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A restriction factor of direct DNA extraction from volcanic ash soils

Hiroki Rai[A], Saki Yokoyama[A], Shinmei Hashimoto[A], Shin-ichi Nakamura[A] and Hiroyuki Hattori[A]

[A]Faculty of Bioresource Sciences, Akita Prefectural University, Akita city, Akita Prefecture, Japan, E-mail raihiro@akita-pu.ac.jp

Abstract
The methods of direct DNA extraction from soils made dramatic improvements in analysis of soil microbial communities. Andosol, the volcanic ash soils, are very important soils which account for approximately 50 % of the field crop in Japan. Andosol contains a large amount of humic substances up to 10 % w/w. We tried to extract DNA from these volcanic ash soils directly, but it was impossible to extract DNA using earlier methods. Andosols have special characteristics such as high phosphorus adsorption and humic substances because the soils contain an amorphous aluminium in a silicate called allophane. In our research, the amorphous Al adsorbed DNA contains phosphorus bases in nucleotides. We tried to eliminate DNA adsorption using a high concentration of EDTA as chelator and phosphoric acids as masking material. In this paper, we show the effects of EDTA addition. By the removal of Al from soils in the extraction stage, the DNA also could be extracted. It was a proof that the restriction factor of the difficulty of DNA extraction from Andosol was amorphous Al. We improved the extraction buffer composition and combined with an easy purification step for acquiring purified DNA at high yield.

Key Words
Direct extraction, soil DNA, volcanic ash soils, amorphous aluminium, humic substances.

Introduction

Various microbes inhabit in soils and the diversity of the soil microbes is great. However, it is considered the 90 % or more of the soil microbes are unable to be cultivated at present. Consequently the cultivation methods of soil microbes, limit in the analysis of microbial community structure and it is clear that gene analysis of the un-culturable microbes in soil cannot be performed.

As a recent approach instead of the culture-dependent analysis of microbial community structures, PCR amplification products of crude DNA which was extracted from environmental samples is used for denaturing gradient-gel electrophoresis (DGGE), temperature gradient-gel electrophoresis (TGGE) or cloning techniques.

If the soil DNA can be extracted and analysed directly, it will become possible to know what microbes are living in soil and to obtain the information on new genes of them as DNA sequences whether the soil microbes are culturable or not. It is thought that the potential utility value of soil DNA including the information on new genes of these microbes is very high. The trial of extracting DNA from soils was performed for the first time by Torsvik and Goksoyr (1978). They carried out separation and recovery of the fraction which contained soil microbes with using pyrophosphate buffer, etc from soils, and then DNA was extracted from the fraction including microbes washed out from soil. They used the term "soil DNA" for DNA obtained using the method described above. This method once collects fraction of microbes derived from soils, and so it is called indirect extraction method now. By this method, the DNA of the unrecoverable microbes even after washing soil particles with buffer solution could not be obtained. There were microbes attached to soil particles such as actinomyces, which were living in the dead plant body, and inhabiting the inside of soil particles, whose DNA could not be extracted by this method.

Then, the direct extraction method was developed successively by Ogram *et al.* (1996), Tsai and Olson (1987) and Zhou *et al.* (1991), etc. Without separating microbes from soils, in these methods, soils were treated directly with alkaline solution containing enzyme such as lysozyme and proteinase K and surfactant denaturing protein such as SDS. Microbes were lysed in extraction solution and DNA was extracted in the presence of soil substances (sand, silt, clay, humic-substances, etc) as matrix. This method is considered to reflect more the actual microbial community structure than the indirect extraction methods, and to obtain in good yield. However, by this method, because of heat-treatment of soils in an alkaline solution for a long time, the contamination of humic-substances cannot be ignored.

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On the other hand, the method using a beads beater for the purpose of extracting DNA from more soils microbes was newly developed. It destroys microbial cells with beads mechanically (Kuske *et al.* 1998). Conditions of beads-beating were considered in detail by Burgmann *et al.* (2001). By this method, the microbial cells are mechanically crushed even if gram positive bacteria whose cells cannot be easily destroyed by surfactant such as SDS are included, because of the polysaccaride membranes surrounding their cells. Therefore the DNA can be extracted from such bacteria with extremely high yield by this method. Moreover, since the extraction procedure is for a short time, a crude soil DNA can be obtained with less contamination of humic-substances compared to heating methods.

There are some kits which prepare DNA from soils by the original methods over a short time, such as Bio101 Fast DNA spin kit (Qbio USA) and UltraClean Soil DNA kit (MoBio USA), produced commercially in this decade. These kits required a beads beater and the soil DNA extraction operation is finished in a short time.

We tried to extract DNA directly from volcanic ash soils in the Kanto region in Japan but it was impossible to extract DNA using earlier methods. Volcanic ash soils have special characterics such as high phosphorus adsorption and much humic substances because the soils contain an amorphous aluminium silicate called allophane. We tried to improve the DNA extraction method for volcanic ash soils, especially examining the composition of the extraction buffer. We also tested an easier purification step for acquiring purified DNA.

Material and Methods

Chemical analysis of sample soils

We sampled 31 soils in Kanto, Kinki and Tohoku regions which represented typical soil types in Japan. Especially, we sampled volcanic ash soils (Andosol), which are very important soils which account for approximately 50 % of crop area in Japan. Chemical analysis of soil pH (H_2O), pH (KCl), total nitrogen and carbon determined by N/C analyzer (NC-90A, Sumika Analytical Center) were carried out. We measured the amounts of amorphous aluminium and iron contained in Andosols and which distinguish Andosols from other soils, by a selective dissolution method (Blackmore *et al.* 1981). The results of chemical analysis of the soils which were used for the detailed experiments are shown in Table 1.

Soil DNA extraction with conventional extraction buffer

We tried to extract DNA from 31 soil samples with a conventional buffer which consisted of 1 % SDS, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA. Extraction buffer was added to 0.5 g soil and beads for beating for 30 sec (at 5 m / sec). After bead beating, the test tubes were centrifuged at 10000 x g for 10 min and the supernatants were collected. Denaturing proteins were removed by chloroform and the DNA was precipitated with 2-propanl.The DNA was subjected to electrophoresis in 1 % agarose gel and stained with ethidium bromide.

The effects of EDTA concentration in extraction buffer

EDTA has ability to chelate metal cations strongly. We tested the improvement of the extracted DNA yield by adding EDTA to extraction buffer especially from volcanic ash soils. The extraction solution consisted of 1 % SDS, 100 mM Tris-HCl (pH 8.3) and EDTA. The concentration of EDTA ranged between 0 ~ 300 mM. The extraction buffer was added to 0.5 g soil, beating beads and denaturing proteins were removed with chloroform and the DNA was precipitated with 2-propanol. The agarose gel was stained with SYBR Green I and quantitative analysis of the DNA concentration used fluorescent intensity by reference to the λ Hind III digest markers signal.

The relationship between soil DNA yields and quantities extracted metal ions from soils.

The extraction solution consisted of 1 % SDS, 100 mM Tris-HCl (pH 8.3) and EDTA. The concentration of EDTA ranged between 0 ~ 400 mM. After bead beating and centrifugation, a part of the supernatants was collected for quantitative analysis of metal cations. The metal cations (Al, Fe, Ca, Mg) in extracted supernatants were determined by ICP-AES (SPS-6000 Seiko).

The comparison of the original method with the other methods and soil DNA extraction kits

Five methods (the original, heating extraction (Zhou *et al*. 1996), beads beating extraction (Cullen *et al.* 1998), and two commercial kits (UltraClean Soil DNA kit and Bio101 Fast DNA spin kit) were tested for soil DNA extraction from 7 volcanic ash soils, 5 non-volcanic ash soils (one of them, Souti res. pasture was contaminated with a little volcanic ash by volcanic eruption)

The original method was carried out in the procedure. 0.5 g fresh soil and 1 g of silica-zirconia beads (0.1 mm : 0.5 mm =3 : 1 mix) were added to 2 ml screw-capped tubes and 1200 µl of lysis buffer was added (1 % SDS, 100 mM Tris-HCl, 200 mM EDTA, 500 mM Na_2HPO_4 (pH 8.6)). The high concentration of EDTA and sodium phosphoric acid could chelate and mask amorphous aluminium. After beating with beads at 5 m / sec for 30 sec the supernatants were collected by centrifugation at 12,000 x g for 5 min. 750 µl of supernatant was transferred to a new 2 ml tube and add 250 µl of 5 M NaCl and 250 µl of 10 % CTAB (cetyltrimethylammonium bromide) added and incubated at 65 °C for 5 min. After mixing vigorously with a vortex mixer for 15 sec, the tube was centrifuged at 15,000 x g for 10 min. 1,000 µl of the aqueous layer was transferred to a new 2 ml tube and an equal volume of 12 % PEG was added. The DNA was precipitated by centrifugation at 20,000 x g for 20 min at 4 °C. The DNA pellet was washed with 70 % cold ethanol and dissolved in 100 µl TE buffer.

Results

Soil DNA adsorption to volcanic ash soils.

We show the result of DNA extraction from several soils (volcanic and non-volcanic soils) with low EDTA concentration buffer in Figure 1. The soil DNA could be extracted from non-volcanic soils but little or no soil DNA could be extracted from volcanic ash soils and the soil contaminated with volcanic ash. It showed the volcanic ash soils strongly interfered in the extraction of DNA. The effects of EDTA for DNA extraction from soils is shown in Figure 2. From Osaka crop field soil (non-volcanic soil), the DNA could be extracted easily with 30 mM EDTA at least. However, from Tochigi forest soil, the DNA could not be extracted with 100 mM EDTA. The addition of 300 mM EDTA made the extraction of soil DNA possible. The concentration of EDTA was higher than usual for in molecular biological experiments.

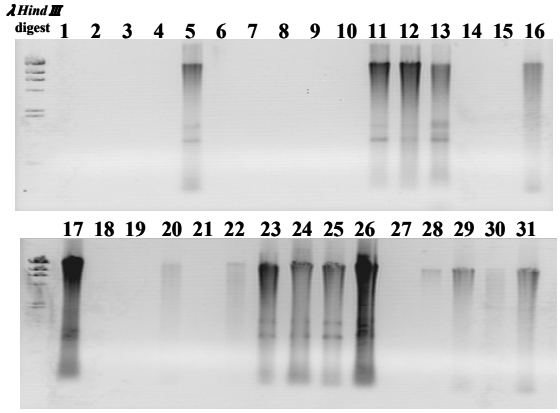

Figure 1. The result of DNA extraction from several soils (volcanic and non-volcanic soils). The extracted DNA were measured by electrophoresis in agarose gel with *λ Hind III* digest marker (ethidium bromide stained). (Volcanic ash soil: 1~4,6~7,9~10,14~16,18~22,27~31; Non-volcanic soils: 5,11~13,17,23~26; Non-volcanic soil contaminated with a little volcanic ash: 8).

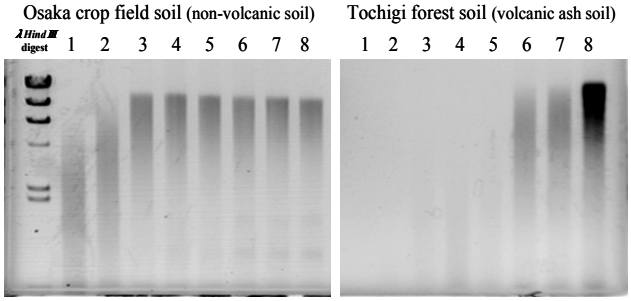

Figure 2. The effects of EDTA for DNA extraction form soils. The extraction solution was consisted of 1% SDS/ 100mM Tris-HCl (pH 8.3) and EDTA. The concentration of EDTA was changed. (1;10 mM, 2;10 mM, 3;20 mM, 4; 30mM, 5;50 mM, 7;200 mM, 8; 300mM).

A restriction factor of soil DNA extraction from volcanic ash soils.

The effects of the concentration of EDTA in extraction buffer for soil DNA yield and extracted metal ions are shown in Figure 3. It was thought that the high concentration of EDTA released soil DNA from adsorption by the soil particles. The volcanic ash soils contain high amounts of active aluminium and iron (amorphous mineral called allophane). To measure amorphous aluminium and iron adsorbed DNA, we determined the quantities of the extracted aluminium and iron in the supernatants after beads beating by ICP-AES.

In Figure 3, the removal of aluminium from soils by chelating reaction of EDTA made soil DNA recovery higher. It was suggested that amorphous aluminium in volcanic ash soils adsorbed DNA in the extraction buffer and made the direct soil DNA extraction difficult.

Comparison between the original direct soil DNA extraction method and the other methods

The DNA yields were showed in Figure 4. The earlier methods and two commercial kits could extract the soil DNA from non-volcanic soils, but not extract from volcanic ash soils at all. The original method could extract soil DNA at high yield from not only non-volcanic soils but also volcanic ash soils.

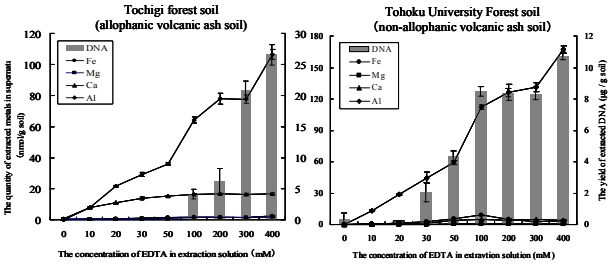

Figure 3. The effects of the concentration of EDTA in extraction buffer on soil DNA yield and extracted metal ions. The DNA yields were determined by stained agarose gel. The metal ions in extracted suspensions were determined by ICP-AES.

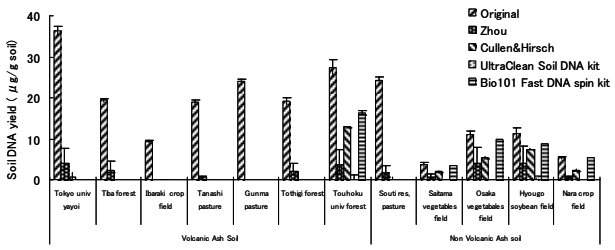

Figure 4. A result comparing the original method in this research with earlier two methods and two extraction kits. The DNA yields were determined by SYBR-Green I stained agarose gel electrophoresis.

Table 1. The results of soil analysis. Total N, C were determined by N/C analyzer. The selective dissolution extracts were determined by ICP-AES. Allophanic Al was calculated (Acidic oxalate extractive Al – Pyrophosphate extractive Al).

soils	Soil Taxnomy	pH (H_2O)	pH (KCl)	Total N (%)	Total C (%)	Acidic oxalate extraction (mg/g soil)			Pyrophosphate extraction (mg/g soil)		Allophanic Al (mg/g soil)
						Si	Fe	Al	Fe	Al	Al
Tokyo univ yayoi	Andisol (Allophanic)	6.98	5.58	0.27	3.12	26.17	25.45	47.52	1.01	2.38	45.14
Tiba forest	Andisol (Allophanic)	6.2	5.11	0.43	5.40	15.77	26.75	62.88	1.57	6.03	56.85
Ibaraki crop field	Andisol (Allophanic)	6.3	5.69	0.34	4.64	10.41	19.79	50.56	1.33	5.41	45.15
Tanashi pasture	Andisol (Allophanic)	4.96	4.3	0.41	4.90	14.98	27.12	62.58	2.00	7.15	55.43
Gunma pasture	Andisol (Allophanic)	5.82	5.45	0.37	3.97	9.67	8.61	39.07	1.08	3.56	35.51
Tothigi forest	Andisol (Allophanic)	5.28	4.38	0.68	8.76	6.25	12.42	38.35	5.00	14.22	24.13
Touhoku univ, forest	Andisol (un-Allophanic)	4.97	4.24	0.45	9.19	4.07	16.39	38.62	8.13	17.80	20.82
Souti res, pasture	Inceptisol	6.11	5.35	0.27	3.64	1.44	7.91	10.99	3.77	5.17	5.83
Saitama vegetables field	Inceptisol	5.01	3.98	0.13	1.18	0.45	4.10	2.09	1.79	0.87	1.22
Osaka vegetables field	Inceptisol	6.9	6.37	0.15	1.05	0.21	3.22	0.90	2.53	0.30	0.59
Hyougo soybean field	Inceptisol	7.81	7.4	0.16	1.44	0.42	2.11	0.89	1.51	0.32	0.56
Nara crop field	Inceptisol	5.11	3.98	0.22	1.47	0.45	6.25	1.52	2.50	0.62	0.89

Conclusion

We developed new direct DNA extraction method which extracts soil DNA from volcanic ash soils. The quantity of soil DNA in volcanic ash soils was higher than non-volcanic soils. The soil DNA was up to 20～40 μg / g soil and it suggested the population of microbial communities of volcanic ash soils were 10^9 orders as bacteria (calculated at 10 fg DNA / one bacteria). We expect the new method could reveal the microbial community structure in volcanic ash soils and be applied to measuring the biomass in soils through the quantitative analysis of extracted soil DNA.

Acknowledgement

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References

Torsvik V L , Goksoyr J (1978) Determination of bacterial DNA in soil. *Soil Biology and Biochemistry* **10,** 7-12

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. *Journal of microbiological methods* **7,** p57-66.

Tsai YL, Olson BH (1992) Rapid method for separation of bacterial-DNA from humic substances in sediments for polymerase chain-reaction. *Applied and Environmental Microbiology* **58,** 2292-2295.

Zhou JZ, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. *Applied and environmental microbiology* **62,** p316-322.

Kuske CR, Banton KL, Adorada DL (1998) Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Applied and Environmental Microbiology* **64,** 2463-2472.

Bürgmann H., Pesaro M, Widmer F, Zeyer J (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods* **45,** 7-20.

Blackmore LC, Searle PL, Daly BK (1981) 'Methods for Chemical Analysis of Soils'. New Zealand Soil Bureau Scientific Report 10A (DSIR: New Zealand).

Cullen DW, Hirsch PR (1998) Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biology and Biochemistry* **30**, 983-993.

Changes of enzymatic activity in soil supplemented with microbiological preparation UGmax®

Anna Piotrowska,[A] Jacek Dlugosz[B] Ryszard Zamorski[A], Piotr Bogdanowicz[C]

[A]Department of Biochemistry, University of Technology and Life Sciences, Bydgoszcz, Poland, ap03@wp.pl
[B] Department of Soil Science and Soil Protection, University of Technology and Life Sciences, Bydgoszcz, Poland, jacked@utp.edu.pl
[C]"Bogdan" Trade-Service Company, Ltd., Boleslawowo, Poland, bogdan@bogdan.agro.pl

Abstract
The influence of the microbiological fertilizer UGmax® on soil biological activity measured as dehydrogenase and cellulases activities was determined. The research was carried in 2005-2008 on a productive field of 2 ha under winter wheat and winter rape. One half of the field was supplemented with UGmax® every year after harvest on the stubble and as top-dressing in spring, while the other part was the control. Ten soil samples localized with the GPS were collected every year (2005 – 2008) from the humus horizon. Analyses of basic soil chemical parameters and enzymatic activities were done prior the experiment (in 2005). The results showed that the microbiological preparation UGmax® significantly promoted the initial phase of the decomposition of post-harvest residues what was confirmed by a clear decrease of cellulase activity in the soil samples taken from the field with UGmax® as compared with control field. Cellulases activity decreased gradually year after year. The most significant drop of this activity was noted in the specimen taken in the last year. The application of UGmax® has not influenced the activity of dehydrogenases. The tendency observed over the experiment was similar in both sets of soil samples. Furthermore UGmax application increased soluble carbon content significantly which was confirmed by higher organic carbon and total nitrogen contents.

