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Evaluation of soil enzyme activities as soil quality indicators in sludge-amended soils

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Abstract

Soil enzymatic activities are commonly used as biomarkers of soil quality. Several organic and inorganic compounds found in municipal wastewater sludges can possibly be used as fertilizers. Monitoring and evaluating the quality of sludge amended soils with enzyme activities accepted as a beneficial practice with respect to sustainable soil management. In the present study, variation of some enzyme activities (Alkaline phosphatase, dehydrogenase, urease and β-glucosidase activities) in soils amended with municipal wastewater sludge at different application rates (50, 100 and 200 t ha^{-1} dry sludge) was evaluated. Air dried sludge samples were applied to soil pots and sludge-soil mixtures were incubated during a period of three months at 28ºC. The results of the study showed that municipal wastewater sludge amendment apparently increased urease, dehydrogenase, alkaline phosphatase and β-glucosidase activities in soil by 48-70%, 14-47%, 33-66% and 9-14%, respectively. The maximum activity was generally observed in sludge amended soil with dose of 200 t ha^{-1}. Urease activity appeared to be a better indicator of soil enhancement with wastewater sludge, as its activity was more strongly increased by sludge amendment. Accordingly, urease activity is suggested to be soil quality indicator best suited for measuring existing conditions and potential changes in sludge-amended soil.

Key words

Enzyme activities, Incubation, Soil amendment, Wastewater sludge

Introduction

Application of organic additive is a favourable option to achieve soil improvement in some areas where organic matter content and biological activity are low. Organic matter is important for water retention, formation and stabilization of aggregates and formation of microhabitats, all factors with a strong influence on the survival of micro-organisms in soil. Wastewater sludge consist of essential nutrients (nitrogen and phosphorus) and is potentially beneficial as fertiliser for plants (Tejada *et al.,* 2006; Topaç *et al.,* 2008; Franco-Otero *et al.,* 2012). In this respect, addition of wastewater sludge is a method of replenishing degraded soil quality through improvement of biological status of soil, which usually implies an increase in both microbial and enzyme activity (Albiach *et al.,* 2000, Bastida *et al.,* 2006).

Soil enzymatic activity assay act as potential indicator of soil quality being operationally practical, sensitive and integrative. Besides, they are measures of soil microbial activity and therefore, they are strictly related to nutrient cycles and transformation (Drijber *et al.,* 2000, Nannipieri *et al.,* 2002, Puglisi *et al.,* 2006). Moreover, as claimed by several authors, (Dick and Tabatabai, 1993; Dick 1997; Van Belen and Doelman 1997; Trasar-Cepeda *et al.,* 2000) soil enzyme activities may be considered as an early and sensitive indicator of soil alteration in both natural and agro-ecosystems, thus being well suited to measure the impact of pollution on the quality of soil.

Dehydrogenase activity is recognized as a useful indicator in evaluating the metabolic activity of soil microorganisms. As dehydrogenases are not active independent of parent microbial cell as extracellular enzymes in soil, estimation of dehydrogenase is overall a good indicator of microbial activity and oxidation of organic matter (Kızılkaya and Hepşen, 2004). β-glucosidase activity is involved in degradation of soil organic matter and its activity may be useful in monitoring

soil quality because of its central role in soil organic matter cycling, which is generally regarded as an important component of soil quality (Turner *et al.,* 2002). Urease activity plays a very important role in mineralization of nitrogen compounds. It forms stable complexes (urease-humus) and is important in contributing to soil fertility (Nannipieri *et al.,* 1980). Phosphatase is an enzyme of great agronomic value because it hydrolyses compounds of organic phosphorus and transforms them into different forms of inorganic phosphorus, which are assimilable by plants (Alef *et al.,* 1995; Pascual *et al.,* 2002).

In light of the above, the objectives of the present study was to evaluate the response of soil dehydrogenase, alkaline phosphatase, β-glucosidase and urease activities to municipal wastewater sludge amendment and to determine the most appropriate sludge dose with respect to soil enzyme activities and to suggest the best-suited enzyme activity as a soil quality indicator for sludge amended soil.

