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ORIGINAL PAPER

The effect of bacteria and fungi on chemical weathering and chemical denudation fluxes in pine growth experiments

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Abstract Vascular plants and associated microbial communities affect the nutrient resources of terrestrial ecosystems by impacting *chemical weathering* that transfers elements from primary minerals to other ecosystem pools, and *chemical denudation* that transports weathered elements out of the system in solution. We performed a year-long replicated flow-through column growth experiment to isolate the effects of vascular plants, ectomycorrhiza-forming fungi and associated bacteria on chemical weathering and chemical denudation. The study focused on Ca^{2+}, K^{+} and Mg^{2+}, for which the sole sources were biotite and anorthite mixed into silica sand. Concentrations of the cations were measured in input and output solutions, and three times during the year in plant biomass and on exchangeable cation sites of the growth medium. Weathering and denudation fluxes were estimated by mass balance, and mineral surface changes, biofilm and microbial attachments to surfaces were investigated with scanning electron microscopy. Both bacteria and fungi increased weathering fluxes compared to abiotic controls. Without a host plant denudation rates were as large as weathering rates i.e. the weathering to denudation ratio was about one. Based on whole year fluxes, ectomycorrhizal seedlings produced the greatest weathering to denudation ratios (1.5). Non-ectomycorrhizal seedlings also showed a high ratio of 1.3. Both ectomycorrhizal hyphal networks and root hairs of non-ectomycorrhizal trees, embedded in biofilm (microorganisms surrounded by extracellular polymers), transferred nutrients to the host while drainage losses were minimized. These results suggest that biofilms localize both weathering and plant nutrient uptake, isolating the root-hypha-mineral interface from bulk soil solution.

Keywords Biofilm · Chemical weathering · Chemical denudation · Column experiment · Ectomycorrhiza · Root-hypha-mineral interface

Abbreviation
EMF Ectomycorrhiza-forming fungi

Introduction

Mineral weathering is the primary source of non-nitrogen, mineral-derived nutrients necessary to sustain productivity in terrestrial ecosystems. It is known that vascular plants and associated microbial

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communities increase soil mineral weathering and mineral-derived nutrient availability through physical and chemical processes (Leyval and Berthelin 1991; Barker et al. 1997; Bormann et al. 1998; Kelly et al. 1998; Banfield et al. 1999; Landeweert et al. 2001; Marschner 2002). Maintenance of soil fertility and long-term productivity of terrestrial ecosystems require that rates of chemical denudation—the transport of weathered elements in solution to groundwater, rivers, and the oceans—do not exceed weathering rates and internal cycling of cations. Large weathering rates and large ratios of weathering to denudation are important in the establishment of primary successions (Bormann et al. 1998; Kelly et al. 1998).

Regulation of local hydrology has been widely recognized as a key means by which ecosystems prevent nutrient loss (e.g. Bormann and Likens 1979). However, rhizospheric processes may also play a key role in this regulation (Fernandez and Caldwell 1975; Marschner 2002). Rhizospheric soil solutions generally have lower pH and more available cations than the bulk soil solution (Caldwell and Camp 1974; Marschner 2002), and Rosling et al. (2004) suggest that mycorrhizal plants can weather minerals and take up nutrients independent of bulk soil solution chemistry. Both laboratory and field studies provide evidence that ectomycorrhizal fungi are able to extract nutrients such as P, K, Ca, Mg, and Fe from apatite, biotite, feldspars and other silicates and promote plant growth under nutrient-limited conditions (Leyval and Berthelin 1991; Paris et al. 1995, 1996; Jongmans et al. 1997; Crawford et al. 2000; Wallander and Wickman 1999; Hoffland et al. 2003; Nezat et al. 2004; Rosling et al. 2004). Our observations in the "sandbox" experimental ecosystems at Hubbard Brook Experimental Forest (Bormann et al. 1987; 1998; O'Brien et al. 2004; Keller et al. 2006) showed that tree growth and small rates of nutrient loss in drainage were associated with ectomycorrhizal hyphal and biofilm coverage of mineral surfaces (Balogh-Brunstad et al. 2008). [Biofilm is defined here as microorganisms concentrated on the root-hypha-mineral interface and surrounded by extracellular polymers they produce, utilizing plant and fungal exudates in soils (Banfield et al. 1999; Ghannoum and O'Toole 2004; Gadd 2007).] All of these findings imply that there may be bio-physico-chemical processes, involving root-microbe associations operating at mineral surfaces, that both enhance weathering and isolate the mineral-to-plant transfer of weathered nutrient mass from flowing soil water.

The purpose of this study was to test the foregoing idea by means of a replicated column experiment in which microbes were provided the nutrient and energy sources needed for growth, with and without a vascular host. The sole sources of Ca^{2+}, K^{+}, and Mg^{2+} were minerals in the growth medium, so that these three cations could only be obtained by weathering. We hypothesized that (a) ectomycorrhiza-forming fungi (EMF) and associated bacteria without a host would increase both total weathering and chemical denudation relative to abiotic controls; (b) ectomycorrhizal seedlings would strongly increase the rate of mineral weathering, while reducing denudation relative to non-ectomycorrhizal seedlings.

Materials and methods

Growth medium and inoculation

The growth medium was prepared in 164-ml plastic Ray Leach tubes (3.8 cm inside diameter, 21 cm depth; with bottom cut and sealed with 100-μm mesh nylon; Stuewe & Sons Inc., OR) and consisted of a mixture of 200 g acid-washed silica sand amended with 3% anorthite and 1.5% biotite by weight. One control contained only silica sand. The composition of the minerals was determined by X-ray fluorescence spectrometer (XRF; Thermo-ARL, Lausanne, Switzerland) following the procedure of Johnson et al. (1999) at Washington State University, Pullman, WA, USA. Biotite contained 9 wt% K, 14 wt% Mg and 0.2 wt% Ca; anorthite contained 0.1 wt% K, 11 wt% Mg, and 13 wt% Ca (both from WARD'S, Rochester, NY); silica sand contained less than 0.1 wt% of the three cations (Lane Mountain Company, Valley, WA). Biotite and anorthite were ground and the 250–500 μm size fraction was separated out by dry sieving, then it was sonicated and rinsed in DI water five times to remove small adhered particles and pasteurized at 90°C. Silica sand, which was coarser than 500 μm, was acid washed with 20% HCl and rinsed 10–15 times with de-ionized (DI) water before drying at 90°C. The initial growth medium of

each column was leached with DI water for 2 weeks prior to sowing the pines to reduce the effect of freshly disturbed and broken mineral surfaces.

