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Pollution Induced Community Tolerance (PICT) of Microorganisms in Soil Incubated with Different Levels of PB

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***Abstract*—**Soil microbial activity is adversely affected by pollutants such as heavy metals, antibiotics and pesticides. Organic amendments including sewage sludge, municipal compost and vermicompost are recently used to improve soil structure and fertility. But, these materials contain heavy metals including Pb, Cd, Zn, Ni and Cu that are toxic to soil microorganisms and may lead to occurrence of more tolerant microbes. Among these, Pb is the most abundant and has more negative effect on soil microbial ecology. In this study, Pb levels of 0, 100, 200, 300, 400 and 500 mg Pb [as $Pb(NO_3)_2$] per kg soil were added to the pots containing 2 kg of a loamy soil and incubated for 6 months at 25°C with soil moisture of -0.3 MPa. Dehydrogenase activity of soil as a measure of microbial activity was determined on 15, 30, 90 and 180 days after incubation. Triphenyl tetrazolium chloride (TTC) was used as an electron acceptor in this assay. PICTs (ΔIC50 values) were calculated for each Pb level and incubation time. Soil microbial activity was decreased by increasing Pb level during 30 days of incubation but the induced tolerance appeared on day 90 and thereafter. During 90 to 180 days of incubation, the PICT was gradually developed by increasing Pb level up to 200 mg kg^{-1}, but the rate of enhancement was steeper at higher concentrations.

***Keywords*—**Induced tolerance, Soil microorganisms, Pb, PICT, Pollutants.

I. Introduction

THE soil microbial community fulfils a vital role in the maintenance of soil quality and fertility. It is responsible for organic matter cycling and various energy production processes [1], [2]. It also is a potentially sensitive indicator of environmental pollution which is adversely affected by pollutants and may result in detrimental effects on long-term soil sustainability [3]. Soil may become contaminated with metals from a variety of anthropogenic sources. Heavy metals specially Pb are a serious treatment to soil quality due to their toxicity and persistence after entering the soil. Elevated concentrations of these elements are known to affect soil microbial populations and their associated activities [4],[5]. Several studies have demonstrated that microbial parameters may be useful as indicators of changing soil conditions caused by chemical pollution [6], [7]. Thus risk assessment associated with heavy metal-polluted soils should therefore be evaluated to preserve the environment.

A number of studies have shown that the bacteria and fungi isolated from sites exposured to long-term heavy metal are more tolerant than isolates from less polluted sites [8],[9],[10] but community-level adaptation to increased metal concentrations is not well studied. The concept of pollution-induced community tolerance (PICT) was introduced by Blanck et al. [11]. It is based on community shifts towards more tolerant populations in response to the presence of a toxicant in an ecosystem and allows ecological effects to be related to the occurrence of a specific pollutant. As pollution increases, sensitive species gradually will be lost from the system until only tolerant organisms remain. It has been suggested that the degree of PICT in a community can be used for risk assessment purposes as a quantitative measure of ecological stress [12]. Many authors regard PICT as an indicator of deleterious effects within the microbial community due to the expected alteration in the genetic and species composition of the soil [13], but PICT also could be seen positively as the community adapting to maintain soil function and enhance sustainability. In the context of metal contamination, three different mechanisms are suggested as causes of the increased tolerance: (1) an immediate, toxic effect killing sensitive species, (2) a selection for metal tolerance due to different competitive abilities of surviving organisms, and (3) acclimatization/adaptation of organisms developing in these polluted soils due to physiological and/or genetic changes. The presence of PICT can be considered as evidence for toxic effects on organisms under field conditions. Metal resistance is therefore an undesirable phenomenon when quality of the environment is considered [14].

Soil enzymes activity is also used as a sensitive indicator of the effect of pollutants, including metals in soils [15],[16], [2],[17]. The soil microbial component and soil enzymes activity are attractive as indicators for monitoring disturbance or pollution of soils because of their central and crucial role in the functions of the soil ecosystem. Enzymes may rapidly respond to the changes caused by both natural and anthropogenic factors [18]. The strong inhibition of the activities of a variety of enzymes has been reported in metal polluted soils [19],[20],[21],[22],[23] and these effects vary considerably. Heavy metals may inhibit enzyme activities by masking catalytically active groups, having denaturing effects on the conformation of proteins, competing with the natural ions involved in the formation of enzyme-substract complexes [18], [24], [25] or by affecting the synthesis of the enzymes within the microbial cells . For these reasons soil enzymes activities have been suggested as suitable indicators of soil

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quality since they have been considered sensitive indicators to measure the degree of soil degradation in both natural and agro-ecosystems, being thus well suited to measure the impact of pollution on the quality of soil [16],[2],[26].

