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Interaction of copper and 2,4,5-trichlorophenol on bioremediation potential and biochemical properties in co-contaminated soil incubated with *Clitocybe maxima*

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Abstract: Bioremediation of soil co-contaminated with heavy metal and organic pollutants has arisen considerable attention in recent years. *Clitocybe maxima* (*C. maxima*), a species of mushroom producing ligninolytic enzyme, was introduced into this work to evaluate the interaction of copper and 2,4,5-trichlorophenol (2,4,5-TCP) on bioremediation potential and biochemical properties in co-contaminated by pot experiments. Results indicated that *C. maxima* could be considered as a candidate for the bioremediation of soil co-contaminated with copper and 2,4,5-TCP. Copper was accumulated in fruiting body of *C. maxima* and showed a positive correlation with the initial copper concentration in soil. A significant enhancement was found on dissipation of 2,4,5-TCP incubated with *C. maxima*, and removal ratios varied from 82.6 to 90.9% with the level of co-contaminations, which were associated with the production of manganese peroxidase and dehydrogenase. Invertase, urease and dehydrogenase activities in rhizosphere declined varying with pollutants levels before the bioremediation, and recovered to a certain level after bioremediation process, which demonstrated that soil enzyme activity could be an accessible indicator for reflecting remediation effects.

Key Word: Bioremediation, co-contamination, mushrooms, ligninolytic enzyme, soil enzyme activity, *Clitocybe maxima*

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1. Introduction：

Extensive agricultural and industrial activities brought about a majority of various pollutants remaining in soil. Heavy metals and persistent organic pollutants (POPs), well characterized as highly toxic, mutagenic and carcinogenic, have been two kinds of priority pollutants in soil throughout the world (1, 2). Co-occurrence of organic and metal pollutants in soil now becomes widespread and has been considered to be one of the severest environmental issues. Great concern was focused on remediation of metal-organic co-contaminated soil and to explore the interaction of mixed pollutants on soil remediation (3-5).

Bioremediation technology has been suggested to be a feasible approach for removal of pollutants in soil due to its cost-effectiveness and sustainability(2, 6, 7). In this field, phytoremediation has ever been an emerging technology for treatment of heavy metal - organic co-contamination, played a dominant role and was recognized as an ideal strategy for biological detoxification (8, 9). Plants possessed the remediation potential of soils contaminated with heavy metal and PAHs in recent scientific literatures, but conventional phytoremediation existed a limitation that dissipation of organic pollutants enhanced quite slightly when growing plants, because of its lack of organic degradation capacity (10, 11).

Here we introduce mushroom as a bioremediator to deal with co-contaminated soil due to its high biomass, heavy metal tolerance and organic-degrading capacity (12-14). Mushrooms have been reported to be capable of metal bioaccumulation in our previous study (15, 16). And white-rot fungi are microorganisms with a well-known capacity for degrading a wide range of organic compounds on account of the attribution of the extracellular ligninolytic enzymatic system (17). The ligninolytic enzymes attack the polyphenolic molecule of lignin or molecule structurally similar to lignin under natural conditions. But there is lack of information on interaction of metal and organic pollutants on their fate by mushrooms in soil. Accordingly, attempt has been made to apply *C.maxima*, a species of mushroom with high production of laccase and MnP, to the bioremediation for soil co-contamination (18).

Copper and 2,4,5-TCP were studied as the representatives of heavy metal and persistent organic contaminants in this paper. Copper pollution has actually worsened with the excessive exploitation of copper mining and the wide application of feed additives, organic fertilizers, irrigations, fungicides, and urban sewage sludge compost utilization(19). Furthermore, 2,4,5-TCP is an important kind of chlorinated organic compounds that has been widely used as antifungal agents to slow down the decomposition of cut timber in forests, and as precursors of different herbicides in agriculture. 2,4,5-TCP became typical of chemicals on the USEPA's Persistent Bioaccumulative Toxics list on account of its significant toxicological effects and potential carcinogenicity(20, 21). Co-occurrence of copper and 2,4,5-TCP probably existed where soil encountered the abuse of pesticides and contamination of copper mining. It is therefore necessary to conduct a research on remediation of soil co-contaminated with copper and 2,4,5-TCP.

