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Metalloid and Insecticides-Induced Modifications in the Key Soil Enzymes Regulating Biogeochemical Cycling

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ABSTRACT

Activities of soil enzymes are closely associated with its fertility. Extensive agricultural practices firmly based on unethical pouring of agrochemicals, which adversely affects soil microflora, and interferes with their associated enzymatic activities. Therefore, current study was aimed to monitor single and combined impacts of insecticides chlorpyrifos and cypermethrin, and metalloid arsenic, on activities of microbial enzymes, in clay loam soil, under *in vitro* conditions, for 50 days. Data revealed that applied treatments resulted in both stimulatory and inhibitory impacts on dehydrogenase, amylase, protease and acid phosphatase, as a function of days of incubation. However, addition of arsenic alone and with cypermethrin, exerted a synergistic effect over tested enzymes. Other side, addition of chlorpyrifos with arsenic or cypermethrin, and/ or both, exhibited antagonistic or inhibitory impacts. Overall results conclude that application of insecticides and arsenic, in suitable proportion, would possibly exert limited toxicity to microbial enzymes thereby preserve soil fertility.
KEYWORDS: Acid Phosphatase; Amylase; Arsenic; Chlorpyrifos; Cypermethrin; Dehydrogenase; Protease

I. INTRODUCTION

The major constraints in agricultural soil are pesticides and heavy metals/ metalloids. Agriculture is the main occupation of the world. To protect crops from different types of insect in order to preserve its high yield and productivity is the major goal of agriculture. This can be achieved by frequent use of pesticides and insecticides. But, indiscriminate addition of pesticides resulted in contamination of agricultural soil and became a major risk of soil health [1]. Now-a-days, pesticides are essential components of agricultural practices and are categorized into a range of compounds depending on their chemical constituents and usage. These are majorly classified into organophosphate, synthetic pyrethroides, carbamate, organochlorine and inorganic pesticides [2]. At the time of application, these compounds are quite specific, but later on penetrates into the soil and adversely affect both target and non-target organisms including microbial flora, and their enzymatic activities [1]. After prohibition, on the usage of organochlorine, the organophosphates and pyrethroid compounds are popularly used in the agriculture sector. Of these, chlorpyrifos (Chl) is one of the largely used organophosphate insecticides. It has low miscibility in water, moderate hydrophobicity and binds strongly to both soil and organic matter. Its half-life ranges from a few days to more than four years, depending upon the rate of application, type of ecosystem, and existing environmental conditions. Higher dose of it was shown to cause nervous disorders in the organisms [3]. Likewise, cypermethrin (Cyp), a synthetic pyrethroid, has neurotoxic property and persists in both the soil and sediments. Its half-life ranges between 14 to 76 days, depending upon the physicochemical properties and microbial activity of the soil [4].

Additionally, contamination of agricultural soils by an array of heavy metals (HMs)/ metalloids such as Pb, Cd, Cr, Cu, Hg, Zn, As, *etc*., released from the sewage sludge, industrial wastes, fertilizer application, *etc*., is another potential risk of soil health [5]. Among these, arsenic (As) is one of the most abundant elements in the earth's crust, which comes from both natural (released from pyrite ores in water by geochemical factors) and men made (through insecticides, herbicides, phosphate fertilizers, *etc.*) sources [6]. Adsorption of As on soil particle is a major determinant of its immobilization and persistence in nature. Its toxicity and speciation depends on the pH of the soil [7]. Arsenic is a carcinogen and lethal to all the organisms including plants, even in trace amount [7]. A significant part of arable land was marked as the HM/ metalloid polluted, resulting in considerable loss in the grain yield, every year [7]. Moreover, presence of it adversely affects the activities of important soil enzymes (SEs) of Carbon (C), Nitrogen (N), Phosphorus (P) and Sulphur (S) transformation [8]. The HM/ metalloid-induced inhibition of SEs are due to 1) their complexation with the substrate, and 2) their binding with the protein-active groups of the enzymes [9].

Usually, SEs are synthesized by native microorganisms, which serves as biological catalysts to different reactions and metabolic processes of the soil, especially of different biogeochemical cycles [9]. A number of enzymes like protease, amylase, dehydrogenase, urease, phosphatase, *etc*., are widely shown to be present in the soil, and are key operators of various biogeochemical cycles and metabolic processes [10]. Out of these, dehydrogenase is part of all living microbial cells, and is a reflection of microbial respiratory status in the soil. It