Dehydrogenase activity (DHA) typically occurs in all viable microbial cells [18]. Thus, its measurement is usually related to the presence of viable microorganisms and their oxidative ability has been often used as a functional indicator of soil health This enzyme is found in all living organisms and takes part in many metabolic reactions involved in oxidative energy transfer in microbes. As dehydrogenase is not active as extracellular enzyme in the soil, the management of DHA has been used as a good overall indicator of microbial activity and of the capacity of microbes to oxidize soil organic matter [27],[16]. Several studies have demonstrated that dehydrogenase enzyme activity of microorganisms is among the most sensitive parameters for the evaluation of toxicity [28].

This study was aimed to demonstrate whether the tolerance of soil microorganisms to Pb is increased in soil polluted artificially with this metal. This was made by measuring PICT using DHA measurement in soil containing different levels of Pb.

II. Materials and Methods

A. Soil Preparation

The soil used in this study was collected from the top layer (0–20 cm) of a field in Agricultural Research Station of the University of Tabriz at northwest of Iran. The soil was ground, sieved through 4 mm and kept at 4°C .

B. Measurement of Available Pb

After addition of soluble Pb to the soil, its availability decreases with time due to precipiation and adsorption processes, therefore, microbial community is exposed to Pb levels of lower than their initial concentrations. For obtaining available concentrations of Pb in treatments, lead concentrations of 0, 100, 200, 300, 400 and 500 mg $Pb.kg^{-1}$ soil, as $Pb(NO_3)_2$ were added to the soil and mixed throughly. After 4, 10, 13, 15, 30 and 90 days incubation at 25˚C and soil moisture of 70% water-holding capacity (WHC) the DTPA-available Pb was determined in soil [29]. After 30 days of incubation, Pb concentration in solution phase equilibrated with solid phase and consequently, available Pb in soil reached constant levels of 0, 30.0, 81.0, 136.0, 200 and 260 $mg.kg^{-1}$ (Fig.1). These concentrations were used in detection phase of PICT determination.

C. PICT Measurement

Pollution induced community tolerance was estimated using DHA assay in Pb amended soil as described below

PICT determination consists of two stages, the selection phase and the detection phase. During the selection phase soil microbial community exposed to different levels of Pb (0, 100, 200, 300, 400 and 500 $mg.kg^{-1}$ soil). The selection phase followed by a detection phase during which the soil microbial community extracted from each Pb level, exposed to the Pb concentrations of 0, 30.0, 81.0, 136.0, 200 and 260 $mg.L^{-1}$ in nutrient solution to estimate the community tolerance.

D. Soil Treatments and Incubation Conditions (Selection Phase)

A sandy clay loam soil containing 9.1% total carbonate calcium, EC of 0.7 $dS.m^{-1}$ and pH of 8.3 was used in this experiment. Pots were filled with 2 kg of air dried soil and the moisture was adjusted to 50% WHC by adding distilled water and then pre-incubated for 14 days at 25°C to stabilize microbial activity (Liao et al., 2005). After pre-incubation, the lead concentrations of 0, 100, 200, 300, 400 and 500 mg $Pb.kg^{-1}$ soil, as $Pb(NO_3)_2$ solution were sprayed to the pots and mixed thoroughly to ensure homogeneous distribution of Pb. Pots were kept at room temperature (approximately 25˚C) for 180 days. Distilled water was added regularly to maintain the soil moisture at a constant level of 70% WHC (-0.3 Mpa). Dehydrogenase activity of soil as a measure of microbial activity was determined on 15, 30, 90 and 180 days after incubation.

E. DHA Assay in Soil (Detection Phase)

Soil microorganisms were extracted from soil accorrding to the method described by [30]. Briefly, 3 g of soil was mixed with 30 ml sterile tris (Hydroxymethyle aminomethane) buffer solution, pH 7, and shaked for 20 min at 150 rpm on a rotary shaker. The soil suspension was centrifugated at 3000 rpm for 5 min and DHA was determined in supernatant. DHA assay was performed using 2,3,5-triphenyltetrazolium chloride (TTC) as artificial electron acceptor which is reduced to the red-colored triphenyl formazan (TPF) by the enzyme. Portions (0.3 ml) of microbial suspension were inoculated into four replicate glass tubes containing 2.5 ml of nutrient broth-glucose medium amended with Pb (0, 30.0, 81.0, 136.0, 200 and 260 $mg.L^{-1}$) and pre-incubated on a rotary shaker-incubator at room temperature for 30 min. Thereafter, 0.2 ml of 0.4% (w/v) TTC in deionized distilled water was added to each tube. The final concentrations of nutrient broth, glucose and TTC in the medium were 2, 2 and 0.267 mg ml^{-1}, respectively. The controls consisted of inoculated media without Pb. The reaction mixtures were further incubated in the dark at room temperature for 72 h. The TPF produced was extracted in 5ml of ethylacetate and determined spectrophotometrically at 460 nm. [31]. The recorded absorbances were expressed as relative activity of microbial community in test tubes.