Materials and Methods

Surface soil samples (0–20 cm) were taken from an experimental station in the campus of Uludağ University. The site is located in the Marmara Region, Northwestern Turkey (Latitude, 40°15'N; longitude, 28°53'E). The soil used in this experiment contained 62.3% clay, 14.5% silt and 23.2% sand (clay soil). Soil texture can accordingly be classified as an order of "entisol" and suborder of "fluvent" (Soil Taxonomy, 1999). The municipal wastewater sludge sample was obtained from municipal wastewater treatment plant in Bursa which treats wastewater at a flow rate of 64.000 $m^3 day^{-1}$. Initial values of enzyme activities in soil were as follows: Dehydrogenase activity (DHA): 32.85 μg g^{-1} $24h^{-1}$, β-glucosidase activity (β-GA): 145.72 μg g^{-1} h^{-1}, Alkaline phosphatase activity (APA): 246.21 μg g^{-1} h^{-1}, Urease activity (UA): 26.06 μg g^{-1} h^{-1}.

Experimental procedure : Soil samples were air-dried in laboratory and sieved through 2 mm screens. Then 500 g of soil was placed in cylindrical plastic container. The wastewater sludge was thoroughly mixed with soil at rates equivalent to 50, 100 and 200 t ha^{-1} on dry weight basis. A control treatment without wastewater sludge was also included. The incubation study was planned as a completely randomised design and each pot was replicated thrice to give a total of 12 pots. The pots were incubated for 90 days in dark at 28±0.5 °C. The moisture content in soil was maintained at 60% of field capacity throughout the incubation period.Soil samples were collected and analysed at each month of the incubation period.

Chemical analyses : Sample extracts were collected by shaking the samples with distilled water in 1:5 ratio (w/v). Electrical conductivity ($EC_{25°C}$) and pH of the sample extracts were measured with conductivity and pH meters respectively. Nitrate and ammonium nitrogen concentrations were determined by steam distillation with MgO and Devarda alloy (Keeney and Nelson 1982) in samples that were extracted with 2 M KCl. The Kjeldahl digestion method was used to measure total nitrogen concentration (Bremner and Mulvaney, 1982). In addition, dichromate oxidation was used to measure total organic carbon (Nelson and Sommers, 1982). Total heavy metals in sludge and soil samples were analyzed from HCl extracts, using ICP OES (Perkin Elmer OPTİMA 2100 DV).

Enzymatic activity of urease, dehydrogenase, alkaline phosphatase and β-glucosidase were determined according to the methods described by Tabatabai (1994). Urease activity was based on determination of NH_4^+ released by urease when soil was incubated with THAM buffer (pH = 9), urea solution (0.02M) and toluene at 37°C for 2 hrs. Ammonium formation was determined by steam distillation and the results were expressed as μg NH_4^+ Ng^{-1} h^{-1}. In order to determine dehydrogenase activity, triphenyl tetrazolium chloride solution (3%) was added to soil and suspension was incubated at 37°C for 24 hrs. Triphenyl formazan formation was determined spectrophotometrically at 485 nm and the results were expressed as μg TPF g^{-1} h^{-1}.

Alkaline phosphatase activities were performed by addition of modified universal buffer (pH = 11), toluene and p-nitrophenyl phosphate solution (0.025 M) to soil and incubation of soil suspension at 37°C for 1 hour. Released (PNP) p-nitrophenol was determined spectrophotometrically at 410 nm and the results were expressed as μg PNP g^{-1} h^{-1}. β-glucosidase activity test was based on colorimetric determination of p-nitrophenol released by the enzyme when soil was incubated with buffered p-nitrophenyl-β-D-glucoside solution as substrate and toluene. Released p-nitrophenol was determined spectrophotometrically at 410 nm and the results were expressed as μg PNP g^{-1} h^{-1}.

Statistical analysis : All the statistical calculations were performed using STATISTICA 6.0 software. Two way ANOVA (repeated-measures of ANOVA) was performed to test the effect of sludge application doses (0, 50, 100 and 200 t ha^{-1}) and incubation time (Table 2). The effect of incubation time on enzyme activities was further tested with one-way ANOVA for each sludge dose. When significant effects were indicated by ANOVA, post hoc analysis was performed using Tukey's HSD multiple comparison test. In addition, Pearson correlation coefficients (r) were calculated between sludge dose and soil enzyme activities (Table 3).

