Are enzymes transported in soil by water fluxes?

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Highlights

- Hydrolytic enzymes are transported convectively, attached to soil colloids
- This transport is soil and enzyme specific
- Soil colloids released jointly with ions which alter ionic strength in effluents
- Enzyme activity demonstrated a bell-shape relationship with the ionic strength

ABSTRACT

Transport of extracellular hydrolytic enzymes in soils has always been a subject of doubt. The considerations against its importance are that (i) enzymes benefit their producers the most when they remain in close proximity; and (ii) enzymes are large molecules with low mobility due to high affinity to fine soil particles and organic matter. However, soil mineral colloids (SMC), to which extracellular enzymes also have an affinity and which are known to facilitate transport of a broad variety of chemicals and microorganisms in soils, can serve as vehicles for enzyme transport as well. Since current literature lacks information on enzyme transport in soils, our goal was to determine whether enzymes are transported and, if so, whether they are transported in a free- or in a colloid-associated form. We conducted column transport experiments with four hydrolytic enzymes, namely, β-glucosidase, acid-phosphatase, cellobiohydrolase, and xylosidase, in soils with contrasting textures. The eluents containing enzymes were applied on top of soil columns, while enzyme activities, SMC, and electrical conductivity were measured in the effluents from the columns. Our results provided evidence of joint enzyme transport with soil colloids. The enzymes associated with the coarse SMC (1 μm < Ø) contributed 52 - 88% of the total enzyme activity in the effluents. The remaining enzyme activity was attributed to the enzymes associated with organic colloids, fine SMC (Ø < 1 μm) and free enzymes in solution. This study suggested a dual effect of ionic strength in the soil suspension on enzyme activity and their release from soils with soil colloids.

Keywords: Soil hydrolytic enzymes, colloid-facilitated transport, column experiments, ionic strength.

Abbreviations: IS, ionic strength; EC, electrical conductivity; POM, particulate organic matter; SMC, soil mineral colloids; CMC, coarse mineral colloids; FMC, fine mineral colloids; TN, total nitrogen; TC, total carbon; SL, Sandy loam; SL-S, sandy loam soil from summit; SL-D, sandy loam soil from depression.

INTRODUCTION

Most plant and microbial cell debris present within soil as polymeric molecules are quickly transformed by extracellular enzymes to oligo- and monomers, which then become readily available to microbial decomposers. Like most proteins, soil extracellular enzymes are capable to diffuse away from their parent cell in free solutions due to Brownian motion (Burns et al., 2013). Moreover, several studies in artificial solutions have reported a self-propelled diffusion of enzymes, which enhanced their movement by 30-80% during substrate catalysis (Yu at al., 2009; Muddanaet al., 2010; Riedel et al., 2015; Jee et al., 2018; Günther et al., 2018). Still, there is no consensus on the enzyme diffusivity. On one hand, diffusivity of free enzymes should increase as substrate availability decreases, thus the enzyme producer can potentially access more distant substrates (Allison et al., 2011). On the other hand, competition for products between enzyme producers suggests relatively low enzyme diffusivity (Burns et al., 2013). The amount of reaction products captured by a microbial cell per unit of enzyme produced declines with increasing distance between the cell and the produced enzymes due to diffusion losses of the product to the environment, cell competition for reaction products, and decreasing enzyme and substrate concentrations. These product losses increase exponentially with the distance between the enzymes and microbial cells, and less than 4% of the product can reach the microbial cells located at a distance > 200 μm from the enzymes (Guber et al, 2021). Therefore, low enzyme diffusion, and thus lower diffusion losses of the product might represent a beneficial strategy for microbial cells.

The enzyme's capacity to move within an unrestricting volume of liquid does not imply that enzymes can similarly freely diffuse through soil pores. Among factors restricting such diffusion are: (i) enzyme attachment to soil particles and soil colloids (Nannipieri et al., 2003); (ii) relatively low water retention and fast draining of large pores resulting in reduction of pore volume available for enzyme movement (Allison et al., 2011); (iii) hydraulic discontinuity of water pathways in partly saturated pores and layers of extracellular polymeric substances, created by microorganisms on surface of soil particles, restricting free enzyme motion (Or et al., 2007). Moreover, most free enzymes survive only briefly in pore solutions due to fast denaturation by physical and chemical factors or consumption by proteolytic microorganisms (Sarkar and Burns, 1984; Burns, 1986). However, adsorption confers protection against microbial degradation (Lähdesmäki & Piispanen, 1992; Kedi et al., 2013). Therefore, most viable enzymes and microorganisms in soil are bound to organic surfaces (Ahmed and Oades, 1984; Christensen and Bech-Andersen, 1989; Jocteur Monrozier et al., 1991) and fine soil particles (Singh and Singh, 1995; Schulten et al., 1993; Stemmer et al., 1998; Kandeler et al., 1999a). Association of microbial cells and enzymes with soil particles and organic surfaces precludes their free diffusive transport due to relatively large size of these particles and low pore volume available for the diffusion in partly water-saturated soil. However, rapid water infiltration after heavy rainfalls or overland irrigation causes both physical and chemical perturbations in the soil and results in a release of colloids from soil matrix and their transport via large pores (Ryan and Elimelech, 1996). These colloids are composed of organic and mineral particles (including clay and partly silt soil fractions) with effective diameters < 10 μm (McCarthy and Zachara, 1989; Sposito, 2016). The colloids within size range 0.01-10 μm are regarded as the most stable (Buffle and Leppard, 1995) and are common carriers of soil microorganisms, organic substances (Smith et al., 1985; McCarthy & Zachara, 1989; Natsch et al., 1996), and environmental contaminants (Ryan & Elimelech, 1996; de Jonge et al., 2004). Strong adsorption of enzymes on fine soil particles and their low extractability in free form

(Fornasier et al., 2011), suggests possibility of their transport by moving colloids. However, despite extensive study of enzyme interaction with clay minerals and soil colloids during last 4 decades, surprisingly, enzyme transport in soils has never been reported. Therefore, the goal of this study was to explore the possibility of enzyme transport in intact soils with contrasting textures under water flow conditions that mimic those during heavy rainfalls.

The activity of four hydrolytic enzymes involved in C and P acquisition were studied in the transport experiment: β-glucosidase, acid-phosphatase, cellobiohydrolase, and xylosidase. The former two participate in the last step of decomposition, i.e., release of monomers (glucose and phosphate) that are easily available for microorganisms. The latter two are involved in the early stage of decomposition destroying long polymeric chains of cellulose and hemicelluloses.

2. MATERIALS AND METHODS

2.1. Soil properties

Soil for the column experiments was collected at three experimental sites with contrasting soil texture located in Michigan, USA. Sandy soil of Riddles-Hillsdale series (fine-loamy, mixed, active, mesic Typic Hapludalfs) was obtained from Michigan State University's (MSU) Sandhill farm site, East Lansing, MI. We further refer to this soil as Sand. Two sandy loam soils of Capac series (fine-loamy, mixed active, mesic Aquic Glossudalf) were obtained from summit and depression topographical positions at MSU's Mason experimental farm, East Lansing, MI. We refer to them as sandy loam soils SL-S and SL-D, for summit and depression respectively. Loamy soil of Kalamazoo series (fine-loamy, mixed, active, mesic Typic Hapludalfs) was from Cellulosic Biofuel Diversity Experiment at Kellogg Biological Station Long-Term Ecological Research site, Hickory Corners, MI, referred to further as Loam soil. At each site, undisturbed soil cores (Ø=2.5 cm, height=10 cm) were taken from 5-15 cm depth and stored at 4°C for 2-3 days prior to experiments. In addition, disturbed soil samples in amounts of approximately 300 g were taken from the immediate vicinity of the intact cores for basic soil analyses. At the time of sampling, Sand soil was under corn, SL-S and SL-D soils were under corn-soybean rotation, and Loam soil was under long-term (>10 years) native prairie vegetation. We collected 3 cores from each site and one additional core from Loam soil site, for a total of 13 cores. The number of soil cores was selected arbitrarily solely for exploring purposes.

The following soil analyses were conducted using disturbed soil samples. Soil texture and soil mineral colloids (SMC), operationally defined as mineral particles Ø < 10 µm, were measured using the pipette method (Gee & Or, 2002). Total nitrogen (TN) and total carbon (TC) were measured using an elemental analyzer ECS 4010 CHNSO (Costech Analytical Technologies Inc., Valencia, CA, USA). Soil particulate organic matter (POM) was measured by wet sieving (Cambardella and Elliot, 1992). Soil pH and electrical conductivity (EC) were measured using SevenCompact Duo s213 meter (Mettler-Toledo LLC, Columbus, OH USA) in Soil-DI water suspensions at 1:1 solid/liquid ratio. General characteristics of the studied soils are given in Table 1.

2.2 Applied suspensions

The compositions of applied suspensions, referred to as eluents, for the enzyme transport experiment were designed to minimize artificial effects of solution chemistry on the transport and transformation processes within the soil cores. This composition mimicked soil suspensions generated in the field during heavy rainfalls by kinetic energy of rain drops or runoff water. Therefore, the eluents were prepared individually for each soil by adding 1 g of fresh soil to 100 ml of DI water followed by 5 min low-energy sonication using Fisher Scientific FS20 Ultrasonic Cleaner (Thermo Fisher Scientific Inc., Waltham, MA, USA). The sonication settings were chosen to break up soil aggregates, while preserving SMC (Stemmer et al., 1998), soil organic colloids, microorganisms and enzymes (Kandeler et al., 1999b). Soil suspensions were kept for 30 min to allow settling of sand particles, and the supernatant solutions separated from the sediment were used as eluents. Thus, the prepared eluents contained dissolved chemicals, soil particles, microorganisms, and enzymes native to the respective soils. The activities of *β*-glucosidase, phosphatase, xylosidase and cellobiohydrolase were measured in the eluents before and after precipitating the coarse mineral colloids (CMC) as described in Sections 2.3 and 2.4. Soil mineral colloids were measured in the eluents as described in Section 2.2.