Soil enzyme activity is an important biochemical indicator to reflect soil quality and toxicity of pollutants in soil, because they are sensitive to the presence of pollutants. Soil enzymes are the catalysts of important metabolic processes, including the detoxification of xenobiotics and heavy metals (22). Dehydrogenase activity, invertase and urease are the most frequently used biological tests for determining the effect of various pollutants on the microbiological quality of soil(22, 23). Pollutants introduced into the soil exert an influence on the microbiota, which is manifested by changes of enzyme activities. The changes of soil enzyme activities are essential for evaluation of bioremediation effects.

The main objectives were to study the interaction of copper and 2,4,5-TCP on (1) growth response of *C. maxima*; (2) bioremediation effects; (3) soil biochemical properties in co-contaminated soil. The results of this research would provide valuable information for the application of mushrooms in metal and organic co-contaminated soil remediation.

2. Materials and Methods

2.1 Materials

Analytical standard of 2,4,5-TCP (97.0% purity) was purchased from Labor Dr. Ehrenstorfer-Schäfers (Augsburg, Germany), while other chemicals were of analytical reagent grade and purchased from Chengdu Kelong Chemical Reagent Company (Chengdu, China). Mycelia bags of *C. maxima* were purchased from a production site for the edible mushroom in Shuangliu County, Chengdu. Experiments were carried out in a greenhouse by pots (pot size: 20 cm base, 22 cm in height, 29 cm in calibre) at the temperature of 30-34ºC during daytime and 24-30ºC during night.

2.2 Soil sampling and experimental design

Soil samples used in the present study were collected at depth of 0-20 cm in Wangjiang Campus, Sichuan University (30°38′N, 104°05′E). The samples were air-dried, sieved through a 2 mm mesh and then analyzed for main physical and chemical properties. Soil testing results showed pH (1:2.5 water) 6.28 ± 0.05, OM content 18.38 ± 0.40 g kg^{-1}, CEC 10.28 ± 0.52 cmol kg^{-1} and total Cu 22.90 ± 1.21 mg kg^{-1}.

Totally 16 treatments were tested and the detailed experimental design was shown in Table 1. The levels of copper and 2,4,5-TCP were selected on basis of several literatures and policy formulated in China and Europe (21, 24-26). Cu was spiked in form of $Cu(NO_3)_2$ to the resulting soil to obtain the soil sample with Cu(II) initial concentration of 0, 100 or 300 mg kg^{-1} , while acetone stock solution of TCP was added subsequently with initial concentration of 0, 40, 80 or 120 mg kg^{-1} , which was to create artificially single or mixed contaminated soil. Briefly, the bulk soil was first mixed thoroughly with $Cu(NO_3)_2$ in an aqueous solution, equilibrated at a moisture condition for 5 weeks until the samples were air-dried naturally, fully homogenized. Then high purity 2,4,5-TCP in acetone was added into the soil. After acetone had evaporated off, the spiked soils were sieved again through 2 mm sieve to ensure the homogeneity and stored for use.

Experiments were carried out in pots containing 4.0 kg of contaminated soil and

1.7 kg of *C. maxima* mycelia bags. A fraction of soil samples were autoclaved 1 hour for 3 times so as to obtain sterilized soil. The soil moisture content was kept at 70 percent water holding capacity by quantitative watering once every day. After 30 days' growth under treatment in each pot, *C. maxima* was harvested and prepared for analysis.

