, Holland et al. 1996). Such exudates are assumed to stimulate soil microbes and subsequently benefit the plant through better nutrient availability (Lovett and Ruesink 1995, Frost and Hunter 2004). Likewise, soil microbes may benefit directly from nutrient leaching with throughfall from plants under herbivore attack. Overall, the microbial community is likely to respond to insect herbivory, but how plant diversity modifies this response is largely unknown.

In this study, we tested for insect herbivore effects on the fast cycle and tested if these change with plant diversity. We used the common meadow grasshopper *Chorthippus parallelus* to carry out a greenhouse experiment with plant communities of one to six plant species composed of grasses and legumes to address the following questions: 1) does grasshopper herbivory depend on plant species richness and the composition of the plant community? 2) Does grasshopper herbivory increase concentrations of nutrients in throughfall? 3) Do changes in throughfall due to herbivory vary with plant diversity or composition? And 4) is the influence of herbivory significant enough to induce a measurable response of the soil microbial community?

Methods

Plant material and experimental plant communities

Plant seeds of six grassland species, three grasses (*Dactylis glomerata*, *Poa trivialis*, *Holcus lanatus*) and three legumes (*Trifolium pratense*, *Lotus corniculatus*, *Trifolium campestre*) were obtained and sown on soil–sand mixture on 29 Jan 2009. These species are part of the species pool of the Jena Experiment, a large grassland biodiversity experiment on the effects of plant diversity on nutrient cycling and trophic interactions (Roscher et al. 2004).

We filled pots of 2.72 l volume with approximately 3 kg topsoil from the site of the Jena Biodiversity Experiment and stored the soil for one month in the greenhouse prior to the start of the experiment. Pots had holes at the bottom to prevent anaerobic conditions in soil and allow the drainage of water.

To form plant mixtures of one, two, three, or six species we transplanted twelve pre-grown seedlings into each pot, 18 days after sowing. In plant communities containing only grasses (grass communities) or legumes (legume communities), the gradient ranged from one to three plant species, and in communities containing both groups of plants (mixed communities) the gradient ranged from two to six plant species (Table 1). All species were grown in monoculture. For two- and three-species combinations, we chose species of a particular functional group randomly out of the three possible species and balanced their occurrence within each diversity level. In total, there were 27 different plant species compositions ('plant communities'), each replicated in three pots except for the six-species mixture, which was replicated nine times, resulting in 87 pots in total (Table 1). We selected the position of plant individuals in the pots randomly and kept it constant across replicated identical communities. For the first four weeks after transplantation,

Table 1. Plant diversity levels, plant community compositions and number of replicates in the microcosm experiment.

	No. of communities of different composition					
No. of plant species	Communities with grasses only	Communities with legumes only	Communities with grasses and legumes (mixed)	Total no. of unique communities	Replicates per unique community	Total no. of pots
1	3	3	–	6	3	18
2	3	3	6	12	3	36
3	1	1	6	8	3	24
6	–	–	1	1	9	9
Sum	7	7	13	27	–	87

we replaced dead individuals by new seedlings from the same batch.

The experiment was conducted in a greenhouse with additional lighting to achieve long-day conditions (L:D 16:8) with lights switched on when outside light intensity fell below 3000 Lux. Daily temperature ranged between 15 and 35°C.

We distributed the 27 different plant communities over six greenhouse tables that were considered blocks in the statistical analysis. Each table received all replicates of four or five plant communities (i.e. 12 or 15 pots), at least one from each of the four plant species richness levels. The six-species community was replicated nine times and therefore distributed over three of the tables. Within a table, we rotated pots every third day to avoid potential effects of heterogenic light conditions in the greenhouse. To ensure identical growing conditions, we closely kept together the three replicate pots of the same plant community within a block.

Herbivory treatments

We used the meadow grasshopper *Chorthippus parallelus* (Orthoptera, Acrididaea) as model herbivore in the experiment. This univoltine species of the subfamily Gomphocerinae has a widespread distribution and occurs in many habitat types throughout central Europe (Köhler 2001). It is a generalist herbivore preferentially feeding on grasses, but legumes also constituted about 5–10% of its diet in experiments under semi-natural conditions (Unsicker et al. 2008, 2010). *Chorthippus parallelus* is abundant on the field site of the Jena Experiment and was previously used in herbivory experiments (Specht et al. 2008, Scherber et al. 2010b). Grasshoppers used for this study were the offspring of adult females we caught between July and August 2008 in grasslands in Jena, Thuringia, Germany. Females oviposited in the lab in a 1:1 mixture of moistened sand and soil. We kept egg clutches at room temperature for two weeks, allowing for the embryos to develop before transferring them to the refrigerator for overwintering at 5°C. On 13 Mar 2009 we removed egg clutches from the refrigerator and placed them under UV lights with a light regime from 6:00 am to 4:00 pm for two weeks. Hatched *C. parallelus* nymphs, we kept on *D. glomerata* and transferred them to the greenhouse for adaption to the new climate ten days prior to use in the experiment.

Each of the three replicates per plant community was subjected to one of three herbivory treatments: either 1) no grasshopper = control; 2) three grasshoppers (low herbivore pressure); or 3) six grasshoppers (high herbivore pressure). We introduced herbivores into plant communities as nymphs, in their second/third nymphal stage selecting them randomly from rearing cages and noting the instar of each individual at the beginning and end of the experiment. We placed round acrylic glass cages (diameter = pot width, height = 100 cm, wall-thickness = 800 μm) on all pots. Each cage had three rectangular windows (in total 840 cm^2) and an open top (314.2 cm^2) closed with gauze of mesh size 0.2×0.2 mm to allow for air ventilation, watering, and throughfall collection.

The experiment started on 27 Apr 2009 with the introduction of grasshoppers into the herbivory cages, and lasted for 28 days when we harvested plant communities destructively.