Key words
Biological preparation, cellulases, dehydrogenases, enzymatic activity, soil, UGmax®

Introduction

Organic matter is the most important soil component, always taken into account in all issues of environmental protection. Soil organic matter quality and quantity is of importance for many soil features, such as soil water retention, sorption and buffering potential, etc. Moreover, it is the source of nutrients and energy for living organisms. Since soil organic matter is one of the elements of the global matter and energy circulation, being both the producer and the emitter of CO_2, and finally its natural sequestration complex system. That is why decreasing quality and quantity of soil organic matter is extremely important, not only because of soil fertility, but also from the point of view of climate protection.

One of the methods for increasing organic matter content in arable soils is application of microbiological preparations. They increase soil microbial activity and in consequence humus compound formation. UG max produced by the "Bogdan" Trade-Service Co, Ltd. is one of microbiological preparations available in Poland. It is composed of lactic acid, *Pseudomonas* and *Penicillium* bacteria plus actinomycetes. Preliminary results showed that UG max can increase the decomposition rate of post-harvest residues. It was confirmed by a parallel decrease of cellulase activity. Moreover, UGmax increased and stabilized organic matter content and available phosphorus content.

Soil enzymes play an important role in the catalysis of some important reactions essential for soil microorganisms, decomposition and formation of organic matter, and are responsible for nutrient cycling and decomposition of organic wastes [Dick and Tabatabai 1993], what of course is of special agricultural significance. Total soil enzymatic activity is composed of both intracellular and extracellular enzymes. The dehydrogenase group of enzymes is the best example of exclusively intracellular enzymes. Dehydrogenases play a significant role in the biological oxidation of soil organic matter by transferring the proton from substrates to acceptors (Rossel *et al*. 1997). That is why their activity is considered an indicator of the oxidative metabolism in soil and thus also of microbial activity [Quilchano and Maraňón 2002]. However, the largest part of soil enzymes is extracellular and excreted to the soil solution. They are extremely important in the hydrolysis of substrates that are too large or insoluble to be taken up directly by cells (Dick

1997). One of them is cellulose, the most abundant organic compound in the biosphere, comprising almost half of the biomass synthesized by photosynthetic fixation of CO_2 (Eriksson *et al*. 1990). The hydrolytic enzymes that mediate cellulose degradation in soil are known as cellulases. There are three kinds of cellulase which can hydrolyse cellulose in different ways. Due to importance of the cellulase complex in the global recycling of cellulose it is important to understand the factors that affect the enzyme, so that it may be used more often as an index of the soil fertility status.

Material and Methods

The soils were collected from the experimental field in 2005-2008.The research concentrated on the surface (humic) horizon of eutric, gleic Cambisols of a productive field of 2 ha under winter wheat (2005, 2006, 2008) and winter rape (2007) localized in the southern part of the Sepopolska Plain near the Budniki village (54° 11' 54" N and 20° 38' 12" E). One half of the field was supplemented with UGmax® every year after harvest on the stubble (0.7 l per ha) and as top-dressing in spring (0.3 l per ha), while the other part was the control. Ten soil samples localized with GPS were taken every year (2005 – 2008) from the soil humus horizon. Results of the analyses of basic soil chemical parameters were done prior the experiment (2005) and after UGmax® application (2006-2008) (Table 1). The data on dehydrogenases and cellulases avtivities determined in 2005 were published earlier (Smolinski *et al*. 2008). Since our idea was to reach the soil equilibrium and to learn on a long-term soil quality status we analysed the soil not earlier than after six months of the UGmax application.

Soil dehydrogenases activity was measured as described by Casida *et al*. [8] with some small modifications. In general, soil samples of 6g were placed in 16mm x 150 mm test tubes and incubated with 1ml 3% 2,3,5-triphenyltetrazolium chloride (TTC), 60mg $CaCO_3$ and 2.5ml distilled water at 37°C for 24h. After incubation the red 2,3,5-triphenyltetrazolium formazan (TPF) was extracted with 50 ml of ethanol and read colorimetrically at 485nm for quantification. Cellulolytic activity was measured according to Deng and Tabatabai [1994] with some modifications as given herewith. 5 g of air-dried soil samples were weighed and rinsed with 1 ml toluene in order to stop microbial growth. Than the samples were suspended in 5ml 10% carboxymethyl cellulose (CMC - sodium salt) in 50 mM acetate buffer of pH 5.5 and incubated at 30°C for 24 hours. After that the amount of reducing sugars released was determined with the standard Nelson-Somogyi colorimetric method as described by Deng and Tabatabai [1994]. All determinations were made in triplicate and expressed on a dry weight basis (DM). Results of dehydrogenase and cellulolytic activities were expressed as □M TPF/g DM ·/24h and □M glucose ·/g DM ·/h x 10^{-3}, respectively.

Results

As is shown in Table 1, UGmax® application caused an increase of soil organic matter content and also stabilized it, what was confirmed as increased organic carbon and total nitrogen concentrations. Preliminary results showed that the microbiological preparation (UGmax®) under study clearly accelerated the initial phase of post-harvest residues decomposition. It was confirmed by a distinct decrease of cellulose activity in the soil samples taken from the field where the UGmax® was applied as compared with the control field. Cellulases are known to be the inductive enzymes and their activity depend on the substrate availability and products content. UGmax accelerated the fresh organic material (straw) decomposition and simultaneously declined the amount of the substrate available for cellulose degradation. As the result a significant enzymatic activity decreasing was noted after second (spring) UGmax® application. A significant decrease of cellulolytic activity was observed in the soil samples collected after one year of the UGmax® application with the mean activity of 0.737μM glucose /1g d.m. soil/1h 10^{-3}. Enzymatic activity decreased systematically year after year. The most significant decrease of its activity was noted in the soil taken in the last year of the experiment (Figure 1).

Table1. Some chemical properties of soil under study (2005-2008)

Parameter	2005				2006-2008			
	with UGmax®		without UGmax®		with UGmax®		without UGmax®	
	mean	min-max	mean	min-max	mean	min-max	mean	min-max
C_{ORG} [g/kg]	15.44	14.15-16.46	15.42	12.30-19.51	17.3	13.28-27.16	14.76	12.01-19.40
N_{TOT} [g/kg]	1.52	1.41-1.64	1.51	1.23-1.85	1.70	1.32-2.51	1.44	1.41-1.64
Clay fraction [%]	24.0	15.0-29.0	22.4	16.0-31.0	24.0	15.0-29.0	22.4	16.0-31.0
pH 1M KCl	6.16*	5.82-6.51	5.94*	5.51-6.62	6.19*	5.51-6.84	5,35*	4.46-5.96

*geometric mean

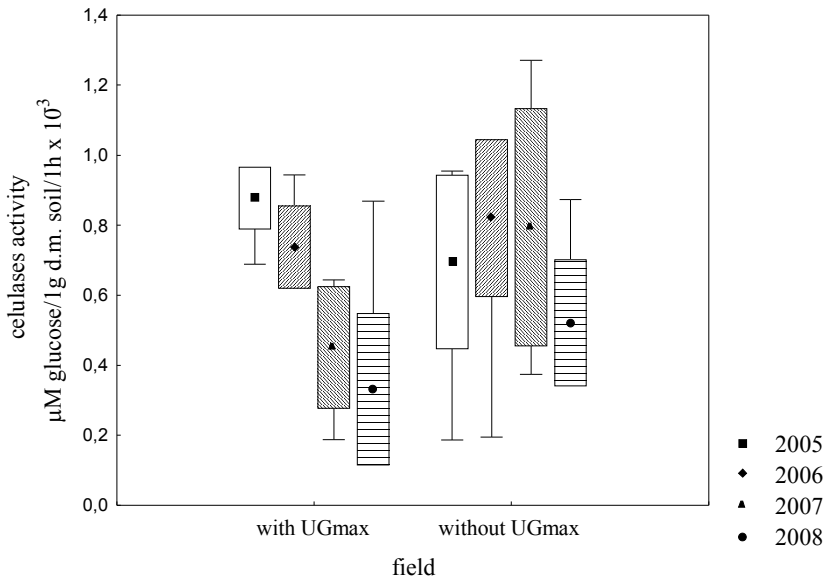

Figure 1. Changes of cellulases activity in the field with and without UGmax®

The mean value of cellulases activity in the soil samples taken from the field with UGmax® in 2008 amounted 0.331µM glucose /1g d.m. soil/1h 10^{-3}. The total reduction of studied enzymes activity after UGmax® using reached 0.55µM glucose /1g d.m. soil/1h 10^{-3} (Figure 2), while cellulases activity determined in the soil samples taken from the control plot changed negligibly. The only significant change in their activity was observed in samples analyzed in the last year of the experiment. In this case the activity decreased about (of) 0.17 µM glucose /1g d.m. soil/1h 10^{-3} (Figure 1).

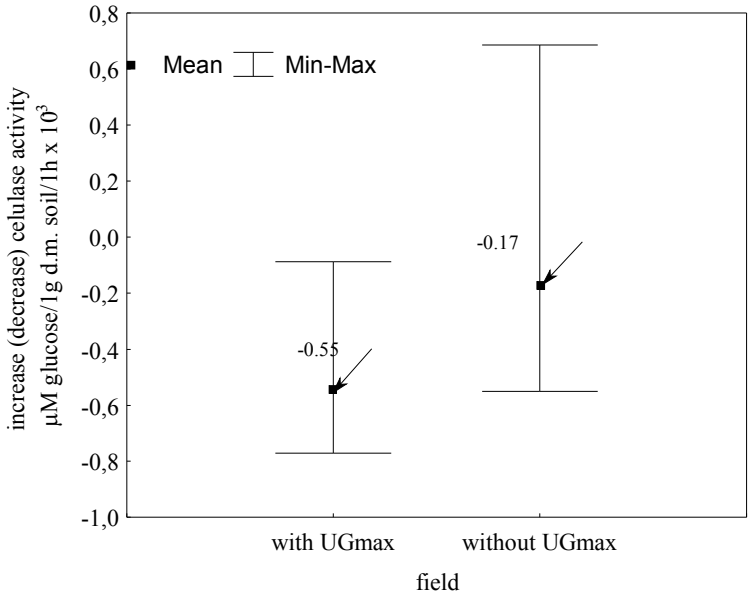

Figure 2. Cellulases activity before and after the UGmax® application (2005-2008)

Results of cellulolytic activity exhibited a high spatial variability within the analyzed area. It was confirmed by a significant dispersion of results among soil samples. The reason for that phenomenon could be probably differentiation of chemical parameters in the surface soil horizon (Table1).
The application of the UGmax® did not affect the activity of dehydroegenases activity very little. The differences occurring in soil samples taken every year were similar in both fields. The highest dehydrogenases activity was noted in the soil samples taken in 2007, both in samples with UGmax® and without it. Dehydrogenases activity, similarly to that of cellulases, disclosed a significant spatial variability.

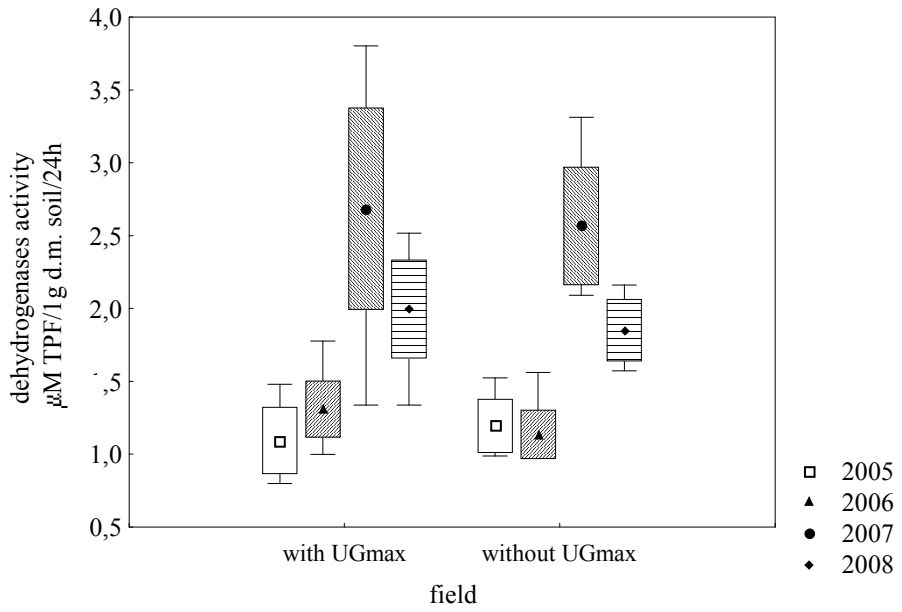

Figure 3. Changes of dehydrogenases activity in the field with and without UGmax

Conclusions

The results showed that UGmax® is the preparation determining the decomposition rate of post-harvest residues and increasing significantly the amount of soil organic matter. It can be concluded that out of the enzymes studied only the activity of cellulases can be a good indicator of soil changes after the UGmax® use because these enzymes clearly and univocally respond to the preparation application. The application of the UGmax® had basically no influence on the activity of dehydrogenases. There was no clear tendency in the dehydrogenases activity in soil samples taken from both UGmax® field and the control one.

References

Casida LE, Klein DA, Santoro T (1964) Soil dehydrogenase activity. *Soil Science* **98**, 171-176.

Deng SP, Tabatabai MA (1994) Cellulase activity of soil. *Soil Biology and Biochemistry* **26**, 1347-1354.

Dick WA, Tabatabai MA (1993) Significance and potential uses of soil enzymes. In 'Soil microbial ecology'. (Ed FB Metting Jr.) pp. 95-127. (Marcel Dekker, New York)

Dick RP (1997) Soil enzyme activities as integrative indicators of soil health. In 'Biological indicators of soil health'. (Eds C Pankhurst, VVSR Gupta) pp. 121-156. (CAB International: London, UK)

Eriksson KEL, Blanchette RA, Ander P (1990) Biodegration of cellulose. In 'Microbial and enzymatic degradation of wood and wood components'. (Eds KEL Eriksson, RA, Blanchette, P Ander) pp. 89-180. (Springer-Verlag: New York)

Quilchano C, Maraňón T (2002) Dehydrogenase activity in Mediterranean forest soil. *Biology and Fertility of Soils* **35**, 102-107.

Rossel D, Tarradellas J, Bitton G, Morel JL (1997) Use of enzymes in soil ecotoxicology: a case for dehydrogenase and hydrolytic enzymes. In 'Soil ecotoxicology'. (Eds J Tarradellas, G Bitton, D Rossel) pp. 179-206. (Lewis Publishers CRC Press).

Smolinski S, Dlugosz J, Piotrowska A, Zamorski R (2008) Spatial variability of soil dehydrogenases and cellulases activities in a field scale. *Polish Journal of Soil Science* **41**, 73-80.

Characterisation of organic phosphorus compounds in soil by phosphatase hydrolysis

Kathrin Eva Annaheim[A], Emmanuel Frossard[A] and Else Katrin Bünemann[A]

[A]Institute of Plant Sciences, Swiss Federal Institute of Technology Zürich, Eschikon 33, CH-8315 Lindau, Switzerland, Email kathrin.annaheim@ipw.agrl.ethz.ch

Abstract
Many organic and condensed forms of phosphorus (P) can be found in soils. Phosphate groups have to be hydrolysed from these P compounds to become available to plants and microorganisms. This chemical reaction is catalysed by several hydrolytic enzymes, which can be used experimentally to investigate the nature and hydrolysability of soil organic P. As a prerequisite to this approach, phytases, alkaline phosphatases, acid phosphatases, and an inorganic pyrophosphatase were tested for their specificity against eight P substrates (monoesters, diesters, inorganic and organic phosphoanhydrides, and a phosphonate). The inorganic pyrophosphatase preparation was specific against pyrophosphate.The other enzymes hydrolysed simple monoesters and condensed phosphate completely and differed only in their ability to hydrolyse phytate, DNA and RNA. None of the enzymes hydrolyzed the model phosphonate. The implications for the interpretation of the experiments planned with soils are discussed.

Key Words
Enzymes, organic phosphorus, soil, phosphomonoesterase, inorganic pyrophosphatase.

Introduction

An important part of P in soils is represented by organic and condensed P compounds, which comprise several chemical classes: orthophosphates, pyrophosphates, polyphosphates, orthophosphate monoesters, orthophosphate diesters and phosphonates (Turner *et al.* 2003). These forms of P may function as a source of P for soil organisms and plants after the release of phosphate. The release of phosphate is promoted by enzymatic activity. Several studies using enzyme additions to soils have been published in the recent decades (Jackman and Black 1952; Shand and Smith 1997; Hayes *et al.* 2000; Turner *et al.* 2002; George *et al.* 2007). In these, many different hydrolytic enzymes have been employed. Widely varying conditions during the assay, including choice of buffer and pH value, added enzyme activity, duration and temperature, presence of a microbial inhibitor, end of the reaction and method for determination of product assay conditions make comparison between previous studies difficult or impossible (Bünemann 2008). For this reason, Bünemann proposes a standard protocol for enzyme addition assays. To achieve this, however, it would be necessary to use commercially available preparations that can be easily purchased. A prerequisite to interpret the hydrolysis data correctly is accurate characterisation of the enzymes. Substrate specificity, kinetic properties, and the isoelectric point must be known, and information about the inhibition and inactivation of the enzymes is needed. This fundamental information has been absent in many publications. Furthermore, there is no study that characterizes and compares a comprehensive selection of commercially available phosphatases under the same conditions.

Our ultimate goal is to gain more information about the bioavailability of soil organic P by the use of enzyme addition to soil. Here we compare several commercial enzymes, using model substrates in order to characterize them for their substrate specificity. In subsequent steps, the preparations will be verified for purity, and diesterases will be included in the enzyme list as well. Having characterized the enzyme preparations, tests on soil extracts and subsequently on soil suspensions will be carried out.

Methods

Principle of enzyme addition
Commercially available enzymes are added to model P compounds, and the amount of molybdate-reactive P released is measured using malachite green (Ohno and Zibilske 1991). To ensure completion of the reaction, the release must reach a plateau, and several time points must therefore be sampled.

Enzymes, buffers and model substrates

Aqueous solutions of alkaline and acid phosphomonoesterases, phytases and inorganic pyrophosphatase were prepared and added to the appropriate buffers (
Table 1). Four dilutions of each enzyme were pretested on two substrates. The first dilution was chosen based on the indication of enzymatic activity against *p*-nitrophenol given by the supplier and diluted 10, 100 and 1000 times. In order to have the lowest possible consumption of enzyme preparations, and to avoid interferences with the colorimetric analysis, the highest dilution that still achieved maximum hydrolysis was chosen for each enzyme (data not shown).

Table 1. Hydrolytic enzymes and their respective abbreviation, supplier, source, preparation of the commercial product, activity against *p*-nitrophenol indicated by the supplier, and buffer type and concentration in the well. Freeze dried powder, F; liquid preparation, L; granules, G.