Red pine was chosen for this study based on its hardiness on base-poor soils, because it grows well under laboratory conditions (e.g. Richter and Bruhn 1986; Koide and Kabir 2001), and to relate our results to the Hubbard Brook sandbox experiment in which it was also used (Bormann et al. 1998; Keller et al. 2006; Balogh-Brunstad et al. 2008). Red pine seeds were obtained at Sheffield's Seed Co, Inc., Locke, NY. Seeds were surface sterilized with 30% hydrogen peroxide containing a drop of Tween 20 (a surfactant) for 30 min and then rinsed several times with sterilized DI water (Yamanaka et al. 2003). Five seeds were sown in each pine treatment column, and then the 1-month-old seedlings were thinned to one per growth column by lifting out all but one whole seedling.

Suillus tomentosus and *Pisolithus tinctorius* were selected as EMF, because they were able to colonize red pine roots and grow well under laboratory conditions (Koide and Kabir 2001; Balogh 2006). The *S. tomentosus* sporocarps were collected about 20 miles North-East of Potlatch, ID (47°00′N, 116°39′W), and *P. tinctorius* sporocarps were collected in Corvallis, OR (44°34′N, 123°16′W). Small pieces of inner tissue were incubated at room temperature on Melin-Norkran's medium (MMN, Marx 1969) to obtain pure cultures of each fungus. Discs of mycelium were cut from the plates and blended in 150 ml DI water for use as EMF inocula, and then 3 ml were applied to each fungal column.

The bacterium *Ewingella Americana* was isolated from the *S. tomentosus* sporocarps and two bacteria, *Bacillus megaterium* and *Pantoea agglomerans* were isolated from the *P. tinctorius* sporocarps. The bacteria isolates were identified, based on 16S rRNA gene sequence similarity, by MIDI labs (Newark, DE, USA). Bacteria were grown on TSA medium (DIFCO, Detroit, MI, USA) for 3 days at room temperature then suspended in 150 ml DI water. Three ml of this suspension (4×10^7 CFU ml^{-1}) were applied to each bacterial column as inoculum.

Experimental design and conditions

The experiment was set up following Colpaert et al. (1999) and Yamanaka et al. (2003) with some modifications. The factorial experiment had four variables: ±bacteria (B), ±fungi (F), ±pine tree (P), and ±minerals. The combination of the variables and species resulted in 11 treatments (Table 1). We replicated each treatment 15 times to be able to destructively sample 5 tubes 3 times over the course of a year of growth. The tubes were placed in a growth chamber for 12 months with 24–16°C (day–night) regime and under 16–8 h photoperiod. The columns were inoculated 4 weeks after sowing. Treatments called ectomycorrhizal contained pine trees inoculated with both bacteria and fungus; and treatments called non-ectomycorrhizal contained pine trees inoculated with only bacteria (Table 1). Treatments without pine were referred to as bacteria-only or fungus-only and treatments without biotite and anorthite were called NoMin. One treatment was left abiotic (Table 1).

All columns received 20 ml of DI water 3 times a week. A 1/4 strength modified Hoagland's solution that lacked Ca^{2+}, K^+ and Mg^{2+} [all in mg l^{-1}: $NaNO_3$ (128), NH_4NO_3 (80), $NH_4H_2PO_4$ (29), Na_2SO_4 (71), $MnCl_2*4H_2O$ (0.45), H_3BO_3 (0.73) $ZnSO_4*7H_2O$ (0.06) $CuSO_4*5H_2O$ (0.02), $H_2MoO_4*H_2O$ (0.03) and $FeSO_4$ (0.75); pH 4.5] was applied once a month to each treatment. Bacteria-only and fungus-only treatments received 1 ml of 8% glucose solution (pH 4.0) as a carbon source once a week starting at inoculation. To minimize contamination among the treatments (a) antibiotics (pH 3.6; 0.3 ppm Ca, 0.1 ppm Mg) Penicillin G (500 μg/ml) and Rifampicin (50 μg/ml; Sigma Chemical Company, St. Louis, MO, USA), were applied to treatments without bacteria once every 2 months (Gerhardt et al. 1981), and (b) fungicides (pH 5.5; 0.5 ppm Ca, 0.1 ppm Mg) Steptomycin Sulfate (100 μg/ml) and Nystatin (40 μg/ml; Sigma Chemical Company, St. Louis, MO, USA) were applied to treatments without fungi once every 2 months (Juhnke and Jardiu 1989; Karwowski et al. 1996). The abiotic control received both antibiotics and fungicides. Algae growth was not eliminated by these chemicals and 10% $CuSO_4$ solution (pH 5.5; 27 ppm Cu, 1.7 ppm Ca; Bruneau and Lewis 1995) was used occasionally in treatments without fungi to eliminate algae growth.

Sample collection and analyses

Drainage water was continuously collected from each column. The volume of drainage water was measured every 2 weeks and an aliquot of each treatment was

Table 1 Experimental design and treatment setting

Treatment ID	Biotite and anorthite[a]	*Ewingella americana* (B1)[b]	*Suillus tomentosus* (F1)	*Bacillus megaterium* and *Pantoea agglomerans* (B2)[c]	*Pisolithus tinctorius* (F2)	Red pine (P)
Abiotic	✔	–	–	–	–	–
B1[d]	✔	✔	–	–	–	–
F1[e]	✔	–	✔	–	–	–
B1/P[f]	✔	✔	–	–	–	✔
B1/F1/P[g]	✔	✔	✔	–	–	✔
NoMin1	–	✔	✔	–	–	✔
B2[d]	✔	–	–	✔	–	–
F2[e]	✔	–	–	–	✔	–
B2/P[f]	✔	–	–	✔	–	✔
B2/F2/P[g]	✔	–	–	✔	✔	✔
NoMin2	–	–	–	✔	✔	✔

[a] All treatments had silica sand as a physical growth medium

[b] The bacteria *Ewingella* (B1) was isolated from sporocarps of the fungus *Suillus* (F1)

[c] The bacteria *Bacillus* and *Pantoea* (B2) were isolated from the sporocarps of *Pisolithus* (F2)

Also referred in text as [d] bacteria-only; [e] fungus-only; [f] non-ectomycorrhizal; [g] ectomycorrhizal

saved for cation analysis by inductively coupled argon plasma optical emission spectrometer (ICP-OES; Thermo Jarrell Ash, model 61) with about 5% accuracy and with detection limits about 0.2 ppm for Ca and Mg and 0.4 ppm for K.