2.3 Column experiment

Soil column experiments were conducted to quantify possible activity of the four hydrolytic enzymes in the suspensions passing through the intact soil cores, which we will refer to as effluents. We used undisturbed soil cores to preserve distribution of enzymes and water flow pathways unchanged in the soils. For Sand soil the entire soil cores were used as experimental columns, that is, the column height was 10 cm. For finer textured SL-S, SL-D, and Loam soils, due to their low water infiltration rates, the intact cores were cut to 5 cm experimental columns. Longer Sand columns as compared with those for SL-S, SL-D, and Loam soils reduced the water flow velocity in the Sand columns and prevented mechanical detachment of colloids from soil. The eluents were applied by a pipette to the top of the columns in 1 ml increments to prevent water ponding on the soil surface (Fig. 1). A pressure head of -30 kPa, which is an equivalent to the field capacity, was maintained at the bottom of the columns during the experiment to keep steady-state flow through the columns. Coarse porous filters with particle retention Ø > 40 μm (Filter Paper Grade 417, VWR®, Radnor, PA, USA) were installed at the bottom of the columns. The filters, permeable for SMC, microorganisms, and enzymes, prevented detachment of soil particles from the columns. The effluents were collected in 4 ml increments from the bottom of Loam, SL-S and SL-D columns, and in 8 ml increments from Sand columns. Collected effluents were analyzed for activity of the four enzymes, SMC contents, pH, and EC. The enzyme activities were measured in the effluents with all colloids and in the supernatant solutions which contained only the colloidal particles smaller than 1 μm (Fig. 1). To precipitate the coarse mineral colloids (CMC) size of large 1 μm, the effluents were centrifuged for 5 min at 5000 rpm using a Heraeus Megafuge 16 centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA). Diameter of precipitated CMC was calculated (Gee and Or, 2002) as:

$$d = \frac{1}{60}\sqrt{\frac{18\eta ln\,(\frac{r_2}{r_1})}{(\rho_s - \rho_l)\omega^2 t}} \qquad (1)$$

where r_1 to r_2 are distances from the axis of centrifuge rotor to the particle [cm], η is the fluid viscosity [g cm^{-1} sec^{-1}], $\rho_s \sim 2.6$ g cm^{-3} is the density of soil mineral particles, ρ_l is the liquid density [g cm^{-3}], ω is the centrifuge angular velocity [rpm], t is time for particle of diameter d to settle from r_1 to r_2 [sec]. It was confirmed in a preliminary study that selected centrifugation settings had a negligible effect on the activity of colloid-free almond β-glucosidase and wheat germ acid phosphatase (CALZYME Laboratories, Inc., San Luis Obispo, CA U.S.A.) dissolved in DI water. Precipitating mineral colloids smaller than 1 μm (e.g., 0.45 mm) and organic colloids ($\rho_s < 2.6$ g cm^{-3}) requires increasing of angular velocity of centrifugation proportionally to ω or increasing the duration of centrifugation as t^2. Such changes in the centrifugation settings results in partial precipitation of bio colloids and free enzymes in the effluent suspension in amounts that are generally unknown. Therefore, most organic colloids, bio colloids enzymes and mineral colloids size of smaller 1 μm remained in supernatants, while CMC were precipitated by centrifugation.

For comparison purposes the results of the column experiments were expressed in a normalized form. Specifically, volumes of eluent that passed through the columns were expressed in total pore volumes, where the total pore volume in each soil column was calculated from its bulk density, particle density [~2.6 g cm^{-3}], and column's volume. The SMC contents, enzyme activities and EC values in the effluents from the columns were normalized by the corresponding values in the eluents. To evaluate the effect of ionic strength in the effluents on the enzyme activity, the measured EC values were transformed using the Marion-Babcock equation (Sposito, 2016):

$$log_{10}(I) = -1.841 + 1.009\ log_{10}\ (\mathrm{EC}) \qquad \text{for } I \leq 0.2\ \mathrm{M} \qquad (2)$$

where I is the ionic strength in the solution [M], and EC is the electrical conductivity in the solution [dS m^{-1}].

2.4. Enzyme assay

The activity of *β*-glucosidase, acid-phosphatase, xylosidase and cellobiohydrolase were measured in the soil, eluents, and effluents collected in the column experiments before and after precipitating CMC as described in Section 2.3 (Fig. 1). For the activity measurements we used a microplate fluorometric assay technique described in Saiya-Cork et al. (2002) and Deng et al. (2011) with minor modifications of the substrate concentrations. Specifically, we used substrates based on 4-methylumbelliferone (MUF) fluorescent reagent (i.e. MUF-β-D-glucopyranoside, MUF-Phosphate, MUF-b-D-xylopyranoside, and MUF-β-D-cellobioside) in quantities of 40 nmol well^{-1} per 50 μl well^{-1} soil suspensions with 5.0 μmol well^{-1} of sodium MES buffer ($C_6H_{13}NO_4SNa_{0.5}$, pH 6.1 at 25°C) solution. The calibration was performed using 0, 100, 200 and 400 pmol well^{-1} MUF solutions with the same aliquots of soil suspensions. The fluorescence intensity in the plates was measured using a Multilabel Plate Reader Victor^3 (PerkinElmer Inc., Waltham, MA, USA) every 15 min for 2 hours. The enzyme activities were calculated from linear parts of the intensity time series with correction for the substrate autohydrolysis (Deng et al., 2011).

The enzyme activity associated with CMC in the effluents v_c^{ef} was calculated as:

$$v_c^{ef} = v_t^{ef} - v_s^{ef} \quad (3)$$

were v_t^{ef} is the total enzyme activity in the effluent [pmol min^{-1} ml^{-1}], and v_s^{ef} is the enzyme activity in the supernatant from centrifuged effluent [pmol min^{-1} ml^{-1}].

Since the mass of SMC differed in the effluents from the four soils and changed over time, to evaluate the effect of ionic strength on the enzyme activity associated with SMC, the activity was normalized by the dry mass of colloids in the effluent:

$$v_c^S = v_c^{ef} / S_c \quad (4)$$

where v_c^S is the activity of enzymes associated with CMC per mass of dry colloids [nmol min^{-1} g dry colloids^{-1}], and S_c is the dry mass of CMC in the effluent [g L^{-1}]. Note, that the dry mass of colloids in supernatants from the effluents was within the accuracy range of analytical balance (0.1 mg). Therefore, the dry mass of SMC measured in the effluents was attributed to CMC.

2.5. Data processing and statistical analysis.

Differences among the soils in terms of the studied properties were conducted via analysis of variance, with soils as the only studied factor, using *lm* function of R (version 4.1.2). To compare the enzyme activities within the soils we fitted the data with a statistical model that consisted of (i) the fixed effects of the soils and the enzymes and their interaction, and (ii) a random effect of the replicated samples, nested within the soils, also used as an error term for testing the soils' effect. Model fitting was conducted using *lmer* function from R's *lme4* package. When the effects of the studied factors were found to be statistically significant ($p<0.05$) we performed t-tests to conduct all pairwise comparisons among the means using *emmeans* package.

We used correlation analysis to explore possible relationships between enzyme activity associated with CMC and colloid contents in the effluents from the soil columns using Analysis tool of SigmaPlot software (Systat Software Inc., San Jose, California, USA). Pearson correlation coefficients were reported as statistically significant and $p < 0.05$ and marginally significant at $p < 0.1$.

3. RESULTS

3.1. Properties of the soils and applied suspensions.

The soils of the four locations differed in their properties. The bulk density was the lowest in the sandy soil (Sand) due to its loose structure. The highest sand content was expectedly observed in Sand, lowest in Loam, and intermediate in the sandy loam SL-S and SL-D soils (Table 1). An inverse trend was observed for SMCs, which contents were the highest in Loam, lowest in Sand, and intermediate, though close to Loam, in SL-S and SL-D soils (Table 1). The POM content was rather low in all soils (0.11 % – 0.31%) but tended to be the highest in Sand. TN and TC increased in the soils in the order of decreasing sand content. The pH values ranged from 5.4 to 6.0. The EC values were 2 to 3 times higher in SL-S and SL-D as compared to Sand and Loam, indicating overall higher contents of dissolvable ions in sandy loam soils.

The SMC content in the eluents increased in the order Sand < SL-D < SL-S < Loam. The pH values were slightly higher than in the soil suspensions and ranged from 5.9 to 6.7, while the EC values were much lower (6-9 μS cm^{-1}) in the eluents as compared to those in the soils, as an expected result of a 100-fold dilution of the soil suspensions (Table 1).

The differences in the soil properties were mirrored by enzyme activities, which for β-glucosidase and phosphatase were the highest in Loam and SL-S, and the lowest in Sand (Table 1). For xylosidase and cellobiohydrolase the highest activities were observed in SL-S, and the lowest in Sand. The enzyme activity in the eluents followed the same general trend as that in the soils, with much smaller differences between β-glucosidase and phosphatase activities as compared with those in the soils. Among the four enzymes, the activities in most soil samples and eluents increased in the order xylosidase < cellobiohydrolase < phosphatase < β-glucosidase and were up to one order of magnitude higher for phosphatase and β-glucosidase than for xylosidase and cellobiohydrolase (Table 2). Precipitation of CMC by centrifugation considerably reduced enzyme activity in the eluents. The enzyme activity associated with CMC calculated using Eq.(3) ranged from 33% to 100% in the eluents in the four soils. The lowest activities of CMC-associated enzymes were observed in Sand (Table 2), while the highest activities were observed in Loam, where the enzyme activities in the supernatants after eluent centrifugation were below the detection limit.

3.2.Column experiment

The relative concentrations of SMC in the effluents were the highest in the first portions of effluent collected from the columns and decreased with the relative volumes of the solution passed through the columns (i.e. pore volumes) (Fig. 2 and Fig. S1). The recovered SMC differed in the four soils and ranged from 7% of that applied in Loam to 49% in Sand, with intermediate values for SL-S and SL-D (Table 2). The relative concentrations of SMC were the highest for Sand and the lowest for Loam columns (Fig. 2ab). Noticeably, in the Sand columns the relative concentrations of SMC from the first portions of the effluent were >1 (Fig. 2a), indicating that the effluents contained more SMC than the applied eluents. For Loam (Fig. 2b), SL-S (Fig. S1a), and SL-D (Fig. S1b) columns, the SMC contents in the effluents were always smaller than in the eluents.

The EC dynamics in the effluent resembled that of SMC, though unlike SMC, the highest relative EC were observed in SL-S and SL-D columns (Fig. S1). The relative EC values in the effluents were >> 1 in all samples of all studied soils, indicating much higher concentrations of soluble chemicals in the effluents as compared to those in the applied eluents.