Enzyme	Abbr.	Supplier	Source	Prep.	Activity	Buffer
Alkaline phosphatase [a]	AlPS	Sigma	*Escherichia coli*	F	0.05 – 1.00 µkat/mg protein	0.2 M Glycine pH 9.0
Alkaline phosphatase [a]	AlPR	Roche	Calf intestine	L	33.34 µkat/mg	0.2 M Glycine pH 9.0
Acid phosphatase [b]	AcPS	Sigma	Potato	F	0.05 – 0.17 µkat/mg solid	0.2 M Mes pH 5.2
Acid phosphatase [b]	AcPR	Roche	Potato	F	~0.03 µkat/mg lyophilizate	0.2 M Mes pH 5.2
Phytase [c]	PhyN	Novozyme	*Peniophora lycii*	L	≥ 83 µkat/mg	0.2 M Mes pH 5.2
Phytase [d]	PhyB	BASF	*Aspergillus niger*	G	n.s.	0.2 M Mes pH 5.2
Pyrophosphatase [e]	PyPS	Sigma	*Saccharomyces cerevisiae*	F	≥8.34 µkat/mg protein	0.2 M Hepes pH 7.0

[a] EC 3.1.3.1, [b] EC 3.1.3.2, [c] EC 3.1.3.26, [d] EC 3.1.3.8, [e] EC 3.6.1.1

Enzymes were tested against eight model P substrates belonging to different functional classes (
Table **2**). The concentration of total P in the model substrate solutions was determined by autoclaving 0.1 ml of substrate with 1.0 ml of 0.9 M sulfuric acid containing 0.14 M ammonium persulfate. Enzyme solutions, buffers und substrates were prepared with autoclaved Nanopure® water.

Table 2. The eight P substrates tested in this experiment, their abbreviations, suppliers, functional class and concentrations used (means and standard deviations shown).

Substrate	Abbr.	Supplier	Functional class	Conc.(mmol P /L)
Myo-inositol hexakiphosphate	Ins6P	Sigma Chemicals	Phosphate monoester	9.0 ± 0.1
D-Glucose 6-phosphate	G6P	Sigma Chemicals	Phosphate monoester	9.8 ± 0.1
Glycerol phosphate	GP	Sigma Chemicals	Phosphate monoester	6.7 ± 0.1
Deoxyribonucleic acid	DNA	Sigma Chemicals	Phosphate diester	6.5 ± 0.3
Ribonucleic acid	RNA	Sigma Chemicals	Phosphate diester	5.5 ± 0.1
Pyrophosphate	PP	Riedel de Haën	Phosphoanhydride	9.7 ± 0.1
Adenosine 5'-triphosphate	ATP	Roche	Organic phosphoanhydride	9.9 ± 0.01
2-Aminoethyl phosphonic acid	AEP	Aldrich	Phosphonate	9.8 ± 0.2

Assay procedure

Assays were made in flat-bottomed microtiterplates made of polystyrene (Greiner Bio one GmbH, Frickenhausen, Germany). Microtiterplates were put on ice, and the following volumes were added to each well: 60 µl of buffer, 20 µl of different P compounds, 20 µl of enzyme preparations and water to make the total volume up to 300 µl. The assay was performed with 5 analytical replicates. Controls with enzyme only or substrate only were included. An internal orthophosphate standard curve was prepared in duplicate. Plates were incubated at 40 rpm and 30°C (Vortemp™ 56, Labnet international Inc.). Measurements were made just after the addition of enzyme, when the microplate was still on ice, after 30 min., 60 min., 180 min., 24 h and 48 h, in order to verify the completion of the reaction. With each measurement, 20 µl were removed from the incubation plate and diluted 6 times. Finally, 20 µl diluted liquid were transferred to a new plate and made up to 200 µl with water. Molybdate-reactive P was determined using malachite green (Ohno and Zibilske 1991). No additional reagent was used to stop the reaction, as it had been demonstrated in the case of PhyN that the first reagent of the colorimetric measurement stops the enzymatic reaction. Plates were read at 620 nm at room temperature in a computerized microplate spectrometer (Biotek ELx800 Absorbance Microplate Reader, BioTek Instruments, Inc., USA).

Results

Completion of the reaction was reached for almost all substrates after an incubation period of 24 h. Absorption values of enzyme controls were all below the detection limit; thus, the amount of P released by enzyme hydrolysis was calculated from the difference between the assay with substrate and enzyme and the

control with only substrate.
AEP was not hydrolyzed by any of the enzyme preparations, whereas PP was hydrolyzed almost completely by all enzymes (Table 3). The two alkaline phosphatases gave very similar results: they were active against G6P, GP, and phosphoanhydrides, giving complete recovery, whereas Ins6P, DNA and RNA were hydrolyzed at rates below 12%. The two acid phosphatases hydrolysed monoesters, DNA and phosphoanhydrides approximately at the same rate as the alkaline phosphatases. However, the AcPR showed higher hydrolysis rates for Ins6P and RNA (62.5% and 64.1%, respectively). The two phytases hydrolyzed Ins6P at high levels (93.2% and 77.3%, respectively), and they also hydrolyzed monoesters and phosphoanhydrides. RNA was hydrolyzed to a greater extent by PhyN (86.9%) than by PhyB (11.7%). Finally, PyPS was very specific against PP, which was hydrolyzed at 92.8%, and did not hydrolyze any of the other compounds.

Table 3 Proportion of P hydrolyzed by enzymatic catalysis after 24h. Means and standard deviations (n=5).

Substrate	AlPS	AlPR	AcPS	AcPR	PhyN	PhyB	PyPS
	Substrate recovery as hydrolyzed P (%)						
Ins6P	8.9 ± 0.3	4.1 ± 1.3	5.7 ± 0.5	62.5 ± 8.0*	93.2 ± 3.2	77.3 ± 2.2	-0.7 ± 0.3
G6P	97.5 ± 1.4	95.9 ± 3.0	92.4 ± 3.9	96.7 ± 2.2	105.6 ± 3.9	94.0 ± 3.1	-0.4 ± 0.2
GP	94.9 ± 2.4	95.0 ± 3.4	90.0 ± 3.0	95.7 ± 1.8	103.9 ± 3.3	76.1 ± 2.0	-0.4 ± 0.5
DNA	9.0 ± 0.5	11.7 ± 1.1	9.4 ± 0.5	10.7 ± 0.8	11.5 ± 1.3	9.3± 0.7	-1.3 ± 0.1
RNA	10.7 ± 1.5	10.8 ± 1.9	13.8 ± 1.3	64.1 ± 2.5*	86.9 ± 4.1	11.7 ± 1.0	-0.3 ± 0.2
PP	95.9 ± 1.8	88.2 ± 1.8	90.3 ± 0.9	95.9 ± 2.3	109.0 ± 4.9	93.4± 4.5	92.8 ± 4.0
ATP	96.9 ± 1.9	95.1 ± 3.5	90.7 ± 4.7	98.3 ± 4.2	129.4 ± 0.6	71.4 ± 1.8	-0.1 ± 0.3
AEP	0.3 ± 0.6	-0.5 ± 0.5	-0.1 ± 0.7	0.3 ± 0.7	0.7 ± 0.2	-0.1 ± 0.6	-0.9 ± 0.6

* Completion of the reaction was not reached

Discussion

The most specific enzyme tested was PyPS, which released phosphate only from PP. The other enzymes were less specific, but they released Ins6P and RNA at different rates. The poor specificity of AcPR could be due to an impurity of the preparation. The fact that PhyN is able to hydrolyze RNA to an extent of 86.9% is also an indication that impurities are present. The phosphate release from DNA was on average about 10%. This phenomenon could be explained by the fact that monoesterases are able to hydrolyze just the 5' and 3' phosphate residues (Gasmi *et al.* 1991), while the other phosphate groups are not accessible. None of the enzymes triggered P release from the studied phosphonate.

All enzymes tested show a high release of at least one substrate tested. Thus, they are all good candidates for the tests on soil extracts and soil suspensions. The release of PP from solutions can be determined directly with the PyPS. For the enzymes that hydrolyze more than one chemical class, the release of phosphate from soil has to be calculated by measuring the difference in the phosphate release resulting from two or more enzymes. In this way we will be able to calculate the amount of Ins6P available to enzymes by comparing the results from AcPR, PhyN or PhyB with AlPS, AlPR and AcPS. Phosphate released from DNA would be calculated in the same way (Feuillade and Dorioz 1992). Therefore it is important to add a phosphodiesterase to the enzyme set. The further characterization (kinetic properties, inhibition and inactivation) will be made on soil samples. The isoelectric point will be a tool to determine the reason for a low enzymatic activity in soil suspensions, where several factors have to be considered. For example, adsorption on clay surfaces and the presence of proteases play a role in terms of stability and in the ability of an enzyme to hydrolyze organic P compounds in soil (Nannipieri *et al.* 1996).

The use of enzymes with different degrees of substrate specificity and the combination of some of these enzymes enables the characterization of the hydrolysable amount of organic P in the soil. Together with the additional information collected for the single enzymes a better understanding of the role of enzymes in the soil can be obtained.

References

Bünemann EK (2008) Enzyme additions as a tool to assess the potential bioavailability of organically bound nutrients. *Soil Biology & Biochemistry* **40,** 2116-2129.

Feuillade M, Dorioz JM (1992) Enzymatic release of phosphate in sediments of various origins. *Water Research* **26,** 1195-1201.

Gasmi G, Pasdeloup M, Pratviel G, Pitié M, Bernadou J, Meurier B (1991) 31P NMR characterization of

terminal phosphates induced on DNA by the artificial nuclease 'Mn-TMPyP/KHSO5' in comparison with DNases I and II. *Nucleic Acids Research* **19,** 2835-2839.
George TS, Simpson RJ, Gregory PJ, Richardson AE (2007) Differential interaction of Aspergillus niger and Peniophora lycii phytases with soil particles affects the hydrolysis of inositol phosphates. *Soil Biology & Biochemistry* **39,** 793-803.
Hayes JE, Richardson AE, Simpson RJ (2000) Components of organic phosphorus in soil extracts that are hydrolysed by phytase and acid phosphatase. *Biology and Fertility of Soils* **32,** 279-286.
Jackman RH, Black CA (1952) Hydrolysis of Phytate Phosphorus in Soils. *Soil Science* **73,** 167-172.
Nannipieri P, Sesqui P, Fusi P (1996) Humus and enzyme activity. In 'Humic substances in terrestrial ecosystems'. (Ed A Piccolo) pp. 293-328.
Ohno T, Zibilske LM (1991) Determination of low concentrations of phosphorus in soil extracts using malachite green. *Soil Science Society of America Journal* **55,** 892-895.
Shand CA, Smith S (1997) Enzymatic release of phosphate from model substrates and P compounds in soil solution from a peaty podzol. *Biology and Fertility of Soils* **24,** 183-187.
Turner BL, Mahieu N, Condron LM (2003) The phosphorus composition of temperate pasture soils determined by NaOH-EDTA extraction and solution P-31 NMR spectroscopy. *Organic Geochemistry* **34,** 1199-1210.
Turner BL, McKelvie ID, Haygarth PM (2002) Characterisation of water-extractable soil organic phosphorus by phosphatase hydrolysis. *Soil Biology & Biochemistry* **34,** 27-35.

Effect of rice straw application on hydrolytic enzyme activities in Chinese paddy soils

Beke Gredner[A] and Rolf Tippkötter[A]

[A]Institute of Soils Science, University of Bremen, Germany, Email: bgredner@uni-bremen.de

Abstract
The cultivation of rice (*Oryza sativa* L.) produces large quantities of straw. Although the burning of straw is illegal it is a commonly used method of rice straw management in China, causing a series of problems. An alternative is the incorporation of rice straw into the soil.
In this study different rice straw management practices were simulated in a microcosm experiment under controlled conditions in a climate chamber. The decomposition of incorporated straw and straw ash was determined by monitoring the hydrolysis of polysaccharides by extracellular enzyme activities. As a result, the actual hydrolytic activity, the cellulose-induced activity as well as the xylan-induced activity caused by rice straw decomposition indicate significant differences between two investigated soils of the regions Wujia and Jinjiaba (Jiangsu Province, China).

Key Words
Soil enzymes, rice straw management, paddy soils, microcosms

Introduction

The cultivation of rice produces large quantities of straw, as an agricultural waste, ranging from 2 - 9 t/ha world wide. In many countries the traditional management practice of post harvest rice residues is the elimination of straw by open-air burning (Becker *et al*. 1994; Cao *et al*. 2008). Although this includes advantages such as disease and pest control (Ponnamperuma 1984), disadvantages related to the release of greenhouse gases and the production of particulate matter are obvious (Cao *et al*. 2008). Therefore, as an alternative to burning of straw the incorporation into the soil stands to reason, which has been widely accepted (Cassman and Pingali 1995), although it may reduce yields (Cassman *et al*. 1995). In contrast to straw of wheat or barley the main components of rice straw are cellulose and hemicellulose, encrusted by lignin in addition to only small amounts of proteins. Consequently, it is very resistant to microbial decomposition compared to other cereals (Parr *et al*. 1992).

The soil enzyme activities are supposed to be direct expressions of the soil microbial community which is required for the metabolic transformation of the organic substances added to the soil and the nutrients supplied by the soil itself (Caldwell 2005). Since decomposition of plant residues is accompanied by extracellular enzymes such as cellulases and xylanases, the activity of these can act as a reliable indicator of decomposition (Stemmer *et al*. 1999). In addition, several studies have shown that the enzyme activity can be used as an early indicator for changes in soil soil properties initiated by soil management practices (e.g. Dick 1992; Lal *et al*. 1998).

The intension of this research is to evaluate the effect of rice straw and straw ash in paddy soils of different texture on the activities of hydrolytic enzymes, cellulases and xylanases. The study was carried out under controlled conditions in a microcosm experiment according to Eickhorst and Tippkötter (2009).

Materials and Methods

Soils
The microcosm experiment was set up in a climate chamber with two different soils from the Jiangsu Province, China: a. Gleyic Fluvisol (Wujia, WU), and b. Stagnic Anthrosol (Jinjiaba, JI). The soils were analysed for selected physical and chemical characteristics, presented in Table 1. Samples were incubated in microcosms (15 cm in diameter, height 25 cm), filled with an artificial plough pan of about 3 cm thickness (Db 1.4-1.6 g/cm³) and a puddled Ap-horizon of 14 cm height. Climatic conditions were simulated according to the mean temperatures of the original location during the rice growing period with a night to day ratio of 13.5 to 10.5 hours.

Table 1. Physical and chemical properties of the investigated soils.

Location	Texture (WRB)	Sand [%]	Silt	Clay	pH ($CaCl_2$)	EC [µS/cm]	C_t [%]	C:N
Wujia	Silty loam	27.1	64.2	8.7	6.3	268	1.23	11.1
Jinjiaba	Silty clay	0.9	58.1	41.0	5.1	418	1.88	10.9

Plants and straw application
The two soils were planted with irrigated short period rice (*Oryza sativa* L., type *Japonica*, cultivar 中选 181 (Zhong Xuan 181), vegetation period 90 days after transplanting (DAT), two plants per microcosm).
Four scenarios of straw application have been chosen:

A	Rice	Rice straw	8 t/ha dry matter
B	No plants	Rice straw	8 t/ha dry matter
C	Rice	Rice straw ash	8 t/ha straw dry matter
D	Rice	No plant residues	

Plant residues were incubated directly before transplanting of rice to simulate intensive cropping. Fertilization was held at equal levels for all variants. The soils were under submerged conditions until DAT 112, and subsequently drained for two weeks.

Sample collection
Soil samples for the analysis of enzymes were taken from the whole soil profile randomly and analysed photometrical for a) actual hydrolytic activity (act. hyd.) according to Boschker *et al*. (1995) with modifications as incubation temperature (40°C) and time (16 hours) are concerned, b) cellulose-induced activity (cell.-ind.) according to Hope and Burns (1987) and c) xylan-induced activity (xyl.-ind.) similar to the method of cellulose-induced activity (according to Hope and Burns (1987); modified). The hydrolysis under actual and substrate-saturated conditions was measured by the release of monosaccharides after inhibition of microbial uptake with ~~a~~ the determination of the produced reducing sugars according to Lever (1973).
The frequency and time of sampling of the enzymes was due to the plant development stages with a high sampling density at the beginning and the end of the vegetation period.

Statistical analysis
Statistical analysis was performed using SPSS software (SPSS Inc., 2008). All data were expressed as mean values including the standard deviation. Statistical significance of the treatment effects was established by the analysis of variance (ANOVA; Kruskal-Wallis) and the comparison of means. Levels of probability were 5% (*), 1% (**) and 0.1% (***), respectively.

Results
Enzyme activities, expressed as mg glucose produced per g soil (dry matter) and hour are presented in Figure 1. The results of the analysis of variance are given in Table 2.
The actual hydrolytic activity showed maximum values during the first week after transplanting with highest values in the straw incubated variants (A, B). From DAT 21 on the values decreased and stayed at a constant level until harvest. Over the vegetation period the actual hydrolytic activity of JI (clayey soil) was higher than WU (sandy soil).
The cellulose-induced activities in the soil WU showed significant strong correlations to the time, but there was no influence of the straw treatment. In contrast, the soil JI showed significant differences between the variants but not with the time. The cellulose-induced activities of both soils were not subjected to significant fluctuations during the vegetation period. The xylan-induced activities were similar to the cellulose-induced activities, but resulted in higher values of activity, especially of the straw treated variants (A, B). Differing from the other enzyme activities, the xylan-induced activity was similar in both investigated soils.

Table 2. Results of the analysis of variance. Effect of the investigated soils, the variants of treatment, and the days after transplanting on the enzyme activities.

Location		Act.hyd.	Cell.-ind.	Xyl.-ind.
	Soils	***	***	n.s.
Wujia	Variant	n.s.	n.s.	n.s.
	DAT	n.s.	***	***
Jinjiaba	Variant	n.s.	**	***
	DAT	**	n.s.	n.s.

(DAT: days after transplanting, Act.hyd.: actual hydrolytic activity, Cell.-ind.: cellulose-induced activity, Xyl.-ind.: xylan-induced activity; n.s.: not significant, *: significant with p = 0.05, **: significant with p = 0.01, ***: significant with p = 0.001)

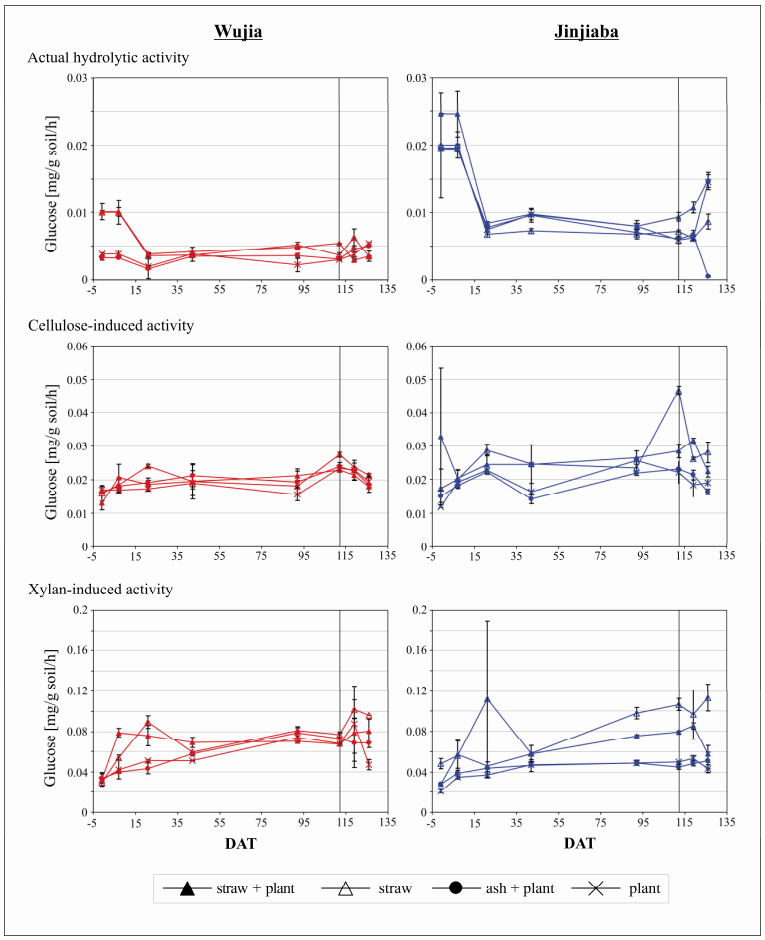

Figure 1. Results of investigated enzyme activities during the vegetation period.
(DAT: days after transplanting)

Discussion

The actual release of reducing sugars was increased by the addition of rice straw to soil especially in the silty clay. This is reasonable since rice straw is a natural substrate for microorganisms in rice field soil (Stemmer *et al*. 1999) and is presumably caused by the presence of easily decomposable carbohydrates. From DAT 21 on there was no difference between the diverse straw treatments in both soils and the decomposition of polysaccharides was apparently limited by the availability of carbohydrates. Constant levels of carbohydrates were presumably accessible and the remaining were subjected to only little changes during the vegetation period.