Five columns of each treatment were destructively sampled at 6, 9 and 12 months. Cation content of seeds and the exchangeable cation pool of the growth medium were measured at the beginning of the experiment in order to estimate changes in the pine biomass (ΔB) and the soil exchangeable cation (ΔS) pools. Both ΔB and ΔS were similar for 6–9 and 9–12 month periods, so we combined those values and only compared the first 6 months to the second 6 months. At each destructive sampling, the root system of each pine tree was scanned at 1,200 dpi on a flat bed scanner. Total root length and ectomycorrhizal root length were determined using ImageJ image analyzing software (available at http://rsb.info.nih.gov/ij). Two copies of each root system image were saved. The total visible root system was traced on one image and the length of all traced lines yielded the total root length. On the other image, only ectomycorrhizal roots were traced. We selected ectomycorrhizal roots based on the assumption that roots with typical ectomycorrhizal morphology and anatomy are mycorrhizal (Peterson et al. 2004). This method gave us a minimum estimate of both total and ectomycorrhizal root length.

The pine biomass was separated into aboveground and belowground parts after scanning the root systems and oven dried at 65°C until it reached a steady weight. The seedlings were ground after recording the dry mass. A few mineral particles could not be removed from the roots by shaking. We estimated the amount of these minerals for each pine treatment by ashing a subsample of the homogenized belowground biomass of the largest and the smallest root samples out of the 5 replicates in a muffle furnace (Ameel et al. 1998). The approximate ash content value was subtracted from the dry belowground biomass weight to correct for mineral contamination of the roots in each pine treatments, and then all biomass results were reported as ash-free dry weight (Ameel et al. 1998). The ground seedling biomass was digested according to SW-846 EPA 1995 protocol using concentrated hot nitric acid and hydrogen peroxide. The diluted digests were analyzed by ICP-OES.

Bulk and rhizospheric growth media were collected separately during destructive pine sampling. Each growth medium was subsampled for exchangeable cation and various microscopic analyses. The subsamples for exchangeable cations were oven dried at 65°C then conventional ammonium acetate extraction procedure was used (McIntosh 1969). The extracts were analyzed for cation composition by ICP-OES.

To determine bacterial and fungal morphology, colonization and biofilm coverage on mineral surfaces, growth medium subsamples were freeze dried (Hayat 2000). In all dried subsamples, biotite and anorthite minerals were separated from the growth medium by hand, mounted on stubs with a double sticky carbon tape and carbon or gold coated. A Hitachi S-570 SEM (at WSU) and a Zeiss Leo 982 field emission SEM (at Pacific Northwest National Laboratory, Environmental Microbiological Science Laboratory, Richland, WA) were used for imaging all treatments and replicates. The total of 8 samples among all treatments were contaminated (2 abiotic samples had algae growth, 3 fungus-only samples had bacteria, 1 non-ectomycorrhizal pine had fungus and 2 ectomycorrhizal pine had algae). The contaminated samples were omitted from all analyses.

Biofilm was removed from mineral surfaces in three subsamples of each column to be able to observe weathering features, such as etching on biotite and anorthite surfaces. Rhizospheric and bulk growth medium subsamples were washed in 30% hydrogen peroxide for 10 min without heating then rinsed with DI water about 10 times and air dried (Douglas and Fiessinger 1971; Mikutta et al. 2005). This process successfully removed the biofilm. To evaluate artifacts caused by the chemical treatment, we tested whether hydrogen peroxide altered mineral surfaces in reserved untreated growth medium. We did not find notable changes on the reserved samples after hydrogen peroxide treatment.

We took a semi-quantitative approach to determine biofilm covered area % and weathered area % differences among treatments, using ImageJ image analyzing software that could estimate affected areas and compare them to the whole area of the SEM images. Hand-separated biotite and anorthite of three subsamples of each column were imaged at 10 random locations at the same resolution (5000× magnification). This yielded 30 images of biotite per column for weathered area evaluation, and 30 images of biotite and anorthite per column for biofilm coverage analyses.

Calculations and statistics

The growth medium was the sole source of Ca^{2+}, K^{+}, and Mg^{2+} in our experiment, but small amounts of these elements were added by antibiotics, fungicides and algaecide in the abiotic and the bacteria- and fungus-only treatments (see above). These inputs were subtracted from the drainage losses. Chemical denudation (D) of Ca^{2+}, K^{+}, and Mg^{2+} was defined as drainage flux (mol m^{-2} $year^{-1}$), where the area basis was the exposed surface area of the growth medium in the tubes, for comparison to previous sandbox and watershed results. Denudation fluxes were estimated at each drainage-collection interval by multiplication of measured elemental concentration by the drainage rate over the interval. Chemical weathering fluxes were defined using the same area basis and estimated using a modified form of the mass balance approach of Bormann et al. (1998):

$$W = D + \Delta B + \Delta S \quad (1)$$

where W is chemical weathering, D is chemical denudation, ΔB is change of cation content in pine biomass, and ΔS is change in the exchangeable cation pool of soil. The ΔB and ΔS terms of Eq. 1 were estimated at the times of destructive sampling, following 0–6 months and 6–12 months. The D term was cumulated so that Eq. 1 could be evaluated for these two periods. The significance of differences in the effects of bacteria and fungus, pine, and the presence or absence of ectomycorrhizae on pine was determined by analyses of variance (SAS 2004). Cation uptake by pine was contrasted between ectomycorrhizal and non-ectomycorrhizal treatments for each element in both time periods. Changes of cations on the exchangeable cation sites of the growth medium and the differences between weathering and denudation fluxes were analyzed in 5 non-orthogonal contrasts including bacteria- to fungus-only treatments; pine treatments to no-pine treatments; and ectomycorrhizal to non-ectomycorrhizal treatments in the 6–12 month period when pines came to dominate energy supply. For the root and electron microscopy results and measurements, we calculated standard errors.

Results

Cation uptake by pine

Pine biomass increased about 1.5–2 times in the second half of the experiment in all treatments (Table 2). The seedlings continued to grow roots to explore the growth medium in treatments with

Table 2 The total amounts of base cations (Ca^{2+}, K^+ and Mg^{2+}) in pine biomass, the total dry weight of pine biomass, total root length and ectomycorrhizal root length were measured at 6 and at 12 months