As was the case with SMC and EC, the relative activity of four enzymes in the effluents decreased with the amount of eluent passing the columns (Fig. 3). Among the four enzymes, the absolute values of activities in the effluents were the lowest for xylosidase and cellobiohydrolase and the highest for phosphatase (Table 3). Enzymatic activity in the first portions of the effluents 1.5 to 8 times exceeded those in the eluents (Fig. 3 and Fig. S2), with exception of β-glucosidase in SL-S and SL-D columns (Fig. S2a) and cellobiohydrolase in the Loam column (Fig. 3b). Among the four enzymes, the relative activity in the first portions of effluents was the highest for phosphatase (Fig. 3b and Fig. S2b), the smallest for β-glucosidase (Fig. 3a and Fig S2a), and intermediate for xylosidase (Fig. 3c and Fig. S2c) except the Loam soil, where xylosidase and cellobiohydrolase were not detected in the effluents. Among the four soils, the relative activities in the effluent were the highest for Sand, the lowest for Loam (Fig. 3), and intermediate for SL-S and SL-D columns (Fig. S2), which was consistent with the dynamics of SMC outflow from the columns (Fig. 3 and Fig. S1). For example, β-glucosidase activities in the effluent corresponding to 1 pore volume in Sand was >2 times higher than that in Loam, while for phosphatase it was >4 times higher than in Loam (Table 3).

For β-glucosidase and phosphatase the activity associated with CMC constituted a substantial portion of the total enzyme activity (Fig. 4, Table 3). For example, for β-glucosidase in the effluent corresponding to the first pore volume, the activity associated with CMC constituted 66% and 52% of the total activity for Loam and Sand, respectively (Table 3). For phosphatase, it was even higher and constituted 79% and 88% for Loam and Sand, respectively. In most tested enzymes, their activity associated with CMC was positively correlated with concentration of

SMC in the effluents (Fig. 5 and Fig. S3). The correlation coefficients between v_c^{ef} and concentration of SMC in the effluents were high at $p < 0.1$ and $p < 0.05$ for all enzymes except xylosidase and cellobiohydrolase in the Loam column where the activities were below the detection level (Fig. 5), and except phosphatase, xylosidase and cellobiohydrolase in SL-D columns (Fig. S3).

The relationships between the activity of enzymes associated with SMC v_c^{ef} and ionic strength in the effluents, as derived from EC using Eq.(2), were rather scattered. Still, a sharp increase of *β*-glucosidase and phosphatase activity associated versus not associated with CMC can be seen in the Sand and Loam columns with increasing ionic strength from 0.1 to 2.3 mM (Fig. 6). The slope of linear regression between the enzyme activity and IS ranged from 4.5 to 43 pmol min^{-1} g^{-1} mM^{-1} and was steeper for Sand as compared with that in the Loam columns. For the SL-S and SL-D columns the activity of all enzymes was somewhat decreasing within the ionic strength range 1.4 - 21.4 mM. This trend was less expressed but still visible for xylosidase (Fig. S4 a,c) and cellobiohydrolase (Fig. S4 b,d) in the SL-S and SL-D columns.

The pH values in the effluents moderately increased with pore volumes during the experiment from 6.0 to 6.6, 6.3 to 7.1 and 5.2 to 6.3 in the Sand, Loam, and both SL-S and SL-D columns, respectively. However, v_c^{ef} did not corelate with pH in the effluents.

4. DISCUSSION

The results of column experiments demonstrated possibility for soil hydrolytic enzymes to be transported by water fluxes during heavy rainfall events in the soils with different soil texture.

4.1. *Associations between enzymes and colloids in the eluents*

Precipitation of CMC in the eluents reduced considerably activity of the four enzymes suggesting their association with colloids (Table 2). CMC are comprised of clay and silt particles, which hydrolytic enzymes are mostly associated with (Feller at al., 1994; Turner et al., 2002; Sinsabaugh et al., 2008; Kandeler et al.,1999a; Kedi et al., 2013). Association of *β*-glucosidase with soil mineral colloids in paddy soil has been reported by Yan et al. (2010 a,b). These authors found approximately 50% higher *β*-glucosidase association with fine (< 0.2 μm) than with the coarse (0.2 – 2.0 μm) SMC. On the contrary, 86% to 100% of *β*-glucosidase activity was associated with CMC in our study. The discrepancy likely results from Yan et al

(2010 a,b) using highly diluted (1:100 solid to liquid ratio) soil suspensions, where all added enzymes freely interacted with all resuspended soil particles. Yet, in intact soils, as used in our study, enzyme-colloid interaction occurs primarily in hydrologically active pores occasionally and not completely saturated by liquids.

It should be noted that enzyme-CMC associations in eluents were enzyme and soil specific. While activity of all enzymes was completely associated with CMS in Loam soil, in the coarser textured soils the percent of CMC associated activity was lower. Of the four studied enzymes, *β*-glucosidase seemed to be the most associated with CMC, while activity of xylosidase and cellobiohydrolase tended to be the least associated, especially in Sand (Table 2). While it is not clear what is the cause for the differences among the enzymes, the results suggest high possibility of joint transport of enzymes with CMC, especially in fine-textured soils, though transport of not associated enzymes cannot be excluded.

4.2. *Colloid and enzyme transport through the soil columns*

The transport experiments demonstrated high mobility and recovery of SMC in sandy and sandy loam soils and low mobility in the Loam (Fig. 2), likely caused by straining of colloids within small pores of the finer textured soil. This result is consistent with other studies (Bradford et al., 2002, 2003), where straining of colloids was shown to increase with increasing colloid size and with decreasing soil grain size (Bradford et al., 2003).

Soil colloids appeared to be involved in enzyme transport, the result that was consistently observed in all cases when the enzymes were present in the soil in appreciable amounts (Table 3). High significant correlation between the activity of these enzymes associated with CMC (v_c^{ef}) and SMC contents in the effluent (Fig. 5, Fig. S3) imply that v_c^{ef} was proportional to the mass of transported colloids for each portion of the solution passed the columns. Closeness of the intercepts of the linear regressions to zero for all enzymes in Sand and Loam columns (Fig. 5), and considerable (from 2 to10-fold) decreases in the enzyme activity after precipitation of CMC in the effluents (Table 3 and Fig. 5) suggested that large fraction of the enzymes was transported convectively attached to SMC.

High correlation coefficients between enzyme activity and contents of SMC in the effluents do not imply that all detected enzyme activity was associated solely with SMC. Non-zero activities of all enzymes were detected in the supernatants after centrifugation of effluents from SL-D columns (Fig. S3). The centrifugation removed mostly CMC (Ø > 1 μm) from the effluents. Fine mineral colloids, organic colloids, microbial cells and macromolecules, with density much smaller than that of soil mineral particles ($\rho_s < 2.6$ g cm^{-3}), were only partly precipitated by centrifugation, but could also act as colloidal carriers (Buffle and Leppard, 1995). The number of fine colloid particles < 0.2 μm (not necessarily mass) can constitute up to 70% of the total number of mobile colloids during a heavy rainfall (Lehmann et al., 2021). Moreover, the activity of β-glucosidase associated with fine (<0.2 μm) SMC can be higher than that associated with coarse (0.2 – 2.0 μm) SMC (Yan et al., 2010 a,b). Therefore, enzymes remaining in the effluents after centrifugation were more likely associated with not-precipitated FMC, viable microbial cells (Nannipieri et al., 2012), and/or organic colloids rather than being free floating enzymes, whose lifetime in soil solutions is rather short (Burns, 1982; Ladd, 1985; Nannipieri, 1994, Nannipieri et al., 2002). Possible enzyme association with soil colloids suggest that in real-world systems enzyme transport depends on the source and composition of moving colloids. Specifically for agricultural environments, colloid-facilitated transport of enzymes can be expected after irrigation by reclaimed wastewater or heavy rainfalls followed manure application.

Colloidal transport is known to affect soil hydraulic properties, e.g., conductivity of soil pores and their sizes (Miller and Baharuddin, 1986; McDowell-Boyer et al., 1986). Precipitation and straining of colloids in soil pores during colloidal transport results in progressive straining of colloids and in the associated decrease of pore volume available for transport. Therefore, it is reasonable to suggest that, with time, the ratio between large and small colloids in the effluents shifts toward small colloids. Since the proportion of organics and the surface area of colloids increases with decreasing sizes of colloidal material (Buffle and Leppard, 1995), higher association of enzymes with fine colloids is expected. This fact can be illustrated by β-glucosidase (Fig. 4a) and xylosidase (Fig. 4c) activities in SL-S columns, where the ratio between activities of enzymes associated (filled bars) and not associated (open bars) with CMC decreased with the number of pore volumes.

The relationship between SMC content in effluents and activity of CMC-associated enzymes was enzyme specific (Fig. 5 and Fig. S3) possibly due to mineralogical composition of transported colloids and differences in enzyme-mineral interactions. The mineral composition of Ap horizon in fine, mixed, mesic, Typic Hapludalfs, used in our study, varies greatly among soil fractions. According to Sparks et al. (1979), quartz and mica dominate (>50%) in > 5 μm and < 2 μm mineral fractions of Typic Hapludalfs. The 2-5 μm soil fraction is composed of quartz, mica and kaolinite in approximately equal amounts. Kaolinite contents vary from 7 to 28% in the soil fractions with approximately two-fold greater contents in < 5 μm than in > 5 μm soil fractions. Feldspar is present only in silt fractions, while vermiculite only in clay fractions, in the amounts of less than 10%. Different enzyme affinity to the substrates in presence of soil minerals has been reported in multiple studies (Ross, 1983; Makboul and Ottow, 1979; Haska, 1975; Pflug (1982); Gianfreda and Bollag, 1994). Therefore, it is reasonable to suggest that SMC-enzyme associations and enzyme activities in the effluents were affected by changing sizes and mineralogy of transporting colloids. For example, the share of quartz particles in the total SMC mass passing the columns likely decreased with time, while the share of mica and kaolinite particles increased. Due to differences in soil texture, it is expected that the ratios between contents of different minerals in the effluents was soil specific. Therefore, the differences in the enzyme activities observed in Fig. 5 and Fig. S3 can likely be attributed to different association of enzymes and their activity on SMC, which mineralogy changed in the effluents with respect to the textural, mineralogical, and hydraulic properties of these soils. We realize that it is infeasible to conduct particle and mineralogical analysis in the effluents, but it is worth to note that the relationship between the enzyme activity and quantities of transported colloids is more complicated than any adsorption isotherm used to model interactions between chemicals, microorganisms, and soil particles.