The cellulose and xylan induces activities displayed higher values compared to the actually hydrolytic activity. This presumably indicates the decomposition of polysaccharides was limited rather by the accessibility of substrate than by the availability of enzyme activity. Hence, for the sandy soil WU an impact of incorporated straw on the enzyme activity could not be detected, but the clayey soil JI presented a strong correlation to the application of straw.

The gradually increasing xylan-induced activity of JI is in accordance to Stemmer *et al*. (1999), who found that straw amendment can stimulate enzyme activities a microbial biomass with varying effects for different enzymes.

Differences of enzyme activities between the both soils occur presumably because of many factors, including texture, organic matter content, and nutrient composition of the soils. Hence may free enzymes complex with humic colloids and may be stabilized on clay surfaces and organic matter, especially in the clayey soil (JI), which results in higher activities.

Conclusion

The incorporation of rice straw or straw ash into paddy soils revealed texture dependent differences of enzyme activities. Straw induced effects are only detectable in the clayey soil. The reaction of the enzymes to the diverse treatments (addition of straw, straw ash, planting) is different.
The varying response of the investigated enzyme activities showed that the enzymes are influenced by various soil properties and therefore are not qualified as early indicators for management induced changes in soil properties.

References

Becker M, Ladha JK, Ottow JCG (1994) Nitrogen losses and lowland rice yield as affected by residue nitrogen release. *Soil Science Society of America Journal* **58**, 1660-1665.

Boschker HTS, Bertilsson SA, Dekkers EMJ, Cappenberg TE (1995) An inhibitor-based method to measure initial decomposition of naturally occurring polysaccharides in sediments. *Applied and Environmental Microbiology* **61**(6), 2186-2192.

Caldwell BA (2005) Enzyme activities as a component of soil biodiversitiy: A review. *Pedobiologia* **49**, 637-644.

Cao GL, Zhang XY, Wang YQ, Zheng FC (2008) Estimation of emissions from field burning of crop straw in China. *Chinese Science Bulletin* **53**(5), 784-790.

Cassman KG, De Datta SK, Olk DC, Alcantara J, Gamson M, Descalsota J, Dizon M (1995) Yield decline and nitrogen economy of long-term experiments on continuous irrigated rice systems in the tropics. In 'Soil Management: Experimental Basis for Sustainability and Environmental Quality'. (Eds R Lal, BA Stewart) pp. 181-222. (CRC Press, Boca Raton, Florida).

Cassman KG, Pingali PL (1995) Intensification of irrigated rice systems learning from the past to meet future challenges. *Geo Journal* **35**, 299-305.

Eickhorst T, Tippkötter R (2009) Management-induced structural dynamics in paddy soils of South East China simulated in microcosms. *Soil and Tillage Research* **102**, 168-178.

Glissmann K, Conrad R (2002) Saccharolytic activity and its role as a limiting step in methane formation during the anaerobic degradation of rice straw in rice paddy soils. *Biology and Fertility of Soils* **35**, 62-67.

Hope CFA, Burns RG (1987) Activity, origins and location of cellulases in a silt loam soil. *Biology and Fertility of Soils* **5**, 164-170.

Lever M (1973) Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. *Biochemical Medicine* **7**, 274-281.

Parr JF, Papendick RI, Hornick SB, Meyer RE (1992) Soil quality: attributes and relationships to alternative and sustainable agriculture. *American Journal of Alternative Agriculture* **7**, 5-11.

Ponnamperuma FN (1984) Straw as a source of nutrients for wetland rice. In 'Organic matter and rice'. (IRRI) pp. 117-137. (IRRI, Los Banos, Philippines).

Stemmer M, Gerzabek MH, Kandeler E (1999) Invertase and xylanase activity of bulk soil and particle-size fractions during maize straw decomposition. *Soil Biology and Biochemistry* **31**, 9-18.

Enzyme activity and adaptation in dry soil

Joshua Schimel[A], Claudia Boot[A], Patricia Holden[B], Dad Roux-Michollet[B], Sophie Parker[A], Sean Schaeffer[A], and Kathleen Treseder[C]

[A]Dept. Ecology, Evolution, and Marine Biology. University of California Santa Barbara. Santa Barbara, CA 93106. USA. Schimel@lifesci.ucsb.edu
[B]Bren School of Environmental Science and Management. University of California Santa Barbara. Santa Barbara, CA 93106. USA.
[C]Dept. Ecology and Evolutionary Biology. University of California Irvine. Irvine, CA 92697. USA.

Abstract
At least 1/3 Earth's land experiences regular drought, and climate models suggest this will increase. However, the biological processes occurring during the dry season have only been studied by inference from what happens when the rains return. Important dry soil phenomena remain unexplained, such as the "Birch Effect"--the pulse of respiration on rewetting a dry soil. Important and surprising processes occur during the dry season. For example, during the California summer, in grasslands, soils are dry and plants are dead, but the biomass and population size of several important groups of microorganisms increase, even though their activity is very limited. These changes appear to result from a combination of microbial drought survival physiology, disconnections in soil water films in dry soil as well as limited substrate diffusion and organismmovement. This talk will discuss the current state of knowledge on microbial drought and dry/wet cycle dynamics.

Keywords
Drought, biogeochemistry, California, grassland.

Introduction
The world is a dry place: roughly 1/3 of the terrestrial land surface has arid, semi-arid, or Mediterranean climates that are characterized by long droughts. Climate models also suggest that drought is likely to become more prevalent with climate warming. However, the biogeochemistry of the dry season has usually been studied only implicitly– as "antecedent conditions" that regulate the pulses of biological activity that occur with the early rains or the chemical characteristics of streamflow. However, rarely have the biogeochemical processes that occur during the dry season been studied explicitly to understand what creates the conditions at the beginning of the wet season.

In California, summer can go 6 months without any rain. During the summer, temperatures can exceed 40° C. It has always been assumed that the dry season was a period of dormancy and mere survival: native grasses senesce, some native shrubs may shed their leaves, and microbial respiration rates drop to levels of 0.1 to 0.3 g $C/m^2/d$ as soils dry to as low as 5% H_2O (Xu *et al.* 2004).

Surprisingly, however, over the summer, microbial biomass increases (Figure 1a; Parker 2006) as do the potentials for nitrification and denitrification (Figure 1d) and even denitrification potentials more than doubled (Figure 1e). These surprising results beg an explanation. Why, at a time when activities are lowest and conditions appear worst, does it appear that many groups of organisms are doing best?

We hypothesized that these surprising summertime dynamics result from two micro-scale phenomena: a) the physiology of microbial drought survival and b) the hydrological disconnectivity of the "microbial landscape." As soils dry, microbes experience direct physiological stress, resource limitation from drying, and hydrological disconnections in their environment. On the other hand, microbes may experience reduced predation pressure (Gorres *et al.* 1999) because microbial predators also rely on a connected landscape for foraging. As water potentials decline, cells must accumulate solutes to reduce their internal water potential to avoid dehydrating and dying. As their primary osmolytes, microorganisms are thought to use simple organics as osmotic agents. In culture, bacteria have been shown to use amino compounds such as proline, glutamine, and glycine betaine (Csonka 1989), while fungi use polyols such as glycerol, erythritol, and mannitol (Witteveen and Visser 1995). Although bacteria are able to accumulate K^+, they only do this after they have exhausted their ability to synthesize or take up preferred compounds (Killham and Firestone 1984).

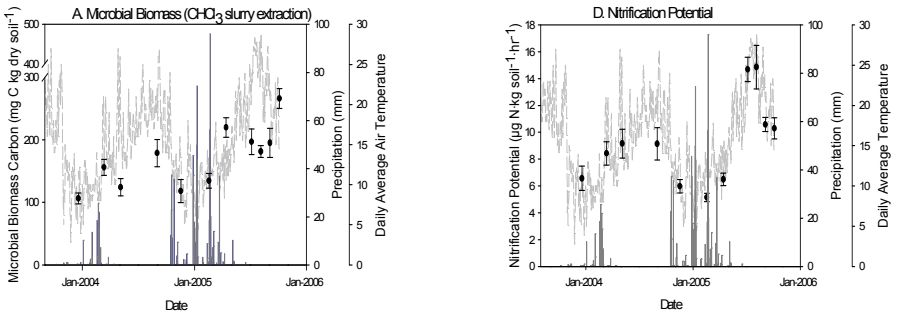

Figure 1. Microbial biomass and nitrification potential during dry and wet periods in a grassland in California.

Accumulating osmolytes however, is energetically expensive. Bacteria can accumulate amino acids to between 7 and 20% of total bacterial C (Killham and Firestone 1984) and between 11 and 30% of bacterial N. In fungi, polyols can account for over 10% of cell mass (Tibbett *et al.* 2002). When extrapolated to an ecosystem scale, the amounts are large. For example, in a grassland soil, osmolyte production to survive a single drought event could conservatively account for 20 g C/m^2, compared to an NPP in the range of 300 - 600 g $C/m^2/y$. The proportional values for N are larger, 0.75 g N/m^2 or more, equivalent to 10-40% of annual net N mineralization.

If summers are stressful, however, it is thought that the rewetting in the fall could be even more damaging, causing up to 50% mortality (Kieft *et al.* 1987). This is in line with the "Birch Effect," the flush of respiration and mineralization on rewetting a dry soil.

In our research, we have explored the dynamics of dry season biogeochemistry, with specific questions being:

What are the changes in microbial populations and processes through the dry summer?
How important are these dynamics in annual C and N cycles?
What mechanisms are responsible for these changes?
What happens on rewetting?
What are the physical and biological mechanisms that regulate drying/rewetting dynamics?

Materials and Methods

Our core research site is at the Sedgwick Reserve in the Santa Ynez Valley of Central California. This is an area with a Mediterranean Climate—cool wet winters and hot dry summers. The soils are Mollisols, typically argixerolls, with pachic argixerolls dominating on valley floors. The vegetation is a mix of open annual grassland, dominated by Mediterranean invasive species dominated by *Bromus diandrus* and *Avena fatua.*

We measured biogeochemical parameters by regular soil sampling throughout several summers. Soil cores were collected to 20 cm depth and returned to the laboratory for analysis. Microbial biomass was measured by a $CHCl_3$ slurry method (Fierer and Schimel 2003). Mineralization potentials were measured by sealing jars and measuring headspace CO_2 accumulation; periodically samples are harvested and analyzed for NH_4^+ and NO_3^-. Nitrification potentials were measured by chlorate slurry (Belser and Mays 1980). Cellular amino acids (osmolytes) were analyzed by HPLC on the $CHCl_3$ extracts. *In situ* fungal growth was measured using minirhizotrons with a microscopic camera and image analysis to evaluate the turnover of individual fungal hyphae. Drying/Rewetting experiments were done in the laboratory with soil samples in canning jars. Soils were allowed to air dry for varying periods of time and then were rapidly rewet.

Results and Discussion

While in situ respiration rates are minimal during the dry summer (data not shown), all indices of microbial biomass and potential are typically highest at the end of the dry season; these include microbial biomass, short-term respiration potential, and nitrification potential. Fungal growth is slow during the summer, averaging < 2 new hyphae/cm^2 /month. Certain bacterial populations, notably proteobacteria, on the other hand, decline strongly with the onset of summer. Pools of NH_4^+ and extractable organic C (EOC) increase through the summer but then decline with the first rains of autumn, the NH_4^+ rapidly being nitrified (Figure 2).

We postulate that NH_4^+ and EOC pools increase because some exo-enzymatic and microbial processes continue in thin water films even in dry soils, but that diffusion is so limited that these materials remain unavailable until soils wet up. What remains unclear is why overall microbial populations increase during the dry summer. We hypothesize that this is because bacteria and fungi that survive the initial dry-down are drought tolerant and so are able to maintain low rates of activity and growth. Predation by protozoa and other microfauna, on the other hand, should be even more sensitive to moisture than is microbial growth. Protozoa require water-filled pores to forage. Thus, in a dry soil, death rates due to predation may decline even more extremely than do growth rates, allowing populations to increase.

We measured the *in situ* concentrations of cellular amino acids throughout the year, anticipating that concentrations of known amino acid osmolytes (proline and glutamate) would increase over the summer. In fact, proline was never measurable, while glutamate remained a relatively constant proportion of the total microbial biomass throughout the year, changing little between summer and winter. Thus, amino acids do not appear to be used as osmolytes in this microbial community. We are measuring other possible compounds, but it also remains possible that in a natural soil, where C is a limiting resource, that microbes are forced to rely on inorganic osmolytes or that a large fraction of the community uses glutamate as a constitutive osmolyte.

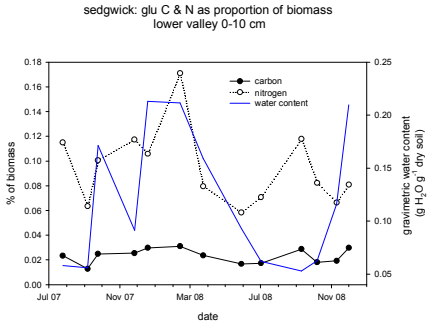

Figure 2: Pools of NH_4^+ and extractable organic C during dry and wet seasons in a grassland in California.

When dry soils are finally rewet, there is a large flush of respiration. An isotope equilibration experiment, in which ^{14}C-glucose is added to soil and taken up by the microbial biomass prior to dry-down and rewet, indicated that the CO_2 released is mainly derived from microbial material, although a substantial amount of extractable organic C was also released (Fierer and Schimel 2003). However, in a number of studies, we have found that through multiple dry-wet cycles microbial biomass does not decline, and may actually increase dramatically (Xiang *et al*. 2008). Additionally, through multiple dry-rewet cycles, more CO_2 may be released than was present in the biomass. Thus, while the C released in a single dry-rewet cycle may be dominated by microbial material, over multiple cycles, the C must be released by physical processes, such as aggregate disruption, desorption, and diffusion of otherwise unavailable material to microbes.

Thus, these results raise some conundrums that are difficult to reconcile: the apparent lack of identifiable organic osmolytes, the apparent microbial source for CO_2 respired in the rewetting flush, and in multiple-cycle it is soil organic matter that fuels successive rewetting pulses. Our current working hypothesis to tie together these different results is that physical and biological processes are closely coupled through multiple dry-rewet cycles.

We hypothesize (Fiugre 3) that during drought, several critical processes occur: 1) microbes accumulate cellular materials that may be respired on rewetting, and 2) desorption, exoenzymes, and microbial turnover produce a pool of easily respired material that accumulates because of diffusion limitation.
On rewetting, several processes occur: 1) microbes respire part of the cellular material, 2) the accumulated soil nutrients becomes bioavailable and is rapidly metabolized, and 3) mass rewetting redistributes organic materials throughout the soil, overcoming diffusion limitation, and 4) desorption and aggregate disruption release an additional fraction of otherwise unavailable soil organic matter. The newly-available resources are used by microbes and accumulate as cellular materials as a new drying cycle begins. Thus, while it is physical processes that ultimately drive C from the soil through multiple dry-rewet cycles, these are proximally mediated by microbial processes associated with stress tolerance and the release from stress.

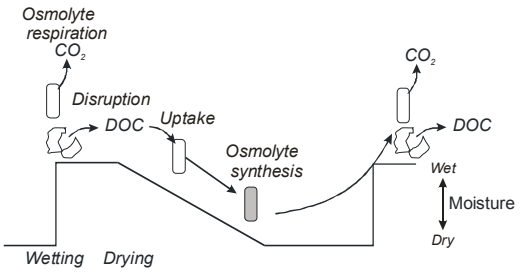

Figure 3. Hypothetical processes during drought and rewetting.

References

Belser LW, Mays EW (1980) Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. *Applied and Environmental Microbiology* **39**, 505–510.

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Review* **53**, 121-147.

Fierer N, Schimel JP (2003) A proposed mechanism for the pulse in carbon dioxide production commonly observed following the rapid rewetting of a dry soil. *Soil Science Society of America Journal* **67**, 798-805.

Gorres JH, Savin MC, Neher DA, Weicht TR, Amador JA (1999) Grazing in a porous environment: 1. The effect of soil pore structure on C and N mineralization. *Plant and Soil* **212**, 75-83.

Kieft TL, Soroker E, Firestone MK (1987) Microbial biomass response to a rapid increase in water potential when dry soil is wetted. *Soil Biology and Biochemistry* **19**, 119-126.

Killham K, Firestone MK (1984) Salt stress control of intracellular solutes in *Streptomycetes* indigenous to saline soils. *Applied and Environmental Microbiology* **47**, 301-306.

Parker S (2006) PhD Dissertation. University of California Santa Barbara.

Tibbett M, Sanders FE, Cairney JWG (2002) Low-temperature-induced changes in trehalose, mannitol and arabitol associated with enhanced tolerance to freezing in ectomycorrhizal basidiomycetes (*Hebeloma* spp.). *Mycorrhiza* **12**, 249-255.

Witteveen CFB, Visser J (1995) Polyol Pools In Aspergillus-Niger. *FEMS Microbiology Letters* **134**, 57-62.

Xiang S-R, Doyle A, Holden PA, Schimel JP (2008) Drying and rewetting effects on C and N mineralization and microbial activity in surface and subsurface California grassland soils. *Soil Biology and Biochemistry* **40**, 2281-2289.

Xu LK, Baldocchi DD, Tang JW (2004) How soil moisture, rain pulses, and growth alter the response of ecosystem respiration to temperature. *Global Biogeochemical Cycles* **18**, GB4002.

Glomalin-related soil protein influence on soil aggregate stability in soils of cultivated areas and secondary forests from Northern Thailand.

Suphathida Aumtong [A], Pongsuda Sirinikorn, Pithawat Susingsa , Nonglux Maungjai

[A]Agricultural Production Faculty, Maejo University, Chiangmai, Thailand 50290, supathida@mju.ac.th

Abstract
Types of land use and soil cultivation are important to maintain soil fertility. This project aimed to identify the relationships between glomalin – related soil protein contents and aggregate stability of various land uses from mountainous areas in Northern Thailand. Soil aggregate and glomalin-related soil protein components responded to land use intensity and landscape characteristics. Land use type with shifting cultivation that occurrs in mountainous areas showed the highest aggregate stability and amount of glomalin-related soil protein, due to lack of tillage and least disturbing managements. This study found positive relationship between soil aggregate stability and easily extractable glomalin content.