Grasshopper performance

Throughout the experiment, we monitored the numbers of grasshoppers every four days, replacing dead individuals in the first two weeks of the experiment only. To compare the development of grasshoppers across the different plant communities we calculated an index representing the average number of developmental stages that the individuals had passed through until the end of the experiment. The index was weighted by the proportion of grasshoppers surviving to the end of the experiment, to account for differences in survival probability. For calculations, we coded nymphal stages as follows: 1st instar = 5, 2nd instar = 4, 3rd instar = 3, 4th instar = 2, adult stage = 1. We then calculated the development index as the 'average stage at the beginning of the experiment' - 'average stage at the end of the experiment' multiplied by the 'proportion of nymphs that survived until the end of the experiment'.

Throughfall sampling, plant and soil variables measured

We regularly watered all plant communities with the same amount of de-ionized water: either 0, 100 or 200 ml per day, depending on the greenhouse temperature and resulting water use by plants. Using plastic funnels (opening area: 31.2 cm^2), covered with synthetic mesh (mesh size: 1 mm) we collected throughfall 5 cm above the soil surface. The funnels led to glass vessels (volume: 26 ml) dug into the ground in the centre of each pot and were emptied every third day. For use in nutrient analysis, for each community we pooled throughfall from the last three sampling dates (15, 19 and 25 May 2009).

Complete throughfall collection in forested ecosystems commonly combines precipitation passing through tree

canopies ('throughfall') and precipitation running down tree stems ('stemflow'). Stemflow sampling is very difficult in grassland systems and was therefore neglected in our study, even though throughfall and stemflow in grasslands may differ in their concentrations of particular compounds (van Dam et al. 1991).

At the end of the experiment we took soil and plant samples for analysis and measured herbivory. Using a corer of 3 cm in diameter we took bulk soil samples from each pot across a horizon from 0 to 7 cm depth. We then passed the soil through a 2 mm sieve and froze it at –20°C until measurements of water content and microbial biomass. As a measure of herbivory, we visually estimated percentage leaf area loss for each plant species by comparison of the particular plant species in the grasshopper treatments with those in the control pot of the respective plant community. We used the following categories of leaf area loss for herbivory estimation: $<1\%$ ($=0.5\%$ in analysis); 1–5% ($=3\%$ in analysis), 5%, 10% etc. in steps of 5%. At the community level, we obtained herbivory values by averaging over all plant species in the plant community and weighting by the number of individuals of a plant species in the community in case an individual had died.

Separated by plant species, we cut all aboveground (shoot) biomass at the soil surface, and dried it for 48 h at 70°C before weighing. We obtained total belowground (root) biomass per plant community by washing out all soil under running water and retention of root components in a 0.5 mm sieve. We then dried root biomass at 70°C for 48 h before weighing. Biomass is reported as gram dry weight.

Analysis of throughfall nutrients and pH, and soil microbial biomass

We stored throughfall samples at 5°C, filtered them within 2 days after collection (quantitative ashless filter papers) and kept them frozen at –20°C until analysis. Prior to sample filtration we rinsed filters with 40 ml de-ionized water. For a rough estimate of faecal pellet input, we pooled grasshopper faeces retained by the filter papers for the last three sampling dates, drying them for 24 h at 70°C before weighing.

We analysed ammonium (NH_4^+) using a SAN^{++} continuous flow device. Quantification of NH_4^+ is based on a modified Berthelot reaction; NH_3 is chlorinated at pH 12.6 forming monochloramine, which in turn reacts with salicylic acid to indophenol blue dye using sodium nitroprusside as catalyst. The absorbance of the blue component is detected at $\lambda = 655$ nm. The typical working range covers NH_4-N concentrations from 0.2 up to 10 mg l^{-1}.

Nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) measurements, we performed on a ion chromatography system. An integrated sample pre-treatment and pre-concentration unit and an ultra-low-pressure trace-anion-concentrator column allow detection of small amounts of the analytes in samples, which are contaminated with aromatic dyes, lipids, aromatic carboxylic acids, hydrocarbons, surfactants, or other contaminants. The applied sample injection volume of 200 µl guarantees high analytical sensitivity. For removing particles, we filtered samples through a 0.45 µm syringe filter prior to analysis. The one-way filters were tested to be interference-free. Nevertheless, we initially rinsed them with about 2 ml of each sample, and the following 1.6 ml of the respective sample were then used for analyses.

For statistical analysis, we set all values below detection limit ([mg l^{-1}] NO_3^-: 0.013; NO_2^-: 0.006; PO_4^{3-}: 0.008) to zero. This affected about 52% of NO_2^- values, 5% of NO_3^- values, and 7% of PO_4^{3-} values. Quantification limits [mg l^{-1}] were: NO_3^-: 0.048; NO_2^-: 0.021; PO_4^{3-}: 0.029. Values in the range between detection and quantification limit, we used as measured. This affected about 42% of NO_2^- values, 14% of NO_3^- values and 3% of PO_4^{3-} values. All measurements of NH_4^+-N and SO_4^{2-} were above quantification limits.

For each nitrogen species, we calculated N from the molecular weight of each element involved and summed up N derived from all three species (NH_4-N, NO_3-N, NO_2-N) to give a value of dissolved mineral N (DMN). Despite the large proportion of values below detection and quantification limits for NO_2, we included this compound of DMN for completeness and we found that results from statistical analyses of DMN excluding NO_2-N were identical to results presented here. PO_4-P and SO_4-S were likewise calculated from the quantified amounts of PO_4^{3-} and SO_4^{2-}. We then used calculated DMN, PO_4-P and SO_4-S values in the statistical analyses. Measurements of pH in throughfall samples, we performed with a pH-meter.