4.3. Whether ionic strength in solution affect enzyme transport?

Soil's ionic strength affected recovery of SMC applied with eluent in the fine-textured soils (Loam, SL-S and SL-D). The recovery was greater in the SL-S and SL-D columns (Table 2), where soil EC and, respectively, IS were much higher than those in the Loam soils (Table 1). Increasing IS causes coagulation, while decreasing IS causes disaggregation and mobilization of soil colloids (Ryan and Elimelech, 1996). Rainfall water has typically much lower IS as compared with that in soil solutions (McCarthy and Zachara, 1989). Therefore, decrease of soil IS, due to dilution of pore solution by rainwater, results in a release of colloids from the soil matrix at early stages of rainwater infiltration into the soil (Flury et al., 2002; Grolimund and Borkovec, 1999). With time the difference between IS in soil pores and the applied solution decreases slowing down soil disaggregation. However, soil, disaggregated by low IS, becomes less conductive and filters out new infiltrated colloids via straining or physical-chemical collection by attractive surfaces of the immobile soil matrix (McCarthy and Zachara, 1989) Visually, this process manifested itself in this study via a noticeable reduction of the infiltration rate in the fine textured soils after 1-1.5 pore volumes of the effluent were collected. The high recovery of colloids from the Sand columns (49.1%) was less affected by soil disaggregation due to much lower clay to sand ration as compared with the other soils (Table 1). Therefore, the effect of IS on colloidal outflow was less pronounced in the Sandy, and more pronounced in Loam and Sandy loam columns.

The IS also affects the activity of transported enzymes. In batch experiments with pure clay minerals and in solutions with controlled pH and EC the relationships between enzyme activities and IS could be well described quantitatively (e.g., Quiquampoix et al., 1993; Leprince & Quiquampoix, 1996). In real soil and transport conditions this relationship is rather scattered (Fig. 6) and likely altered by: (i) possible differences in mineralogy of soil colloids of the four soils and effluents from the columns (Jaber et al., 2018; Nannipieri et al., 1996); (ii) different affinity of enzymes to different minerals and colloids (Makboul and Ottow, 1979; Ross, 1983; Sarkar et al., 1989); (iii) and different and likely changing during the transport experiment ionic and colloidal composition of the effluents. Yet, we attempt to derive a relationship between the activity of adsorbed enzymes and IS for out column studies using dependencies between phosphatase catalytic activity and pH measured for montmorillonite at different IS levels reported by Leprince & Quiquampoix (1996, Fig. 2, central column). The original data were reorganized to depict relationships between the rate of the catalytic reaction (*V*) and IS at pH levels within 5.0 - 7.0 interval (Fig. 7, symbols). The reorganized relationships were fitted using an empirical equation:

$$V = \frac{a}{IS} exp[-b(\ln(IS) - c)^2] \qquad (5)$$

where *a*, *b*, and *c* are the fitting parameters. The regression wizard of SigmaPlot software (Systat Software Inc., San Jose, California, USA) was used to fit Eq.(5) to the data shown in Fig. 7. The similarity in shapes between the regression curves and experimental data was assessed using the two-sample Kolmogorov-Smirnov test in the Real Statistics Resource Pack software (Release 7.6, Copyright (2013 – 2021), Charles Zaiontz, www.real-statistics.com).

Despite a small number of experimental points (6 on each curve), Eq.(5) adequately ($p < 0.05$) reproduced shapes of the *V(IS)* curves (Fig. 7). Therefore, Eq.(5) was applied to reconstruct the shape of experimental curves describing relationships between activities of enzymes (i.e., β-glucosidase and phosphatase associated (Fig. 6 a,b) and not associated (Fig. 6 c,d) with CMC) and IS in effluents from the four soils. Based on the Kolmogorov-Smirnov test the fitted curves reproduced adequately ($p < 0.05$) the shapes of β-glucosidase and phosphatase activity curves in the Sand and Loam columns for enzymes not associated with CMC (Fig. 6 c,d). The shapes of fitted curves differed significantly ($p<0.05$) from those experimental for enzymes associated with the CMC. Fitting Eq.(5) to xylosidase and cellobiohydrolase activity was not successful due to scattered data on these curves (Fig. S4).

Several mechanisms can potentially explain a bell-shape relationship between enzyme activity and IS. The first one is a competition between enzymes and cations in the effluents for adsorption sites on mineral and organo-mineral colloids. Large IS values imply higher contents of anions and cations in the effluents, and stronger competition with enzymes for the adsorption sites on SMC. The increase in IS also alters repulsive electrostatic interactions and weakens enzyme association with colloids. The competition mechanism explains the decrease of enzyme activity but does not explain its increase within the IS range 0.2-1.3 mM. It also does not explain the decrease in activity of the enzymes not associated with CMC (Fig. 6 c,d) which were likely partly associated with unprecipitated colloids and partly were in a free form in the effluents.

The second mechanism, affecting the relationship between the enzyme activity and IS, is based on enzyme activity association with pH, which commonly has a bell-curve shape with the maximum activity at an optimal pH level (Leprince and Quiquampoix, 1996; Turner, 2010; Kedi et al., 201`3). Importantly, the optimal pH level of enzyme activity narrows and shifts toward the alkaline pH when enzymes are adsorbed on mineral surfaces of soil particles (McLaren and Estermann, 1957; Aliev et al., 1976; Leprince and Quiquampoix, 1996). Furthermore, the enzymes activity peaks shift towards more acid pH values with increasing IS in the suspensions (Goldstein et al., 1964). Given almost one unit difference in the pH between the surface of the colloids and the soil solution and changing chemical composition of the effluents, the pH optima for particular enzyme species, as well as the activity of the colloid associated enzymes, can vary strongly and deviate from that in chemically clean laboratory solution. Therefore, less adequate reproduction of our experimental curves (Fig. 6) by Eq.(5) than those derived from Leprince and Quiquampoix (1996) (Fig. 7) can be attributed to variations in mineral composition of soil colloids and chemical composition of effluents.

4.4. Implications of colloid-facilitated transport.

The colloid-facilitated transport of microbial cells and enzymes is likely part of microbial survival strategy. While microorganisms in soil are primarily attached to the solid surfaces and form associations there (e.g., colonies, films, or flocks), their activity is mainly associated with soil solution. Most organic materials entering the soil (plant residue, dead roots, manure) are insoluble or only partly soluble in water, and barely mobile. Therefore, soil microorganisms must either populate most of available pore space or be highly mobile to access new organic inputs. However, microbial cells and free enzymes are unlikely capable to travel far in soil pores in searching for new energy sources due to: (i) their relatively large size and slow diffusion rates (i.e., for 1-10 $\mu m\ s^{-1}$ for enzymes and 7–8 $\mu m\ s^{-1}$ microbial cells according to Young et al., 1980; Dechesne et al., 2010; and Zhang and Hess, 2019); (ii) small pore volumes available for diffusion because of pore discontinuity and low thickness of water menisci in partly saturated soils; (iii) overall short presence of free enzymes in soil solutions due to denaturation and proteolysis, or interaction with clay and organic surfaces (Burns, 1982; Ladd, 1985; Sarkar et al., 1989; Nannipieri, 1994, 2002). Moreover, most enzymes are irreversibly attached to soil solids, as evident through low extractability of enzymes from bulk soil (Vepsäläinen, 2001; Štursová and Baldrian 2011). Therefore, only a small fraction of soil enzymes can potentially freely diffuse in soil pores and reach newly added organic materials.

Mobilization of colloids and their convective transport through soil macropores during fast water flow events, e.g., irrigation or rainfall, as well as in partly saturated soils, are important for soil microorganisms in exploring new sources of energy. The benefits of colloid-facilitated transport for soil microbes are obvious: (i) much shorter times and longer travel distances as compared with the restricted diffusion; (ii) better protection from protozoa on colloid surfaces (Sarkar et al., 1980; Nannipieri et al., 1982); (iii) energy savings for production of new enzymes, since enzymes can be transported by the colloids; (iv) influx of new partly degraded organic materials from soil surface and their joint transport with microbial cells. Such transport explains appearance of new hotspots of microbial activity commonly observed in soil after heavy rainfalls much better than the diffusion theory does. Soil hotspots and hot moments are defined based on time and rates of microbial activity exceeding the average rates in bulk soil (Kuzyakov and Blagodatskaya, 2015). Input of labile substrates to the hotspots triggers microbial activity and thus drives the hot moments. We suggest that, in addition to the substrate-triggered hot spot activation, the colloid-facilitated transport enables enzymes and microbial cells to move quickly and in relatively large quantities to or with the labile substrates, thus forming new transport-triggered hotspots during high precipitation and preferential flow events.

The results of this study and communication with anonymous reviewers have risen research questions that require further in-depth exploration. Among them there are: (i) how to separate the release of colloids and enzymes from soil matrix and their transport through pores; (ii) how sizes and mineralogy of colloids present in soil affect enzymes association with them; (iii) how sizes and mineralogy of colloids are changing in the effluents, and to what extend these changes affect enzyme association and transport with colloids; (iv) what drives the differences between enzymes in their associations with mineral and biological soil colloids and subsequent transport; (v) are enzymes transported with their producers; (vi) how soil structure affects enzyme locations within the soil matrix and their transport with colloids; (vii) which factors are the dominant drivers of enzyme transport in soils?

CONCLUSIONS

This study revealed a possibility for hydrolytic enzymes (i.e. β-glucosidase, acid-phosphatase, xylosidase and cellobiohydrolase) to be transported through soil pores by water fluxes. Strong association of hydrolytic enzymes with fine soil particles and mobility of soil colloids results in their joint convective transport. This transport is affected by ionic strength in pore solution via dissociation and release of soil colloids from soil and alteration of enzyme activity in the transported suspensions. The former effect can be attributed to the shift of optimal pH of enzyme activity near the surface of soil colloids. It remains to be seen how soil texture and structure, colloid size and composition, enzyme properties and location in soil pores contribute to their release and transport in the field conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Selected properties of the soils collected from the four studied experimental sites.

Soil texture	Sand	Loam	Sandy loam (SL-S)	Sandy loam (SL-D)
Sand %	87±2[*] a [**]	38±2 b	58±8 c	56±2 c
Silt %	8±3 a	48±1 c	29±7 b	31±3 b
Clay %	5±2 a	14±1 b	13±1 b	13±1 b
SMC[£] %	7±2 a	29±2 c	24±1 b	26±1 b
POM %	0.31±0.07 a	0.15±0.09 ab	0.15±0.10 ab	0.11±0.01 b
BD, g cm^{-3}	1.41±0.09 a	1.62±0.08 b	1.73±0.05 bc	1.80±0.05 c
pH	5.7±0.2	6.0±0.2	5.4±0.3	5.4±0.2
EC, μS cm^{-1}	75±19 a	86±24 a	148±28 b	247±37 c
TN %	0.03±0.01 a	0.14±0.03 c	0.11±0.01 bc	0.10±0.01 b
TC %	0.49±0.11 a	1.53±0.34 c	1.13±0.01 cb	0.98±0.01 ab
Enzyme activity in soil, pmol min^{-1} g^{-1}				
β-glucosidase	2.23±0.42 aA	6.53±1.01 cA	6.90±1.05 cA	4.94±0.18 bA
Phosphatase	1.09±0.26 aB	3.41±0.31 cB	2.77±0.16 bB	2.46±0.06 bB
Xylosidase	0.20±0.04 aC	0.30±0.08 aC	0.82±0.12 bC	0.66±0.12 bC
Cellobiohydrolase	0.23±0.03 aC	0.46±0.29 aC	0.89±0.04 bC	0.55±0.13 aC

[*] Data presented as mean ± one standard deviation.