2.3 Assay of laccase and MnP Activity in soil

Laccase and MnP were extracted in soil by a modification of the method by Baldrian (3). Briefly, 1g of fresh soil was exacted with 5 mL phosphate buffer (pH 7.0) on ice packs for 1 hour. The aqueous suspension was centrifuged and the clear supernatants were used immediately for estimation of enzyme activities. Laccase activity was measured by the oxidation of ABTS in acetate buffer (27). The reaction mixture (2 mL) contained 1.4 mL of sodium acetate buffer (pH 4.5), 0.2 mL of 1mM ABTS and 0.4mL of supernatants. The activity was defined as the amount catalyzing the production of 1μmol of colored product mL^{-1} min^{-1}. MnP activity was determined spectrophotometrically at 30°C and 468 nm as described by Field (28). The reaction mixture (2 mL) contained 1.38 mL of sodium acetate buffer (pH 4.5), 0.1 mL of 10 mM DMP, 0.1 mL of 0.2 mM $MnSO_4$, 20 μL of 0.1 mM H_2O_2 and 0.4 mL of supernatants. The activity was defined as the amount catalyzing the production of 1μmol of colored product mL^{-1} min^{-1}.

2.4 Determination of soil enzyme activity in rhizosphere

Dehydrogenase activity was indicated by the evolution of triphenolformazan (TPF) (29). 1g of fresh soil was incubated at 37ºC for 24 hours in test tubes containing 4 mL of Tris-HCl buffer (pH 7.6), 2 mL of 0.5% TTC and 2 mL of 0.1 M glucose. After 24 hours, 10 mL methanol was added. The extract with methanol was centrifuged and determined spectrophotometrically at 492 nm. Dehydrogenase activity was expressed as μg TPF g^{-1} soil h^{-1}. Urease activity was determined by mixing 1g of fresh soil samples, 200 μL of methylbenzene, 2 mL of urea solution

(10%), and 4 mL of citrate buffer (pH 6.7) in test tubes. The mixture was incubated for 24 hours at 37ºC. The indophenols were colorimetrically determined at 578 nm. The activity was defined as mg NH_4-N kg^{-1} h^{-1} (30). Invertase activity was measured according to the method by Guan (30). 1g fresh soil samples were incubated for 24 hours at 37 ºC with 3 mL of 8% sucrose solution and 1 mL phosphate buffer (pH 5.5). The amount of glucose in supernatant, as a result of the sucrose hydrolysis, was measured at 508 nm. Invertase activity was expressed as μg glucose g^{-1} soil h^{-1}.

2.5 Exaction and analysis of heavy metal and 2,4,5-TCP

The dried mushroom samples were digested with HNO_3, $HClO_4$, and 30% H_2O_2 (5:3:2, v/v/v), and the concentration of copper was quantified using FAAS (VARIAN, SpectrAA 220FS). 2,4,5-TCP analyses were performed using gas chromatograph-mass spectrometer (SHIMADZU, GCMS-QP2010). 5 g of air-dried soil sample was extracted with 25 mL of n-hexane and 5 mL of 0.05 M H_2SO_4 in shake flasks for 30 min. After ultrasonication, centrifugation and dewatering, the extracts of organic layer were concentrated in a rotary evaporator and moved into a 1.5 mL sample vial. A Hewlett Packard-5 capillary column with a temperature gradient from 100°C to 250°C at a rate of 15°C min^{-1} was used to separate the compounds. The initial and final hold time was 2 min and 5 min respectively, and the detector temperature was 260°C. 2,4,5-TCP recovery was measured by adding a known concentration of it standard (10, 30, 50, 70 and 90 mg kg^{-1}) to uncontaminated soil, and the recoveries of 2,4,5-TCP from spiked samples were 90.15, 88.04, 89.64, 85.81 and 91.02%, respectively.

2.6 Statistical Analysis

Statistical analysis was performed using SPSS 22.0. Results were expressed as mean followed by corresponding standard deviations. One-way ANOVA was to compare the effects of 2,4,5-TCP on copper accumulated in mushrooms. Multiple comparisons were made by Duncan test at $P<0.05$ level. Two-way ANOVA was used to compare the interaction of copper and 2,4,5-TCP in fruiting time, biomass and soil

enzyme activity.