Key Words
Glomalin-related soil protein, soil aggregate stability, Northern Thailand.

Introduction
The mountain areas of Northern Thailand are considered the origin of important natural resources. The prevailing land management practices of the ethnic tribes in the sloping areas, mainly shifting cultivation and zero tillage made it appear that the land was undisturbed (Aumtong *et al.* 2009). Glomalin-related soil protein component is produced by arbuscular mychorrrihza (Rillig 2004;Wright *et al.* 1998), and as a stable glue the hyphae (Wright and Upudhyaya 1998) has an important role in soil aggregate stabilization. Wright and Anderson (2000) indicated that glomalin produced from some crop rotation cropping system could promote aggregate stability. The quantification of glomalin can be divided into two fractions; first, easily extractable glomalin and second, total glomalin. Both of them show different responses to land use change (Wright and Upadhyaya 1998). Wright *et al.* (1999) indicated that arbuscular mycorrhiza (AM) fungi have been related to aggregate formation and stability. Arbuscular mycorrhizal fungi (AMF) occur in the soil of most ecosystems, including polluted soils. AMF form symbiotic networks with host plant roots. The fungi scavenge nutrients from soils and transfer these nutrients to the host plant in exchange for carbohydrates. Host plants rely upon mycorrhizal fungi to acquire nutrients such as phosphorus and nitrogen for growth. The screening of native host plants e.g. weed, shrub and tree in various ecosystems could be done. The benefit from plant hosts at a practical level could then be as a cover crop in agro-ecosystems or agro- forestry systems.

Because AMF infect about 80 % of vascular host plant species and are found in almost every soil type, glomalin is detected in large amounts in numerous soils. Glomalin is defined as organic substance glycoprotein copiously produced by all AMF, measured operationally in soils as glomalin-related soil protein (GRSP) (Rillig 2004). Glomalin is firmly incorporated into the hyphae and spore wall in large amounts (Diver *et al.* 2005) and is highly positively correlated with soil aggregate stability (Wright and Upadhyaha 1998). However, the relationship between the content of glomalin and other soil properties should be studied. The objective of this project is to identify the relationships between glomalin –related soil protein contents and aggregate stability for various land uses in a mountainous area of Northern Thailand.

Methods
The study was carried out in Khiud Chang Watershed (KCW), Ban Pong and Maejo in Chiangmai Province, Northern Thailand. The mean annual rainfall of this area from 1998 to 2009 was about 1200 mm. The mean monthly air temperature was about 29°C. Being mountainous, it has an undulating landscape. KCW is a tributary of Mae Nam Mae Rim watershed and is located in an area that has an elevation of 300 to 1,100 meters above sea level (ASL).

Land uses
From each site, a composite soil sample of 8-10 subsamples was collected from each land-use. Samples were taken at a depth of 0-15 cm at 39 sites.

Table 1 shows the characteristics of the main land uses in the area.

Land use	Abbrevation	Number of replications	Characteristics
Secondary conservation forests	(SCF)	4	15 years old, much of the plant cover is bamboo and has nearly 50 % closed canopy. Some villagers harvest nontimber forest products (NTFP's).
Fruit tree plantations	(FTP)	12	mostly longan, mango and lychee (*Litchi chinensis)*, which are now 7 to 30 years old.
Fallow areas	(FA)	4	covered with bush and grass; free of agricultural activities for 1- 4 years.
Afforestated areas	(Teak)	4	young 15 year old teak (*Tectona grandis*) areas.
Wetland rice (Paddy soil)	(PAD)	4	a practice that started about 30-years ago, wetland rice is grown using machine tillage in soil preparation.
Upland rice	(UPR)	3	planted upland rice for home consumption, and maize for livestock feed, annually or rotated.
Vegetable	(VEC)	8	planted vegetable and ornamental crop for selling and consumption, high input of chemical fertilizer and frequency of land use.

Glomalin related soil protein analysis

Easily-extractable and total glomalin related soil protein were extracted by procedure adjusted from Wright and Upadhyaya (1998). One gram of air dried soil was placed into a 50 ml centrifuge tube and mixed with 8 mL of 50 mM sodium citrate buffer (pH = 8), then samples were autoclaved for 30 minutes. The supernatant was collected by centrifugation at 2510 g for 15 minutes. Total glomalin related soil protein was extracted with 8 mL of 50 mM sodium citrate buffer (pH = 8), then samples were autoclaved for 90 minutes. The supernatant was determined for related- soil protein as glomalin by the Bradford assay with bovine serum albumin standard.

Aggregate stability

The soil was transferred along with the filter paper into an aluminum can and dried at 105 °C for 24 hours. The oven-dry soil aggregates (5 g) were transferred into the wet sieving apparatus. The dispersing agent of 10 ml of 5 percent solution of sodium hexametaphosphate and enough distilled water were added to fill the cup to within 4 cm of the rim, and then the suspension was stirred for 5 minutes. Aggregate stability is the amount of aggregated soil > 0.25 mm remaining after 5 min of wet sieving (Kemper and Rosenau 1986).

Soil organic carbon (SOC)

Soil organic carbon content was determined after sieving (0.5 mm) by the wet combustion method described in Nelson and Sommers (1986).

Statistical analysis

The amounts of glomalin, SOC and aggregate stability differed among land-use types were analyzed by one way ANOVA. Evaluation of the relationships between glomalin and other soil indicators was by regression analysis.

Results

The results showed that the amount of total glomalin was lowest in paddy soils or soils that were intensively disturbed by tillage (Table 2.). The tillage practice caused loss of soil organic matter leading to low soil biological activity. Microbial activity could include mychorrhizal fungi activity. Meanwhile, the upland soil planted with rice or less cultivated and disturbed than other arable soils, showed high amounts of easily extractable glomalin and total glomalin. Wright and Anderson (2000) indicated that glomalin changes quickly in response to tillage practices and crop rotation. The basic method for planting field crops such as corn for animal feed and upland rice, involved dropping seeds directly into a hole in the soil. These practices seen as economical in terms of seeds, convenient in terms of the sloping land characteristics, were an important local wisdom to manage production factors, and may be a means to protect and maintain soil resources besides preventing soil erosion. From the high content of glomalin upland rice, the upland rice plantation could be considered as a non-tillage practice for mountainous area in Northern Thailand, and could be included in the shifting cultivation cropping system. Crop rotation and non-tillage could be found in

this area. The transformation of agricultural land (e.g. upland rice) to fallow area and secondary forest is the pattern of land use change in shifting cultivation. This pattern agrees with the diversity of host plants that produce glomalin. This study found a significant positive relationship between soil aggregate stability and easily extractable glomalin content (Figure 1).

Table 2. Land use types affected on amount of easily-extractable glomalin and total glomalin related soil protein, soil organic carbon and aggregate stability for soils from a mountainous area northern Thailand.

Land use (see Table 1)	Number of samples	Easily-extractable glomalin	Total glomalin	SOC (%)	Aggregate Stability (%)
		(mg/kg)			
VEC	8	6.16 cd	7.16 b	1.36 cd	29.69 cde
PAD	4	5.70 cd	6.30 b	1.55 bcd	21.35 e
UR	3	8.77 a	11.22 a	1.73 bc	51.75 b
FT	12	7.31 b	10.37 a	1.62 bc	35.51 c
Teak	4	5.65 cd	7.63 b	1.11 d	28.13 cde
FA	4	6.81 bc	8.19 b	2.04 b	34.20 cd
SCF	4	7.96 ab	10.81 a	4.31 a	78.16 a

For each variable different letters are indicative of statistical differences (P<0.05)

Conclusion

The level of disturbed land uses affected the soil quality due to the decreasing soil organic carbon and amount of glomalin related soil protein. These fractions could be related to soil aggregate stability. The land use types minimally disturb soils, like upland rice cultivation in a mountainous landscape, had the highest amount of easily extractable and total glomalin. Meanwhile, the land use of the highly accessible lowland had the lowest glomalin contents of soils which showed positive relationships between soil aggregate stability and easily extractable glomalin content.

References

Aumtong S,Sirinikorn P,Susingsa P,Maungjai N (2009) Relationship between glomalin and aggregtae stability of various land use.The final report; Agricultural Production Faculty, Maejo University Thailand,11 pp.

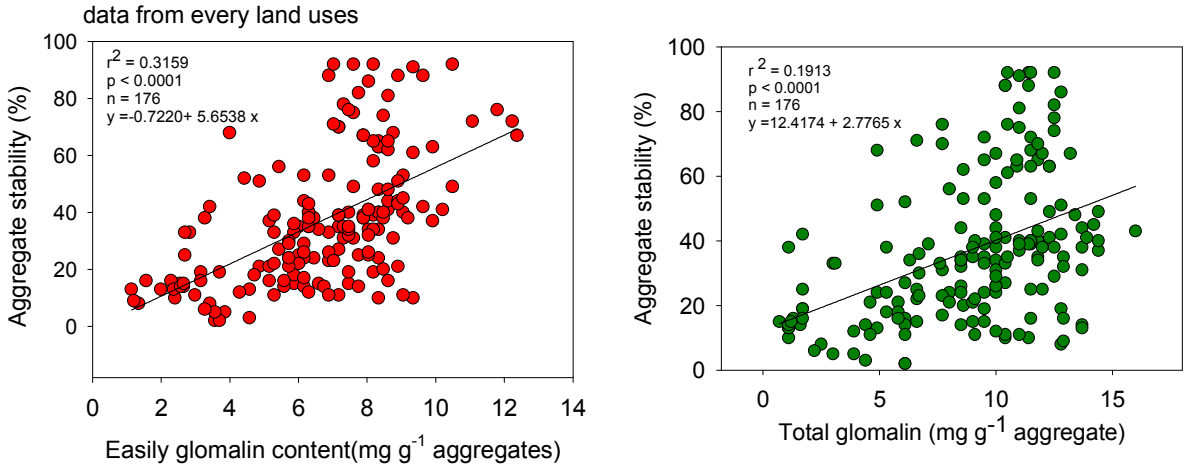

Figure 1. Relationship between easily extractable, total glomalin and stability of 1-2 mm size aggregates in 0-15 cm soil samples for a variety of land uses in Chiang Mai province, Northern Thailand.

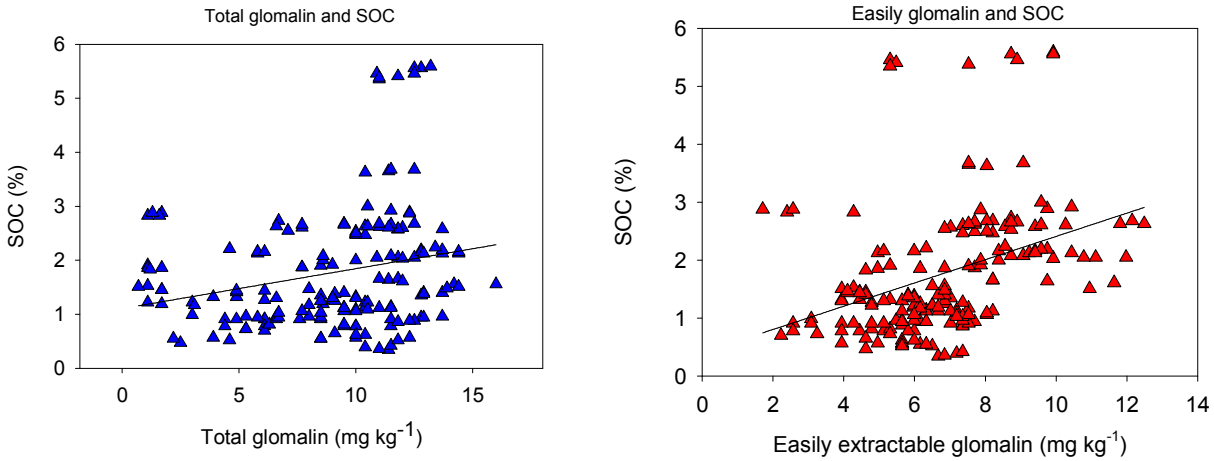

Figure 2. Relationship between easily extractable glomalin, total glomalin and SOC in 0-15 cm soil samples for a variety of land uses in Chiang Mai province , Northern Thailand.

Reference

Driver JC, Holben WE, Rillig MC (2005) Characteristic of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. *Soil Biological Biochemistry* **37**,101-106.

Kemper, WD, Rosenau RC (1986) Aggregate stability and size distribution.. In 'Methods of soil analysis. Part1' 2nd ed. SSSA Book Ser. 5 (Ed A Klute) pp. 425–442. (SSSA,Madison,WI.).

Nelson DW, Sommers LE (1986) Total C, organic C and organic matter. In 'Methods of Soil Analysis' (Ed AL Page) (Soil Science Society of American Journal, Madison, Wisconsin)

Rillig MC (2004) Arbuscular mycorrhizae and terrestrial ecosystem process. *Ecology Letter* **7**, 740-750.

Wright SF, Upadhyaya A (1998) A survey of soil for aggregates stability and glomalin, a gylcoprotein produced by hyphea of arbuscular mycorrhizal fungi. *Plant and Soil* **198**, 97-107.

Wright SF, Starr JL, Paltineanu IC (1999) Changes in aggregate stability and concentration of glomalin during tillage managements transition. *Soil Science Society American Journal* **63**, 1825-1829.

Wright SF, Anderson RL (2000) Aggregates stability and glomalin in alternative crop rotations for the central Great Plains. *Biology and Fertility of Soils* **31**, 249-253.

Interaction of enzymes with soil colloids: adsorption and ectomycorrhizal phosphatase activity on tropical soils.

Brice Kedi[A,B], Josiane Abadie[A], Joseph Sei[B], Hervé Quiquampoix[A], **Siobhán Staunton**[A]

[A]UMR Eco&Sol, INRA-IRD-Supagro, 2 place Viala 34060 Montpellier cedex 02, France. staunton@supagro.inra.fr
[B]Laboratoire de Chimie de Matériaux Inorganiques, Université Cocody Abidjan, Côte d'Ivoire

Abstract
Ectomycorrhizal fungi phosphatases may play a significant role in phosphorus nutrition by the solubilisation of soil organic phosphorus, and this may be of particular importance for highly weathered tropical soils. The expression of phosphatase catalytical activity is highly pH dependent and may be modified to various degrees when in contact with soil colloids. This modification is expected to depend on the nature of the organo-mineral surfaces. The adsorption and modification of acid phosphatase activity, of both intra and extracellular enzymes obtained from three ectomycorrhizal fungi strains in contact with three tropical soils, were studied. The effect of physical fractionation (clay-sized fraction and sieved soil) and chemical cleaning (treatment with H_2O_2 or dithionite-citrate-bicarbonate to remove organic matter or iron oxides) of the organo-mineral surfaces has been studied. Measurements of catalytic activity were made in the range of pH 3 – 7. The extent of adsorption and the resulting change in catalytic activity varied between fungal strains, and even for a given strain between extra and intracellular enzymes. The difference between soils was rather small, and neither physical nor chemical treatments had marked effects on the interaction with enzymes.

Key words
Intracellular, extracellular, organic matter, iron oxides; coatings, catalytic activity.

Introduction

Ectomycorrhizal fungi contribute to phosphorus nutrition by synthetising and secreting phosphatases that solubilise soil organic phosphorus, which is not directly assimilated by plants (Quiquampoix and Moussain 2005). The action of these enzymes may be important for highly weathered tropical soils which are depleted in mineral phosphorus. In contact with soil phosphatases are adsorbed and their catalytic activity is modified to varying degrees (Leprince and Quiquampoix 1996). Activity depends strongly on pH, nature of mineral surfaces and organic adsorbents. Several studies have investigated the adsorption and activity of enzymes on pure clay minerals (Quiquampoix and Leprince 1996), clay-organic matter complexes (Gianfreda 1991; Kelleher 2004), and oxides (Shindo 2002) but few studies have compared natural soils. We have investigated the behavior of phosphatases produced by ectomycorrhizal fungi, in contact with contrasting tropical soils in order to understand better the role of orano-mineral coatings in soil.

Materials and methods

Preparation of phosphatases
Phosphatases used from three strains of ectomycorrhizal fungi: *Suillus collinitus* , denoted ,and two strains of *Hebeloma cylindrosporum* , denoted H_1 and H_2. Fungi were cultivated *in vitro* at 25 ° C in the dark for 30 days (Leprince 1995). Composition of the nutrient solution was: 0.1 mM NaCl ; 4 mM KNO_3 ; 1 mM KCl ; 2 mM NH_4Cl ; 1 mM Mg SO_4 ; 1 mM $CaCl_2$; 0.3 µM thiamine-HCl ; 125 mg/l ferric citrate; 10 g/l glucose ; trace elements as recommended by Morizet and Mingeau, (1976) . At the end of culture, the nutrient solution containing the extracellular phosphatase was filtered and frozen until required. Thalli were crushed in a mortar with 10% polyvinylpolypyrrolidone (PVPP) and sand. The mixture was taken up in a solution of acetate buffer (pH 5.5) containing 5 mM dithiotrietol. The suspension was centrifuged and the supernatant frozen until required.

Soils colloids
Soil samples from three tropical regions were selected for this study, all had clayey texture and were sampled from the A horizon 0-10 cm. A vertisol (V) was taken from Martinique, French West Indies and two ferralsols were taken from Niari, South Congo (F_1) and Paraná, South Brazil (F_2). The air dried soils were crushed and sieved to 200 µm. The clay fraction of the soils (≤ 2µm) was separated by sedimentation. Subsamples of the sieved soils also underwent chemical treatments with either H_2O_2 or citrate-bicarbonate-dithionite (Mehra & Jackson, 1960). After chemical treatments, soil residues were rinsed with water and $CaCl_2$ solution to remove excess chemical reagents.

Phosphatases activity measurements

The enzyme activity was measured as function of pH using para-nitrophenyl phosphate (pNPP) as substrate. The pH of the solutions was adjusted in acetate or MOPS buffer (300mM). The catalytic reaction was stopped after 20 minutes with glycin buffer. The reaction product, para-nitrophenol was quantitatively measured in an UV-visible spectrophotometer at 405 nm. Three procedures for measuring phosphatases activity denoted A, B and C (Quiquampoix 1987a; Quiquampoix and Leprince 1996) were used to distinguish the contributions of adsorbed and solution phase phosphatase. Procedure A simply measured the catalytic activity of the enzyme in solution. Procedure B measured the activity in the presence of soil or soil clay, due to both adsorbed and non adsorbed enzyme. In procedure C, the activity in a supernatant solution after contact with soil was measured. A comparison of A and C allows the extent of adsorption to be calculated. Phosphatase activity for each procedure was expressed as the velocity of the catalytic reaction, V, in nKatal.g^{-1} fungus. We calculated the proportion of enzyme remaining free in solution, F as V_C/V_A and the relative activity of enzymes in the adsorbed state, R as $(V_B-V_C)/(V_A-V_C)$.