[**] Means within the same row followed by the same low case letter are not significantly different from each other ($p<0.05$); means of enzyme activity within the same column followed by the same upper-case letter are not significantly different form each other ($p<0.05$).

[£] Particle size < 50 μm

SMC denotes the soil mineral colloids, POM denotes the particulate organic matter, BD denotes the soil bulk density, EC denotes the electrical conductivity, TN denoted the total nitrogen, and TC denotes total carbon.

Table 2. Selected properties of the applied suspensions generated from the soils from the four studied experimental sites

Soil texture	Sand	Loam	Sandy loam (SL-S)	Sandy loam (SL-D)
SMC, g l^{-1}	0.68±0.16* a **	2.89±0.15 c	2.42±0.09 b	2.28±0.11 b
pH	5.9	6.7	6.2	6.4
EC, µS cm^{-1}	6	7	8	9
Enzyme activity in the applied suspensions, pmol min^{-1} ml^{-1}				
β-glucosidase	6.03±1.13 aA	9.66±1.22 bA	14.95±2.28 cA	14.06±0.52 cA
Phosphatase	3.13±0.75 aB	10.63±1.23 bA	10.26±0.60 bB	12.61±0.30 cB
Xylosidase	1.09±0.24 bC	0.28±0.08 aB	1.81±0.26 cD	1.60±0.30 cC
Cellobiohydrolase	0.69±0.09 aC	0.48±0.30 aB	3.43±0.16 cC	1.77±0.41 bC
Average enzyme activity associated with coarse mineral colloids (CMC) in the applied suspensions, %				
β-glucosidase	85.7 aA	100.0 aA	94.1 aA	97.3 aA
Phosphatase	97.6 bA	100.0 bA	57.4 aB	99.5 bA
Xylosidase	33.1 aB	100.0 bA	65.4 abB	57.3 abB
Cellobiohydrolase	32.9 bB	100.0 aA	99.5 aA	78.0 aAB
SMC recovered in the column experiment				
SMC recovered, % of applied	49.1±11.8 c	7.3±2.5 a	31.3±3.3 b	40.5±15.0 bc

* Data presented as mean ± one standard deviation.

** Means within the same row followed by the same letter are not significantly different from each other ($p<0.05$); means of enzyme activity within the same column followed by the same upper-case letter are not significantly different form each other ($p<0.05$).

Table 3. Estimated enzyme activities in the effluent from the column experiments in Sand and Loam soils obtained for the eluent amount that replaced 1 pore volume in the columns. Shown are averages from all columns for the total enzyme activity in the effluent and the proportion of the total activity from the enzymes associated with CMC.

Enzymes	Soil	Total enzyme activity in the effluent, pmol min^{-1} ml^{-1}	Activity of the enzymes associated with CMC, % of total
β-glucosidase	Loam	1.4	66
	Sand	3.0	52
Phosphatase	Loam	1.8	79
	Sand	7.6	88
Xylosidase	Loam	0.0	-
	Sand	1.0	50
Cellobiohydrolase	Loam	0.0	-
	Sand	0.9	30

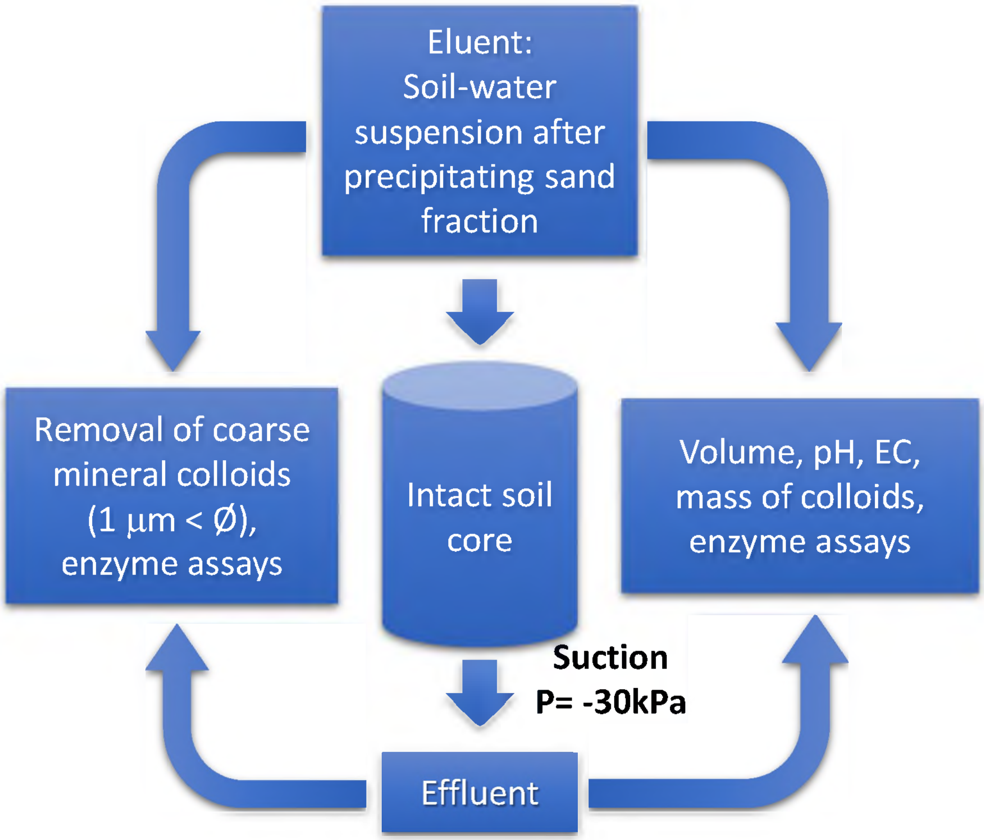

Figure 1. Experimental design and measurements conducted in the eluents and effluents from the columns.

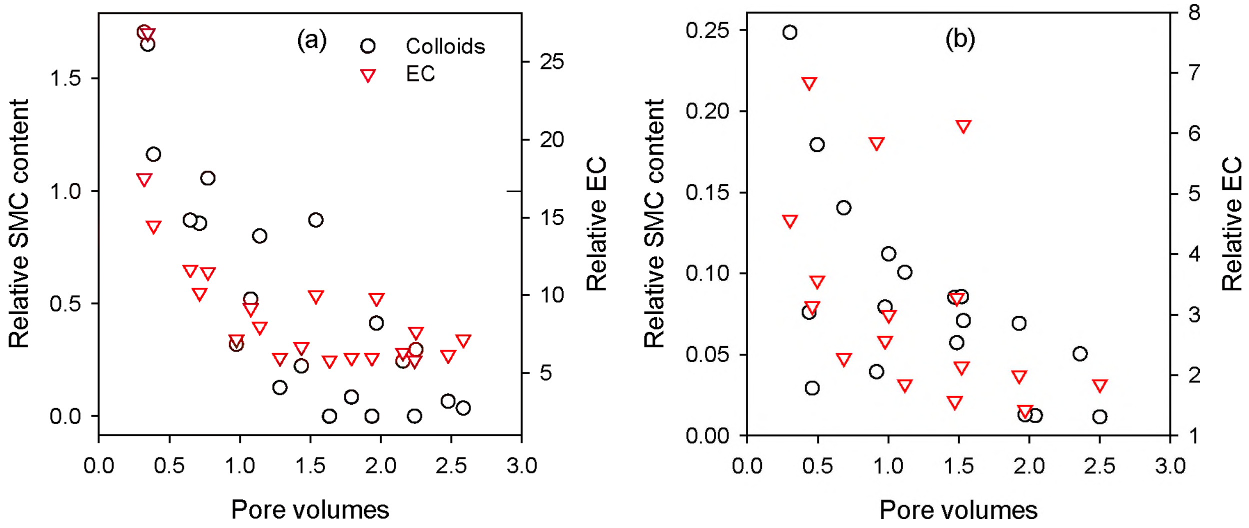

Figure 2. Concentrations of soil mineral colloids (SMC) and electrical conductivity (EC) in the effluents relative to those in the applied solutions for Sand (a) and Loam (b) soil columns for eluent volumes that passed the columns relative to the pore volumes. Data are combined from 3 columns from each soil. Horizontal line (a) marks the relative SMC in the effluent corresponding to that in the eluent. Different scales are used in (a) and (b) for better data visibility.