Results and Discussion

The results of physical and chemical properties of soil and wastewater sludge are presented in Table 1. Chemical properties varied among the sludge. Sludge was slightly acidic, with 6.22 pH. EC of wastewater sludge was 3650 dS m^{-1}. In addition, the results showed that sludge was an important potential source of plant nutrients. Total N and P content in wastewater sludge was 5.14%

and 1.65%, respectively. It contained relatively low extractable quantities of mineral N as compared with total N content (122.27 and 20.38 mg kg^{-1} ammonium-N and nitrate-N, respectively). Organic carbon content in wastewater sludge was 30.26% and could provide organic matter to soil, if applied in high concentration.

Total Cd, Cr, Ni, Pb, Cu and Zn concentration in sludge samples is given in Table 1. The concentration of heavy metal in sludge was lower than the permissible limit for agricultural use.

Soil used in experiment was clay loam texture and pH indicated that soil was slightly alkaline, which is common for Turkey soil. It had moderate lime and organic matter content and low salt content.

Alkaline phosphatase activity : Phosphatases are considered the key enzymes in phosphorus cycling in soil (Pascual *et al.,* 1998). Variation in phosphatase activity, apart from indicating changes in the quantity and quality of soil phosphorated substrates (Rao and Taraftar 1992; Kızılkaya and Bayraklı, 2005), are also good indicators of soil biological status. Variation in alkaline phosphatase activity (APA) levels in sludge amended soils during the incubation period of 90 days are shown in Fig. 1.

Statistical results revealed that APA in soil was significantly dependent on sludge doses and incubation time. Wastewater sludge application generally increased APA in soil for all treatments ($p<0.001$). This increment in activity levels is probably related to utilization of organic phosphorus (George *et al.,* 2006; Mohanty *et al.,* 2006; Criquet *et al.,* 2007, Hirzel *et al.,* 2007). APA levels in sludge amended soils increased with increasing sludge doses ($p<0.001$).

When time-dependent variation was evaluated, it was found that APA level in soil significantly decreased for all the treatments. This may be attributed to depletion of organic substrates and accumulation of metabolic toxins. In addition, phosphate ions, products of phosphatase hydrolysis, are competitive inhibitors of this enzyme in soil (Garg and Bahl, 2008; Gianfreda *et al.,* 2005; Ewulo *et al.,* 2008). Therefore, decreased amount of APA in soil may have resulted due to inhibition of enzymatic activities by inorganic phosphorus (Kızılkaya *et al.,* 2004; Criquet *et al.,* 2007).

According to the results, higher APA values in incubated soil were observed in soil pots amended with 200 t ha^{-1} sludge. The maximum value of 432 ug PNP g^{-1} h^{-1} was determined in those pots after incubation period of first month. On the other, hand there was no significant difference between 50 and 100 t ha^{-1} sludge treatment with respect to APA levels.

Fig. 2 depicts variation of dehydrogenase activity (DHA) in soil during the incubation period. Development of microbial biomass is sustained by biosynthesis of enzymes that provide energy and nutrients for microbial development. Dehydrogenase activity in soil is considered as a general index for evaluating soil microbial activity (Bastida *et al.,* 2012) and the effects caused by addition of municipal wastes (Reddy and Fazza, 1989; Pascual *et al.,* 1998).

Table 1 : General characteristics of wastewater sludge and soil

Properties	Values	
	Sludge	Soil
pH (1:5 deionized water)	6.22	7.76
$EC_{25°C}$ (1:5 deiyonized water, dS m^{-1})	3650	161.3
Organic C (%)	30.26	1.23
Total-P (%)	1.65	0.17
Total-N (%)	5.14	0.15
NH_4^+-N (mg kg^{-1} d.wt.)	122.27	29.63
NO_3^--N (mg kg^{-1} d.wt.)	20.38	19.75
Mineral-N (mg kg^{-1} d.wt.)	142.65	49.38
Zn (mg kg^{-1} d.wt.)	416	1.35
Cu (mg kg^{-1} d.wt.)	237	2.05
Ni (mg kg^{-1} d.wt.)	94	<1.00
Cr (mg kg^{-1} d.wt.)	286	<1.00
Cd (mg kg^{-1} d.wt.)	12.47	<1.00
Pb (mg kg^{-1} d.wt.)	25.81	<1.00