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oxidizes soil organic matter by oxido-reductase activity, and is an indicator of total microbial activity in the soil [11]. Amylase, a starch hydrolyzing enzyme, is widely distributed in the soil, and plays a vital role in carbon cycling. By this, starch is converted into glucose monomers that serve as source of C for microorganisms [12]. Similarly, protease, an extracellular enzyme, is linked with N mineralization, and therefore determines its availability in the soil. Phosphatases, key players of P cycling are a group of enzymes responsible for hydrolyzing anhydrides and esters of phosphoric acid [13]. One of the predominant groups of it found in the soil is phosphomonoesterases, which includes both acid and alkaline phosphatases [13]. Extensive use and accumulation of insecticides/ HMs in the soil imposes neutral, negative or positive impacts over functioning of SEs. Thus, enzymic activities are popularly recognized as a sensitive indicator of any natural or anthropogenic disturbances in the soil properties [14]. Further, conventional practice and risk assessment have generally been focused on exposure of single synthetic chemical or additive to the agricultural fields [15]. However, in reality, contaminants do not exist alone instead invariably occurs as mixtures of it [16]. In order to investigate the amendments in the activities of key SEs, in response to insecticides and HMs, efforts should urgently be needed to assess combined impacts of these pollutants in the agricultural soils [17]. Recently, few studies dealt with adverse impacts of chemical mixtures in the environment [16]. But, toxic impacts of insecticides-metalloid interaction on the activities of SEs are less scrutinized.

Considering these facts, proposed study has been designed to investigate the adverse impacts of 1. As, 2. Chl+As, 3. Cyp+As, and 4. Chl+Cyp+As, on the activities of SEs. In this study, dehydrogenase, amylase, protease and acid phosphatase were chosen, considering them as representatives of the total metabolic activity of the soil, as well as their involvement in the biogeochemical cycling of C, N, and P, respectively.

II. MATERIALS AND METHODS

A. Soil: sampling site and physicochemical analyses

In this study, surface soil (0-15 cm) sample was collected from the paddy growing fields of Kumhari, Durg (21.27ºN, 81.52ºE, 285 MSL), 14 Km to West of Raipur. Soil samples collected from different (5 Nos) locations of same field were then pooled and were brought to the laboratory within 1 h. The soil sample was air dried at room temperature (temperature 26 ± 2ºC, 52% relative humidity), sieved through 2 mm mesh, and stored in sterile polypropylene boxes at 4ºC. The soil pH was determined by making a soil suspension, maintaining a ratio of 1:2.5 (soil: water), and was measured to be pH 7.1. Data regarding physicochemical properties of the soil were obtained from Department of Soil Science, Indira Gandhi Agriculture University, Raipur.

B. Insecticides and metalloid

All chemicals and reagents are of analytical grade. In this study, pure forms of both the insecticides Chlorpyrifos (98%) and Cypermethrin (92%) were obtained from the M/s Gharda Chemicals, Pvt. Ltd., Mumbai, India. The molecular formula and chemical names of Chl and Cyp are $C_9H_{11}cl_3NO_3PS$ [O,O-diethyl O-(3,5,6-tricholoro-2-pyridyl) phosphorothioate] and $C_{22}H_{19}Cl_2NO_3$[(+/-)-a-cyano-3-phenoxybenzyl(+/-)-cis, trans-3(2,2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate]. In addition, sodium arsenite ($NaAsO_2$, Sigma, USA) was used as a source of As.

C. Experimental design

The experiments were conducted in air tight sterile polypropylene boxes (20cm×5cm×6cm). Each box contained 500 g (dry mass basis) of fine, dry, sieved soil sample. The soil samples were added (separately or in combination) with aqueous solutions of As (3.5 mg Kg^{-1} soil, T1), Chl+As (50+3.5 mg Kg^{1} soil respectively, T2), Cyp+As (40+3.5 mg Kg^{-1} soil respectively, T3), and Chl+Cyp+As (50+40+3.5 mg Kg^{-1} soil respectively, T4). The moisture content of each of the soil sample was adjusted to 60% water holding capacity. These boxes were then incubated in the darkness at 26 ± 2ºC temperature and 52% relative humidity, maintaining the same moisture content for next 50 days. The control soil sample was mixed with distilled water only. To determine activities of SEs, soil samples were harvested after 1, 10, 20, 30, 40 and 50 days of incubation (DOI).

D. Soil enzymes: extraction and assay

Dehydrogenase (EC 1.1.1.1) activity was determined following the method of Casida *et al*., [18]. In this method, soil sample (1 gm) was mixed properly with substrate [1 ml of glucose (0.03%, w/v), 4.5 ml of 2,3,5-triphenyl tetrazolium chloride (0.5%, w/v) prepared in 0.1M Tris buffer (pH 7.8), and 3.5 ml of MilliQ water (MW, Millipore Gradient A-10, USA)] and allowed to stand at 37ºC for 24 h. The triphenyl formazan (TPF) thus formed was extracted with acetone and quantified at 485 nm using a UV-Vis spectrophotometer (Lambda-25, Perkin Elmer, USA) against methanol, as a blank. Dehydrogenase activity was expressed in terms of µg TPF formed g^{-1} soil h^{-1}.

Activity of amylase (EC 3.2.1.1) was determined by the method of Cole [19]. The soil sample (1 gm) was mixed with 50 mM Tris-HCl buffer (pH-8) consisting 1% (w/v) sucrose, and incubated at 37ºC for 24 h. In the filtrate of this, reducing sugar was estimated by dinitrosalicyalate method [20]. A standard curve of maltose was prepared and activity of amylase was expressed in terms of mg maltose g^{-1} soil h^{-1}.