To measure microbial biomass carbon (C) in soil samples we used an O_2-microcompensation apparatus (Scheu 1992). We measured the microbial respiratory response at hourly intervals for 12 h at 22°C and then calculated substrate-induced respiration from the respiratory response to D-glucose (Anderson and Domsch 1978). We added glucose according to preliminary studies to saturate the catabolic enzymes of microorganisms (4 mg g^{-1} dry weight solved in 400 µl deionized water, Eisenhauer et al. 2010). We took the mean of the lowest three readings within the first 10 h as maximum initial respiratory response (MIRR; µl O_2 h^{-1} g^{-1} soil dry weight) and calculated microbial biomass (µg C g^{-1} soil dry weight) as $38 \times$ MIRR (Beck et al. 1997). To determine gravimetric soil water content we compared fresh soil samples with samples after drying.

We tested potential nutrient inputs from filters and detected minor concentrations of NH_4-N and SO_4-S in the range of the minimal values measured in throughfall samples (NH_4-N min: 0.288 mg l^{-1}, SO_4-S min: 0.042 mg l^{-1}, Supplementary material Appendix 1). Because these concentrations were very similar across analysed replicates and because comparable volumes of throughfall samples were filtered, potential nutrient addition by filtering is assumed to be minor and constant across all samples, consequently not affecting the statistical outcome. Nevertheless, we did not subtract these background levels of nutrients for statistical analysis, due to these various sources of error.

Statistical analyses

We analysed data using split-plot ANOVA type I sum of squares (Schmid et al. 2002) with 'plant species mixture' as error term to investigate herbivory effects between identical communities, using the statistical software 'R' with packages 'MASS' (Venables and Ripley 2002) and 'plotrix'

(Lemon 2006). Main effects (diversity, functional composition) were based on means across identical communities and were tested sequentially. We excluded cases in which replicate data was missing from the analysis (i.e. one case in root analysis).

Models contained block, log-transformed number of plant species (henceforth 'diversity'), functional composition (henceforth 'composition', levels: legumes, grasses, mixed), and herbivory treatment (control, three or six grasshoppers) as explanatory variables in this order, as well as the interactions of diversity and composition with herbivory. In this model, composition effects were investigated under 'constant' diversity, since diversity and composition were not fully independent (Schmid et al. 2002). To also investigate diversity effects under 'constant' functional composition of the plant community, we interchanged the order of diversity and composition in an alternative model. We first report results from models with diversity fitted and list these in Table 2 to 4. Deviating results from the alternative model, we report second and statistical values are listed in the Supplementary material Appendix 3.

We analysed the full set of communities to investigate main effects of diversity and composition, and to test for herbivory effects and possible interactions between these two factors and herbivory. We analysed sole grasshopper communities to test for differences between the two levels of herbivore pressure and for investigation of plant and grasshopper responses.

Where general directions of diversity and composition effects are reported (as mean ± one standard error (SE)) data was averaged across identical plant communities and therefore across the three herbivory treatments. For conciseness, we present results of the statistical analyses mainly in tables. Figures were produced with 'R'.

Results

Herbivory, plant biomass and response

Mean leaf area loss was significantly lower in the three- ($14.7 \pm 1.9\%$) than in the six-grasshopper treatment ($24.7 \pm 3.1\%$, Table 2). Grasshopper feeding was stronger in grass communities than in mixed or legume communities, and grass communities also suffered the greatest change in leaf area loss with increasing grasshopper density (Fig. 1, Table 2). The grass effect was not driven by a single species (average herbivory across all replicates with grasshoppers: *D. glomerata* 25%, *H. lanatus* 29%, and *P. trivialis* 49%). Shoot biomass, which increased with diversity in the control treatment, was reduced by herbivory and this effect was strongest in grass communities and at high grasshopper density (Fig. 2, Table 3). In contrast, root biomass showed a slight overall decrease with increasing grasshopper density, the effect being marginally non-significant (Fig. 2, $p = 0.057$ see (*) in Table 3).

The log response ratio of plant biomass [log (biomass under herbivory/control biomass)] illustrates the plants' response to herbivory. Values below zero indicate biomass losses in response to herbivory, while values above zero indicate a biomass gain as compared to untreated control communities. Log response ratio for community shoot biomass was close to zero in legume communities and slightly negative in mixed communities. The lowest values were seen in grass communities with the most negative response at the highest level of herbivory (significant composition × herbivory, interaction Table 3, Supplementary material Appendix 2 Fig. A1). In the alternative model, the interaction turned non-significant, but composition effects were still detected (Supplementary material Appendix 3 Table A3). For

Table 2. Effects of herbivory, plant diversity and community composition on herbivory damage and herbivore performance: response variables in columns, explanatory variables in rows in their order of entering the model, Diversity = log transformed plant species richness; degrees of freedom (DF), mean squares (MS), and F-values are given; significant results are indicated by *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.♦

	Average leaf area loss [%] (control vs combined grasshopper treatments)			Average leaf area loss [%] (grasshopper treatments only)			Grasshopper survival [%] (grasshopper treatments only)			Grasshopper faeces [mg][a] (grasshopper treatments only)			Grasshopper development [index] (grasshopper treatments only)		
No. of observations (i.e. pots)	87			58			58			58			58		
Source	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F
Between mixes															
Block	5	245	6.39**	5	367	6.39**	5	652	0.41	5	0.001	0.49	5	0.28	0.40
Initial grasshopper stage													1	0.60	0.87
Diversity	1	13.8	0.36	1	20.7	0.36	1	8090	5.03*	1	0.001	0.30	1	3.63	5.24*
Composition	2	1852	48.4***	2	2778	48.4***	2	4918	3.06	2	0.005	2.08	2	1.79	2.58
Residuals	0	38.3		20	57.4		20	1608		20	0.002		19	0.69	
Within mixes															
Initial grasshopper stage													1	0.055	0.10
Herbivory	1	7512	95.6***	1	1451	21.6***	1	692	0.48	1	0.007	5.18*	1	0.53	0.96
Herbivory : diversity	1	0.40	0.005	1	62.4	0.93	1	689	0.47	1	0.002	1.93	1	0.061	0.11
Herbivory : composition	2	1134	14.4***	2	232	3.44*	2	907	0.62	2	0.003	2.48	2	0.79	1.43
Residuals	54	78.6		25	67.3		25	1455		25	0.001		24	0.55	

♦Sequential ANOVA type I, the upper table 'Between mixes' refers to effects between differently composed communities, the lower table 'Within mixes' refers to herbivory effects on communities of identical composition. Interactive effects (Within) are superior to main effects (Between) of the respective explanatory variable.
[a]data square root transformed.