3. Results and Discussion

3.1 Growth response of *C. maxima*

Compared with the control, the contamination levels of copper and 2,4,5-TCP exhibited no significant effect on biomass of fruiting body among non-sterilized treatments (Table 2), while sterilized soil seemed to affect the fruiting of the mushrooms because none of fruiting body was discovered in sterilized treatments (T10, T16). Indigenous microorganisms may alleviate the stresses mushroom suffered in non-sterilized treatments, and high toxicity of pollutants restrained the generation of the fruiting body in sterilized soil. The fruiting bodies of *C. maxima* were insensitive and able to tolerate the mixed contaminants within certain levels.

Fruiting time in each treatment was recorded to investigate the effect of various factors on the growth of the mushrooms. Two way- ANOVA results showed that there was an interaction of copper and 2,4,5-TCP concentrations on fruiting time, in spite of no significant impact of single copper or 2,4,5-TCP on fruiting time (Table 2). The fruiting time was delayed distinctly and accounted for 107 to 152.94% of the control with the co-contamination levels increasing. But T14 (300 mg kg^{-1} copper and 120 mg kg^{-1} 2,4,5-TCP) did not meet with the increasing trend, which harvested for 33.23 days, only accounting for 126.8% of the control. It is probably high-producing laccase and MnP (shown in Fig. 3) during the whole process in T14 that alleviated the stress from mixed pollutants, leading to reduce fruiting time of mushrooms.

All above results indicated that the mixed toxic pollutants had synergetic effect on fruiting time but no significant effect on biomass of fruiting body of *C.maxima*, which had a certain resistance to mixed pollutants in soil. Hong et al. (31) showed the same results that no distinct change of biomass for *Hypocrea lixii* was observed in copper and pyrene co-contaminated soil compared to the control. Similarly, Ji et al. (32) found that the presence of toxic pollutants in contaminated soil had no effect on the growth of *Tricholoma lobynsis*. Previous study and present findings uncovered a fact that white rot fungi have the capacity resistant to toxic pollutants to a certain

degree, though fruiting time was mildly influenced. Predictions are therefore made that mushroom, as a result of its higher yield than plants in short time, are possibly capable to uptake more heavy metal in co-contaminated soil.

3.2 Copper accumulation in fruiting body

Initial copper concentration in soil significantly influenced copper accumulation in fruiting body of *C. maxima* rather than 2,4,5-TCP concentrations. The mean amounts of copper accumulated in *C. maxima* were calculated and plotted in Fig. 1 using ORIGIN 8.0. It exhibited that copper accumulation strikingly increased following the growing concentration of copper in tested soil. A significant positive correlation was found between copper in soil and copper bioaccumulation in *C. maxima*, and the corresponding relationship can be expressed using the following regression equations:

$$Y = 0.034X + 6.609 \quad (R^2=0.873, P< 0.001)$$

Where Y presents the concentration of copper in *C. maxima*; X presents the concentration of copper spiked in soils. On average, approximately 5.88, 10.69, 16 mg kg^{-1} dry mushroom in treatments spiked copper at concentration of 0 (T1-T4), 100 (T5-T8) and 300 mg kg^{-1} (T11-T14) respectively, were accumulated in *C. maxima* from tested soil. The bioconcentration factors (BCF) of *C. maxima* towards copper were observed to be lower than hyperaccumulator plants, of which was usually greater than 1(33). Given their higher yield within several months, mushrooms are possibly capable to uptake more total amounts of heavy metal in a short time (14, 34). As shown in Table 2, the presence of 2,4,5-TCP barely affected on the biomass of *C. maxima*, making it possible to accumulate copper efficiently in soil co-contaminated with copper and 2,4,5-TCP. And the BCF of *C. maxima* towards copper was almost as high as those mushroom species reported in literatures(35, 36). For further study, metal accumulator mushroom species need to be discovered, and the application of chelating agent and surfactant ought to be taken into consideration for enhancement on bioremediation of co-contaminated soil, especially for removal of heavy metal.