Protease (EC 3.4.21-24) activity was determined following the method of Jayamadhuri [21]. Soil sample (1 gm) was mixed with Tris-HCl buffer (0.1 M, pH 7.5) comprising 1% (w/v) bovine serum albumin, and allowed to stand for 24 h at 37°C. To it, added trichloroacetic acid (17.5%, w/v), and centrifuged (10,000 rpm, 10 min). Supernatant thus obtained was mixed rigorously with Na_2CO_3 (1.4 M) and Folin ciocalteu reagent (33.3%, v/v). The intensity of the blue colour developed was read at 700 nm. A calibration curve was prepared using tyrosine and activity of protease was expressed in terms of µg tyrosine formed g^{-1} soil h^{-1}.

Activity of acid phosphatase (EC 3.1.3.2) was assayed by the following protocol [22]. Soil sample (1 gm) was initially mixed with toluene and disodium phenyl phosphate {prepared in acetate buffer (1.4 M, pH-5)} and allowed to stand for 24 h at 37°C. To it, added ammonium chloride buffer (1M, pH 9.6) and then filtered. In this filtrate, 8% (w/v) potassium ferrocyanide and 2% (w/v) 4-aminoantipyrine were added, and absorbance of it was read at 510 nm. A calibration curve was prepared using phenol, and enzyme activity was referred in terms of µg phenol g^{-1} soil h^{-1}.

E. Statistical analysis

Data obtained were analyzed by two-way ANOVA at $P < 0.05$ level using SPSS software (Ver 16.0). Regression analysis between activities of SEs and treatments applied were also performed.

III. RESULTS AND DISCUSSION

The agricultural soil mainly used for cultivation in Kumhari, Durg district of Raipur, India was analysed to consist of sand: silt: clay in the ratio of 32:34:34. Considering this soil texture analysis, the soil was found to be of clay loam type. Soil analysis also revealed the presence of organic carbon, electrical conductivity, and water holding capacity as 0.68%, 0.19 d S m^{-1}, and 24.47 respectively. Type of soil and its granulometric composition analysis is important because it was found to influence the responses of insecticides/ metalloids and activities of SEs [13]. Studies suggested that in clay loam soil pesticides persists for a bit longer time compared to in any other soil types [23]. Usually, clay loam soil has more organic matter content as well as clay content. In this type of soil, metals and insecticides adsorbed more strongly or form complexes reducing the mobility of these contaminants and enhancing their persistence [23]. Considering these facts, clay loam soil (sand:silt:clay; 32:34:34) was taken in this investigation which was also shown to possess good abundance of microflora and higher activities of SEs. These days, agricultural soil invariably contains mixtures of contaminants like pesticides, fertilizers, HMs/metalloid, *etc*. Thus, current investigation was planned to study the effects of As (T1), insecticides and As (Chl+As, T2) and (Cyp+As, T3), and combination of above three (Chl+Cyp+As, T4) on the activities of dehydrogenase, amylase, protease and acid phosphatase of soil. The selection of these insecticides is based on their frequent usage in the paddy growing fields, throughout [24]. The concentrations of insecticides (Chl 50 mg kg^{-1} and Cyp 40 mg kg^{-1}) chosen were according to their reckless use against recommended field rates. While, selection of As and concentration of As (3.5 mg kg^{-1}) was decided on the basis of its availability into the agricultural soil [25]. Until recently, studies undertaken in this avenue demonstrated the ill effects of insecticides or HMs alone over the SEs, but their interactive impacts are not resolved fully. The results of linear regression analysis of dehydrogenase, amylase, protease and acid phosphatase are given in Table 1."

A. Treatments *vs* Dehydrogenase

The activity of dehydrogenase was observed to be sensitive towards all the applied treatments (T1, T2, T3 and T4). Its activity was found to fluctuate in between 0.01 to 0.24, 0.02 to 0.12, 0.02 to 0.18 and 0.03 to 0.08 µg TPF formed g^{-1} soil h^{-1}, in response to addition of T1, T2, T3 and T4 respectively (Table 2). Two-way ANOVA revealed significant variation in the activity with respect to DOI ($F = 40.139$, $p < 0.001$), treatments applied ($F = 12.80$, $p < 0.001$), as well as interaction between treatments and DOI ($F = 5.118$, $p < 0.001$). Highest activity of it was recorded on 40 DOI in response to all the treatments (Figure 1). It was observed to differ significantly with their respective controls in treatments (T1, T2, T3 and T4) (Table 2). However, comparison between the treatments was significant and revealed that till 30 DOI, all the treatments exhibited similar responses over dehydrogenase, and thereafter T1 and T3 showed stimulatory effects, while T2 and T4 showed antagonistic responses, compared to the control (Table 3). On the whole, activity of dehydrogenase was seen to be raised considerably with increased DOI (Figure 1). Dehydrogenase was considered to be the most sensitive parameter to the presence of disturbances or xenobiotics in soil [26]. The production and optimum activity of enzyme was fairly related to the time/ day length required for its microbial counterpart to adjust to its prevailing environmental conditions. Also, the increase or decrease in its activity is related closely with stimulation or inhibition in the microbial activities in response to treatments applied. Addition of chlorpyrifos in the treatment (T2 and T4) imposed more toxic impacts due to its complex chemical structure [26]. Stimulation of enzymatic activity could be attributed to the use of the insecticide as a source of electron and energy [23]. Incubation time in the presence of various synthetic agrochemical treatments was considered by many other researchers. Increased dehydrogenase was detected in all the pesticide treated soils after 21 DOI [27]. Likewise, Nweke *et al*., [23] revealed that low doses of both Atrazine and Northrin enhanced the dehydrogenase activity