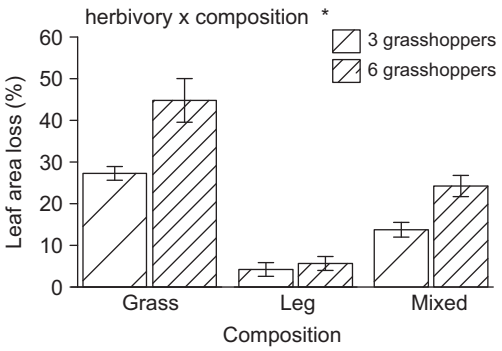

Figure 1. Interactive effect of plant community composition and herbivore pressure on percentage leaf area loss. Plant communities contained grass species only, legume species only (leg) or both functional groups (mixed). Mean ± SE on original data. Significance marked * with $p \leq 0.05$.

responses in root biomass and total community biomass, no significant effects were detected (data not shown). Separate analyses of legume and grass shoot biomass responses revealed that only for grass biomass the herbivory treatment had significantly negative effects, while plant community composition (i.e. the presence or absence of the other plant functional group) had no detectable effects (Supplementary material Appendix 2 Fig. A2, Table A1).

Grasshopper performance

Grasshopper survival did not vary with grasshopper density but increased with plant diversity (Table 2, Fig. 3). Increasing shoot biomass with increasing plant diversity did not explain the diversity effect on survival since shoot biomass of control communities did not affect the parameter ($F_{(1,19)} = 0.010$, $p = 0.921$). Moreover, low grasshopper survival in monocultures was not driven by a particular plant functional group since values were comparably low for both legumes and grasses (about 25% on average in both functional groups). In the alternative model, survival was affected by functional group composition only (Supplementary material Appendix 3 Table A2), being highest in mixed and lowest in legume communities (Fig. 3). In accordance with these results, the development of grasshoppers was also independent of grasshopper density, but grasshoppers developed faster in more diverse plant communities (Table 2): in monocultures, grasshoppers passed through half a developmental stage over the course of the experiment (0.52 ± 0.21), and through 1.56 ± 0.40 in the six-species communities. In the alternative model, only functional group composition was significant (Supplementary material Appendix 3 Table A2), and grasshoppers passed through more developmental stages in mixed communities (1.39 ± 0.15) than in grass (1.15 ± 0.23) or legume communities (0.64 ± 0.13).

More grasshopper faeces were collected in funnels of the six-grasshopper treatments (16 ± 2 mg) than in the three-grasshopper treatments (10 ± 1 mg, Table 2). When fitting composition first in the alternative model a significant interaction with herbivory occurred (Supplementary material Appendix 3 Table A2): in the three-grasshopper treatments most faeces accumulated in grass communities, while in six-grasshopper treatments, most faeces accumulated in mixed communities.

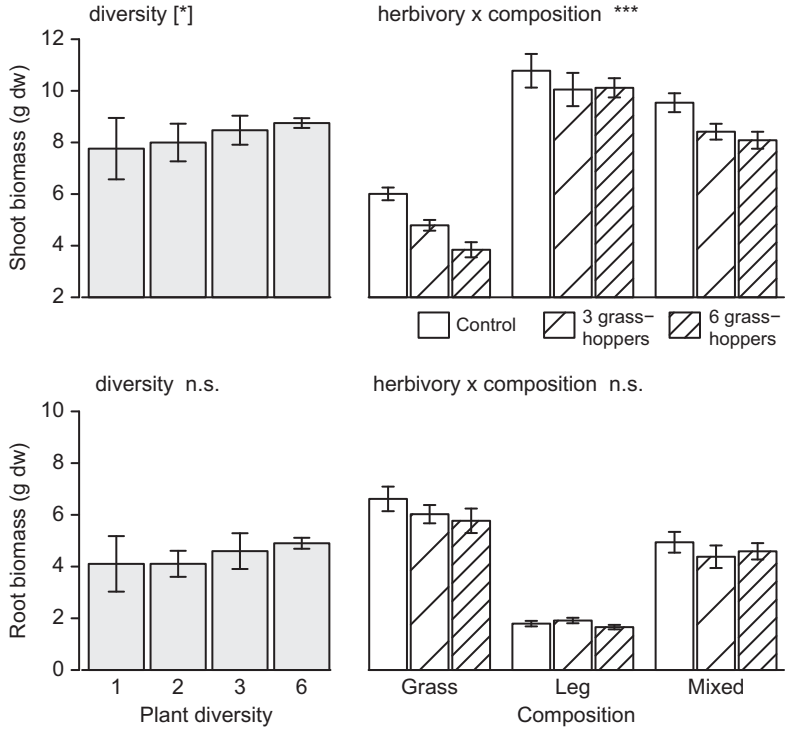

Figure 2. Plant community above (shoot) and belowground (root) biomass as affected by plant diversity and the interaction between community composition and herbivory. Plant communities contained grass species only, legume species only (leg), or both functional groups (mixed). Mean ± SE on original data. Significant effects are marked: * $p \leq 0.05$, *** $p \leq 0.001$, square brackets indicate significance only, when plant diversity was fitted first in the statistical model. Non-significance: n.s. For numbers of replicates per factor level see Table 1.