Treatment ID	At 6 months					At 12 months					
	Pine-biomass (g)	Amount of cations (μmol)			Root length (cm)	EMR length (cm)[#]	Pine-biomass (g)	Amount of cations (μmol)			Root length (cm)	EMR length (cm)[#]
		Ca	K	Mg				Ca	K	Mg		
B1/P	0.26 ± 0.01	23 ± 2[a]	27 ± 2[a]	36 ± 5[a]	339 ± 15	n.a.	0.39 ± 0.06	41 ± 12[a]	48 ± 9[a]	51 ± 19[a]	538 ± 100	n.a.
B1/F1/P	0.24 ± 0.02	17 ± 3[b]	18 ± 2[b]	20 ± 2[b]	248 ± 38	78 ± 8	0.49 ± 0.09	60 ± 11[a]	45 ± 9[a]	56 ± 8[a]	406 ± 50	92 ± 9
NoMin1	0.09 ± 0.01	0.8 ± 0[c]	3 ± 0[c]	1.5 ± 0[c]	125 ± 19	12 ± 2	0.13 ± 0.02	1.6 ± 0[c]	3 ± 1[c]	2 ± 0[c]	133 ± 16	12 ± 4
B2/P	0.29 ± 0.01	17 ± 2[b]	22 ± 2[b]	32 ± 7[a]	358 ± 26	n.a.	0.41 ± 0.04	34 ± 6[a]	42 ± 5[a]	36 ± 5[a]	626 ± 80	n.a.
B2/F2/P	0.27 ± 0.01	22 ± 2[a]	28 ± 3[a]	34 ± 5[a]	362 ± 28	17 ± 9	0.58 ± 0.16	47 ± 14[a]	70 ± 15[a]	73 ± 24[a]	646 ± 105	16 ± 5
NoMin2	0.12 ± 0.01	0.6 ± 0[c]	3 ± 0[c]	1.3 ± 0[c]	120 ± 2	14 ± 2	0.22 ± 0.03	2 ± 0[c]	6 ± 1[d]	3 ± 0[c]	198 ± 26	8 ± 2

The means ± standard deviations are shown. The different letters represent significant differences within each time period for each element ($P < 0.05$)

[#] Length of root colonized by ectomycorrhizal fungi (n.a.: not applicable)

minerals during this period. The B2 and F2 promoted greater root growth by the end of the year than B1 and F1, with slightly more total biomass (Table 2). On the other hand, ectomycorrhizal root length increased in the second 6 months only in the B1/F1/P treatment indicating better association between F1 and P than between F2 and P (Table 2). However, root hair formation was increased in B2/P and B2/F2/P treatments by end of the experiment (data not shown). During 0 to 6 months, B1/P and B2/F2/P immobilized the most cations, and B2/P and B1/F1/P accumulated smaller but significant amounts of all three cations (Table 2). The cation content nearly doubled in non-ectomycorrhizal pine biomass between 6 and 12 months, and it more than doubled in the ectomycorrhizal seedlings over this period (Table 2). Almost all NoMin pine seedlings survived the experiment with low growth and very low Ca^{2+}, K^+ and Mg^{2+} content. These trees apparently substituted sodium into the biomass to keep charge balance (data not shown), which indicates that the only source of Ca^{2+}, K^+ and Mg^{2+} in other treatments were the amendment biotite and anorthite.

Drainage water chemistry

Cation concentrations in drainage water generally decreased over time in all treatments, but notable differences developed among the treatments. Drainage water chemistry is only shown for treatments with B1 and F1 (Fig. 1), because treatments with B2 and F2 yielded very similar results. Drainage water flows were not substantially different among treatments—i.e. water uptake via evapotranspiration by the seedlings was small relative to the irrigation rate—so the concentration changes in time and differences among treatments are proxies for denudation changes and differences. After inoculation, large pulses of Ca^{2+} were lost from the bacteria- and fungus-only treatments for about 3–4 months, while Ca^{2+} concentrations of drainage water were 10 times lower in the abiotic and in the pine treatments, and continuously decreased with time (Fig. 1a). The drainage-water Ca^{2+} concentrations stayed higher in abiotic, bacteria- and fungus-only treatments than in pine treatments in the second 6 months (Fig. 1b). Magnesium concentrations were about 50% of Ca^{2+} concentrations throughout the experiment in all treatments, and therefore are not shown.

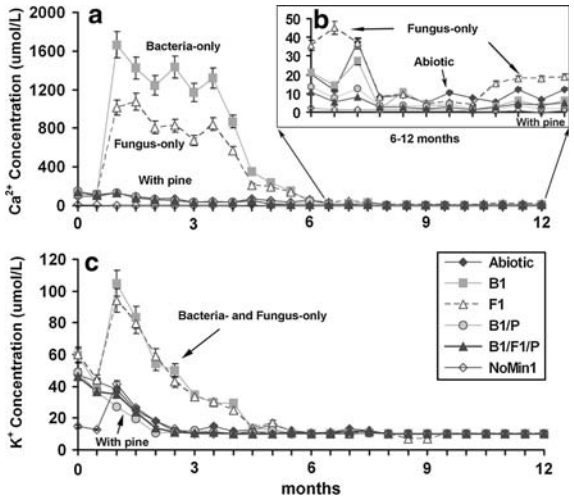

Fig. 1 Time series of Ca^{2+} concentrations (μmol l^{-1}) are shown for the whole year (**a**) and for the second 6 months; (**b**) in drainage water (see Table 1 for treatments). (**c**) K^+ concentrations (μmol l^{-1}) in drainage water are shown for the whole year. Only B1 and F1 treatments are graphed, because no differences were found between species in drainage water results. Error bars represent standard deviation and analytical error

By contrast, K^+ concentrations were much lower than Ca^{2+} concentrations and reached the detection limit (0.4 ppm) much faster in all treatments (Fig. 1c). Drainage water K^+ concentrations were elevated in the bacteria- and fungus-only treatments compared to the pine treatments, but were only 2–3 times as high.

Exchangeable cations

The initial exchangeable cation pool of the growth medium contained high Ca^{2+} (77 μmol) and Mg^{2+} (21 μmol) but no K^+. There was negligible Ca^{2+} and Mg^{2+} on the exchange sites of the NoMin controls, and that did not change (Table 3). In the first 6 months, Ca^{2+} and Mg^{2+} concentrations on the exchange sites decreased dramatically and caused large negative changes in the total soil exchangeable cation pool in every treatment, while K^+ accumulated (Table 3).

In the second 6 months, changes in the soil exchangeable cation pools were positive in most of the treatments. However, the Ca^{2+} and Mg^{2+} values did not fully recover to their initial condition, whereas K^+ continued to accumulate on the exchange sites. The largest gains for all three cations occurred in B1, F1 and F2 treatments, but both ectomycorrhizal treatments gained significant amounts of base cations ($P = 0.001$; Table 3). The abiotic and the non-ectomycorrhizal treatments continued to lose Ca^{2+} from their exchange sites.