after 10 DOI. Application of atrazine stimulated the respiratory activity in soil as a function of dose and day length [28]. Activity of dehydrogenase was also found to be increased on 10 DOI, and onwards in response to Cyp and Quinalphos application in the soil [29]. Interactive study between treatment and DOI on dehydrogenase activity was studied by Rasool et al. [30] and was found to induce significant impact on the enzyme activity.

B. Treatments *vs* amylase

The activity pattern of amylase in regard to different treatments T1, T2, T3 and T4, ranged from 0.002 to 0.08, 0.001 to 0.07, 0.001 to 0.06 and 0.001 to 0.08 mg maltose g^{-1} soil h^{-1} respectively (Figure 2). Significant impact of length of incubation ($F = 101.36$, $P < 0.001$) and applied treatments ($F = 7.37$, $P < 0.001$) over the activity of amylase was discernible. Moreover, interaction between the treatments and length of incubation also influenced amylase activity considerably ($F = 2.56$, $P < 0.01$). Among different days of analyses, on 20 DOI, the activity of amylase was comparatively high in response to all the treatments (Figure 2). Moreover, when all the treatments were compared with their respective controls, significant change in amylase activity was detected on 20, 30 and 40 DOI (Table 2). When we compared the amylase activity in regard to applied treatments, the difference noted was almost non-significant (Table 3). Stimulation in enzymatic activity at a particular time period was due to time of adjustment. Effect of treatments on amylase activity was in concomitant to populations of amylolytic microbes in soil [31] and use of contaminant as substrate for their energy and growth [32]. Prolong incubation showed declining trend as the contaminant which was used as substrate before was degraded by microbial population and no longer available as substrate [32]. In regard to amylase (Figure 2), our finding partially agrees with the results of several researchers who have observed an increment in its activity, than that of control, in response to single or joint application of pesticide(s), and DOI [33]. Nasreen *et al*., [33] mentioned that the activity of amylase increased on 20 DOI, thereafter declined gradually with extended time of exposure. Similar trend in the activity of amylase was also recorded by Gundi *et al*., [34]. Mohiddin *et al*., [35] observed enhancement in amylase during initial days of Flubendiamide and Spinosad application, but was declined later.

C. Treatments *vs* protease

In response to applied treatments (T1, T2, T3 and T4), the *in vitro* protease was detected to fluctuate in between 0.88 to 1.59, 0.53 to 1.50, 0.63 to 1.11 and 0.44 to 1.33 µg tyrosine formed g^{-1} soil h^{-1} respectively (Table 2). A significant change in soil protease was discernible due to the interaction between length of incubation and treatments applied, as revealed by F test ($F = 3.43$, $P < 0.001$). Moreover, applied treatments were also found to be impacted remarkably ($F = 3.49$, $P < 0.05$) over the soil protease. The activity of protease enzyme recorded significantly lesser in different treatments when compared with the control. In control on day 10 only, the protease activity was observed maximum (Figure 3). Hence, length of incubation was found to be inhibitory over protease till 10 DOI, and thereafter stimulatory to it. Additionally, comparison between the treatments was significant and revealed T1 as more prominent over protease, particularly during initial DOI, than the other three treatments (Table 3). Initial decline in its activity was due to sudden exposure of insecticides and heavy metal to the soil. Thereafter, the protease activity recovered largely as it gets adjusted with its prevailing environment. In the current study, declining trend of protease in respect to applied treatments was observed (Figure 3), which is in coherence with the observations recorded by Kumar and Prakash [36] and Srinivasulu *et al*., [37], in respect to joint application of Thiobenecarb and Butachlor, Monocrotophos and Moncozeb, and Chl and Carbendazim into the soil. Similarly, Rasool *et al*., [30] also documented initial decline in the protease of herbicide added soil, but was seen to be enhanced abruptly after 21 DOI. Further, they have also studied the interactive pattern between day length and treatments applied. The interaction between the treatments and DOI highlights whether the contaminant is used as substrate/ inducer for mineralization to use it as nitrogen source or acts as inhibitor with the increasing time of incubation.