Results and discussion

Different interactions of phosphatases with soil colloids

Figure 1 shows the results of measurement procedures A, B and C of the intra and extracellular phosphatases with clay fraction of ferralsol F_1 at pH 5. For a better comparison of A, B and C, the histogram is represented as a percentage of maximum activity of each phosphatase. The observed behavior can be classified into four types: First, $V_A > V_B$ and $V_C = 0$, for H_{2i} (intracellular). Zero values of V_C indicate that the enzyme is completely adsorbed. Since $V_B < V_A$, some activity has been lost on adsorption. In a second case, observed for $V_A > V_B > V_C$ for H_{2e} (extracellular): some of the enzyme was adsorbed and adsorbed enzyme lost part of its activity. Thirdly, for H_{1e}, S_i and S_e, $V_A = V_B > V_C$ indicates that a part of the enzyme was adsorbed but that adsorbed enzyme retained its catalytic activity. Finally, $V_A = V_B$ and $V_C = 0$ for both intracellular enzymes of the *Hebeloma cylindrosporum* fungi, $\mathbf{H_{1i}}$, and $\mathbf{H_{2i}}$, indicating that despite complete adsorption, adsorbed enzyme completely retained catalytic activity. These results show that adsorption of enzymes is not complete as assumed by many studies. Only two of the enzymes studied (H_{2i} and H_{1i}) were completely adsorbed. Furthermore, phosphatases retain over 80% activity in contact with soil colloids in contrast to previous observations where adsorbed enzyme retained little activity (Quiquampoix and Leprince 1996).

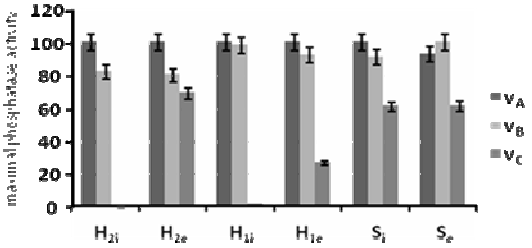

Figure 1: Effect of clay fraction of ferralsol (F_1) at pH5 on expression of phosphatase activity

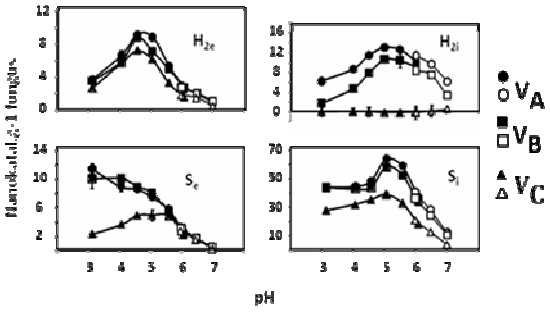

Figure2: Comparison of interactions between intra and extracellular enzymes of *Hebeloma cylindrosporum* H_2 and *Suillus collinitus* S with ferralsol F_1 (clay fraction). Closed symbols acetate buffer, open symbols MOPS buffer

Behavior of intra and extracellular phosphatases from the same fungus

Figure 2 shows the behavior of intra and extracellular phosphatases from **S** (*Suillius collinitus*) and $\mathbf{H_2}$ (*Hebeloma Cylindrosporum*) with clay fraction of ferralsol F_1. $\mathbf{S_e}$ and $\mathbf{S_i}$ have different behavior although from same fungus. While extracellular $\mathbf{S_e}$ was adsorbed weakly with little loss of activity, all intracellular $\mathbf{S_i}$ are adsorbed and part of their activity is lost. In contrast, $\mathbf{H_{2i}}$ and $\mathbf{H_2e}$ had similar behavior, partial adsorption and no loss of activity. This result shows that intra and extracellular phosphatases may differ both in intensity of enzymatic activity and in interaction with soil colloids. Phosphatases from different fungi may have markedly different behaviors. This study shows that the adsorption and modification of enzyme activity depend not only on nature of adsorbent surface but also on the enzyme.

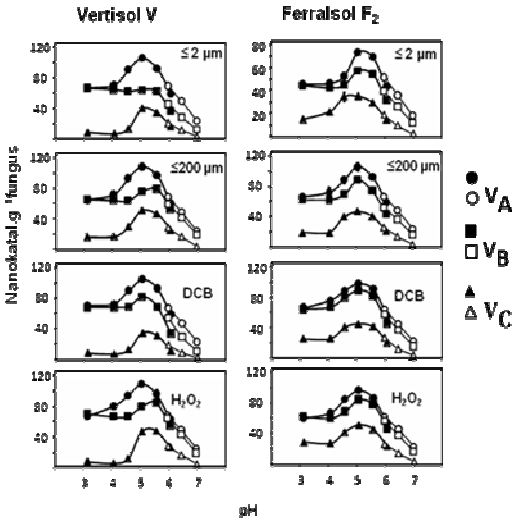

Figure 4: Comparison interactions of enzyme (S_i) with different fractions of soil V and F_2. Closed symbols acetate buffer, open symbols MOPS buffer

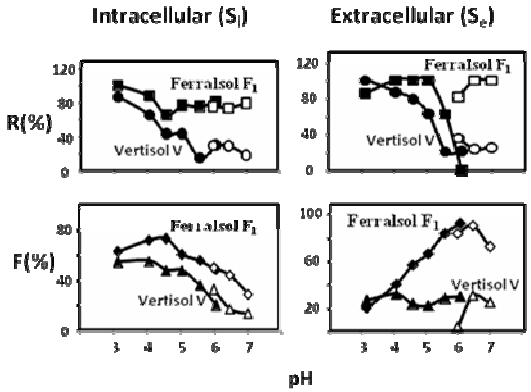

Figure 3: Relative activity (R) and free fraction of enzymes (F) of S_i (*Suillus collinitus*-intracellular) and Se (*Suillus collinitus*-extracellular) on two soils (V and F_1). Closed symbols acetate buffer, open symbols MOPS buffer

Effect of soil type

Figure 3 shows for S_i and S_e, effect of two soil types V (vertisol) and F_1 (ferralsol) on the proportion of enzyme remaining free in solution, F, and the relative activity of adsorbed enzyme, R as a function of pH. For this enzyme, both R and F are greater for the ferralsol F_1 than the vertisol V. This comparison suggests that stronger interaction with the vertisol leds to both greater adsorption and greater inactivitation of the enzyme.

Effect of granulometry, iron oxides and organic matter

Figure 4 shows effect of granulometry, iron oxides and organic matter of two soils (V and F_2) on Si catalytic activity. There are no marked differences between curves. Soil granulometry (<2µm and 200µm) and chemical treatments to remove iron oxides and organic matter have little effect on phosphatase activity. This is surprising, as previous studies have reported contrast in both adsorption and modification of catalytic activity when reference minerals are compared to synthetic organo-mineral complexes (Quiquampoix 1987b; Rao *et al*. 2000; Kelleher *et al.* 2004). The absence of a particle size effect, in contrast to the study of Huang *et al.* (2005)on a Ultisol is less surprising since the soils in the present study are clay textured and so the clay fraction may dominate the interactions with soils.

Conclusion

This study shows that the extent of adsorption and the activity of adsorbed fungal phosphatase varies considerable between enzymes. Behaviour may differ between fungal species and strains, and even for a given fungus between intracellular and extracellular enzymes. In contrast to observations for synthetic organo-mineral complexes, smaller differences were observed between contrasting soils and almost no effect of removal of organo-mineral coatings was detected. More work is required to understand the origin of the interactions of enzymes with natural soil surfaces in order to predict the expression of catalytic activity in complex systems.

References

Gianfreda L, Rao MA, Violante A (1991) Invertase (β-fructosidase): Effects of montmorillonite, Al-hydroxide and $Al(OH)_x$-montmorillonite complex on activity and kinetic properties. *Soil Biology and Biochemistry* **23**, 581–587.

Huang Q, Liang W, Cai P (2005) Adsorption, desorption and activities of acid phosphatase on various colloidal particles from an Ultisol. *Colloids and Surfaces B: Biointerfaces* **45**,209-214.

Kelleher BP, Simpson AJ, Willeford KO, Simpson MJ, Stout R, Rafferty A and Kingery WL (2004) Acid phosphatase interactions with organo-mineral complexes: influence on catalytic activity. *Biogeochemistry* **71**, 285-297.

Leprince F (1995) Mécanismes mis en œuvre par différents champignons ectomycorhiziens dans la mobilisation du phosphor*e. Thèse de Doctorat*, ENSA de Montpellier.

Leprince and Quiquampoix (1996) Extracellular enzyme activity in soil: effect of pH and ionic strength on the interaction with montmorillonite of two acid phosphatases secreted by the ectomycorrhizal fungus *Hebeloma cylindrosporum. European Journal of soil Science* **47**, 511-522.

Mehra and Jackson (1960) Iron oxides removal from soils and clays by dithionite-citrate system buffered with sodium bicarbonate. *Clays and Clay Minerals* **7,** 317-327.

Morizet and Mingeau (1976) Influence des facteurs du milieu sur l'absorption hydrique (étude effectuée sur tomate décapitée en exsudation). Facteurs nutritionnels. *Annales Agronomiques* **27,** 183-205.

Quiquampoix H (1987a) A stepwise approach to the understanding of extracellular enzyme activity in soil. I. Effect of electrostatic interactions on the conformation of a β-D-glucosidase on different mineral surfaces. *Biochimie* **69**, 753-763.

Quiquampoix H (1987b) A stepwise approach to the understanding of extracellular enzyme activity in soil. II. Competitive effects on the adsorption of a β-D-glucosidase in mixed mineral or organo-mineral systems. *Biochimie* **69,** 765-771.

Quiquampoix and Moussain (2005) Enzymatic hydrolysis of organic phosphorus. In 'Organic Phosphorus in the Environment'. (Eds BL Turner, E Frossard, DS Baldwin). pp. 89-112. (CAB international).

Rao MA, Violante A, Gianfreda L (2000) Interaction of acid phosphatase with clays, organic molecules and organo-mineral complexes: kinetics and stability. *Soil Biology and Biochemistry* **32**, 1007-1014.

Shindo H, Watanabe D, Onaga T, Urakawa M, Nakahara O, Huang QY (2002) Adsorption, activity, and kinetics of acid phosphatase as influenced by selected oxides and clay minerals. *Soil Science and Plant Nutrition* **48,** 763-767.

Structural characterization of natural nanomaterials: potential use to increase the phosphorus mineralization.

Marcela Calabi[a], Alejandra Jara[b], James Bendall[c], Mark Welland[c] and María de la Luz Mora[d]

[a] Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera. [b] Departamento de Ciencias Químicas, Universidad de La Frontera. [c] Nanoscience Centre of The University of Cambridge, England. [d] Scientific and Technological Bioresource Nucleus (BIOREN-UFRO), Universidad de La Frontera. Av. Francisco Salazar 01145, P.O. Box 54-D, Temuco, Chile. **mcalabi@ufro.cl**

Abstract
Nanomaterials like nanoclays occur widely in nature. The use of nanomaterials as immobilization support improves enzymatic stability and catalytic activity against other materials. The aims of this study were: i) to characterize natural allophanic clay and nanoclay fraction with and without organic matter from an Andisol and ii) to evaluate allophanic and montmorillonite nanoclays as support to immobilize acid phosphatase. The used Andisol belongs to Piedras Negras Series from Southern Chile. Clays and nanoclays extracted were characterized by TEM, SEM, EDX, ED and AFM. The enzymatic activities were measured with □-nitrophenylphosphate (□-NPP) as substrate. The kinetics parameters (V_{max} and K_m) were calculated according to Michaelis–Menten equation. The microscopy analysis showed that the methodology of the nanoclays extraction allowed obtaining aggregates with high proportions of mesoporous, which are suitable to enzymatic immobilization. The nanoclay fractions showed that organic matter governs the feature behavior and is very recalcitrant, suggesting that this nano-fraction plays an important role in carbon sequestration. Finally, we demonstrated that the immobilized phosphatase has a significant increase both in catalytic efficiency (V_{max}/K_m) and in substrate affinity ($1/K_m$) when allophanic and montmorillonite nanoclays were used as material support.

Key Words
Natural nanoclays; allophane; aluminosilicate, enzyme immobilization, acid phosphatase.

Introduction

Nanoparticles occur widely in the natural environment (*Theng and Yuan* 2008). Particles within nanoscale range have been studied in soil sciences a few decades ago to understand the behavior of soil environment (Wada 1987; Parffit *et al.* 1983). However, these particles have not been studied from the nanoscience point of view. In nanoscience, material design and surface feature of nanomaterials play an increasing role in many fields of technological application such as: electronics, medicine, biocatalysis, material science and other. Although, synthetic nanomaterials are relevant for a wide possibility of applications in nanoscience (Garrido *et al.* 2010), the production of nanoparticle with small diameters and a narrow size distribution is expensive and difficult (Hofmann *et al.* 2008). However, in the environment we can find many types of morphologies that actually are synthesized, thereby an interesting alternative is to obtain and use natural nanomaterials. Nanoclays is a real alternative to get a material with nanometric size. In soils derived from volcanic ashes as Andisols (Calabi *et al.* 2009), different structures of nanoparticles like aluminosilicate with nano-ball (allophane) and nano-tube (imogolite) morphology (Wada 1987; Abidin *et al.* 2007) within clay fraction are possible to find. The most important constituent of Andisols is allophane which is a non-crystalline ('short-range order') aluminosilicate with Al/Si ratio varying between 1 and 2. Independent of composition the unit particle of allophane is a hollow spherule with an outer diameter of 3.5–5.0 nm. Imogolite is more crystalline than allophane. The unit particle of imogolite is a hollow tubule with an outer and inner diameter of about 2 and 1 nm, respectively (Wada 1987; Abidin *et al.* 2007). Allophane clays have a high specific surface area. These nanoparticles form stable microaggregates with pores within the nanoscale range with similar physical characteristics to silica nanomaterials which are very important in biocatalysis (Kim *et al.* 2006), because these improve the catalytic efficiency of the enzyme. Previous results from our investigation group showed that acid phosphatase immobilized on allophanic clays from Andisols of Southern Chile increase its catalytic efficiency (Rosas *et al.* 2009). The aims of this study were: i) to characterize natural allophanic clay and nanoclay with and without organic matter from an Andisol and ii) to evaluate allophanic and montmorillonite nanoclays as support material to immobilize acid phosphatase.

Methods

Extraction of nanoclay and Structural and chemical characterization

An Andisol, from Piedras Negras Series, taken from Southern Chile was sampled within 0–20 cm of depth, sieved to 2 mm mesh and air-dried. One part of the soil was treated with 30% hydrogen peroxide to remove the soil organic matter. The separation of particle-size < 2 μm fractions was performed by sedimentation

procedures based on Stoke's law. The nanoclays were extracted from allophanic clays and montmorillonite clay (AppliChem A6918, LOT 7W007719), these were extracted using clays pre-treated in the similar way as described by Li and Hu (2003). 5 g of clay were suspended in 100 ml of 1 M NaCl. This solution was dispersed applying 214 J by ultrasonic dispersion. The clay suspension was centrifuged at 3000 rpm for 40 min. The pellet was collected, and nine-fold washed with 50 ml of distilled water (these supernatant is the nanoclays suspension). Previous to washing nanoclays, these were moderately stirred for 40 min. The nanoclay suspension was dialyzed (1000 kDa membrane) in pure water until conductivity in ranging 0.8-0.5 μS, and later it was vacuum lyophilized. Allophanic clays and nanoclays were characterized throughout energy dispersive X ray (EDX), transmission electron (TEM) and scanning electron (SEM) microscopy, electronic diffraction (ED) and atomic force microscopy (AFM).

Phosphatase assay

The complexes were formed by interaction between acid phosphatase and allophanic and montmorillonite nanoclays, and used as model systems to simulate enzymatic reactions in heterogeneous environment. The enzymatic activities of free and immobilised phosphatase were assayed with 6 mM *p*-nitrophenylphosphate (*p*-NPP) and the concentration of *p*-nitrophenol was determined by measuring the absorbance at 405 nm with a spectrophotometer (Rao *et al.* 2000, Rosas *et al.* 2009). To avoid interference by turbidity, the samples were centrifuged at 14,000 *g* for 4 min prior to measurement.. The kinetics parameters (V_{max} and K_m values) were calculated according to Michaelis–Menten equation varying *p*-NPP concentrations ranging from 0 to 6 mM.. The enzymatic determinations were made in triplicate and analyzed using the SPSS software, version 14.0. The Tukey test was applied to data using a 0.05 significance level of probability.

Results

Structural and chemical characterization

The TEM analysis showed the allophanic nanoclays in the samples, with predominant size < 50 nm with organic matter (OM). We could appreciate the high occurrence of allophane nanoparticles (outer diameter around 5 – 3 nm) with two kinds of morphology: as gel (arrow short) or condensed aggregates (arrow long), which are commonly reported for allophane materials. The Figure 1a showed the presence of high nanoclay content in the clay samples and some crystalline material. Also, we observed the occurrence of amorphous particles by electron diffraction due to the presence a diffused ring (Figure 1b).

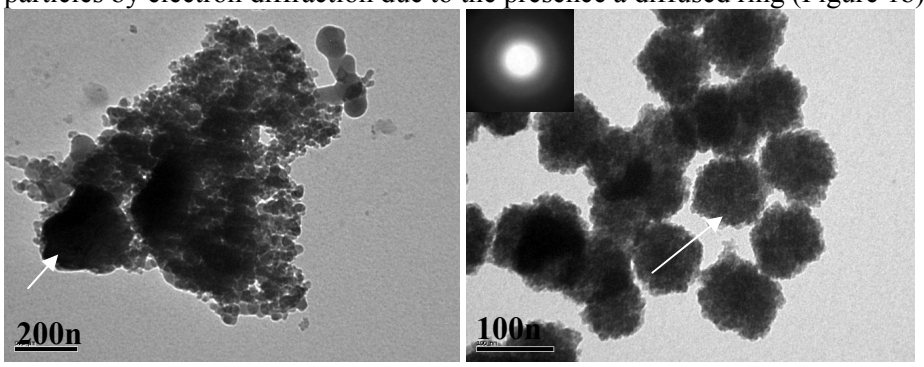

Figure 1. TEM and ED of nanoclays and clays in the presence of organic matter.

The SEM analysis showed difference between clays and nanoclays from allophanic soil with OM and after treatment to remove OM in size (Figure 2). The SEM images showed that nanoclay aggregates with OM (Figure 2a) have high amount of mesoporous with different sizes, this can absorb water from surrounding environment (Okada *et al.* 2008). Thus, providing an aqueous microenvironment which could be useful for biotechnological applications (Li and Hu 2003), allowing an adequate mass transport for both substrates and products. The OM removal exhibited nanoclay aggregates (Figure 2c) of size less than 50 nm and showed cavities in the nanoscale.

The AFM analysis confirmed the presence of allophane and imogolite (Figure 3), and the abundance of aggregates with similar size such as those observed by TEM. AFM observations from allophanic clay samples showed clearly a high content of OM forming networks between particles, and also inside the aggregates as coating (Figure 3c) but, we can not observed clearly the OM content in nanoclays (Figure 3a). However, the elemental analysis showed that the nanoclays (28%) have a 20% higher OM content than clays (19%). This could be explained due to the strong interaction between OM and nanoclays, as we evaluated when we tried to remove it repeatedly with hydrogen peroxide. The analysis of AFM images of allophanic nanoclays after treatment to remove OM (Figure 3b) allowed identify smaller and cleaner allophane and imogolite nanoclays.