Mineral surfaces

The investigation of biotite and anorthite with SEM confirmed colonization by bacteria and fungi on the mineral surfaces (Fig. 2a and b). Treatments did not affect biofilm coverage ($P > 0.05$; Table 4), which varied from 18 to 39% of mineral surfaces in the bulk soil. The presence of pine and fungi did not increase biofilm beyond what formed with bacteria alone. In the rhizosphere of pines with or without fungi, 57–60% of mineral surfaces were covered with biofilm (Table 4). Bacteria and fungal hyphae were part of the biofilm or were covered by the biofilm as shown in the ectomycorrhizal treatments at 6 (Fig. 2a) and at 12 months (Fig. 2b).

Removal of biofilm revealed dissolution features (weathered areas) on biotite and anorthite surfaces, but the features were more distinguishable on the biotite particles (Fig. 2c and d) because biotite has perfect one directional cleavage (Deer et al. 1992). This permitted estimation of surface changes on biotite surfaces, but not on anorthite because of initially rough surfaces that obscured changes from weathering. Curved and branched, fungal-hypha-sized dissolution channels (2–6 μm in diameter) were observed on biotite surfaces in both fungus-only and ectomycorrhizal treatments at 6 and at 12 months (Fig. 2c and d). In all pine treatments, some larger (>10 μm in diameter) dissolution channels were observed that were probably formed by root hairs and small root tips. The largest weathered areas of bulk soil biotite were found in treatments with fungi, with or without pine (Table 4). Treatments with bacteria, with or without pine also caused some biotite surface weathering in the bulk soil, but the weathered areas were smaller than with fungi (Table 4).

Although biofilm coverages were similar in the rhizosphere of ectomycorrhizal and non-ectomycorrhizal treatments, the weathered areas were significantly larger ($P = 0.04$) in the rhizosphere of the ectomycorrhizal treatments than in the non-ectomycorrhizal treatments (Table 4). Some dissolution and precipitation of secondary phases occurred on biotite surfaces in the abiotic control treatment,

Table 3 Changes of the total amount of Ca^{2+}, K^+ and Mg^{2+} on the soil exchangeable cation sites for 0–6 and 6–12 months

Treatment ID	Change on the soil exchangeable cation sites (μmol)					
	0–6 Months			6–12 Months		
	Ca	K	Mg	Ca	K	Mg
Abiotic	−30 ± 9	13 ± 2	−14 ± 1	−7 ± 11	2 ± 7	7 ± 1
B1	−56 ± 3	11 ± 1	−13 ± 1	14 ± 7	23 ± 2	8 ± 1
F1	−57 ± 1	14 ± 2	−14 ± 1	18 ± 1	15 ± 2	3 ± 1
B1/P	−36 ± 4	11 ± 1	−14 ± 1	−1 ± 10	8 ± 1	5 ± 1
B1/F1/P	−25 ± 5	11 ± 1	−13 ± 1	11 ± 7	8 ± 13	8 ± 2
NoMin1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
B2	−55 ± 1	11 ± 1	−14 ± 1	2 ± 4	13 ± 2	3 ± 1
F2	−56 ± 3	11 ± 1	−13 ± 1	20 ± 10	18 ± 3	10 ± 2
B2/P	−36 ± 9	12 ± 1	−13 ± 1	−7 ± 12	10 ± 1	2 ± 2
B2/F2/P	−29 ± 10	11 ± 1	−13 ± 1	13 ± 13	9 ± 1	5 ± 1
NoMin2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Initial values are given in the text. The mean changes ± standard deviations are shown

but no distinct dissolution channel formation was observed (Fig. 2e). The anorthite feldspar surfaces also underwent dissolution and precipitation of secondary phases during the course of the experiment, but the changes were hard to detect because the initial surfaces were uneven and rougher than those of biotite. However, some areas of anorthite grains were extensively weathered (Fig. 2f).

Chemical weathering and denudation mass-balance

Chemical weathering and denudation varied among treatments and between 0–6 month and 6–12 month periods (Table 5). For all treatments, the fluxes were significantly higher in 0–6 months than in 6–12 months ($P = 0.01$), and during the first 6 months the weathering and denudation fluxes were equal, i.e. generation of cations by weathering was balanced by loss of cations in drainage ($P = 0.3$; Table 5). The weathering and denudation fluxes were 10–20 times higher in bacteria- and fungus-only treatments during 0–6 months than in the other treatments (Table 5). These very large early fluxes dominated the totals, such that the bacteria- and fungus-only treatments caused the largest total weathering and denudation fluxes (Table 5). Both fluxes were nearly zero in the NoMin controls, which indicated that the base cations were weathered from biotite and anorthite minerals in the other treatments.

Chemical weathering and denudation fluxes substantially decreased, between 5 and 50 times, with the most dramatic decrease in the bacteria-and fungus-only treatments, after 0–6 months. During 6–12 months, when pines had larger foliage and their roots dominated the columns more completely, the rates of weathering in all columns with pines become slightly higher than the columns with bacteria- or fungus-only ($P = 0.31$). Ectomycorrhizal columns had the highest weathering rate during this period (0.25 mol m^{-2} $year^{-1}$) contrasted with bacteria- and fungus-only, and non-ectomycorrhizal columns (0.15 mol m^{-2} $year^{-1}$; $P = 0.004$). The average of all bacteria- and fungus-only treatments had over 4 times greater denudation that those with pine ($P < 0.001$, Table 5).

When weathering and denudation fluxes are compared to each other within a treatment, the effect of ectomycorrhizal seedlings became clear for both the whole year and 6–12 month periods (Fig. 3). For the whole year the ratios of weathering to denudation were the highest, around 1.5, in the ectomycorrhizal treatments, and also high, around 1.3, with non-ectomycorrhizal seedlings, while in other treatments the ratios remained approximately 1 (Fig. 3a). Differences among treatments became even larger during 6–12 months. Weathering fluxes were greater than denudation in every treatment except the abiotic and the NoMin controls. However, the ratios of weathering to denudation remained lower (1–2.4) in