D. Treatments *vs* acid phosphatase

Activity of acid phosphatase showed variations in between 0.29 to 0.66, 0.21 to 0.64, 0.21 to 0.72, and 0.20 to 0.68 µg phenol g^{-1} soil h^{-1} in response to T1, T2, T3 and T4 respectively (Table2). Incubation time ($F = 22.68$, $P < 0.001$), applied treatments ($F = 2.866$, $P < 0.05$) and interaction between these two ($F = 2.31$, $P < 0.01$) exhibited considerable impacts over activity of acid phosphatase (Figure 4). In general, length of incubation was found to be stimulatory towards the activity of acid phosphatase, more precisely till 40 DOI (Figure 4). Further, when different treatments were compared with their corresponding controls, significant change in acid phosphatase activity was detected on 10, 30, 40 and 50 DOI in respect to T1, T2 T3 and T4 respectively (Table 2). Moreover, when treatments were compared in regard to their stimulatory/ inhibitory impacts, no significant difference was observed among them (Table 3). Just like ours data (Figure 4), in comparison to non-treated control, enhancement in the acid phosphatase activity was observed in black and red soil after 10 days of Monocrotophos addition [38]. While, an initial decline in it was recorded after field application of Chl (2-10 kg ha^{-1}), but was seen to be recovered fully on and after 14 DOI [39]. However, the two organophosphate pesticides Quinolphos and Monocrotophos, and two pyrethroids Fenvalerate and Cyp showed stimulatory effects, when applied at a field rate of 1-5 kg ha^{-1} over the acid phosphatase activity [40]. Likewise, combinations of Mancozeb and Monocrotophos, and Carbendazim and Chl exhibited increased acid phosphatase activity in

respect to 1.0 and 2.5 kg ha^{-1} respectively, of field application. Overall mechanism regarding effect of treatments and day length on acid phosphatase activity is based on the time required for adjustment, toxicity of the chemical, use of contaminant as substrate and time required for degradation of substrate.

Actual impacts of applied treatments on activities of SEs cannot be ascertained precisely, particularly in laboratory scale, because SEs shows complex interaction in their natural habitat. However, till now studies have been conducted to assess the impacts of pesticide alone, combination of pesticides, or combination of pesticide and HM, over activities of SEs. But, comparative study between different treatments is rarely conducted. In this study, comparison between applied treatments revealed significant variation in SEs. By and large, T1 and T3 exhibited stimulatory impacts over SEs, as compared to T2 and T4 (Figures 1, 2, 3 and 4). Thus, increasing pattern of toxicity towards SEs was found to be T3>T1>T4>T2. Accumulated data revealed that, As alone caused initial increase in tested SEs, and thereafter declined (Figure 1). Above change might be the resultant of soil bioavailability of this metalloid. In the soil, As exists in several forms, and its speciation depends upon the seasonal variation and bioavailability of it [7]. The inherent toxicity of HMs differs widely particularly in the order Pb>Cu>Hg>Cd>Zn>Cr(III)>Co>Ni>As>Cr(VI), however, the interaction between a HM and the soil strongly influence its toxic impact [41]. Only a part of the total HM content of a soil is soluble and available for different processes [41]. Moreover, lower sorption rate and high solubility of HM make it less toxic to SEs. Compared to several other HMs, As have low sorption rate hence limited toxic impacts [41]. Thus, As application resulted stimulation in the SEs during initial days of incubation (Figures 1, 2, 3, 4). Study on the activities of protease, dehydrogenase, phosphatase, *etc*. in response to different HMs were initially studied by many researchers like Nweke *et al*., [23], Sethi and Gupta [42], *etc*. Their findings concluded that inhibitions in the enzymatic activities were possibly the resultant of HM-induced changes in the molecular configurations of these enzymes. Researchers have also shown that HMs binds transiently with -sulfhydryl groups available in the active sites of SEs, thereby making them non-functional, forming metal-sulfide equivalents [9]. Binding of metal to enzymes active site block this functional site and thereby inhibit the binding of substrate. Hence, the enzymatic activity gets inhibited. On the other hand, when As was blended with Chl, its impact over SEs was anatagonistic, but with Cyp, it showed stimulatory impacts (Figures 1, 2, 3, 4). Similar to our findings, Shen *et al*., [43] showed toxic impacts of HM and polycyclic aromatic hydrocarbons over few of the SEs. In regard, Sikkema *et al*., [44] stated that the hydrophobic pollutants interact closely with lipophilic components of cytoplasmic membranes of the microorganisms, thereby change in the membrane permeability. The Chl and Cyp used in this study are categorized as hydrophobic pollutants. In addition, the organophosphates have higher toxicity compared to the pyrethroid [45]. Thus, blending of As with Chl exhibited more toxic impacts over tested SEs, than with Cyp. Moreover, Cyp was found to induce synergistic effect when mixed with HM [46]. Further, combination of two insecticides (Chl and Cyp) and As revealed inhibitory effect over SEs. This is because the total concentration of contaminants is higher in combined application which imposed greater toxicity to microorganisms as compared to their single addition (Figures 1, 2, 3, 4).