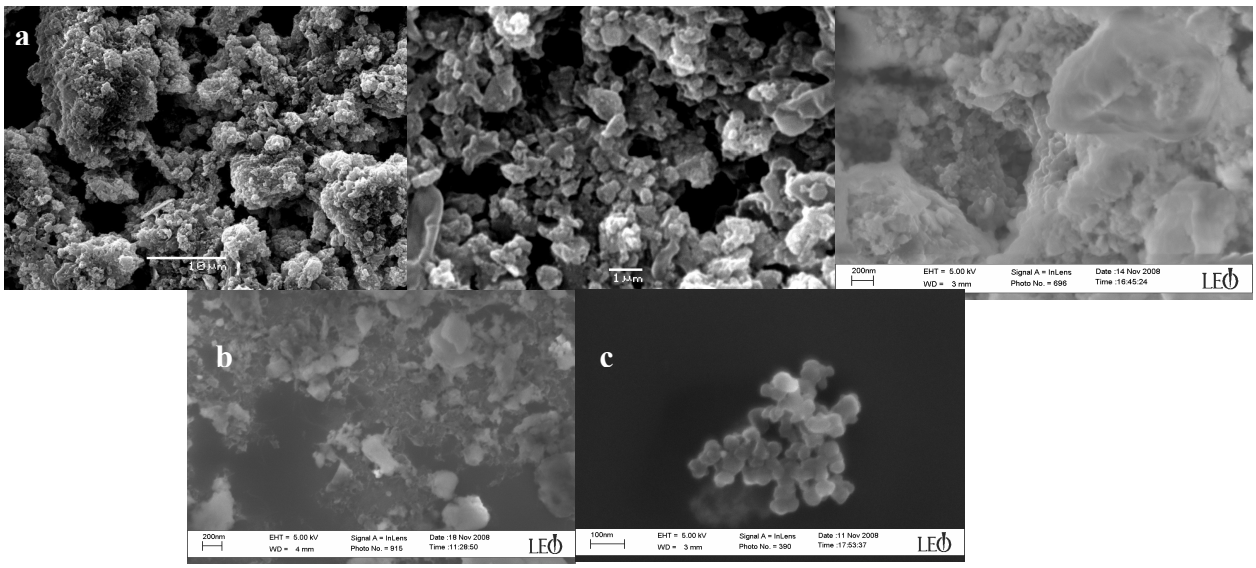

Figure 2. SEM images from allophanic clay and nanoclays. (a) nanoclays with OM at different amplification, (b) allophane clays with OM and (c) allophane nanoclays without OM.

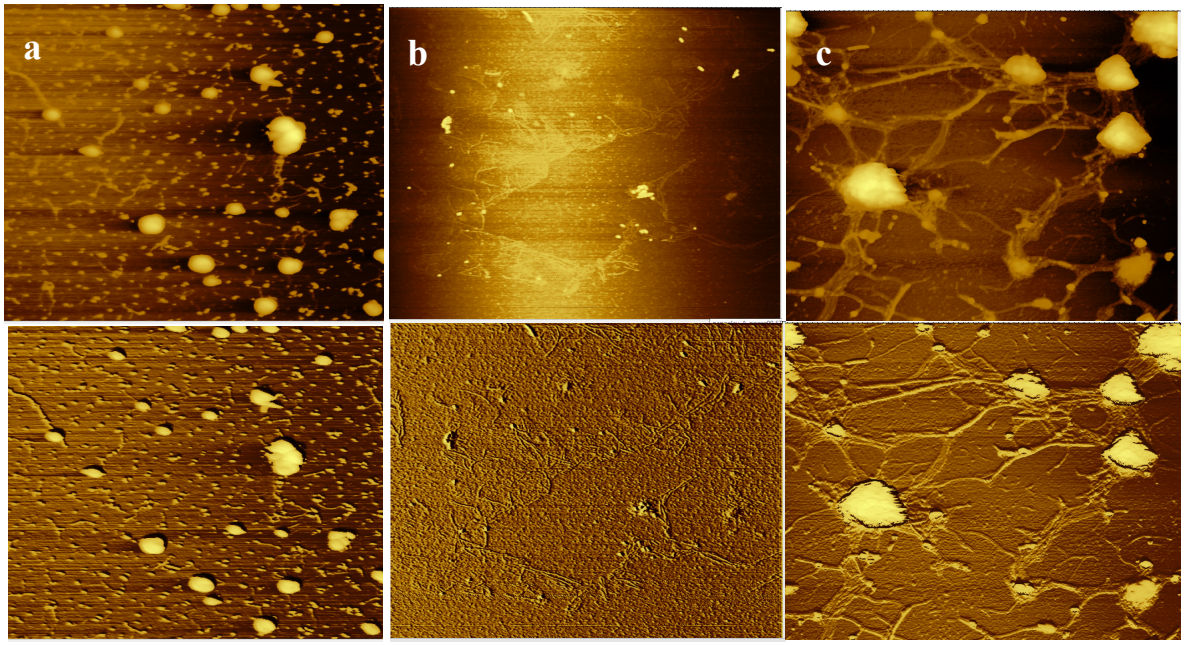

Figure 3. AFM from allophanic nanoclays and clays. (a) nanoclays with OM, (b) allophane nanoclays without OM and (c) allophane clays with OM.

By means of microtopography AFM we confirmed nanoparticles presence with basal length and thickness of single particle aggregates in the nanoscale. The OM removal in nanoclays produced a decrease of maximal-height aggregates from 103.3 to 12 nm for nanoclays (Figure 3a,b). However, we observe no differences in the average values of smaller height. In fact, this is another evidence of the strong interaction between OM and nanoclay. Therefore, the recalcitrant OM content is able to affect the structural and physicochemical features of this material (Mora and Canales 1995). Thus, allophanic nanoclays are a very important fraction for carbon sequestration in Andisols.

Phosphatase assay

Results showed that acid phosphatase immobilization on allophanic and montmorillonite nanoclays reduced the enzymatic activity in 23 and 30 %, respectively. Huang *et al*. (2005) used different support materials from soil colloids to enzyme immobilization and reported an important specific activity reduction between 28 and 61 %. In addition, Rao *et al*. (2000) obtained high deactivation values (37 at 77 %) after acid phosphatase immobilization on montmorillonite clay, tannic acid and organo-mineral complex. Therefore, allophanic and montmorillonite nanoclays are suitable as material support. The kinetic parameters obtained from Michaelis-Menten equation showed that the enzymatic immobilization on allophanic and montmorillonite nanoclays increased both the catalytic efficiency in 55 and 120 %, and the substrate affinity

($1/K_m$) in 50 and 60 % (Table 1). Therefore, nanoclays are suitable support materials to acid phosphatase immobilization.

Table 1. Kinetic parameters of acid phosphatase free (P) and immobilized in allophanic nanoclays (AN-P complexes) and montmorillonite nanoclays (MN-P complexes).

	V_{max} (□NP □mol/ml/min)	K_m (mM)	V_{max}/K_m*	R
P	0,269	0,260	1,04	0,993
AN-P complexes	0,217	0,135	1,61	0,989
MN-P complexes	0,195	0,082	2,37	0,985

*V_{max}/K_m: catalytic efficiency.

Conclusions

The methodology of Li and Hu (2003) modified by our Group, reduced the time of nanoclay extraction by changing the shaking by ultrasonic system and allowed more dispersed particle aggregates. Furthermore, we obtained more homogeneous nanoclay materials. A recalcitrant fraction of OM in the nanoclays was observed. This fraction affected the structural and physicochemical material characteristics.

We observed a significant increase both of the catalytic efficiency in 55 and 120 %, and the substrate affinity in 50 and 60 %, when allophanic and montmorillonite nanoclays were used as support materials for phosphatase immobilization.

In general, the nanomaterials are more promising candidates for enzyme immobilization than conventional supports because they can carry a high enzyme load, while substrate diffusion is relatively unhindered. Then, enzymes immobilized on nanoclay can be show a high mobility and activity, suggesting that the molecules are not rigidly attached to the support materials, also the stereochemistry of the immobilized enzymes is more able to attach to the substrate. Moreover, the good match between the pore dimension of nanomaterials and enzyme molecular size has a stabilizing effect on the immobilized enzyme.

References

Abidin Z, Matsue N, Henmi T (2007) Differential formation of allophane and imogolite: experimental and molecular orbital study. *Journal of Computer-Aided Materials Design* **14**, 5-18.

Calabi M, Theng BKG, Reyes P, Mora ML (2009) Natural nanoclays: applications and future trends – a Chilean perspective. *Clay Minerals* **44**, 161-176.

Garrido-Ramírez EG, Theng BKG, Mora ML (2010) Clays and oxide minerals as catalysts and nanocatalysts in Fenton-like reactions – A review. *Applied Clay Science.* In press.

Hofmann S, Sharma R, Wirth CT, Cervantes-Sodi F, Ducati C, Kasama T, Dunin-Borkowski RE, Drucker J, Bennett P, Robertson J (2008) Ledge-flow-controlled catalyst interface dynamics during Si nanowire growth. *Nature materials* **2140**, 1-4.

Huang Q, Liang W, Cai P (2005) Adsorption, desorption and activities of acid phosphatase on various colloidal particles from an Ultisol. *Colloids and Surfaces B: Biointerfaces* **45**, 209–214

Kim J, Grate JW, Wang P (2006) Nanostructures for enzyme stabilization. *Chemical Engineering Science*, **61**, 1017-1026.

Li Z, Hu N (2003) Direct electrochemistry of heme proteins in their layer-by-layer films with clay nanoparticles. *Journal of Electroanalytical Chemistry* **558**, 155–165.

Mora ML, Canales J (1995) Interactions of humic substances with allophannic compounds. *Commun. Soil Sci. Plant Anal.* **26**, 2805-2817.

Okada K, Matsui S, Isobe T, Kameshima Y, Nakajima A (2008) Water-retention properties of porous ceramics prepared from mixtures of allophane and vermiculite for materials to counteract heat island effects. *Ceramics International* **34**, 345-350.

Parfitt RL, Russell M, Orbell GE (1983) Weathering sequence of soils from volcanic ash involving allophane and halloysite, new zealand. *Geoderma* **29**, 41-57.

Rao MA, Violante A, Gianfreda L (2000) Interaction of acid phosphatase with clays, organic molecules and organo-mineral complexes: kinetics and stability. *Soil Biology and Biochemistry* **32**, 1007-1014.

Rosas A, Mora ML, Jara AA, López R, Rao MA. and Gianfreda L (2008) Catalytic behavior of acid phosphatase immobilized on natural supports in the presence of manganese or molybdenum. *Geoderma* **145**, 77-83.

Theng BKG, Yuan G (2008) Nanoparticles in the Soil Environment. *Elements* **4**, 395-399.

Wada K (1987). Minerals formed and mineral formation from volcanic ash by weathering. *Chemical Geology* **60**, 17-28.

The mobility and persistence of the insecticidal Cry1Aa toxin, Bt (*Bacillus thuringiensis*) in soils

Nordine Helassa[a], Sylvie Noinville[b], Philippe Déjardin[c], Hervé Quiquampoix[a] & **Siobhán Staunton**[a]

[a]UMR 1222 Eco&Sols, INRA, place Viala, 34060 Montpellier, France E-mail : staunton@montpellier.inra.fr
[b]UMR 7075 - LADIR, CNRS, 2 rue Henri Dunant, 94320 Thiais, France
[c] UMR 5635 IEM, CNRS, 2 Place Eugène Bataillon, 34095 Montpellier, France

Abstract
The rapid worldwide expansion of genetically modified crops containing the Bt trait gives rise for concern as to the possible impact on non target species. These plants release insecticidal proteins, known as cry into soil during growth and decomposition of crop residues. The fate of the toxin is strongly influenced by its interactions with soil, in particular adsorption which modifies its biological properties, its mobility and its persistence. We describe three series of studies to investigate (i) the adsorption properties (ii) the mobility of the adsorbed protein and (iii) the decline of toxin in soil as a function of time and microbial activity. We conclude that the toxin will be strongly immobilised in soil and that microbial degradation does not determine the observed decline of toxin over periods of weeks and months.

Key words
Adsorption, protein, genetically modified plants, microbial activity, soil sterilisation.

Introduction
Soil dwelling bacteria, *Bacillus thuringiensis* (Bt), synthesize crystalline proteins (Cry) during sporulation that after ingestion by larvae are solubilised and truncated to produce highly target specific, insecticidal proteins. Target specificity results both from the chemical conditions in the insect mid-gut and the presence of receptors in cell membranes allowing pore formation and rapid insect death. Genes for some of these Cry proteins have been inserted into various plants, and since 1996 such genetically modified (GM) plants have been commercialised. These Bt crops often give better yields and improved crop quality in comparison with non Bt crops. There is considerable public concern about the wide-spread cultivation of these crops. Given the very rapid increase in the area of agricultural land world wild used to grow these crops, understanding of potential negative side effects are necessarily limited. Although the bacteria exist spontaneously in soil and various preparations of spores and crystals are widely used for pest control, including in organic farming, there are important differences between bacterially produced toxin and that of GM crops. Firstly, GM plants synthesise directly the toxin, thus bypassing two of the steps that confer target specificity (solubilisation in the alkaline pH of insect mid-gut and enzymatic activation of the protein). Secondly, GM plants synthesize the protein throughout the plant and continuously during plant growth, whereas the activity of commercial preparations of spores and crystals is rapidly lost.

There has been considerable research on the possible impact of Bt crops on non target organisms. To date there has been no strong evidence of direct negative effects. An important factor in determining the exposition of non target organisms to Bt toxin is the interaction of the proteins with soils, and the resulting mobility and persistence of the toxin in the soil environment.

We have investigated the adsorption, the mobility and the persistence of one Cry protein, Cry1Aa, in contact with various soils and soil minerals.

Materials and Methods
The protein was produced by culture of a genetically modified strain of the bacterium (*B. thuringiensis* subsp. *kurstaki* HD-1 provided by CIRAD, Montpellier) cultivated in a fermentor, then purified and activated as previously described (Vié *et al*. 2001). Concentrated solutions of protein were maintained at high salt concentration and high pH to avoid polymerisation. The monomeric state of the protein was verified using dynamic light scattering (Zetasizer HS3000).

Reference clay minerals, montmorillonite and kaolinite, were size fractionated (<2 µm) by sedimentation, made homoionic with sodium and washed until salt free. Mica was freshly cleaved. Glass was acid-washed and, when required, made hydrophobic by silanisation. Four soils with contrasting texture and organic matter content were selected for incubation experiments. When necessary the protein was labelled with a

fluorescent probe (fluorescein isothiocyanate, FITC). Adsorption isotherms as a function of pH were measured in clay suspension with protein analysis by uv-spectroscopy (Helassa *et al*. 2009). Mobility of FITC-labelled protein adsorbed on montmorillonite was measured using FRAP (Fluorescence Recovery After Photobleaching) which has not hitherto been reported for proteins adsorbed on mineral surfaces. Soils were incubated with trace amounts of Cry1Aa under controlled conditions of temperature and moisture content. Various chemical and physical treatments varied the microbiological activity. Soils were sterilised by γ-irradiation, by autoclaving and by addition of $HgCl_2$, or maintained at 4°C to inhibit microbial activity. Microbial activity was enhanced by addition of trigger solutions of amino acids and/or glucose. Toxin was extracted and analysed using an ELISA test.

Results and Discussion

Adsorption of Cry1Aa on montmorillonite and kaolinite was found to be low affinity and data could be fitted to Langmuir isotherms, as since in Figure 1. Adsorption was measured as a function of pH, but the lowest pH that could be investigated was 6.5, roughly the isoelectric point, since protein oligomerized at acid pH. For both minerals the maximum of adsorption decreased markedly as pH was increased above the isoelectric point. Adsorption was about 40 times greater on montmorillonite than on kaolinite, in line with the difference in their specific surface area. Adsorption capacity with respect to surface area on both minerals followed he same trend as a function of pH, which is surprising given the strong pH dependence of surface charge on kaolinite (Helassa *et al*. 2009). Desorption in water or alkaline buffer was small, thus adsorption was largely irreversible, despite the low affinity isotherms. Desorption was very efficient when non-ionic or zwetterionic detergents were added which is important since current detection techniques of the protein require that it be in solution (Helassa *et al*. 2009).

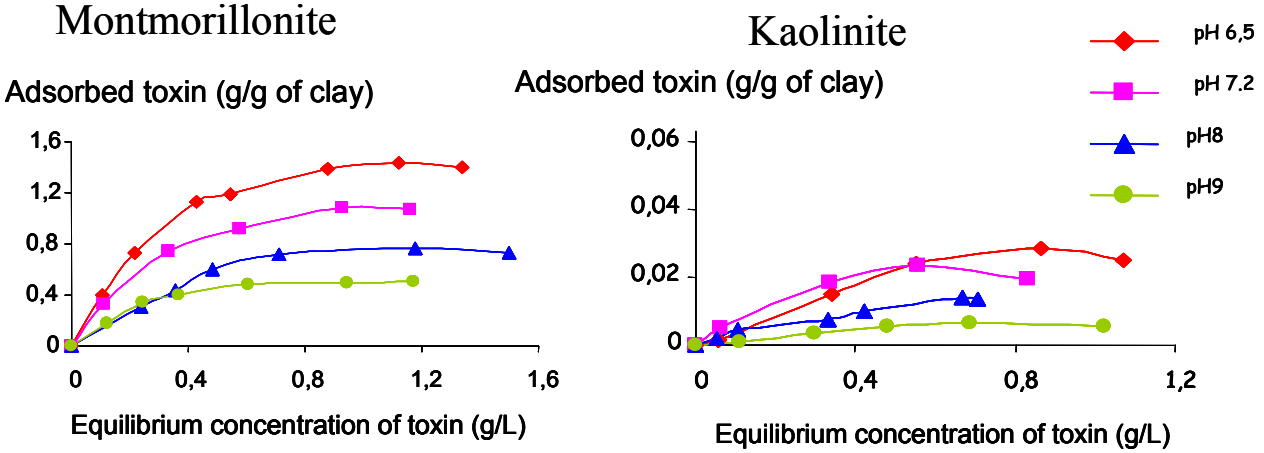

Figure 1. Adsorption isotherms of monomeric Cry1Aa on montmorillonite and kaolinite as a function of pH. The amount of protein adsorbed was calculated by depletion from the initial solutions and proteins quantitatively measured by uv-visible spectroscopy at 280 nm.

It is often assumed that proteins are immobilised by adsorption, however there is little experimental proof of this. The mobility of an adsorbed fluorescent molecule can be deduced by the rate at which fluorescence is recovered after photobleaching. This technique, FRAP, has never previously been applied to environmentally relevant mineral surfaces and proteins. FITC-labelled protein was adsorbed onto montmorillonite in suspension, centrifuged and washed with water to remove excess protein in solution, centrifuged and the pellet smeared onto a glass microscope slide. Protein concentration in remaining solution was negligible. Fluorescence intensity was scanned with a confocal scanning laser microscope and a disc of 12 µm diameter photobleached with and argon laser and the bleached zone scanned for at least 30 minutes. Figure 2a shows a typical image of the bleached zone, and Figure 2b the repeated scans across a section of the bleached zone. There is some variation in the base line of fluorescence intensity, due to small variations in the intensity of the excitation beam. However the shape of the intensity curve did not vary, and this is considered to be a better indicator of the absence of lateral diffusion. We found no evidence of mobility of the adsorbed protein at any pH value between 6.5 and 9 and no mobility at two levels of protein loading on the clay mineral surface. We thus conclude that the protein is unlikely to diffuse far from its point of introduction in the soil and while this limits dispersion, it could lead to the build-up of hot spots.