Table 3. Effects of plant diversity, community composition and herbivory on plant community parameters, throughfall pH, and microbial biomass: response variables in columns, explanatory variables in rows in their order of entering the model, Diversity = log transformed plant species richness; degrees of freedom (DF), mean squares (MS), and F-values are given; significant results are indicated by (*) $p = 0.057$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. ◆

	Shoot biomass [g][b]			Root biomass [g]			Log response ratio – Shoot biomass (grasshopper treatments only)			Throughfall pH			Throughfall pH (grasshopper treatments only)			Microbial biomass [µg g^{-1} soil dw]			Microbial biomass [µg g^{-1} soil dw] (grasshopper treatments only)		
No. of observations (i.e. pots)		87			84			58			87			58			87			58	
Source	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F
Between mixes																					
Block	5	0.17	3.45*	5	26.7	9.52***	5	0.040	1.24	5	0.099	0.69	5	0.11	0.71	5	27511	4.10*	5	15132	2.55
Diversity	1	0.41	8.04*	1	2.16	0.77	1	0.000	0.007	1	0.93	6.54*	1	0.79	4.92*	1	11687	1.74	1	18093	3.04
Composition	2	2.95	58.4***	2	50.7	18.1***	2	0.24	7.58**	2	0.12	0.82	2	0.028	0.17	2	7392	1.10	2	6751	1.14
Residuals	20	0.050		19	2.81		20	0.032		20	0.14		20	0.16		20	6708		20	5944	
Within mixes																					
Herbivory	2	0.33	21.8***	2	1.66	3.03(*)	1	0.084	3.92	2	1.87	13.1***	1	1.44	7.00*	2	6281	1.82	1	1846	0.48
Herbivory : diversity	2	0.005	0.30	2	0.58	1.06	1	0.009	0.43	2	0.39	2.70	1	0.75	3.66	2	21210	6.15**	1	36125	9.33**
Herbivory : composition	4	0.094	6.16***	4	0.42	0.78	2	0.82	3.83*	4	0.094	0.66	2	0.17	0.84	4	552	0.16	2	444	0.11
Residuals	50	0.015		48	0.55		25	0.021		50	0.14		25	0.21		50	3451		25	3871	

◆Sequential ANOVA type I, the upper table 'Between mixes' refers to effects between differently composed communities, the lower table 'Within mixes' refers to herbivory effects on communities of identical composition. Interactive effects (Within) are superior to main effects (Between) of the respective explanatory variable.
[b]data log transformed.

Figure 3. Survival of grasshoppers as affected by plant diversity and community composition. Plant communities contained grass species only, legume species only (leg) or both functional groups (mixed). Mean ± SE on original data. Significant effects are marked [*] with $p \leq 0.05$ only when the respective explanatory variable was fitted first in the statistical model.

Effects of experimental treatments on throughfall nutrients

NH_4-N made up the majority (95.4% averaged across all plant communities) of N species in dissolved mineral N (DMN), followed by NO_3-N (4.3%) and only minute amounts of NO_2-N (0.3%). Grasshopper presence significantly increased DMN concentrations in throughfall, without differences between the two levels of herbivore pressure (Table 4, Fig. 4). There was no effect of diversity or composition on throughfall N. However, fitting the alternative model, revealed a marginally significant interaction between plant diversity and herbivory ($p = 0.051$ see (*) in Supplementary material Appendix 2 and 3, Fig. A3 and Table A4): while in control communities DMN in throughfall was low and tended to decrease with diversity, DMN concentrations were higher and at similar levels in communities with three grasshoppers. For communities with six grasshoppers diversity levels clearly differed, showing increasing DMN concentrations from one to three-species mixtures. DMN concentrations did not correlate with grasshopper faeces weight ($F_{1,56} = 0.96$, $p = 0.331$).

Concentrations of PO_4-P in throughfall were close to zero in control communities and increased substantially in the presence of grasshoppers and with increasing herbivore pressure (Table 4, Fig. 4). When grasshopper communities only were analysed, a positive plant diversity effect became apparent and was strongest at highest herbivore pressure, although the interaction diversity × herbivory was non-significant (Table 4, Fig. 5). In accordance with results from leaf area loss, differently composed plant communities varied in throughfall PO_4-P concentrations and with increased herbivore pressure (significant interaction composition × herbivory; Table 4, Fig. 5). Fitting composition first in the alternative model rendered the plant diversity effect non-significant (Supplementary material Appendix 3 Table A4). PO_4-P concentrations were positively correlated with grasshopper faeces weight ($F_{1,56} = 11.54$, $p = 0.001$, $R^2 = 0.16$).

SO_4-S concentrations in throughfall were low in control communities and increased in the presence of grasshoppers

Table 4. Effects of plant diversity, community composition and herbivory on throughfall nutrients: response variables in columns, explanatory variables in rows in their order of entering the model, Diversity = log transformed plant species richness; degrees of freedom (DF), mean squares (MS), and F-values are given; significant results are indicated by *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. DMN = dissolved mineral N. ♦

	Throughfall DMN [mg N l^{-1}][a]			Throughfall DMN [mg N l^{-1}][a] (grasshopper treatments only)			Throughfall PO_4-P [mg P l^{-1}][b]			Throughfall PO_4-P [mg P l^{-1}][b] (grasshopper treatments only)			Throughfall SO_4-S [mg S l^{-1}][b]			Throughfall SO_4-S [mg S l^{-1}][b] (grasshopper treatments only)		
No. of observations (i.e. pots)		87			58			87			58			87			58	
Source	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F
Between mixes																		
Block	5	0.023	0.92	5	0.033	1.12	5	2.37	2.01	5	4.38	3.89*	5	2.18	2.92*	5	2.65	4.02*
Diversity	1	0.002	0.093	1	0.013	0.45	1	3.70	3.13	1	7.48	6.65*	1	0.26	0.35	1	1.17	1.77
Composition	2	0.015	0.62	2	0.011	0.38	2	13.5	11.5***	2	20.0	17.8***	2	2.79	3.74*	2	2.80	4.25*
Residuals	20	0.025		20	0.029		20	1.18		20	1.12		20	0.75		20	0.66	
Within mixes																		
Herbivory	2	0.16	12.4***	1	0.004	0.24	2	80.0	77.9***	1	5.86	6.82*	2	11.1	28.7***	1	0.69	1.81
Herbivory : diversity	2	0.020	1.56	1	0.016	0.97	2	3.01	2.93	1	0.51	0.60	2	1.07	2.78	1	0.14	0.38
Herbivory : composition	4	0.021	1.63	2	0.035	2.17	4	5.96	5.80***	2	0.66	0.77	4	0.59	1.54	2	0.040	0.11
Residuals	50	0.013		25	0.016		50	1.03		25	0.86		50	0.39		25	0.38	

♦Sequential ANOVA type I, the upper table 'Between mixes' refers to effects between differently composed communities, the lower table 'Within mixes' refers to herbivory effects on communities of identical composition. Interactive effects (Within) are superior to main effects (Between) of the respective explanatory variable.
[a]data square root transformed.
[b]data log transformed.