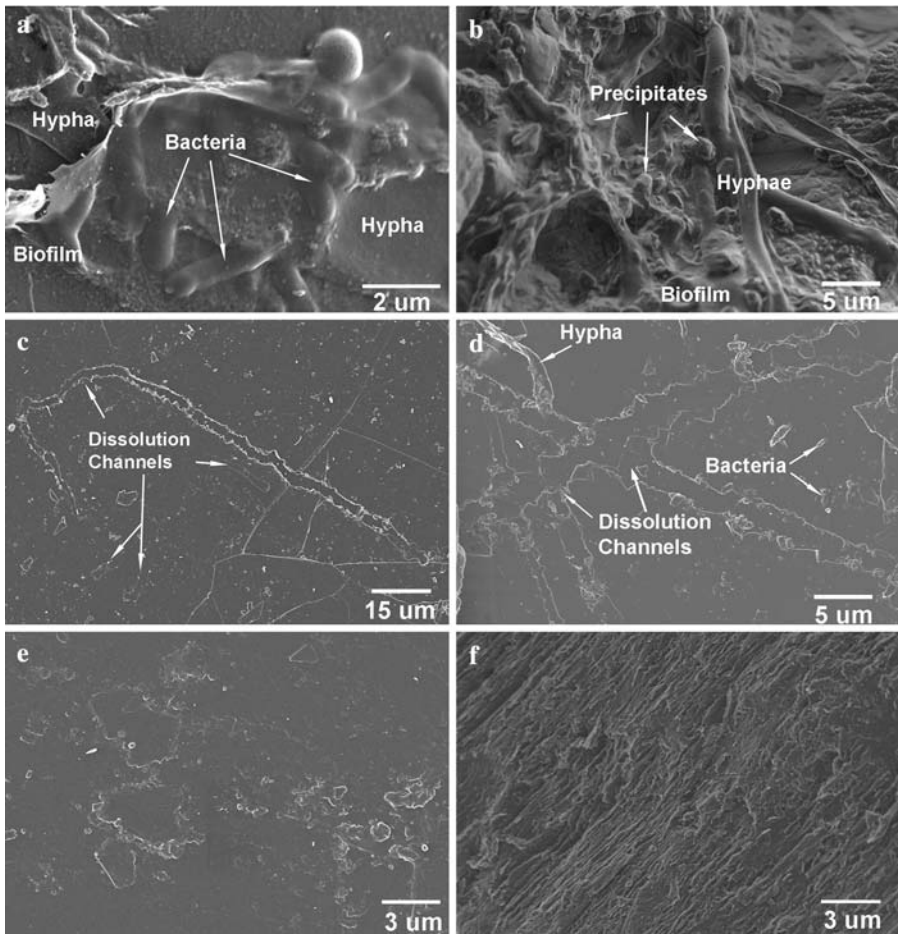

Fig. 2 Examples of SEM images of biotite and anorthite surfaces. (**a**) A biotite surface covered with biofilm containing bacteria and fungal hypha in B2/F2/P treatment at 6 months. (**b**) An anorthite surface covered with biofilm containing fungal hyphae and secondary precipitates in B1/F1/P treatment at 12 months. (**c**) Hypha sized curved dissolution channel on a biotite surface in B2/F2/P treatment at 6 months. (**d**) Multiple branching dissolution channels on a biotite surface in B1/F1/P treatment at 12 months. (**e**) Biotite surface in the abiotic treatment with secondary precipitates. (**f**) A weathered anorthite surface in F1 treatment

the treatments without pine seedlings than in the non-ectomycorrhizal (about 6.5) and ectomycorrhizal (11 and 20) treatments (Fig. 3b). These differences between the fluxes were caused by the recovering soil exchangeable cation sites in most treatments (Table 3) and by the accumulation of cations in biomass in the pine treatments (Table 2).

Discussion

We hypothesized that EMF and associated bacteria without a vascular host would increase both weathering and denudation compared to the abiotic system, and vascular plants associated with EMF would increase mineral weathering and suppress denudation at the same time, compared to non-ectomycorrhizal systems. We also expected to see differences between the two fungal species and between the bacteria species. We found that indeed weathering and denudation fluxes of bacteria- and fungus-only treatments exceeded abiotic controls through the experiment, but in the first 6 months the fluxes of bacteria- and fungus-only treatments were 10 times larger than the fluxes of every other treatment. Differences between weathering and denudation did not develop in any of the treatments until the second 6 months. In that period, significant differences developed among treatments in the ratios

Table 4 Biofilm coverage and weathered area percentages estimated from SEM images of biotite surfaces using ImageJ at the end of the experiment

Each number represents the mean of 30 measurements ± standard deviations

Treatment ID	Bulk soil minerals		Rhizosphere minerals	
	Biofilm coverage (%)	Weathered area (%)	Biofilm coverage (%)	Weathered area (%)
B1	22 ± 9	9 ± 4	n.a.	n.a.
F1	18 ± 17	17 ± 3	n.a.	n.a.
B1/P	30 ± 13	7 ± 1	58 ± 7	5 ± 1
B1/F1/P	38 ± 12	17 ± 4	58 ± 8	17 ± 2
B2	34 ± 4	10 ± 3	n.a.	n.a.
F2	39 ± 8	16 ± 2	n.a.	n.a.
B2/P	28 ± 12	7 ± 1	60 ± 9	6 ± 2
B2/F2/P	38 ± 12	30 ± 6	57 ± 9	14 ± 2

Table 5 Chemical denudation (D) and weathering (W) fluxes (mol m^{-2} $year^{-1}$) were estimated for 0–6 and 6–12 month intervals

Treatment ID	0–6 Months		6–12 Months		0–12 Months	
	D	W	D	W	D	W
Abiotic	0.72 ± 0.12^{d}	0.67 ± 0.14^{d}	0.06 ± 0.01^{b}	0.06 ± 0.04^{b}	0.39 ± 0.13^{d}	0.37 ± 0.18^{d}
B1	9.65 ± 0.63^{a}	9.55 ± 0.63^{a}	0.06 ± 0.01^{b}	0.13 ± 0.03^{a}	4.86 ± 0.64^{a}	4.84 ± 0.66^{a}
F1	6.05 ± 0.56^{c}	5.95 ± 0.57^{c}	0.11 ± 0.04^{a}	0.17 ± 0.04^{a}	3.08 ± 0.60^{c}	3.06 ± 0.61^{c}
B1/P	0.59 ± 0.15^{d}	0.67 ± 0.17^{d}	0.02 ± 0.01^{c}	0.14 ± 0.08^{a}	0.31 ± 0.16^{d}	0.41 ± 0.25^{d}
B1/F1/P	0.55 ± 0.09^{d}	0.60 ± 0.11^{d}	0.02 ± 0.01^{c}	0.26 ± 0.08^{a}	0.28 ± 0.10^{d}	0.43 ± 0.19^{d}
NoMin1	0.08 ± 0.03^{e}	-0.04 ± 0.06^{e}	0.01 ± 0.00^{c}	0.01 ± 0.00^{c}	0.04 ± 0.03^{e}	0.00 ± 0.06^{d}
B2	5.74 ± 0.30^{c}	5.64 ± 0.31^{c}	0.11 ± 0.03^{a}	0.14 ± 0.04^{a}	2.93 ± 0.33^{c}	2.89 ± 0.35^{c}
F2	6.86 ± 0.47^{b}	6.75 ± 0.48^{b}	0.09 ± 0.02^{a}	0.18 ± 0.05^{a}	3.48 ± 0.49^{b}	3.47 ± 0.53^{b}
B2/P	0.59 ± 0.15^{d}	0.65 ± 0.19^{d}	0.02 ± 0.02^{c}	0.11 ± 0.06^{a}	0.31 ± 0.17^{d}	0.38 ± 0.25^{d}
B2/F2/P	0.59 ± 0.11^{d}	0.68 ± 0.15^{d}	0.01 ± 0.00^{c}	0.24 ± 0.10^{a}	0.30 ± 0.11^{d}	0.46 ± 0.25^{d}
NoMin2	0.05 ± 0.01^{e}	0.06 ± 0.02^{e}	0.01 ± 0.00^{c}	0.02 ± 0.01^{c}	0.03 ± 0.01^{e}	0.04 ± 0.02^{e}