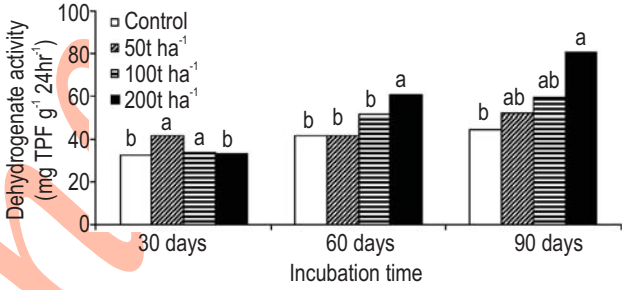

Fig. 1 : Changes in APA in amended soil during the incubation period. Small letter: Variations of alkaline phosphatase activity levels in soil depend on incubation time. Bars labelled with the same letters are not significantly different at the 5% level (each sludge dose was tested separately)

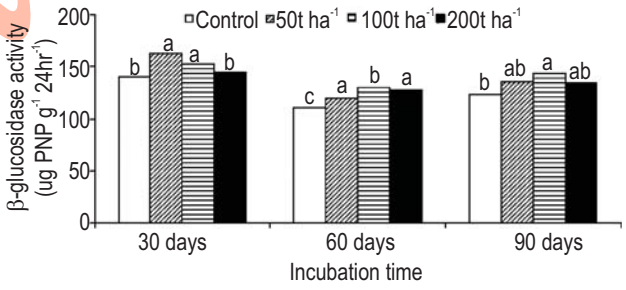

Fig. 2 : Changes in dehydrogenase activity in amended soil during the incubation period. Small letter: variations of dehydrogenase activity levels in soil depend on incubation time. Bars labelled with the same letters are not significantly different at the 5% level (each sludge dose was tested separately)

Dehydrogenase activity was apperantly higher in sludge amended soil than control soil ($p<0.001$), meaning that microbial metabolic activity had increased due to incorporation of compounds capable of activating the soil's autochthonous biomass (Pascual *et al.,* 1998; Brookes *et al.,* 2008; Franco-Otero *et al.,* 2012). In addition, higher DHA in soil can be explained due to increased content of organic carbon and nutrients.

Statistical results revealed that DHA in soil was significantly dependent on sludge doses and incubation time. DHA level in soil did not show significant variation during the first 30 days of incubation and thereafter increased with increasing amount of sludge. When time-dependent variation was evaluated, it was found that DHA level in sludge amended soil showed an increasing trend throughout the incubation period ($p<0.001$). It is possible that sludge amendment stimulated microbial production of DHA in soil or made more of this enzyme accessible to substrate.

Highest DHA activity in soil was obtained with 200 t ha^{-1} sludge treatment after incubation period of 90 days. Higher dehydrogenase activity noted at the high dosage suggests that either the added wastes did not include compounds which were toxic for dehydrogenase activity (Reddy and Fazza 1989, Perucci 1997) or increase due to microbial growth (with the consequent increase in the enzyme activity) and/or addition of microbial cells or enzymes with amendment with organic materials, might have counteracted any inhibitory effect by toxic compounds (Pascual *et al.,* 1998; Moscatelli *et al.,* 2012).

The origin of urease activity is basically microbial and stable complexes (urease-humus) may be formed in soil (Bremner and Mulvaney 1978; Nannipieri *et al.,* 1980). Variation in urease activity in sludge amended soil during incubation period of 90 days is illustrated in Fig. 3. Urease activity levels significantly depended on sludge doses and incubation time ($p<0.001$, Table 2). The results indicated that there was significant interaction between sludge treatment and incubation time. Wastewater sludge application generally increased urease activity in soil for all treatment ($p<0.001$). Highest urease activity in soil was obtained at 200 t ha^{-1} after treatment 3 months of incubation period.