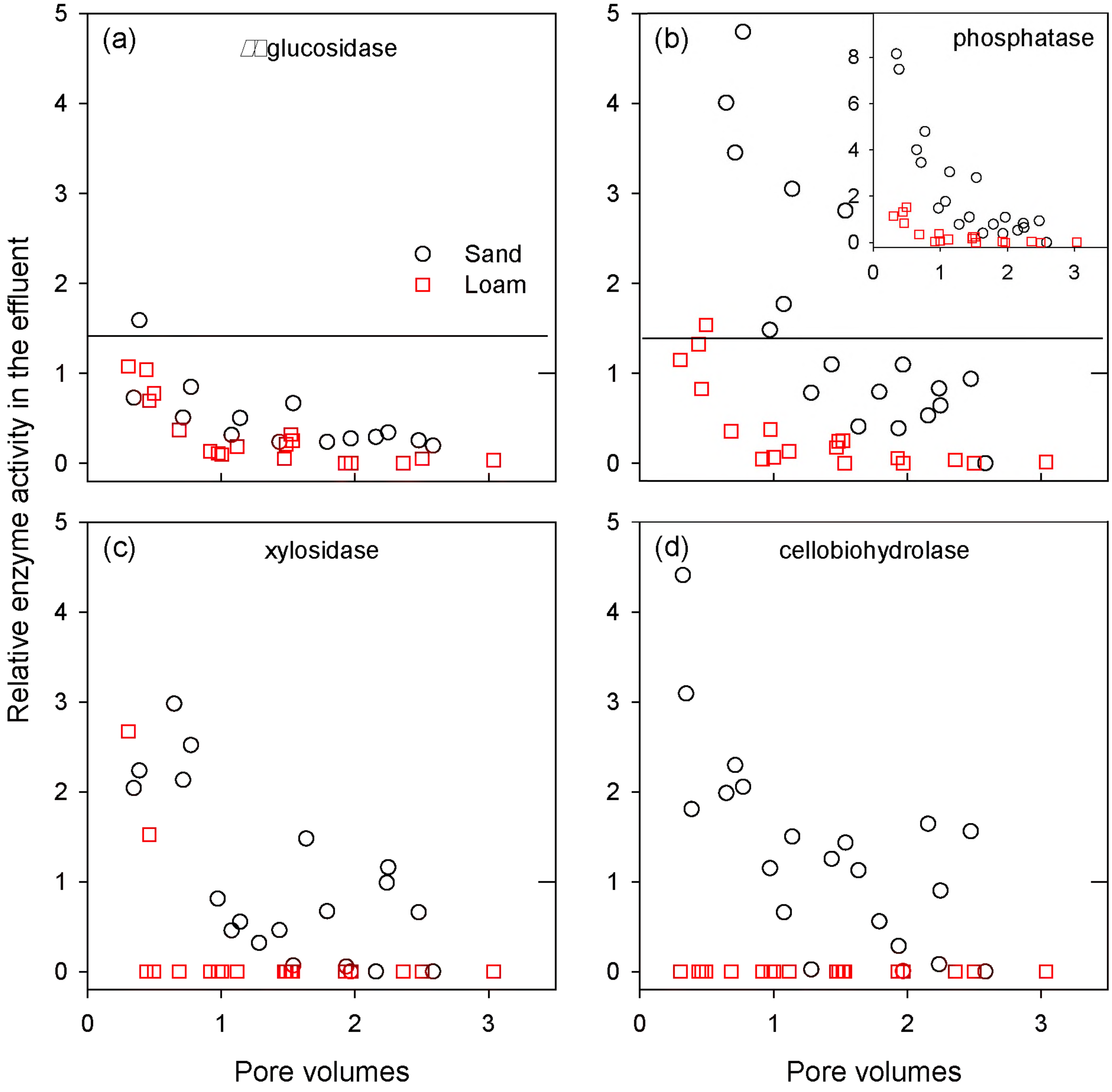

Figure 3. Activity of the four enzymes in the effluents from Sand and Loam soil columns relative to those in the applied solutions for eluent volumes that passed the columns relative to the soil pore volumes. Zero values indicate enzyme activities below the detection limit. Data are combined from 3 columns of each soil. Horizontal lines mark the relative activity in the effluent corresponding to that in the eluent.

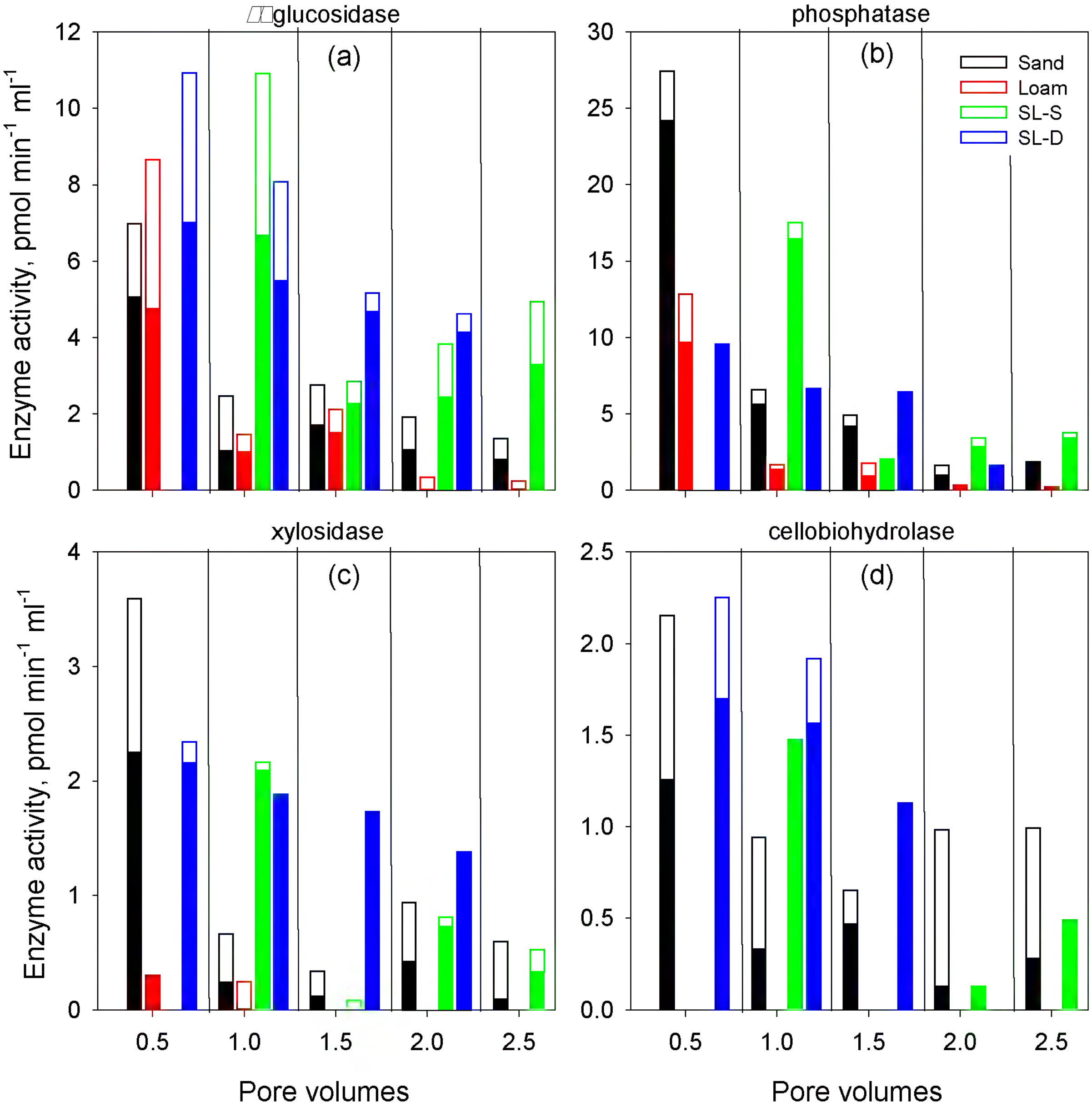

Figure 4. Activity of enzymes associated (filled bars) and not associated (open bars) with CMC in the effluents from Sand, Loam, SL-S and SL-D soil columns for the four enzymes and different eluent volumes passing the columns. Sum of open and filled bars represents the total enzyme activity in the effluents.

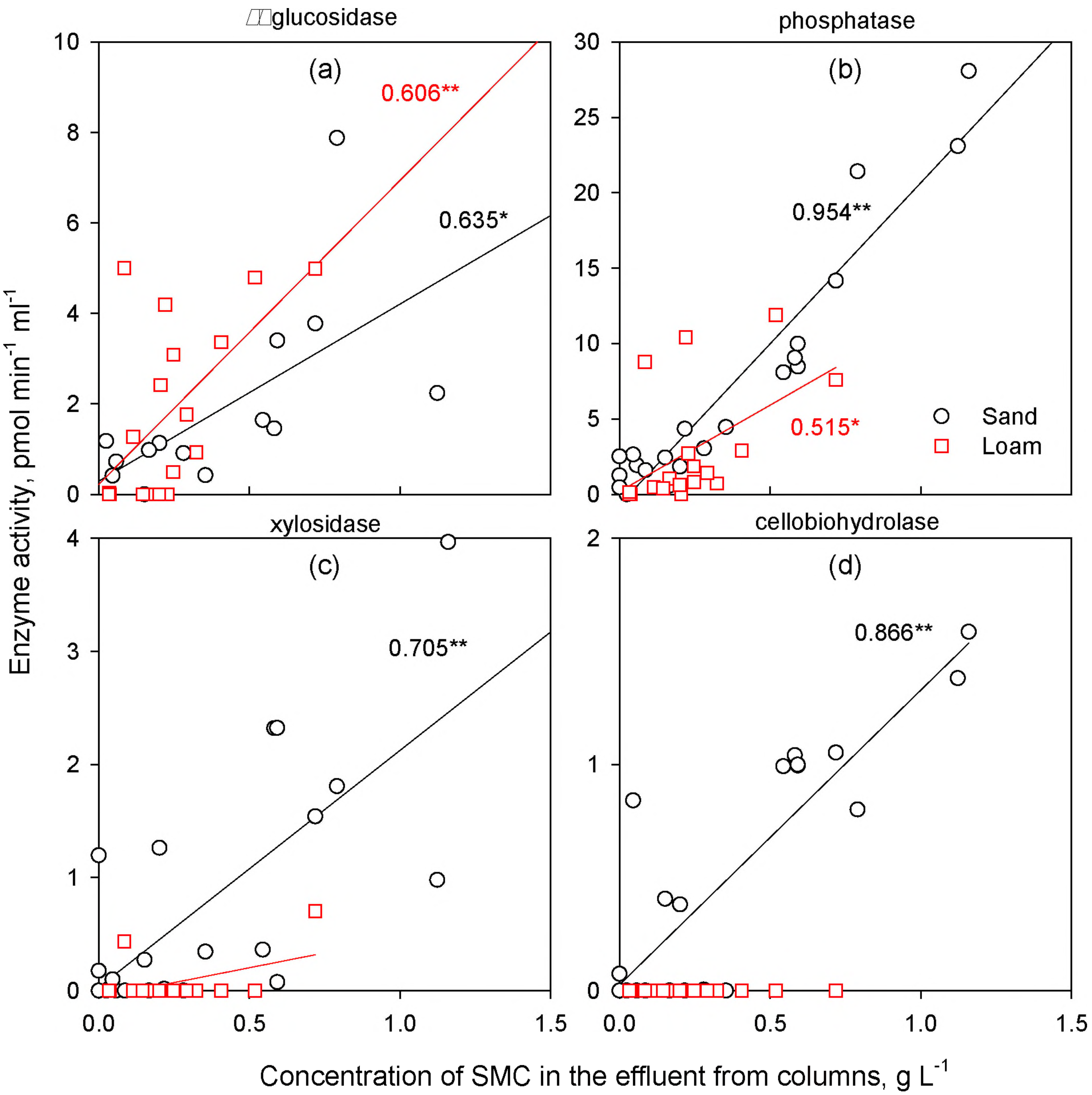

Figure 5. The relationships between activity of the four enzymes associated with CMC and SMC contents in the effluents from Sand and Loam soil columns. Zero values indicate concentration of colloids and enzyme activities below the detection limit. Lines denote linear regressions between enzyme activity and SMC contents. Data are combined from 3 columns for each soil. The numbers show the Pearson correlation coefficients at $p < 0.1$ (*) and $p < 0.05$ (**), respectively.