Figure 4. Herbivory effects on pH and nutrients in throughfall. Mean ± SE on original data. Significant differences between levels of herbivory are indicated by varying lower case letters. Potential inputs of NH_4-N (0.256 ± 0.015 mg l^{-1}) and SO_4-S (0.048 ± 0.007 mg l^{-1}) by filtering are not accounted for. DMN = dissolved mineral N.

(Table 4, Fig. 4). Community composition, but not diversity, influenced SO_4-S in throughfall (Table 4) as legume communities showed lower concentrations (0.296 ± 0.062 mg l^{-1}) than grass (0.806 ± 0.089 mg l^{-1}) and mixed communities (0.859 ± 0.101 mg l^{-1}). SO_4-S concentrations were positively correlated with grasshopper faeces weight ($F_{1,56} = 8.91$, $p = 0.004$, $R^2 = 0.12$).

All nutrient concentrations were positively correlated with each other (simple pair wise regressions, all $p < 0.001$).

Throughfall pH and soil microbial biomass responses

Throughfall pH significantly increased with increasing herbivore pressure and with plant diversity (Table 3, Fig. 4, 6). In the alternative model the plant diversity effect was rendered non-significant and the composition effect turned significant (Supplementary material Appendix 3 Table A3): throughfall pH was lower in grass communities (7.17) than in legume (7.28) and mixed communities (7.38). Throughfall pH was significantly positively correlated with

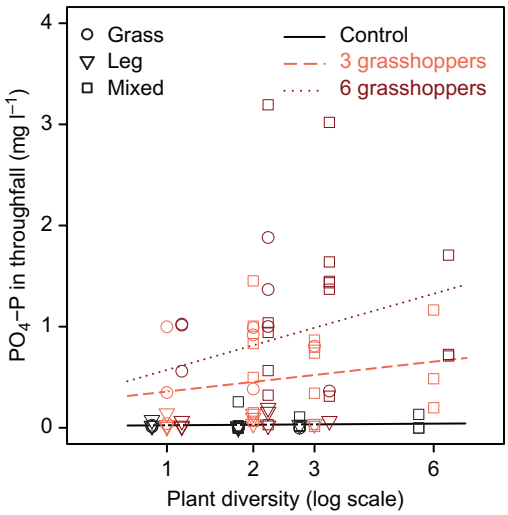

Figure 5. Throughfall PO_4-P concentration in response to plant diversity, composition, and herbivory. Staggered data points depict increasing herbivory levels from left to right. Plant communities contained grass species only, legume species only (leg) or both functional groups (mixed). Data from differently composed communities are given as different symbols (top left legend). Regression lines represent significantly different herbivory levels (Table 4). Original data.

all nutrient concentrations (simple pair wise regressions, all $p < 0.001$) and increased with increasing grasshopper faeces weight ($F_{1,56} = 11.22$, $p = 0.001$, $R^2 = 0.15$).

Soil microbial biomass was affected by a significant interaction between plant diversity and herbivory (Table 3, Fig. 7): soil microbial biomass increased with increasing plant diversity at the highest level of herbivore pressure, but not at lower levels of herbivory (Fig. 7). Microbial biomass was positively correlated with root biomass (linear regression:

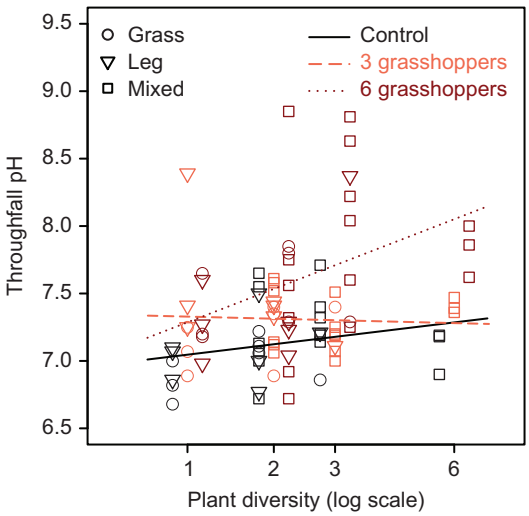

Figure 6. Throughfall pH in response to plant diversity, composition, and herbivory. Staggered data points depict increasing herbivory levels from left to right. Plant communities contained grass species only, legume species only (leg) or both functional groups (mixed). Data from differently composed communities are given as different symbols (top left legend). Regression lines represent significantly different herbivory levels (Table 3). Original data.

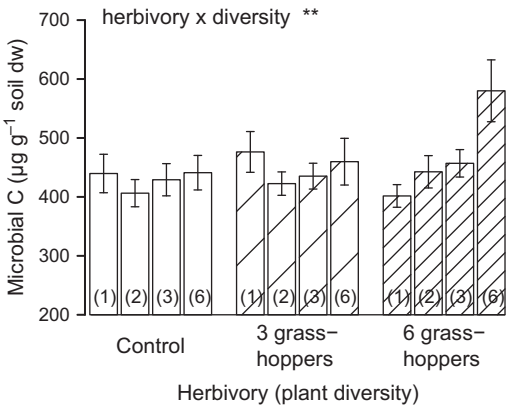

Figure 7. Microbial biomass as affected by the significant interaction between plant diversity and herbivory (** $p \leq 0.01$, Table 3). Mean ± SE on original data.