When time-dependent variation was evaluated, no significant variation was determined in urease activity levels during the incubation period. It is known that in order to increase the biochemical activity of soil and to maintain this effect, the amount of organic matter added to soil must be high (Hueso *et al.,* 2011). Accordingly, it may be concluded that urea-type substrates in the studied sludge was quite enough to maintain the stimulated activity level.

Glucosidase is the rate-limiting enzyme in microbial degradation of cellulose to glucose, playing a crucial role in C cycle of soil (Perez-de-Mora *et al.,* 2005). It is generally sensitive to soil management (Bandick and Dick 1999) and has been suggested to be an integrative measure of physico-chemical and biological properties of soil (Turner *et al.,* 2002).

Fig. 4 shows variation of β-GA in soil at different concentrations of the sludge. In the present study, amended soil showed significantly-higher β-GA than control soil ($p<0.001$), which is in agreement with other reports on the enhancement of hydrolytic enzyme by organic amendments (Jordan *et al.,* 1995; Kremer and Li, 2003; Tejada *et al.,* 2006). Maximum β-GA level in soil was obtained with application of 50 t ha^{-1} at the first stage of incubation period. Similarly, some researchers have reported that wastewater sludge contains easily-degradable compounds that could stimulate the synthesis of hydrolytic enzymes and their release from intracellular media into soil (Bastida *et al.,* 2012). This might be the reason why, after 30 days of incubation, addition of sludge produced high level of total b-glucosidase activity which was able to degrade organic compounds.

β-GA level in soil slightly decreased with increasing incubation period. Presence of high content of degradable organic compounds (available substrate) in sludge might have stimulated enzyme activity: as substrate decreased, enzyme activity decreased as well (Ceccanti and Garcia 1994; Topaç *et al.,* 2008). On the other hand, Pascual *et al.* (1998) reported that

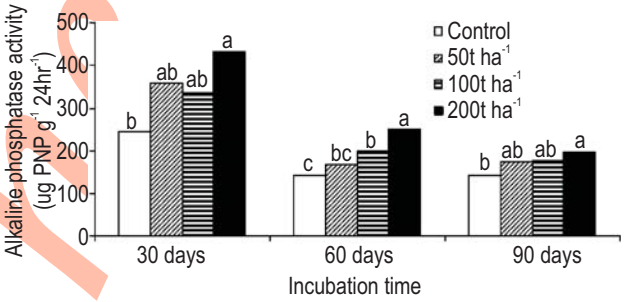

Fig. 3 : Changes in urease activity in amended soil during the incubation period. Small letter: variations of urease activity levels in soil depend on incubation time. Bars labelled with the same letters are not significantly different at the 5% level (each sludge dose was tested separately)

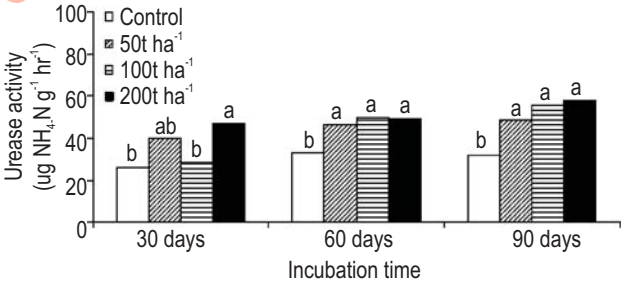

Fig. 4 : Changes in β-GA in amended soil during the incubation period. Small letter: variations of β-glucosidase activity levels in soil depend on incubation time. Bars labelled with the same letters are not significantly different at the 5% level (each sludge dose was tested separately)

Table 2 : Results of repeated measures of ANOVA for the analyses of main effect of sludge dose and incubation time