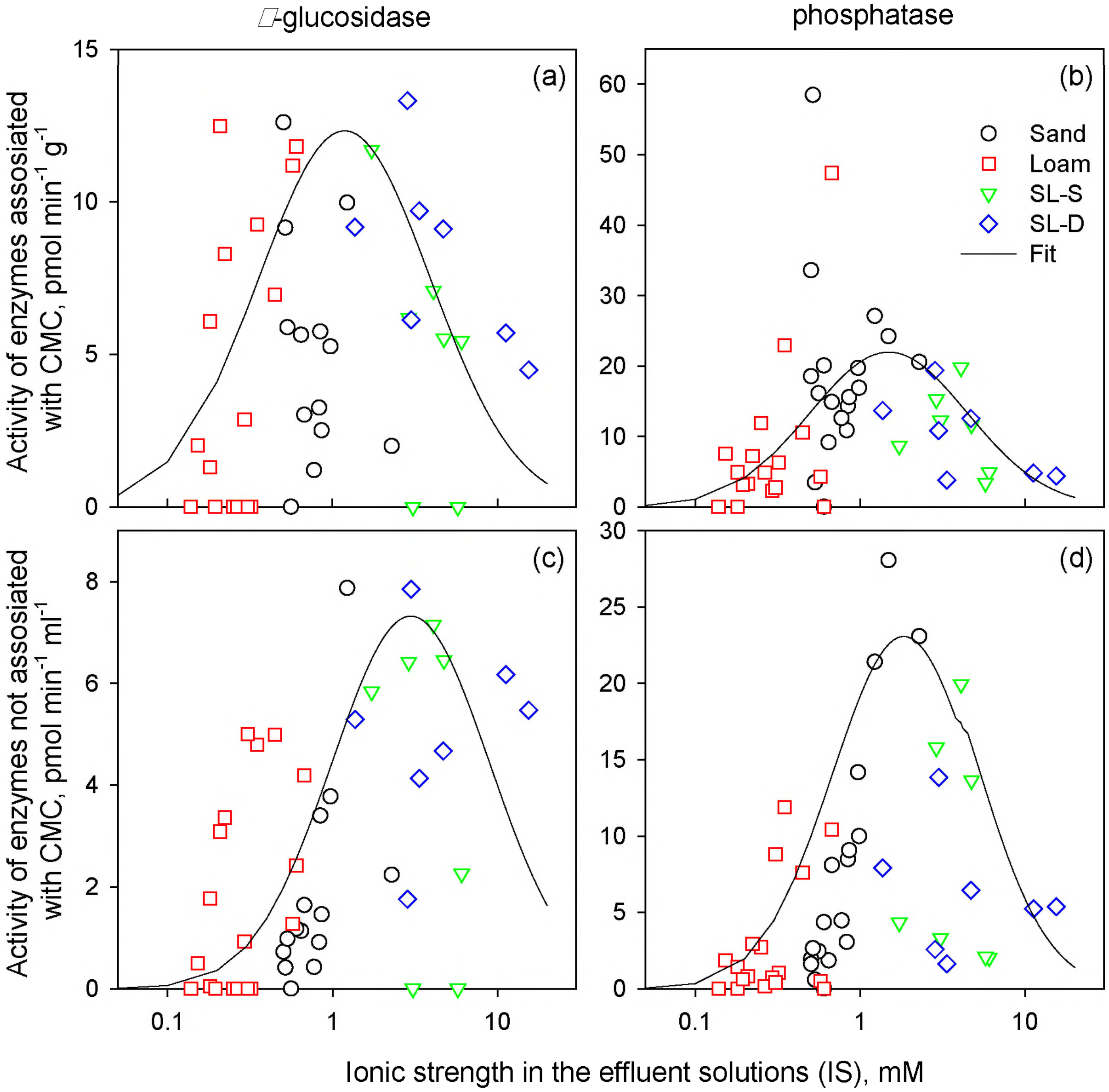

Figure 6. β-glucosidase and phosphatase activities associated and not associated with CMC as a function of ionic strength in the effluents from four soils. The activities are expressed per 1 g of SMC dry mass (a,b) and per 1 ml of effluent (c,d). Black lines show Eq.(5) fitted to the experimental data.

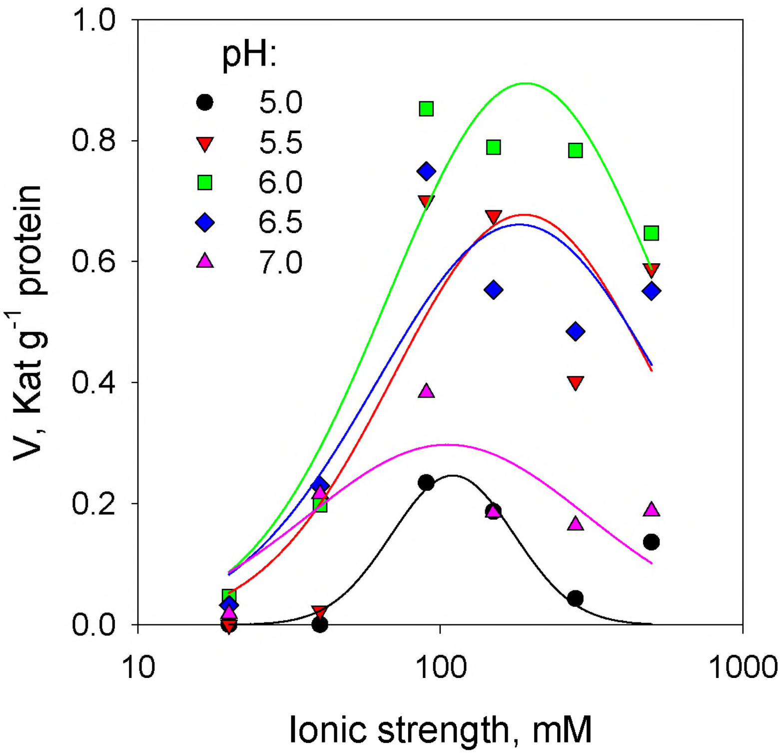

Figure 7. Rate of the catalytic reaction (V) of phosphatase adsorbed on montmorillonite as function of ionic strength in solutions with different pH values (symbols) reconstructed from Leprince and Quiquampoix (1996) (Fig. 2, Central column). Lines show lognormal distribution fit to the experimental data.

Supplemental materials

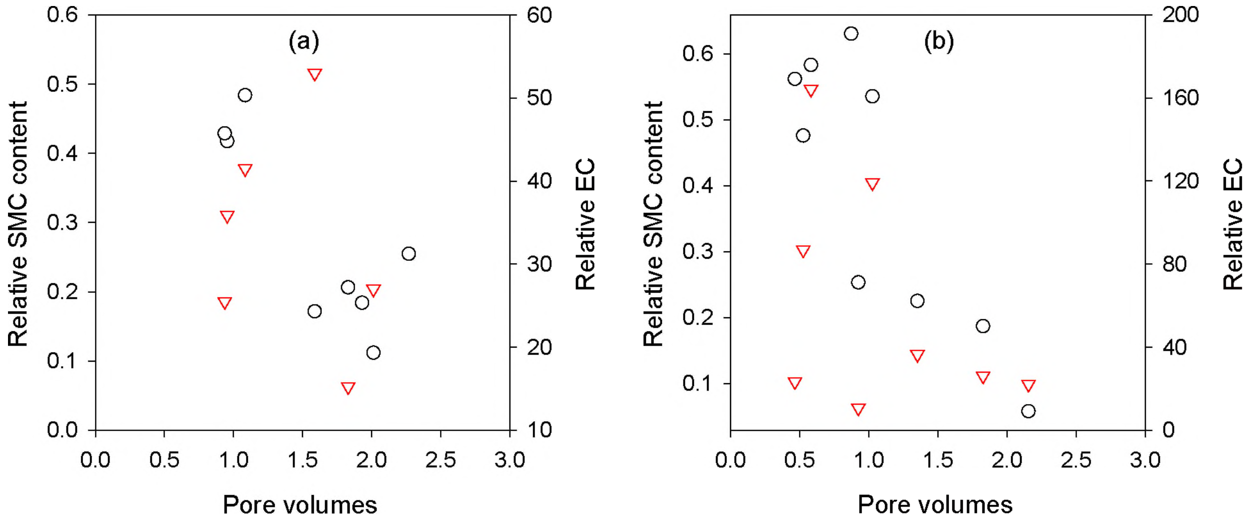

Figure S1. Concentrations of soil mineral colloids (SMC) and electrical conductivity (EC) in the effluents relative to those in the applied solutions for SL-S (a) and SL-D (b) soil columns for eluent volumes that passed the columns relative to the pore volumes. Data are combined from all columns of each soil.