223 3.3 Removal of 2,4,5-TCP in soil

224 Removal ratios of 2,4,5-TCP showed different and varied with the level of
225 contamination. Fig. 2A exhibited the removal ratios of 2,4,5-TCP in 40 days at
226 various concentrations of mixed pollutants incubated with *C. maxima*. Under the same
227 copper level, removal ratios elevated with the increment of 2,4,5-TCP concentrations.
228 Treatments with 300 mg kg^{-1} of copper and 120 mg kg^{-1} of 2,4,5-TCP exhibited the
229 highest removal rates (90.87%) compared with other treatments (82.60-89.16%). Fig.
230 2B showed the effect of sterilized soil and natural conditions on removal of 2,4,5-TCP
231 in two levels of mixed contaminated soil ($Cu_{100}TCP_{80}$, $Cu_{300}TCP_{40}$). Compared to T7
232 and T12), removal ratios increased by 8.23% and 32.12% in treatments without
233 incubation of *C. maxima* (T9, T15), while 6.23% and 32.22% in treatments with
234 sterilized soil (T10, T16). The removal percentages of 2,4,5-TCP in incubated
235 treatments were higher than those of non-incubated group, suggesting that the
236 dissipation of 2,4,5-TCP were enhanced effectively. Compared with non-sterilized
237 groups, higher residual amounts of 2,4,5-TCP were found in sterilized groups,
238 because there may be a reduction of indigenous microorganisms in sterilized
239 contaminated soil. These results demonstrated a phenomenon of an interaction
240 between copper and 2,4,5-TCP initial concentrations on removal of 2,4,5-TCP in soil
241 by *C. maxima*.

242 3.4 Laccase and MnP Activity in rhizosphere soil

243 Laccase activity was determined and the results were exhibited in Fig. 3. An
244 interaction was found between copper and 2,4,5-TCP concentration on laccase
245 activity according to the data (P<0.01) obtained from Two-way ANOVA. Laccase
246 activity in most treatments revealed close within 4 to 5 U g^{-1} soil except T14 (up to
247 30.52 U g^{-1} soil). It is obvious positive co-effects of copper and 2,4,5-TCP on
248 exudation of laccase that led to an enhancement of laccase activity in soil samples.
249 Laccase activity was apparently suppressed by 72.2% and 53.4% in T11 and T12 (300
250 mg kg^{-1} of copper concentration) compared with the control, because there is an
251 inhibition by high concentrations of copper and no distinct stimulation of laccase

activity at low 2,4,5-TCP concentrations. Growth in sterilized environment had no effect on enzyme activity excreted by *C. maxima* in T10 and T16, while laccase activity was not detected among treatments without incubation of *C. maxima* (T9, T15).

As shown in Fig. 3c, both copper and 2,4,5-TCP significantly influenced MnP activity in soil, which ranged from 22.35 U g^{-1} soil to 112.74 U g^{-1} soil among all treatments incubated with *C. maxima*. T14, five times higher than the control, presented the maximum activity (up to 112.74 U g^{-1} soil) as similar as the trend of laccase activity (up to 30.52 U g^{-1} soil), indicating a positive co-effect of high levels of copper and 2,4,5-TCP concentrations on MnP activity. As a whole, both laccase and MnP were stimulated and induced by high concentrations of copper and 2,4,5-TCP in present study, which led to a better degradation of 2,4,5-TCP. Similar phenomenon has been observed in previous reports about enzymatic degradation of xenobiotic compounds in soil by ligninlytic system (37-40). The incubation of *C. maxima* distinctly enhanced 2,4,5-TCP removal and removal rates increased by 12.02% and 23.87% in treatments incubated with *C. maxima* (T9, T15) compared to corresponding non-incubated groups (T7, T12).

To estimate the relationship between ligninlytic system and organic degradation, linear regression equations were established, and the results were showed in Table 3. There is no significant correlation between TCP removal ratio and laccase activity, while a positive correlation was found between MnP activity and the removal ratios of TCP, and R^2 values for linear regressions were 0.891. It is similarly concluded by Francisca et al. that high removal capability of five PAHs by the Chilean white-rot fungus *Anthracophyllum discolor* was associated with the production of MnP production (41). Therefore, fungi-producing MnP activity may be suitable for evaluating TCP degradation in mushroom-incubated soil.