F. PICT Calculations

The community tolerance then quantified as the concentration required to inhibit the activity by 50% in the detection phase. The PICT then determined by comparing the tolerance of the community exposed to Pb to that of an unaffected control community. The PICT thus expressed as the difference between the tolerance of the polluted sample and the unpolluted control [32]:

ΔIC_{50} (or PICT) = IC_{50} polluted sample – IC_{50} unpolluted sample where IC_{50} is the concentration of pollutant at which 50% of the community is inhibited. Fig.1 as an example, shows PICT calculation for 100 mg.kg^{-1} soil after 180 days incubation.

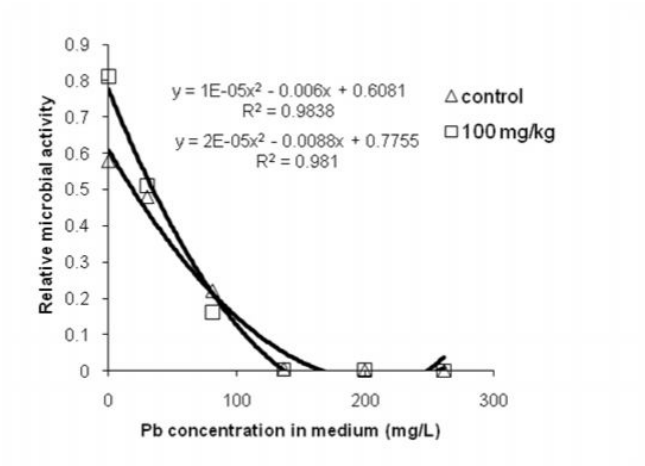

Fig. 1 Relative microbial activity in different levels of Pb in test tubes (detection phase). Microbial communities used in this test have been extracted from soil treated with 100 mg Pb.kg^{-1} in selection phase after 180 days incubation

III. Results And Discussion

A. Availability of Pb in soil

As shown in Fig.2, available Pb in soil was gradually decreased with time and reached a constant level after 30 days at all Pb treatments. When Pb was added to the soil, the Pb ions would then have interacted with the soil through processes of precipitation, ion exchange, complexation and eventually came to equilibrium with prevailing chemical conditions [33]. In this study, available Pb in soil came to equilibrium after 30 days incubation. Concentrations of available Pb in soil after this period were 0, 30.0, 81.0, 136.0, 200 and 260 mg.kg^{-1} in corresponding treatments (0, 100, 200, 300, 400 and 500 mg.kg^{-1}), respectively

Saeki et al. [34] reported that soil solutions with high Cu and Zn concentrations were not necessarily found in the soils containing the largest amounts of these metals. This also consistent with the studies of Almas et al. [35], who found that the correlation of IC_{50} values with total amount of Cd and Zn in soil was much poor than for the labile form of these elements, indicating that total metal concentration is inaccurate for predicting biological effects. Diaz-Ravina et al. [36] pointed out that different soil types have different capacity to fix metal ions. They showed that a soil treated with 5 to 100 mg Cu.kg^{-1} was more toxic to bacterial community than soil treated with 100 to 550 mg Cu.Kg^{-1} . This again confirms that total amount of metal ion in soil can not reflect its effective concentration in soil solution.

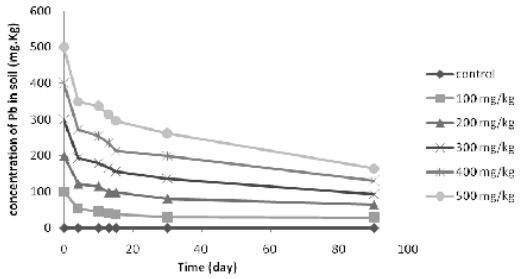

Fig. 2 Changes in available Pb concentration during soil incubation with different Pb levels

Niklinska et al. [37] studied PICT in a forest soil contaminated with Zn and Cu. These researchers found that in sites polluted with Cu and Zn, neither microbial carbon nor bacterial activity differed from the unpolluted controls. They note that the considerable amount of Cu and Zn in this soil are in phosphate or organic-bonded form. Moreover, ion metals present in soil solution are not necessarily bioavailable at all [2]. Soil organic matter usually contains large amounts of water-soluble organic compounds that chelate metals [38]. Wang et al. [39] reported a negative relationship between the activity of soil bacteria, population diversity and heavy metals extractable with NH_4NO_3.