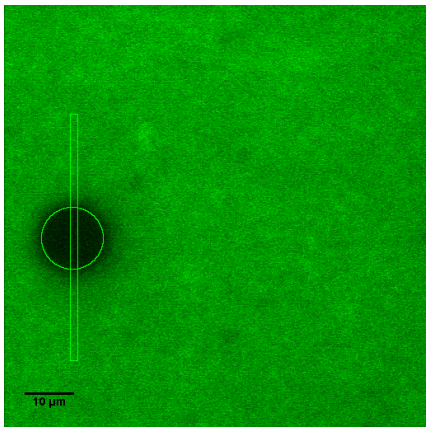

Figure 2a. Typical fluorescence image after photobleaching of a uniform disc of 12 µm of FITC-labelled Cry1Aa adsorbed on montmorillonite.

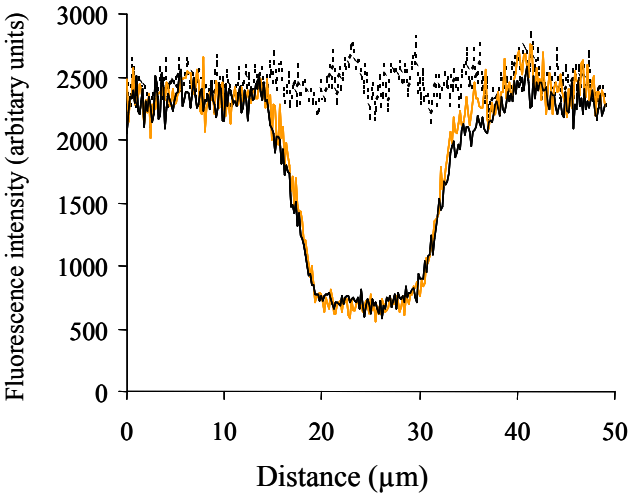

Figure 2b. Fluorescence intensity profiles of FITC-labelled Cry1Aa adsorbed on montmorillonite before (top, flat curve), immediately after and 500 sec after photobleaching

It is often stated that adsorption of proteins on soil mineral surfaces protects them against microbial breakdown and thus prolongs their persistence in soil. We monitored the detection of Cry1Aa incubated in four contrasting soils and varied the level of microbial activity by sterilising or boosting activity. Two of the soils had clayey texture but differed in their organic matter content, whereas the other two were sandy textured with contrasting organic matter content. After different incubation periods, soils were destructively sampled, the protein extracted in an alkaline solution containing surfactant and another protein (bovine serum albumin). This extractant was found to give similar, high extraction yields for all the soils. The protein extracted was quantified using an Elisa test.

Figure 3 shows the trends in proteins extracted and detected for each of the four soils. There are only small differences in the persistence of the toxin in the contrasting soils. The clayey texture does not appear to provide additional protection against degradation, nor enhance irreversible fixation.

If the decline of detectable Bt toxin with time was due to microbial breakdown, then enhancement of microbial activity would increase the rate of decline and conversely sterilisation or inhibition of microbial activity would maintain a higher level of detectable protein. Figure 4 shows the absence of any effect of sterilisation by either γ-irradiation or autoclaving for one soil. Similar results were obtained for all soils. Concurring results were obtained when microbial activity was boosted with amino acids and/or glucose and when activity was inhibited by $HgCl_2$. In marked contrast, at 4°C, when hydrophobic interactions may be favoured, adsorption was smaller and the rate of decline over a 3-week period much slower. We conclude that the decline in extractable, detectable toxin was not determined by microbial activity. However we have not yet been able to distinguish between two possible reasons: firstly that protein becomes more irreversibly bound to soil surfaces with time thus decreasing extractability; secondly that changes in secondary structure of the protein that were maintained after extraction made the protein less well recognised by the Elisa test.

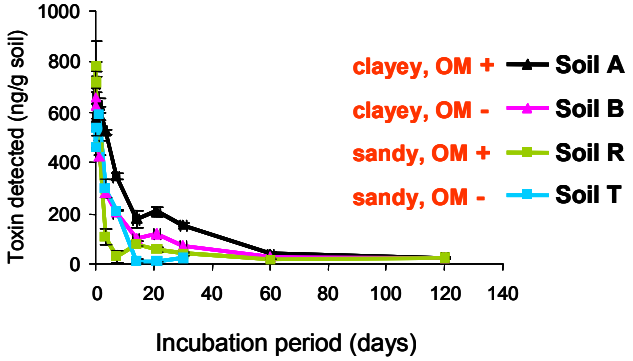

Figure 3. Cry1Aa extracted and detected as a function of incubation period with each soil.

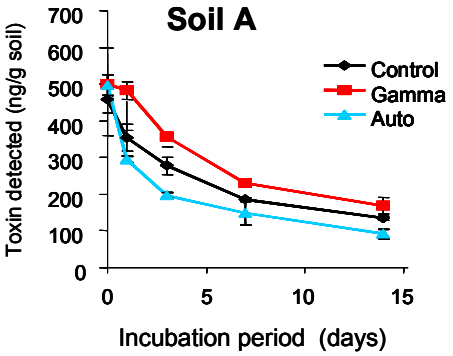

Figure 4. Cry1Aa extracted from Soil A (clayey top soil) and detected as a function of incubation time and sterilisation

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References
Helassa N, Quiquampoix H, Noinville S, Szponarski W, Staunton S (2009) Adsorption and desorption of monomeric Bt (*Bacillus thuringiensis*) Cry1Aa toxin on montmorillonite and kaolinite. *Soil Biology & Biochemistry* **41**, 498-504.

Icoz I, Stotzky G (2008) Fate and effects of insect-resistant Bt crops in soil ecosystems. *Soil Biology and Biochemistry* **40**, 559-586.

James C (2007) Global Status of Commercialized Biotech/GM Crops: 2007. (ISAAA) Brief No 37, Ithaca, NY.

Vié V, Van Mau N, Pomarède P, Dance C, Schwartz JL, Laprade R, Frutos R, Rang C, Masson L, Heitz F, Le Grimellec C (2001) Lipid-induced pore formation of the *Bacillus thuringiensis* Cry1Aa insecticidal toxin. *Journal of Membrane Biology* **180**, 195-203.

The reactivity of carbonates in selected soils from Iran

Adel Reyhanitabar[A] and R. J. Gilkes[B]

[A]Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran. Email areyhani @ tabrizu.ac.ir
[B]School of Earth and Environment, Faculty of Natural and Agricultural Science, University of Western Australia, Crawley, WA 6009, Australia, Email Bob.gilkes @uwa.edu. au

Abstract
This study was conducted to characterize the reactivity of carbonates in the surface horizons of 20 soils (Entisols and Aridisols) from Iran. The samples were analyzed for calcium carbonate equivalent (CCE), active calcium carbonate equivalent (ACCE), non-active calcium carbonate equivalent (N.ACCE), carbonate mineralogy and surface area of carbonates (SSA) by N_2 adsorption. The CCEs ranged from 38 to 228 g/kg of soil, with a mean 105.1g/kg of soil. ACCEs ranged from 13.4 to 99.8 with a mean 37 g/kg of soil. About 34% of $CaCO_3$ in the soils were able to react with oxalate .There is a considerable scatter of data about the regression line ($R^2 = 0.60$) between ACCE and CCE. This observation support the idea that calcium carbonate in the soils does not consist of particles with common particle size distributions. This feature of native carbonates is an important property in determining nutrient elements in calcareous soils. The surface area of carbonates was negatively and significantly correlated with CCE (0.52*, n=20), indicating that with increasing CCE content, surface area decreases. X-ray diffraction (XRD) analysis showed that [Mg_x Ca_{1-x} (CO_3)] is the most commonly carbonate in the studied soils. Dolomite was only present in 7 soils.

Key Words
Calcium carbonate equivalent, active carbonate equivalent, calcareous soils.

Introduction
Carbonates, which are common constituents of many soils of arid and semiarid areas, have a marked influence on soil chemical properties, e.g., pH, cation and anion sorption and physical properties, such as soil structure (Delcampillo *et al*. 1992). For this reason, the measurement of calcium carbonate equivalent (CCE) in calcareous soils is common and is useful for the evaluation of soil processes. In some instances, active calcium carbonate reactivity is related more highly than CCE to soil processes or properties. For example, the severity of iron chlorosis (Morris *et al*.1990) and phosphorous sorption in calcareous soils (Samadi and Gilkes 1999). In several countries, the active calcium carbonate equivalent (ACCE) is determined by reaction with 0.1 M NH_4-Oxalate (Drouneau 1942). Soil carbonate can be estimated from the difference in N_2 adsorption of soil and decalcified soil. This surface area was related to phosphate sorption capacity.
The purpose of the present study was to characterize the carbonate reactivities and carbonate surface area of some calcareous soils of the central part of Iran.

Methods
The 20 samples used in this study were collected from the surface horizons (0-30 cm) of Entisols and Aridisols from Gazvine plain, Tehran and Qom provinces in the central of Iran. Soil physical and chemical characteristics were determined according Method of Soil Analysis (Carter and Gregorich 2008).
Calcium carbonate equivalent was determined by acid digestion method. The ACCE was determined with 0.1 M NH_4 Oxalate using a 1:25 soil: ratio, and shaking 2 h at 250 rpm on a reciprocating shaker (Drouineau 1942). To calculate the N2-accessible SA of the CCE, 10-g samples were treated with HOAC-NaOAC (pH 4.75) buffer to remove carbonate. After this treatment, the samples were washed with water until they began to disperse and were freeze dried and weighted. The surface area (SA) of the intact samples and of their decalcified counterparts was then measured by N2 adsorption.
The "apparent" SA of the CCE was calculated using the equation:

$$SSA = A - B(1 - \frac{\%ACCE \text{ or } CCE}{100})$$

Where SSA is the SA attributable to the CCE, and A and B are the SSA of the intact and decalcified soil materials, respectively.

Results
The CCE ranged from 38 to 228 g/kg and ACCE1 from 9.9 to 90.6 g./kg and ACCE2 from 14.6 to 99.8 g/kg(Table 1).

Table 1. Values of carbonate calcium equivalents for the studied soils.

Soil No.	CCE (g/kg)	ACCE1 (g/kg)	ACCE2 (g/kg)	%ACCE2 / CCE	%ACCE1 / CCE	SSA.CCE (m^2/g)	SSA.ACCE1 (m^2/g)	SSAN.ACCE1 (m^2/g)
1	80	37.2	38.4	48	46.5	157	99	208
2	46	14.9	19.2	41.7	32.4	111	44	143
3	72	26	27.2	37.8	36.1	83	124	61
4	136	60.6	62.6	46	44.6	50	28	68
5	62	43.3	44.5	71.8	70	151	20	450
6	88	15.2	16.1	18.3	17.3	28	36	26
7	98	11.2	13.4	13.7	11.4	127	682	55
8	125	32.2	33.5	26.8	25.8	94	197	58
9	108	19.8	27.8	25.7	18.3	74	141	59
10	228	90.6	99.8	43.8	39.7	79	26	114
11	95	17.4	22.2	23.4	18.3	88	88	88
12	82	39.6	40.8	49.8	48.3	124	13	228
13	38	15.2	27.3	71.8	40	140	110	159
14	94	35.9	40.8	43.4	38.2	102	132	84
15	194	52.8	58.5	30.1	27.2	21	25	19
16	170	50.2	65	38.2	29.5	45	94	25
17	90	21.5	23.5	26.1	23.9	84	39	98
18	48	9.9	14.6	30.4	20.6	403	279	436
19	108	30.9	34.6	32	28.6	49	110	25
20	140	28.5	31.4	22.4	20.3	54	155	28
Mean	105	32.6	37.1	34	31.9	103.2	122	121

ACCE1 is active carbonate calcium equivalent in native soils and ACCE2 is the same property after sieving thorough a 1 mm sieve.

The slope of the regression line between ACCE1 and CCE is 0.60 and for ACCE2 is 0.62, i.e. about 60 % CCE is able to react with Oxalate (Figure 1). As shown in Figure 1, various proportions of CCE are present as ACCE and CCE in the soils indicating that carbonate does not consist of particles with a common particle size distribution. This feature of native carbonate is an important property in determining phosphate and zinc retention (Samadi and Gilkes 1999). X-ray diffraction (XRD) analysis showed that Mg-Calcite is the carbonate most commonly found in the soils (data are not shown). Only in 7 soils was dolomite present.

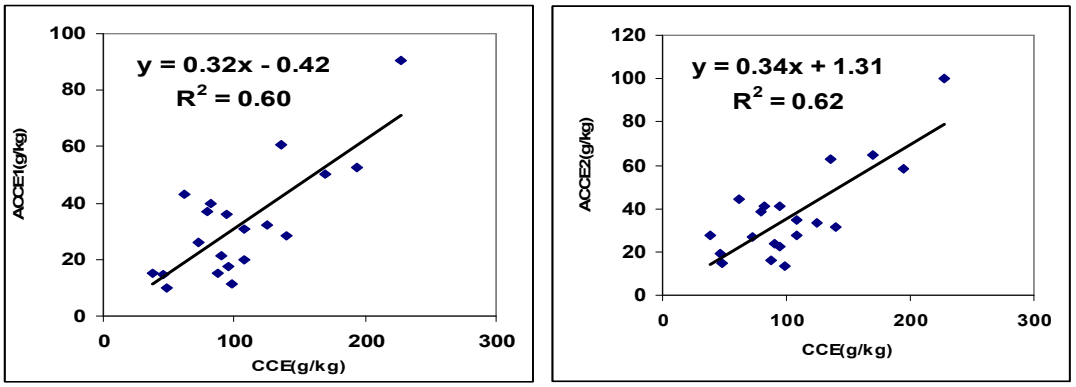

Figure 1. Relationship between active calcium carbonate equivalent and calcium carbonate equivalent.

The calculated SSA of the CCE ranged from 21 to 403 with a mean 103 m^2/g CCE. These values are similar to those found by Holford and Mattingly (1975), but lower than the reported values of Delcampillo *et al*. (1992). Holford and Mattingly (1975) found values up to 500 m^2/g for several soils with about 10 g CCE/kg soil ,but Delcampillo *et al.* (1992) believed that these high values might result from an error in the difference between the SAs of intact and decalcified soil samples, since decalcification could possibly have altered the effective SA of noncarbonated soil components. In this study, it seems that one of soils (soil No.18) was abnormal, so after removing this soil the relationships are better. In these studied soils the SA attributable to the CCE was inversely related to CCE (Figure 2) and positively related to the ACCE/CCE ratio (Figure 3). This result indicates the increasing surface area (decreasing particle size) produced by the progressive dissolution of parent limestone (Delcampillo *et al*. 1992).

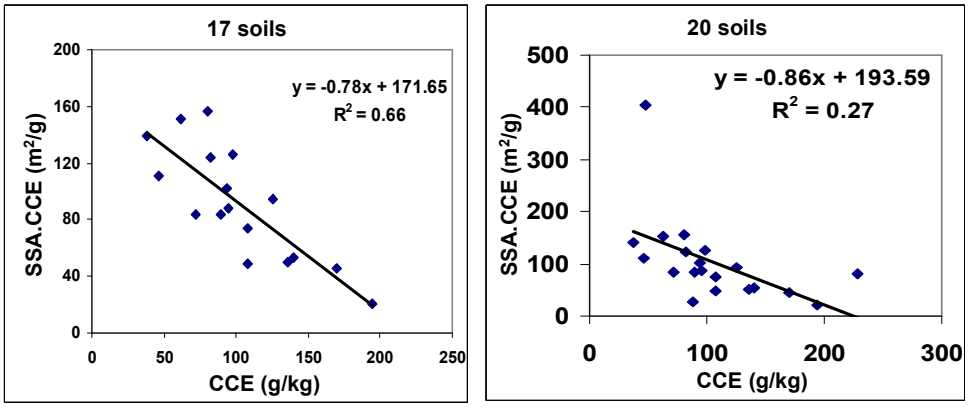

Figure 2. Relationship between SA attributable to the CCE and CCE, in all studied soils (n=20) and after removal of soils no 6, 10 and 18.

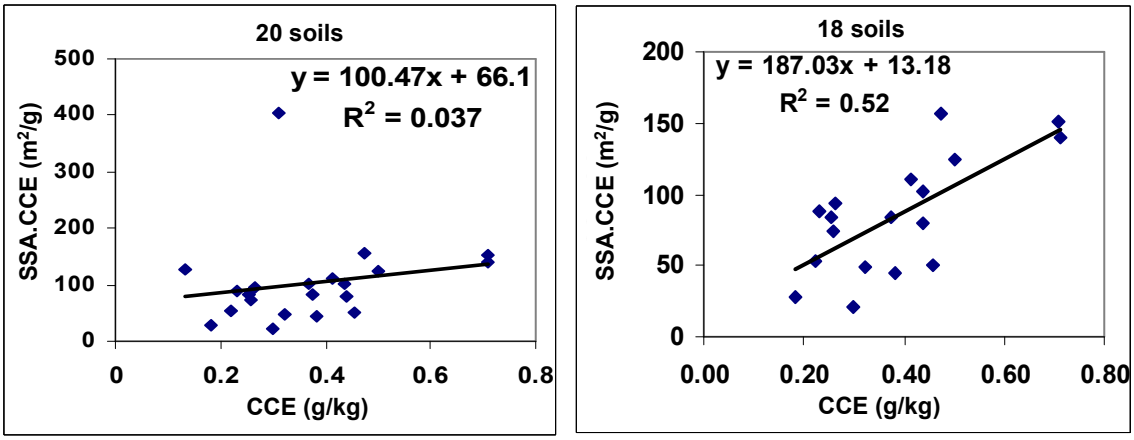

Figure 3. Relationship between SA attributable to the CCE and ACCE/CCE ratio for all the studied soils (no=20) and after removal of two soils (no 7 and 18).

Conclusion

Active calcium carbonate equivalent has been related by many workers with pH buffering and isotopic-exchangeable ions. The ACCE method described in this paper is a fast and provides a good quantitative estimate of reactive soil carbonate. This property should be measured for all calcareous soils of Iran to assist with the better interpretation of soil analysis results.

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References

Borrero C, Pena F, Torrent J (1988) Phosphate sorption by calcium carbonate in some soils of the Mediterreanean part of Spain. *Geoderma* **42**, 261-269.

Carter MR, Gregorich EG (2008) 'Soil Sampling and Methods of Analysis'. (Canadian societyof soil science).

Drouineau G (1942) Dossage rapide du calcaire actif du sol:nouvelles donnees sur la separation et la nature des fractions calcaires.*Ann.Agron*. **12**, 441-450

DelCampillo MC, Torrent J, Looppert RH (1992) The reactivity of carbonates in selected soils of southern Spain. *Geoderma* **52**, 149-160.

Holford ICR, Mattingly GEG (1975) Surface areas of calcium carbonate in soils. *Geoderma* **13**, 247-255.

Morris DR, Loeppert RH, MooreTJ (1990) Indigenous soil factors influencing iron chlorosis of soybean in calcareous soils. *Soil Science Society of America Journal* **54**, 1329-1336.

Samadi A, Gilkes RJ (1999) Phosphorus transformations and their relationships with calcareous soil properties of south western Australia. *Soil Science Society of America Journal* **63**, 809-815.