The total 12 months fluxes are also shown. The W values are the sum of Ca^{2+}, K^{+} and Mg^{2+} in drainage water, change in biomass pool and change on the soil exchangeable cation sites, and the input values are subtracted. Each number is the mean of 5 replicates ± standard deviation. The different letters represent significant differences within each time period ($P < 0.05$)

of weathering to denudation ($P < 0.05$). Overall, the largest weathering and denudation fluxes were observed in the bacteria- and fungus-only treatments over the total 12 months, but weathering to denudation ratios were the highest in the ectomycorrhizal treatments. In general, we did not find significant species-specific differences between the two fungi nor between the bacteria species ($P = 0.2$). In the following discussion we address possible explanations for our observations.

Effect of vascular host on microbial and fungal weathering and denudation

In the 0–6 month interval, relatively high weathering fluxes (0.6–0.7 mol m^{-2} $year^{-1}$) and equally high denudation fluxes characterized all pine treatments (Table 5). The high weathering rate could be attributed to preferential weathering of smaller particles and easily weatherable phases (calcite and apatite inclusions), or simply the fresh and highly active mineral surfaces and edges, or both (e.g. White and Brantley 1995; Bakker et al. 2004). High denudation during 0–6 months was caused in part by depletion of Ca^{2+} and Mg^{2+} from the exchange sites (accounting for 7–10% of leachates) and in part because the small pine seedlings were not a strong photosynthate source yet (little foliage) and their root/hyphal systems had not fully explored the soil volume so that they could not take up large quantities of weathering products. Thus, the high weathering flux was due mostly to the high denudation flux, with biological nutrient storage

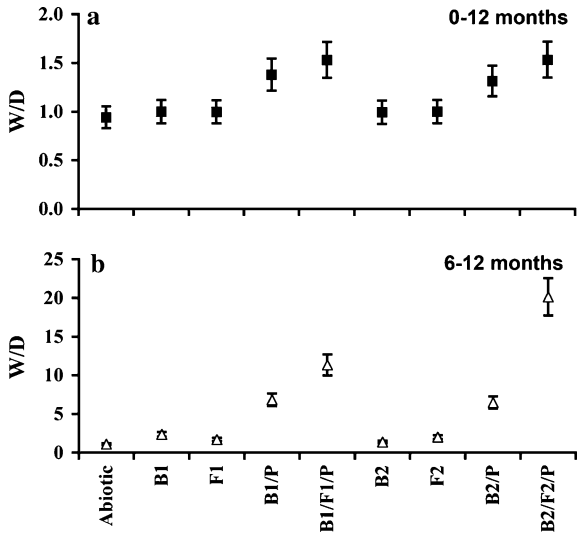

Fig. 3 The ratios of weathering to denudation fluxes are shown for 0–12 months (**a**) and for 6–12 months; (**b**) time periods. Error bars represent standard deviation

pools playing a minor role. The ectomycorrhizal seedlings were not able to regulate or influence weathering more than non-ectomycorrhizal seedlings, and could not increase nutrient uptake in this early growth stage, which was different from some earlier studies (Wallander and Wickman 1999; Colpaert et al. 1999).

The purpose of this study led to a focus on the results for the 6–12 month period, when the effects of seed resources and fresh surfaces were minimized and the effects of tree growth and root development were greatest. Our hypothesis was supported in that we observed reduced denudation losses after 6 months in the ectomycorrhizal treatments (Table 5 and Fig 3). However, we also found suppressed denudation fluxes in the non-ectomycorrhizal pine treatments which indicated that the bacteria and pine association alone could control the losses as effectively as the bacteria–fungus and pine association regardless of species specificity (Table 5). The lowered Ca^{2+} concentrations and fluxes in drainage water of both ectomycorrhizal and non-ectomycorrhizal treatments were similar to the low Ca^{2+} concentrations observed in shallow soil water in the red pine sandbox at Hubbard Brook (Keller et al. 2006; Balogh-Brunstad et al. 2008). The SEM investigation of mineral surfaces confirmed that both bacteria/pine and bacteria/fungus/pine associations produced biofilm coatings on the mineral surfaces (Figs. 2a and b; see also e.g. Barker et al. 1998). Biofilms are known to protect the microbial community from various environmental effects and even from antibiotics (e.g. Ghannoum and O'Toole 2004; Gadd 2007), regulate transport of heavy metals and possibly other cations and nutrients to the microbes (e.g. Gadd 2007), and isolate mineral weathering and nutrient uptake by bacteria from bulk soil processes (Banfield et al. 1999). These characteristics might benefit the surface-to-root nutrient transfer function of ectomycorrhizal fungi and root hairs embedded in biofilms, by cation nutrient adsorption and complexation within the biofilm. These processes could reduce Ca^{2+} mass transfer to bulk soil water, and thus result in low Ca^{2+} concentrations in column drainage waters of the pine treatments. In our experiments no straightforward relationship was found between % biofilm coverage and denudation fluxes among the treatments, because treatments without pine had no rhizosphere, where biofilm coverage might have been most important (Table 4). However, both the ectomycorrhizal and non-ectomycorrhizal treatments exhibited large % biofilm coverage in the rhizosphere (Table 4) coinciding with low denudation fluxes (Table 5).