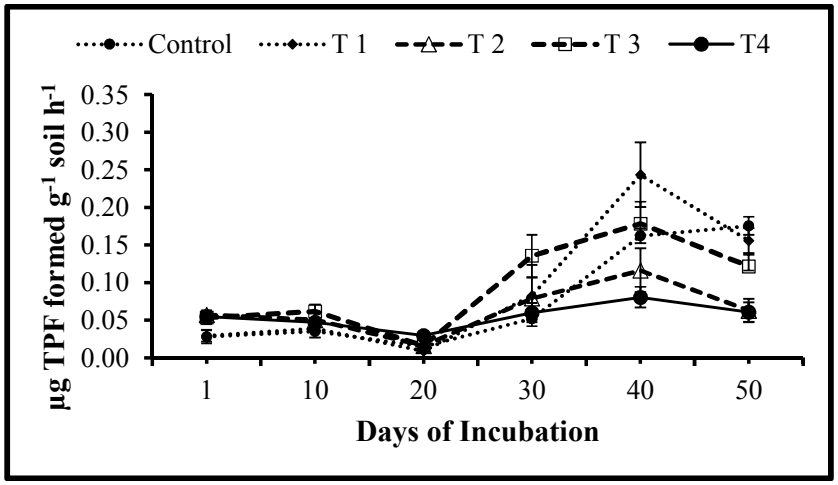

Figure 1 *In vitro* soil dehydrogenase activity in response to single and joint application of arsenic and insecticides (chlorpyrifos and cypermethrin), and days of incubation. Here, T1 = Arsenic, T2 = Chlorpyrifos + Arsenic, T3 = Cypermethrin + Arsenic, T4 = Chlorpyrifos + Cypermethrin + Arsenic. Each bar represents mean ± SE of three independent replicates.

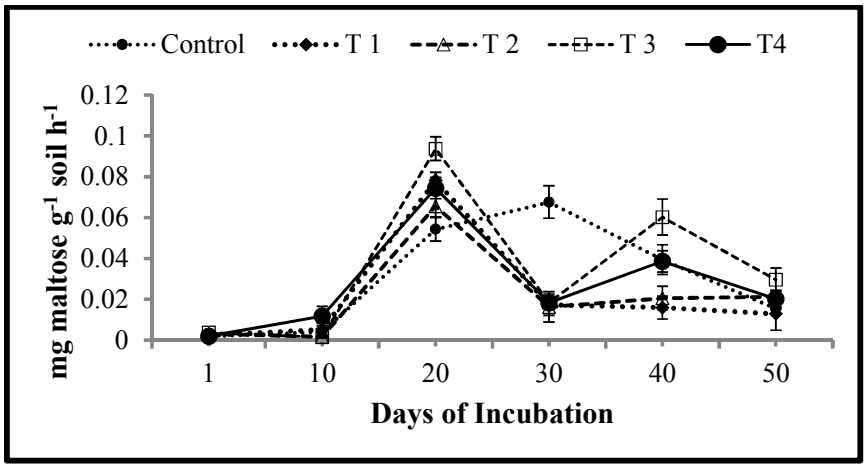

Figure 2 Effect of different treatments and days of incubation on *In vitro* amylase activity of soil. Here, T1 = Arsenic, T2 = Chlorpyrifos + Arsenic, T3 = Cypermethrin + Arsenic, T4 = Chlorpyrifos + Cypermethrin + Arsenic. Each bar represents mean ± SE of three independent replicates.

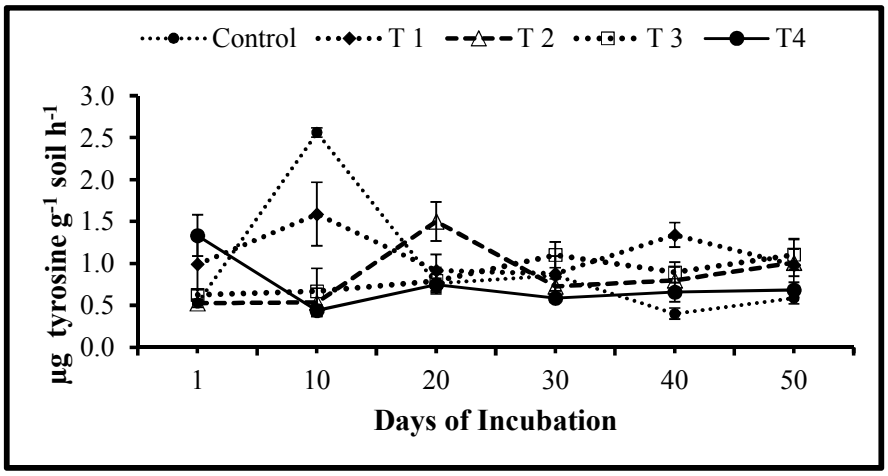

Figure 3 Influence of single and joint application of arsenic and two insecticides (chlorpyrifos and cypermethrin) on *in vitro* protease activity. Here, T1 = Arsenic, T2 = Chlorpyrifos + Arsenic, T3 = Cypermethrin + Arsenic, T4 = Chlorpyrifos + Cypermethrin + Arsenic. Each bar represents mean ± SE of three independent replicates.