Based on these findings, we used Pb concentrations in detection phase which was equal to that of available levels in soil (Figs. 1 and 2)

B. PICT

Soil microbial activity was decreased by increasing Pb level during 30 days of incubation but the induced tolerance appeared on day 90 and thereafter. During 90 to 180 days of incubation, the PICT was gradually developed by increasing Pb level up to 200 mg kg^{-1}, but the rate of enhancement was steeper at higher concentrations (Fig.3).

Kelly et al. [40] found a 87% decrease in viable counts, 47% in microbial biomass and 95% in dehydrogenase activity after 15 days incubation of a soil polluted with Zn. Also, the proportion of zinc resistant bacteria increased from 0.08% to 0.75%.

In our study, PICT values increased with time of incubation and different levels of soil Pb (Fig.3). Liao et al. [41] found that the first time addition of Cd to soil can obviously inhibit soil microbial biomass and its metabolic activity, then favoring a selection of Cd resistant microorganisms with prolongation of addition time [42]. In general, the PICT values increased noticeably over time after soil Cd treatment. Doelman and Haanstra [19] and Speir et al. [43] also reported an increase in PICT over time for different heavy metals.

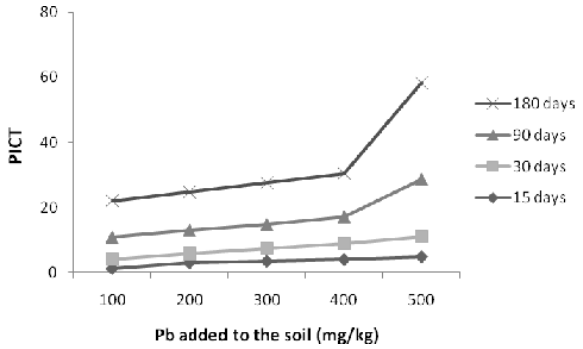

Fig. 3 Changes in PICT values with increasing levels of Pb in soil

Alden Demoling and Bååth [44] have expressed that the differences in PICT values depend on type, concentration, toxicity and bioavailability of pollutants. Of course, among these factors, PICT values more depend on pollutant concentration.

In current study, a marked increase occurred in PICT after 90 and 180 days incubation at 400 mg Pb. Kg^{-1} (equal to 200 $mg.Kg^{-1}$ of available Pb)(Fig.3). This shows that increasing level of available Pb to 200 $mg.Kg^{-1}$ in four incubation periods has not caused important physiological or genetically changes in soil microbial community in term of Pb tolerance .These results suggest that the increase in Pb tolerance of community after adding Pb can be attributed to an immediate effect due to the death of sensitive species and later effect due to different competitive abilities and adaption of surviving bacteria [45]. These researchers also found an increase of tolerance after 14 months of incubation for all metals expect for Pb, but no tolerance was appeared till 7 months. Regarding this finding, 180-day incubation period was appropriate for our study.

Tree different mechanisms are therefore suggested as causes of the increased metal tolerance observed for soil microorganisms in current study: (1) an immediate, toxic effect killing sensitive species; (2) a selection for Pb tolerance due to different competitive abilities of surviving bacteria; and (3) adaption of bacteria developing in the polluted soils due to physiological and/or genetical changes. The predominance of one mechanisms might depend on the level of pollution. In higher concentrations of lead, competition and compatibility is an important factor in increasing the tolerance of microorganisms [46]. However, an increased tolerance to Pb in soil was detected throughout the entire incubation period (Fig.3), suggesting that the level of tolerance of the soil microbial community could be successfully used for detecting metal pollution independently of the time exposure.

IV. Conclusion

The critical points appeared at 200 $mg.kg^{-1}$ of available Pb after 90 and 180 days of incubation (Fig.3) reveal that a drastic change in genetic or structure of soil microbial community may initiate at this points. Thus, it may be entered irreparable damage to soil ecosystems. In fact, sensitive species extinct and create heavy metal tolerant species, thus reduce the functional and genetic diversity [47]. In tolerant communities, the biodiversity may be decreased [35] and the tolerant species may not always be able to perform the same ecological functions as the sensitive ones [2]. As a result, many soil heterotrophic microorganisms are facing problem with food and declines their population. Thus may reduce the quality of soil ecosystems [14].

Critical point for each pollutant is of importance. To prevent adverse effects of pollutants, their concentration in soil should be monitored continuously. All amendments such as organic or chemical fertilizers as well as pesticides containing heavy metals or other pollutants must be added to the soil at a rate that prevent to rise their concentrations to the critical levels. It seems that the detrimental effects of pollutants at concentrations below that of critical points are reversible but it will be harmful and irreversible above these points.

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