The expected weathering flux increase in the pine treatments also became evident after 6 months of growth (Table 5 and Fig. 3). The presence of ectomycorrhizal fungi increased the weathering flux 1.8–2.0 fold compared to bacteria and pine associations. Calvaruso et al. (2006) found a consistent weathering flux increase, a factor of 1.5, by pine with root-associated bacteria compared to pine alone. Our results agreed with findings that ectomycorrhizal seedlings are able to take up base cation nutrient elements from primary mineral sources in larger amounts than their non-ectomycorrhizal counterparts if the sources are limited (Leyval and Berthelin 1991; Paris et al. 1995, 1996; Jongmans et al. 1997; Wallander and Wickman 1999; Hoffland et al. 2003). This may be due to the fact that hyphal mycelia are more effective than root hairs at increasing the absorptive and plant-mineral contact area of the root system (Smith and Read 1997). This idea was also supported by SEM investigation of mineral surfaces, which showed larger percentages of weathered areas in the ectomycorrhizal treatments (Fig. 2c and d; Table 4). Alternatively, it may be that

the "three-way" association among bacteria, fungus and pine is more efficient in nutrient acquisition, transport and storage than the "two-way" association between bacteria and pine. It may be that the bacteria produce the biofilm utilizing the root and fungal exudates (Banfield et al. 1999; Gadd 2007) and the fungi utilize the protection of biofilm while weathering the mineral surfaces and transporting the dissolved elements to the host plant (Jongmans et al. 1997; Wallander and Wickman 1999; Hoffland et al. 2003).

It has been shown that trees and associated fungi use belowground carbon to produce increased amounts of exudates including organic acids, ligands, and enzymes (e.g. Marschner 2002). However, neutral drainage water pH through out the experiment suggested that such acids were consumed during weathering. The larger increase of the soil exchangeable cation pool in the ectomycorrhizal than non-ectomycorrhizal soils also contributed to the higher weathering fluxes of the ectomycorrhizal treatments during 6–12 months (Table 3).

Bacterial and fungal weathering and denudation dynamics without vascular host

The bacteria and fungi we evaluated demonstrated a large capability to weather Ca-bearing minerals (Table 5). Unlike natural environments, our experiment supplied labile carbon as glucose, fresh mineral surfaces for colonization and for nutrient sources, and an environment where the individual species could flourish without competition for resources. Weathering and denudation in bacteria- and fungus-only treatments were initially rapid but then declined sharply (Fig. 1). We suspect that initial conditions caused very high rates of bacteria and fungi growth that could not be sustained. Possible explanations are that the "unlimited" resources ran out after 5–6 months (Fig. 1) and the colonies stabilized with equal growth and turnover rates (Sylvia et al. 1999).

A contributor to the initially large Ca^{2+} losses could be the dissolution of calcite ($CaCO_3$) inclusions because of greatly increased acid production by bacteria and fungi. Another factor contributing to large Ca^{2+} losses might be phosphorus (P) limitation caused by the readily available labile carbon supply. Microbes may preferentially colonize and weather minerals containing P mineral (e.g. apatite) inclusions (Rogers et al. 1998), but the adjacent or hosting minerals (mostly Ca-feldspars) could also be weathered during the target mineral dissolution and ions released to solution (Banfield et al. 1999). This idea is supported by the fact that drainage losses did not contain P (data not shown).

Soil exchangeable Ca^{2+} and Mg^{2+} concentrations also decreased in the first 6 months, but this decrease only could account for 1–2% of the denudation flux and the sites started to recover during 6–12 months (Table 3). By contrast, K^+ accumulated on the soil exchange sites of all treatments during the course of the experiment with the largest increase in the bacteria- and fungus-only treatments (Table 3), and K^+ drainage losses were under detection limit by the 5th month (Fig 1). This observation contrasts with the finding that K^+ was continuously depleted on soil exchange sites in the non-vascular sandbox at Hubbard Brook (Bormann et al. 1998; Keller et al. 2006; Balogh-Brunstad et al. 2008). Here the initial growth medium did not have K^+ on the exchange sites, which might explain a preference for exchangeable K^+ accumulation.

On the whole, weathering and denudation fluxes were equal or nearly equal in bacteria- and fungus-only treatments, which supports the idea that bacteria and fungi, like non-vascular vegetation (moss and lichens), are not able to regulate chemical denudation compared to vascular systems (Bormann et al. 1998; Moulton et al. 2000; Balogh-Brunstad et al. 2008). Such regulation in vascular systems appears to depend on increasing biomass, which acts as a sink for cation uptake and storage. In the bacteria- and fungus-only treatments, the observed 6-10 mol m^{-2} - $year^{-1}$ fluxes early in the experiment were 2 orders of magnitude higher than the weathering flux of the non-vascular sandbox at Hubbard Brook (Bormann et al. 1998; Balogh-Brunstad et al. 2008), and 2–4 orders of magnitude higher than other moss-, lichen-, or bare rock-covered watershed estimates (Bain et al. 1994; Clow and Drever 1996; Moulton et al. 2000; Aghamiri and Schwartzman 2002). These large discrepancies between our laboratory study and field studies were similar to reported lab-field differences in previous studies (for review see White and Brantley 2003). We suggest that the energy source and the inherent weatherability of the particles we used can explain much of the difference between the laboratory and field results.

Summary and conclusions

The experiment allowed us to study, separately and in combination, the effects of bacteria and ectomycorrhiza-forming fungi on chemical weathering and denudation processes, with and without a vascular plant host. Our experiment demonstrated that bacteria and ectomycorrhiza forming fungi can weather Ca-bearing minerals, such as anorthite, but without a vascular host the microbial communities were not able to regulate denudation losses. Chemical weathering and denudation were about equal in each bacteria- and fungus-only treatment similar to abiotic controls.

In the first 6 months of the experiment in all treatments the ratios of weathering to denudation were about the same, all around one, with high weathering and denudation fluxes. The processes here were likely driven by the acidic flushing of the fresh growth medium (pH effect) and weathering was much greater than plant nutrient demands in the pine treatments. However, in the second 6 months the ectomycorrhizal treatments produced the greatest weathering, least denudation, and greatest pine growth among the treatments. In addition, the ratios of weathering and denudation of the ectomycorrhizal treatments were 10–20 times greater than the bacteria- and fungus-only treatments, and even 2–3 times greater than the non-ectomycorrhizal treatments during the 6–12 month period. The denudation fluxes and pine growth were similar in the ectomycorrhizal and non-ectomycorrhizal treatments.

The ability of pine seedlings to retard denudation in both ectomycorrhizal and non-ectomycorrhizal treatments was probably linked to observed biofilm formation on the rhizospheric mineral surfaces. An ectomycorrhizal hyphal network, as part of the biofilm or covered by the biofilm, is able to transport nutrients to the plant with minimum loss to the bulk soil solution. On the other hand, the non-ectomycorrhizal treatment produced more root hairs, stimulated by the associated bacteria (Calvaruso et al. 2006), and this may have partially compensated for lack of hyphal absorbing surfaces in the biofilm. The present study suggests that biofilms not only help to accelerate weathering, but at the same time they could reduce denudation by isolating the root-hypha-mineral interface in the rhizosphere of vascular plants from the bulk soil solutions. Further studies are needed to fully elucidate the role of such biofilms in ecosystem processes.

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