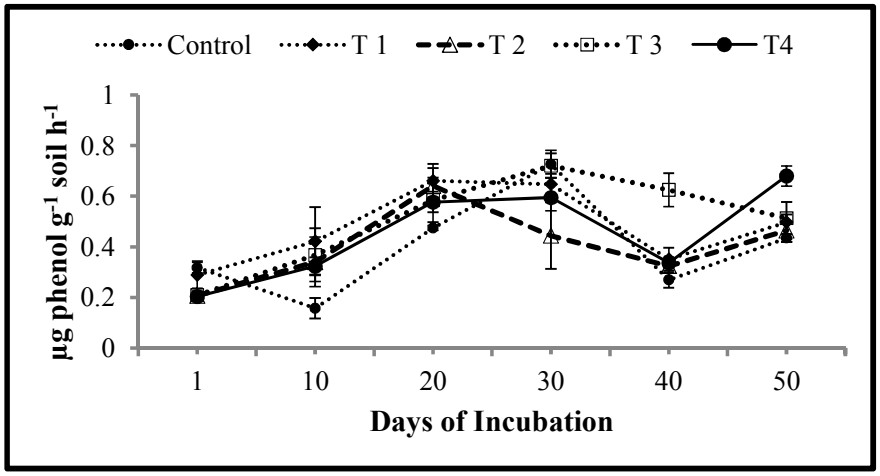

Figure 4 Variation in acid phosphatase activity in response to single and combined addition of arsenic and insecticides (chlorpyrifos and cypermethrin), and days of incubation. Here, T1 = Arsenic, T2 = Chlorpyrifos + Arsenic, T3 = Cypermethrin + Arsenic, T4 = Chlorpyrifos + Cypermethrin + Arsenic. Each bar represents mean ± SE of three independent replicates.

Table 1 Linear regression analysis of Dehydrogenase, Amylase, Protease and Acid phosphatase revealing the best predicted factor.

Dependent variable	Best predictor	r	R^2	F	df	p-value from ANOVA
Dehydrogenase	Day	.321a	0.103	19.097	1,166	0
	Treatment	.396b	0.157	15.33	2,165	0
Amylase	Day	.154	0.024	4.022	1,166	0.047
Protease	Treatment	0.202	0.041	7.065	1,166	0.009
Phosphatase	Day	.476	0.226	48.503	1,166	0

Table 2 Difference in mean values of the four treatments and their respective controls of Dehydrogenase, Amylase, Protease and Acid phosphatase.

Treatments	Days	#Mean Values±Std.Error			
		Dehydrogenase	Amylase	Protease	Acid Phosphatase
T1	D-1	0.03±0.00816^{ef}	0±0.00^{h}	0.99±0.29451^{bcdef}	0.29 ±0.05433^{gh}
T1	D-10	0.04±0.01291^{ef}	0.003±0.0025^{h}	1.59±0.77798^{b}	0.42±0.13399^{defgh}
T1	D-20	0.01±0.00^{f}	0.08±0.0025^{ab}	0.92±0.19063^{cdef}	0.66±0.06579^{abc}
T1	D-30	0.1±0.03924^{de}	0.02±0.005^{gh}	0.88±0.20825^{cdef}	0.65±0.04076^{abc}
T1	D-40	0.24±0.04423^{a}	0.02±0.00645^{gh}	1.34±0.14509^{bcd}	0.35±0.04401^{fgh}
T1	D-50	0.16±0.01658^{bc}	0.01±0.0075g^{h}	0.98±0.31419^{bcdef}	0.50±0.07823^{bcdefg}
T2	D-1	0.06±0.00802^{ef}	0.003±0.00164^{h}	0.53±0.04847^{ef}	0.21±0.0179^{hi}
T2	D-10	0.05±0.005^{ef}	0.001±0.00125^{h}	0.54±0.16931^{ef}	0.34±0.09823^{fgh}
T2	D-20	0.02±0.00313^{f}	0.07±0.00565^{bc}	1.50±0.2336^{bc}	0.64±0.0695^{abc}
T2	D-30	0.08±0.01329^{de}	0.02±0.00756^{gh}	0.73±0.08675^{def}	0.44±0.13159^{cdefg}
T2	D-40	0.12±0.0073^{cd}	0.02±0.00598^{fgh}	0.80±0.15872^{def}	0.32±0.02754^{fgh}
T2	D-50	0.06±0.00944^{ef}	0.02±0.00378^{fgh}	1.01±0.16089^{bcdef}	0.47±0.03485^{bcdefg}
T3	D-1	0.06±0.00598^{ef}	0.004±0.00183^{h}	0.63±0.06041^{ef}	0.21±0.01458^{hi}
T3	D-10	0.06±0.00881^{ef}	0±0.00^{h}	0.67±0.27044^{def}	0.37±0.10543^{efgh}
T3	D-20	0.02±0.00189^{f}	0.1±0.00559^{a}	0.80±0.15754^{def}	0.58±0.08823^{abcd}
T3	D-30	0.14±0.02809^{bc}	0.02±0.0025^{gh}	1.10±0.15334^{bcde}	0.72±0.04814^{a}
T3	D-40	0.18±0.02975^{b}	0.06±0.00934^{bc}	0.90±0.12211^{cdef}	0.63±0.06608^{abcd}
T3	D-50	0.12±0.01552^{cd}	0.03±0.00559^{efg}	1.10±0.17942^{bcde}	0.51±0.01501^{abcdef}
T4	D-1	0.06±0.00585^{ef}	0.001±0.00085^{h}	1.33±0.24594^{bcd}	0.20±0.01188^{hi}
T4	D-10	0.05±0.00312^{ef}	0.01±0.00507^{gh}	0.44±0.06359^{ef}	0.32±0.0337^{fgh}
T4	D-20	0.03±0.00528^{ef}	0.08±0.00508^{ab}	0.75±0.0767^{def}	0.58±0.04153^{abcde}
T4	D-30	0.06±0.01296^{ef}	0.02±0.00209^{gh}	0.59±0.05047^{ef}	0.59±0.05212^{abcd}
T4	D-40	0.08±0.01374^{de}	0.04±0.00512^{def}	0.66±0.11327^{def}	0.34±0.01935^{fgh}
T4	D-50	0.06±0.01312^{ef}	0.02±0.00438^{fgh}	0.69±0.09136^{def}	0.68±0.03941^{ab}
Control	D-1	0.03±0.00^{ef}	0±0.00^{h}	0.53±0.00577^{ef}	0.02±0.00^{i}
Control	D-10	0.04±0.00^{ef}	0±0.0025^{h}	2.56±0.04442^{a}	0.04±0.02363^{i}
Control	D-20	0.02±0.00^{f}	0.1±0.0025^{cd}	0.76±0.05543^{def}	0.01±0.00506^{i}
Control	D-30	0.05±0.00^{ef}	0.1±0.00^{bc}	0.86±0.00^{cdef}	0.05±0.00^{i}
Control	D-40	0.16±0.00^{bc}	0.04±0.00^{de}	0.40±0.00^{f}	0.03±0.00^{i}
Control	D-50	0.18±0.00^{b}	0.02±0.00^{fgh}	0.59±0.00^{ef}	0.02±0.00^{i}