Sources of variation	df	MS	F	p
Dependent variable: Alkaline phosphatase activity				
Sludge dose	3	20 381.0	26.936	<0.001
Incubation time	2	105 443.0	139.357	<0.001
Sludge dose x incubation time	6	2717.0	3.591	<0.05
Error	24	757.0		
Dependent variable: Dehydrogenase activity				
Sludge dose	3	76 669.92	1 197.568	<0.001
Incubation time	2	566.09	8.842	<0.001
Sludge dose x incubation time	6	1786.49	27.905	<0.05
Error	24	353.33	5.519	
Dependent variable: ß-glucosidase activity				
Sludge dose	3	241.9	17.95	<0.001
Incubation time	2	7548.7	560.06	<0.001
Sludge dose x incubation time	6	551.4	40.91	<0.001
Error	24	13.5		
Dependent variable:Urease activity				
Sludge dose	3	721.89	28.765	<0.001
Incubation time	2	553.62	22.060	<0.001
Sludge dose x incubation time	6	88.89	3.542	<0.05
Error	24	25.10		

application of sludge may be attributed to lower cellulose content and/or a lower stability of enzyme in sludge during incubation time. In addition, this decrease in β-GA level througout the incubaion period was probably due to accumulation of metabolic toxins or heavy metals (Hinojosa et al., 2004).

The results of incubation study indicated that addition of sludge generally had positive effects on the studied soil enzyme activities. The average increase in enzyme activities in incubated soil amended with sludge is shown in Fig. 5. The results of the study showed that municipal sludge amendment apparently increased urease, dehydrogenase, alkaline phosphatase and β-glucosidase activities in soil by 48-70%, 14-47%, 33-66% and 9-14%, respectively. Municipal wastewater sludge seems to stimulate soil enzyme activity by increasing the available carbon, nutrients and/or microbial activity (Pontes 2002; Fernandes *et al.*, 2005; Dindar *et al.,* 2010). As compared to alkaline phosphatases, dehydrogenases and β-glucosidases activities, urease activity appeared to be a better indicator of soil enhancement with sludge, as its activity was more strongly increased by sludge amendment. Some authors have reported that application of organic amendments stimulates urease activity (Fernandes *et al.,* 2005; Pascual *et al.,* 2002). The average UA in soil amended with 200 t ha^{-1} was 70% higher than the control treatment. APA and DHA increased by 66% and 47% relative to the control for the sludge dose of 200 t ha^{-1}. As it can be seen from Figure 5, β-GA in sludge amended soils showed a different trend as compared to UA, APA and DHA. Higher doses of sludge amendment did not lead to higher levels of activity. The maximum β-GA level was observed in sludge amended soil with dose of 50 t ha^{-1} (14%).

In a recent study (Tejada *et al.,* 2013) soil samples were mixed with sludge samples at 0.2%, 0.5% and 1% (w/w) and then adjusted to a water-holding capacity of 60%. The results of incubation showed that the application of wastewater sludge to soil caused an apparent stimulatory effect on DHA, BGA and PA. Land application of wastewater sludge serves as a good source of microbial nutrient and the organic constituents providing beneficial soil-conditioning properties (Logan and Harrison, 1995; Singh and Agrawal, 2008).

Similarly, other researcher studied on the effects of addition of different doses (0, 100, 200 and 300 t ha^{-1}) and C:N ratios (3:1, 6:1 and 9:1) of sewage sludge on enzyme activities (β-glucosidase, alkaline phosphatase, arylsulphatase and urease) in a clay loam soil at 25°C and 60% soil water-holding capacity (Kızılkaya and Bayraklı, 2005). The results showed that addition of different doses and C:N ratios of sludge caused rapid and significant enhancement in enzymatic activities of soil. It was mentioned that this increase was specially noticeable in soil treated with high doses of sludge.

Correlation analyses were also carried out between sludge application doses and soil enzyme activities and results are presented in Table 3. Pearson coefficient for urease activity was 0.65 and the correlation was significant at a confidence level of <0.001. This positive correlation coefficient indicated a strong relationship between sludge application dose and soil urease activity level. Increasing sludge application are inclined to produce increasing urease activity level in soil. However, no significant correlation was found for APA, DHA and β-GA.