$F_{1,85} = 17.17$, $p < 0.001$, $R^2 = 0.16$), but not with throughfall nutrients or pH (all $p > 0.05$).

Discussion

In our study we tested if herbivory by a generalist insect herbivore can cause measurable changes in the nutrient content of throughfall, which then can affect fast nutrient cycling, and whether any such effect is influenced by plant diversity and the functional composition of the plant community. Our main results are: 1) grasshopper herbivory resulted in increased concentrations of dissolved mineral N (DMN), PO_4-P, and SO_4-S in throughfall as well as an increased pH, 2) PO_4-P and pH increases were positively affected by plant diversity, especially under high herbivore pressure, 3) plant functional composition affected several throughfall variables, sometimes fully explaining the plant diversity effect. In addition, plant diversity and composition had a number of effects on herbivory, grasshopper survival and plant biomass, some of which underlie the changes seen in throughfall nutrients. These nutrient changes in throughfall may have driven the observed increase in soil microbial biomass at high diversity at the highest herbivory level. In the following, we will discuss these various effects as well as their importance for real-world conditions.

Manipulation of species richness and functional composition of the plant communities

In biodiversity experiments, species and functional diversity, and functional composition of the plant community are often confounded (Schmid et al. 2002), as, at the lower end of a species diversity gradient, representatives of only some of the functional groups are present in a particular plant community (in the one-species level, only one), while at the high end plant communities comprise species from most or all functional groups. This was also the case in our experiment where the six-species communities contained both grasses and legumes. We expected the functional composition of the plant community to be important, because grasses and legumes differ in several aspects relevant to grasshopper feeding and nutrient cycling: legumes contain more

nitrogen while grasses are generally preferred by grasshoppers. While legumes can constitute an important part of *C. parallelus* diet (Unsicker et al. 2008, Franzke et al. 2010), we recorded more feeding on grasses than on legumes in our experiment. Grasses themselves are, however, not a homogeneous group with respect to grasshopper nutrition (Specht et al. 2008) and the herbivory values we recorded did indeed vary between the different grass species we employed (average leaf area loss: *D. glomerata* 25%, *H. lanatus* 29%, *P. trivialis*: 49%). While it was not the aim of our study to analyse the effects of particular plant species on herbivory and nutrients in throughfall, we addressed the effect of functional composition in our statistical analysis. The order of entering the factors plant diversity and functional composition in a sequential ANOVA (type I) affects the outcome of the statistical model: when entering composition after diversity, we aim at testing for composition effects that are independent from diversity effects (as diversity is 'held constant' in the model). Likewise, by entering composition first in the alternative model, we test for diversity effects at constant composition levels (Schmid et al. 2002). Because diversity and composition are, however, correlated and not independent, a strict separation in diversity versus composition effect is not possible. Thus, when plant diversity is significant in the first model and no longer in the alternative model, while composition becomes significant in the alternative model, this does not imply that diversity has no effect at all, as, due to the correlation, the composition effect also contains a diversity effect. If, in the same situation, both composition and diversity are significant in the alternative model, this implies that the diversity effect goes beyond what is already contained in the significant composition term. Testing our results with two alternative models is therefore illustrative to judge the relative importance of functional composition vs. plant diversity even if a full separation of their effects is not possible.

Grasshopper herbivory and performance

As expected, herbivory was affected more by the functional composition of the plant community than by its diversity, which has also been found in some but not all other studies (Scherber et al. 2010b, Loranger et al. 2014). Grasshopper performance was independent of grasshopper density, indicating that there was no food limitation in our experiment. Grasshopper development was faster and survival better in the mixed and more diverse communities. This is consistent with the hypothesis that the possibility of diet mixing, between and within the functional groups of legumes and grasses, can increase fitness in a generalist herbivore such as *C. parallelus* (Unsicker et al. 2008). Grasshoppers can use compensatory feeding when confronted with plants low in N (Berner et al. 2005) and this adaptability in feeding behaviour may be responsible both for the observed higher rates of herbivory in pure grass communities compared to mixed communities and the relatively similar levels of grasshopper survival and development in these communities. While the effects of diversity and composition on grasshopper performance likely were mediated mainly through plant nutrients, plant communities also differed in their architecture. Thus we cannot exclude the possibility that abiotic factors, such as humidity varying with communities, may also have affected survival and developmental time.

Herbivory and throughfall nutrients

When analysing nutrient concentrations in throughfall we found that herbivory released nutrients from plant tissue and that increasing herbivore pressure resulted in increased concentrations of S and P, but also of N compounds in mixtures of two and more plant species. When this throughfall enters the soil it can be used by plants and soil microbes. In contrast to predictions by Seastead and Crossley (1984), phyllosphere microbes seemed of little importance for immobilisation of released nutrients, as indicated by the low concentrations of nutrients in the controls, possibly due to the short time of the experiment (cf. Behrendt et al. 1997). For S and P concentrations, our data suggest a tight correlation with the amounts of faeces released, which therefore seem to represent the main source of these nutrients. Additionally, P concentrations were positively correlated with throughfall pH. Grasshopper faeces have been reported to show partially strong acidity or basicity, depending on food plants and grasshopper species (Frazier et al. 2000). When measuring pH in pure de-ionised water, in water blended with damaged plant parts, or with meadow grasshopper faeces (one sample each) we found pure water and water with plants to have a pH about 6.0 while pH in water with faeces was about 6.8. This observation supports our assumption that pH increase in throughfall was due to an increase in the amount of faeces, which consequently drove P concentrations. Such a correlation implies that nutrient release will vary with the digestive characteristics of the dominant herbivores. While we did not compare the stoichiometric ratios of nutrients in food plants to those in grasshopper faeces and throughfall in the different plant communities, it is likely that there was a correlation as predicted by stoichiometric theory (Elser and Urabe 1999).