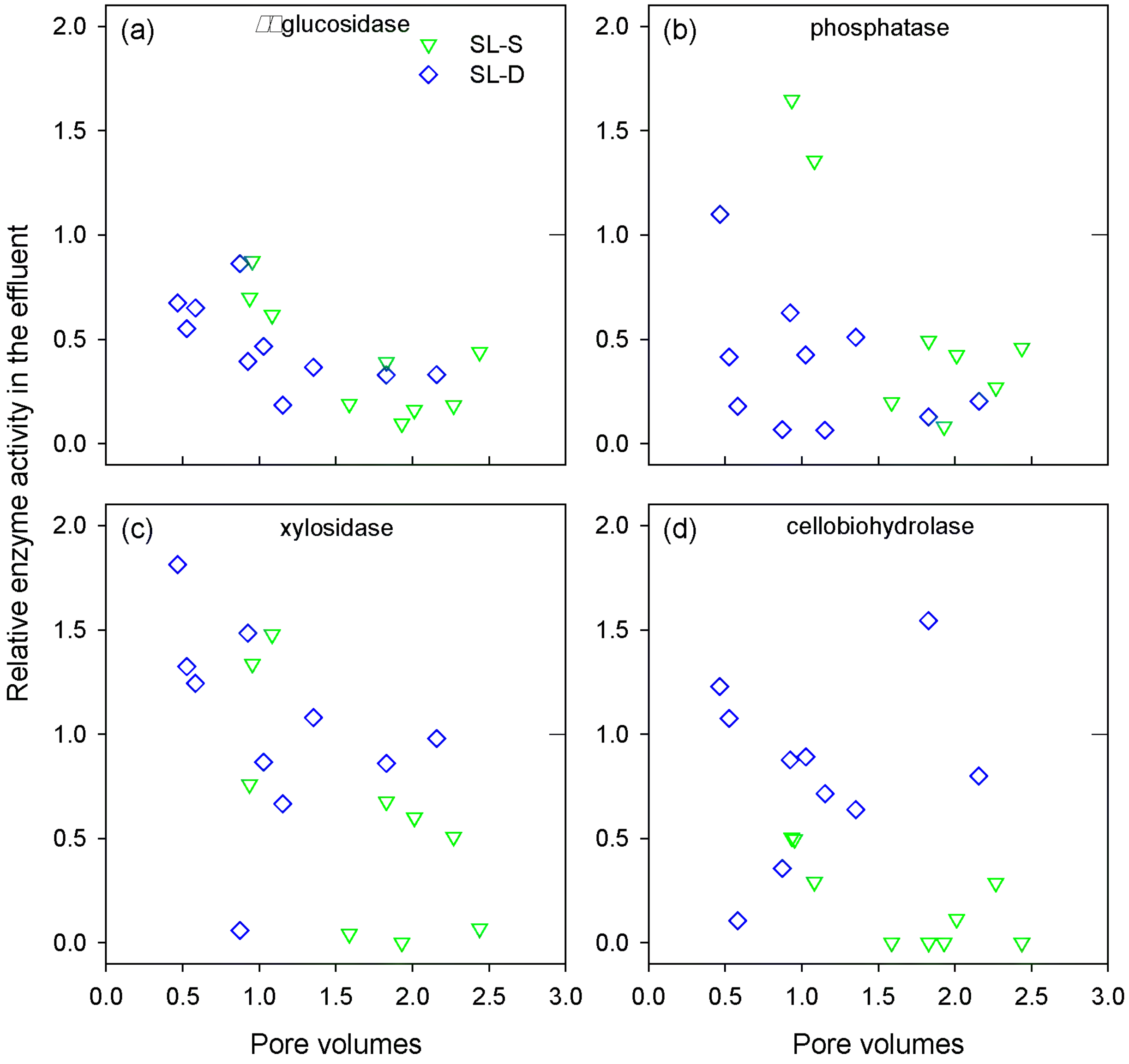

Figure S2. Activity of the four enzymes in the effluents from SL-1 and SL-2 soil columns relative to those in the applied solutions for eluent volumes that passed the columns relative to the pore volumes. Data are combined from all columns of each soil. Horizontal lines mark the relative activity in the effluent corresponding to that in the eluent.

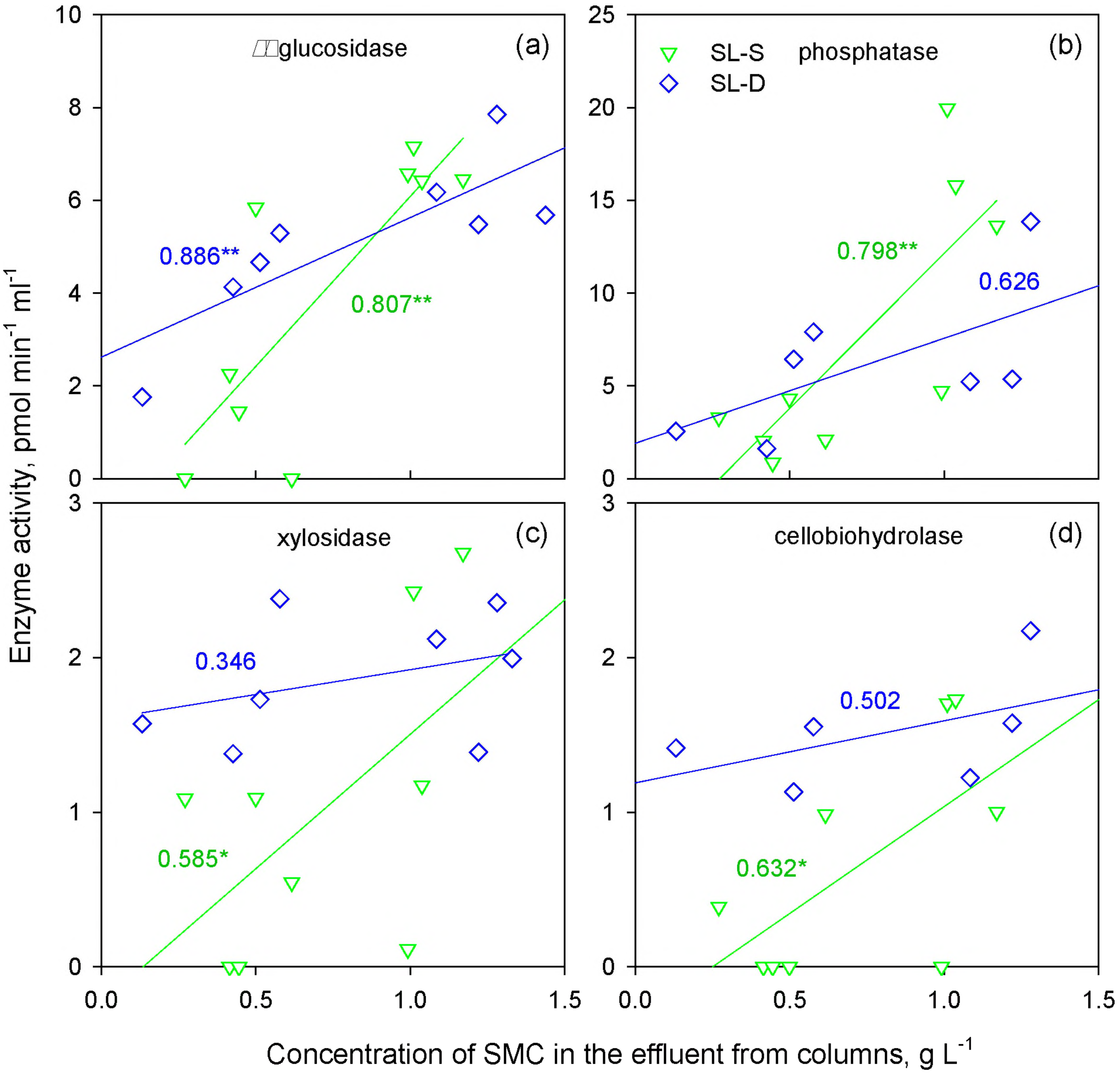

Figure S3. The relationships between activity of the four enzymes associated with CMC and contents of SMC in the effluents from SL-S and SL-D soil columns. Zero values indicate concentration of colloids and enzyme activities below the detection limit. Lines denote linear regressions of enzyme activity on SMC contents. Data are combined from all columns of each soil. The numbers show the Pearson correlation coefficients at $p < 0.1$ (*) and $p < 0.05$ (**), respectively.

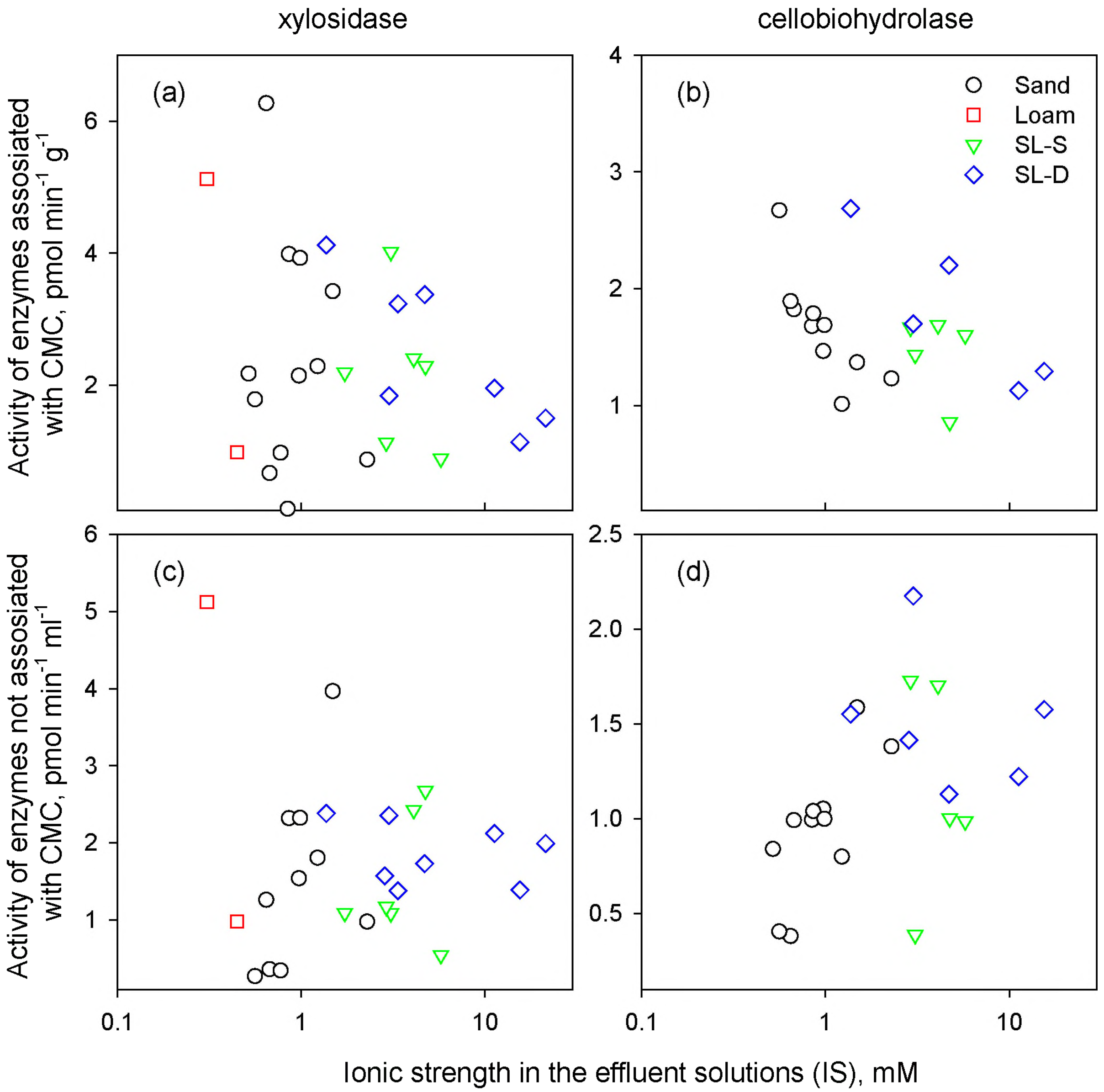

Figure S4. Xylosidase and cellobiohydrolase activities of enzymes associated and not associated with CMC at different ionic strength in effluents from four soils. The activities are expressed per 1 g of SMC dry mass (a,b) and per 1 ml of effluent (c,d).