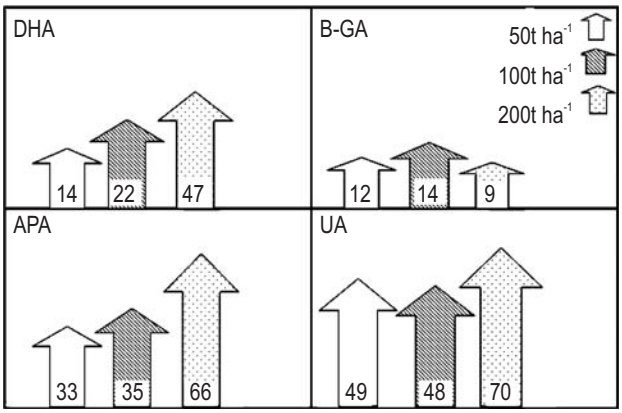

Fig. 5 : The average enhancement (%) of enzyme activities in incubated soils amended with wastewater sludge

Table 3 : The correlation between sludge dose and enzyme activity levels in sludge amended soils

Enzyme activity	Pearson correlation coefficients(r)	p
UA	**0.64**	**p<0.001**
APA	0.43	p=0.009
â-GA	0.18	p=0.281
DHA	0.35	p=0.037

Doubling sludge application did not result in a proportional increase in soil APA, DHA and β-GA level which indicated a nonlinear relationship between enzyme activity level and sludge addition.

Time dependent variation of enzyme activities in sludge amended soils indicated that highest peak of APA and β-GA generally appeared in the early stage of incubation. Thereafter, a decreasing trend was observed, probably due to scarcity of easily-degradable compounds in water-soluble fraction (Benitez *et al.,* 2004). On the other hand, more stable activity was observed for urease and dehydrogenase activities. Sludge application increased activity level within the first month of incubation and these stimulated levels were maintained throughout the follow-up incubation period. The effect of studied municipal wastewater sludge on urease and dehydrogenase activities seemed to be more permanent.

The overall evaluation of the study indicated that application of municipal wastewater sludge at a dose of 200 t ha^{-1} was most effective on enzyme activities (except β-GA). The results apperantly showed that the remedial effect of wastewater sludge was especially obvious for soil urease and dehydrogenase activities. As it was emphasized by many researchers, wastewater sludge additions to agricultural and other soil with background concentrations of heavy metals, raise soil content and degrade soil functions (Stephen and Smith 2009). Although, the studied sludge contains certain amount of heavy metals (Table 1), no negative effects were observed in this short term incubation study. The enzyme activities were not affected by sludge-derived heavy metals even for the highest sludge dose (200 t ha^{-1}). The texture of the soil (clay) used in the present study may have masked the detrimental effects associated with heavy metals. Adsorption of heavy metals is highly dependent on soil components that include silicate clays, organic matter, and iron, aluminium and manganese oxides (Merdy *et al.,* 2009). Lozano Cerezo *et al.* (1999) showed that treatment with wastewater sludge decreased the availability of heavy metals with time in a clay quarry.

Nevertheless repeated application of sludges can create a potential risk of metal accumulation in soil (Heckman *et al.,* 1987) and consequently soil monitoring strategy should be implemented in order to assess the impact of sludge amendments (Carbonell *et al.,* 2009).

The results of the study showed that municipal wastewater sludge amendment apparently increased urease, dehydrogenase, alkaline phosphatase and β-glucosidase activities in soil by 48%-70%, 14%-47%, 33%-66% and 9%-14%, respectively. Maximum activity were generally observed in sludge amended soil with a dose of 200 t ha^{-1}. As compared to the activities of alkaline phosphatases, dehydrogenases and β-glucosidases, urease activity appeared to be a better indicator of soil enhancement with wastewater sludge as its activity was more strongly increased by sludge amendment. Correlation analysis indicated a strong relationship between sludge application dose and soil urease activity level. On the other hand, increasing wastewater sludge application did not result in a proportional increase in soil alkaline phospatase, dehydrogenase and β-glucosidase activities. Accordingly, urease activity is suggested as a soil quality indicator best suited for measuring existing conditions and potential changes in sludge-amended soil.

Consequently, sludge contain nutrients and organic matter that can provide soil microbial activity and are widely used as soil amendments. They also, however, contain contaminants including metals, pathogens and organic pollutants. Although current regulations require pathogen reduction and periodic monitoring for some metals prior to land application. The result of the study related to sludge management, indicated that treatment and disposal of wastes should be evaluated and improved according to legal regulations and laboratory/field studies.

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