Effects of plant diversity and composition on nutrient release

A major finding of our study is that nutrient concentrations in throughfall were not only significantly affected by grasshopper feeding, but also by plant diversity and functional composition of the community. For throughfall S, only composition was significant with grass and mixed communities containing higher levels of sulphate, which corresponded to the higher amount of faeces released in these communities. For N, the interaction between herbivory and diversity was close to significance in the alternative model, i.e. under constant composition an increase in N release with increasing plant diversity became obvious only in communities under highest herbivore pressure. This might imply that under high herbivore abundance, feeding on more than a few selected plant species is more likely, resulting in a release pattern more specific for a level of plant diversity than under low herbivore abundance. For concentrations of P and throughfall pH, there was a positive effect of plant diversity although herbivory damage did not increase at higher levels of diversity. This suggests that mechanisms other than increased consumption play a role in the increase in nutrient concentrations

and pH. One such mechanism may be the grasshoppers' ability of diet mixing: for the same degree of herbivory nutrient intake (and subsequent release) was higher when feeding occurred on several and/or more nutrient rich plants (Unsicker et al. 2008). Effects of plant diversity were generally strongest at the highest level of herbivory. Because our study is the first to report such effects and because results of plant diversity on herbivory are variable (cf. Scherber et al. 2006, 2010b, Loranger et al. 2014), more studies are needed that measure both herbivory and effects on the nutrient cycle at various levels of plant diversity.

Soil microbial and plant response

Plant-derived nutrients that are released with throughfall into the soil should be directly available to the soil microbial community or the plants themselves. Since soil microbes efficiently compete with plant roots for nutrients (Bardgett et al. 2003), they can be expected to respond to additional nutrient inputs by throughfall. Recent microcosm experiments showed that short-term plant diversity effects on soil microbial biomass can be neutral to negative (Eisenhauer et al. 2012), potentially due to competition between plants and microbes, which corresponds with our results for no and low herbivory treatments. However, at the highest level of herbivore pressure we detected an increase of soil microbial biomass with plant diversity. This suggests that high nutrient input levels were necessary to cause short-term changes in microbial biomass. However, our experimental set-up is not sufficient for discerning other potential causes of microbial response. For instance, herbivory on grassland plants was shown to induce root exudation of labile carbon (Dyer et al. 1991, Holland et al. 1996), thereby stimulating the microbial community and consequently improving plant nutrient supply through increased recycling of (also recalcitrant) organic matter in soil (Hamilton and Frank 2001). In this scenario, increasing diversity of carbon substrates released by plant roots at high plant diversity may affect the structure and growth of soil microbial communities (Orwin et al. 2006), thereby potentially explaining the plant diversity effect observed at high herbivory. Moreover, our data indicate a tendency of decreasing root biomass with increasing herbivory which may come along with an increasing amount of dead root material open for microbial decay (Guitian and Bardgett 2000). Therefore, despite some evidence of a soil microbial response, follow-up experiments will have to test the significance of herbivory-induced increased throughfall inputs and root exudation in affecting soil microbial biomass.

Plants may also directly benefit from throughfall nutrient inputs (especially when not having to compensate for severe herbivory-induced biomass losses) as suggested for instance in a recent publication on potential facilitative effects among differently palatable seaweed species (Bracken et al. 2014). We therefore analysed biomass log response ratios of plant communities finding that all communities responded negatively to herbivory, none fully compensating for biomass losses. Neither grasses nor legumes benefitted from the presence (or absence) of the other functional group. This observation likely results from the fact that N-rich legumes did not suffer strong enough herbivory as to produce N-rich throughfall benefitting grasses. Moreover, herbivory in grasses was particularly severe, likely impeding compensation within the time that our experiment ran. In more complex grassland communities, however, proportions of herbivory may differ from our observations here exhibiting potential facilitative impacts on the plant species or functional group level.

Ecological consequences at the field scale

Our microcosm experiment was short-term, i.e. we did not examine longer-term responses of the plant community as they were investigated e.g. by Belovsky and Slade (2000). These authors found in a five-year-study that consistent herbivory of the dominant grasshopper on particular plant species in a grassland community shifted the plant species composition towards species with faster-decomposing litter. The effect of litter decomposition on nutrient cycling has been termed the 'slow' cycle (Hunter 2001) and Belovsky and Slade (2000) found that, as a consequence of the shift in the dominating plant species, both nutrient cycling and net primary productivity (NPP) increased through herbivory. The authors also found effects of grasshopper feeding on the fast cycle as increasing herbivory increased N-availability to plants through frass. Overall, the effects on the slow cycle dominated observed effects on nutrient cycling in the grasslands investigated. In contrast to Belovsky and Slade (2000) who focussed on N, we also found that grasshopper feeding increased the availability of other nutrients, in particular P. It has been suggested that phosphate plays an important role in the status of species diversity in Eurasian terrestrial ecosystems (Wassen et al. 2005) and recent studies emphasize that besides nitrogen also other nutrients affect the composition of species assemblages and ecosystem dynamics (Joern et al. 2012).

In our experiment, the overall effect of herbivory was a decrease in aboveground biomass (Fig. 2), i.e. any short-term positive effect of increased nutrient availability on NPP likely was overcompensated by the relatively high levels of grasshopper herbivory. Since we did not design our experiment with focus on long-term responses and effects on other organisms than microbes, the overall effects of herbivory-induced nutrient release is difficult to judge. Frass and other inputs from herbivory have repeatedly been shown to result in measurable increases in plant growth, though (Kagata and Ohgushi 2012). Longer-term experiments are necessary to determine the diversity- and composition-dependent effects of throughfall nutrient inputs on above- and belowground communities. In our model system of managed grasslands (i.e. meadows), shoot biomass is regularly removed and hence input to the slow nutrient cycle is restricted. Here, herbivory-induced release of plant nutrients likely plays an important role for overall nutrient cycling under field conditions by keeping nutrients in the system.

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Supplementary material (available online as Appendix oik.01476 at <www.oikosjournal.org/readers/appendix>). Appendix 1–3.

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