#Means±SE having similar alphabets, are statistically not significant at $p < 0.05$ level (Based on Duncan's multiple-range test),
SE: Standard Error

Table 3 Comparative analyses of mean values between the four treatments of Dehydrogenase, Amylase, Protease and Acid phosphatase.

ANOVA Summary					
S. No	**Enzyme**	**Treatments**	**F-value (df)**	***P***	**[#]Mean ± SE**
1	Dehydrogenase	T1	6.399 (3, 212)	<0.001	0.093 ± 0.01[a]
		T2			0.064 ± 0.00[b]
		T3			0.094 ± 0.017[a]
		T4			0.06 ± 0.00[b]
2	Amylase	T1	1.85 (3, 212)	>0.05	0.020 ± 0.00[a]
		T2			0.020 ± 0.00[a]
		T3			0.033 ± 0.00[a]
		T4			0.027 ± 0.00[a]
3	Protease	T1	2.91 (3, 212)	<0.05	1.11 ± 0.14[a]
		T2			0.85 ± 0.07[b]
		T3			0.86 ± 0.07[b]
		T4			0.74 ± 0.05[b]
4	Acid phosphatase	T1	1.59 (3, 212)	>0.05	0.48 ± 0.04[a]
		T2			0.40 ± 0.03[a]
		T3			0.50 ± 0.03[a]
		T4			0.45 ± 0.02[a]

[#]Means±SE having similar alphabets are statistically not significant at $p < 0.05$ level (Based on Duncan's multiple-range test), SE: Standard Error

IV. Conclusions

In summary, below inferences were emerged from the conducted study;

1. Application of As and/or insecticides (Chl and Cyp, T1, T2, T3 and T4) in clay loam soil resulted significant variability (inhibition and stimulation) in the *in vitro* activities of dehydrogenase, amylase, protease and acid phosphatase, key enzymes of soil metabolic activity and C, N and P biogeochemical cycling.
2. The adverse impacts of applied treatments were largely dependent on the length of incubation and type of treatment applied. Moreover, regression analysis revealed that dehydrogenase activity was significantly predicted on the basis of DOI and applied treatments ($R^2 = 0.103$ and 0.157, respectively, $P < 0.001$). However, amylase and acid phosphatase were predicted on the basis of DOI ($R^2 = 0.024$ and 0.226, $P < 0.05$ and < 0.001, respectively). While, protease is best predicted based on the treatments applied ($R^2 = 0.041$, $P < 0.01$).
3. Study also revealed that T2 (Chl+As) and T4 (Chl+Cyp+As) were more innocuous over the dehydrogenase and protease, than the T1 (As) and T3 (Cyp+As). This reflects that presence of Chl is more innocuous to the activities of soil enzymes than Cyp.

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