White-rot fungi are macro fungi capable of degrading organic compounds, due to its exudation of laccase and manganese peroxidase (MnP). But the mechanism of chlorophenol degradation in natural soil is complex which is not merely related to ligninolytic enzymatic degradation from white rot fungi (42). The degradation of

organic pollutants may be attributed to the reaction of other cellular and extracellular enzymes from fungi or indigenous microorganism, which may also participate concomitantly with ligniolytic enzymes in degradation processes.

3.5 Assays of soil biochemical properties

Dehydrogenase activity, invertase and urease are commonly used for reflection of soil biochemical quality (22, 23). The changes of soil enzyme activity provided direct information on bioremediation effects of *C. maxima*.

Dehydrogenase activity, conducting a board range of oxidative activity that was responsible for the degradation of soil organic matter, is an important representative of pollution level in soil (11, 43, 44). In present study, dehydrogenase activity presented quite sensitive to the two pollutants, especially copper contamination (Fig. 4A). A sharp decrease was detected in copper-spiking groups, while treatments without spiking copper (T2-T4) were within normal value as near as control (up to 3.55 μg TPF g^{-1} soil d^{-1}). Dehydrogenase activity in all contaminated incubated treatments had been recovered to a relatively normal level after the bioremediation terminated. The ascending values of dehydrogenase activity in contaminated soil might be attributed to the increasing microbial activity as well as the increasing exudation by mushrooms. There is a certain correlation between dehydrogenase activity and the removal ratio of 2,4,5-TCP in soil, and R^2 values for linear regressions were 0.617, which is lower than that of MnP activity (Table 3). The result was supported by a previous demonstration of a positive correlation between dehydrogenase activity and removal ratios of pyrene (11). The continued suppression of microbial activity in the unplanted treatments was similar to results by Mill, but there was no recovery of dehydrogenase activity after 6 weeks in soil contaminated with 250 mg kg^{-1} PCP (44). Most of 2,4,5-TCP could ultimately be removed on account of ligninolytic enzyme and soil dehydrogenase activity.

As demonstrated in Fig. 4B, changes of urease activity exhibited analogous to invertase activity but appeared more sensitive than invertase activity. The inhibition ratios of urease activity (from 24.6-69.1% except sterilized treatments) were higher

than those of invertase activity (from 11.57-42.3% except sterilized treatments). Compared with the control, which showed the highest urease activity (up to 19.09 NH_4-N g^{-1} soil d^{-1}), urease activity in other treatments declined with the contamination levels, particularly the copper concentration. The lowest urease activity (up to 5.91 NH_4-N g^{-1} soil d^{-1}) appeared on T14 soil samples among the non-sterilized and incubated treatments, while Cu and TCP concentration were the highest among the whole tested groups. After the bioremediation process using *C. maxima*, urease activity was found to be increasing significantly in contaminated treatments, and restoring to a normal level only slightly lower than the control. An apparent increase of urease activity was found in each incubated contaminated soil sample, but increments were found to be minor in non-incubated treatments. T1 showed higher activity than previous one, indicating an enhancement of soil microbial activity on account of the incubation with *C. maxima*. Urease activity in T14 apparently enhanced compared with previous T14 but still kept the lowest than other non-sterilized treatments.

Invertase activity, at the beginning, decreased significantly with the increase of contamination levels, especially the copper concentration (Fig. 4C). The control showed the highest invertase activity (up to 6.79 mg glucose g^{-1} d^{-1}), while T14, with the highest-level contamination, presented the least (up to 3.92 glucose g^{-1} d^{-1}) among the non-sterilized treatments. Two treatments in sterilized soil (T10, T16) demonstrated two of the lowest invertase activity (only up to 1.10 and 0.899 glucose g^{-1} d^{-1} respectively) than all treatments, due to an elimination of indigenous microorganisms after soil being sterilized. After bioremediation process, T10 and T16 showed no significant variety compared to other treatments, because *C. maxima* may excreted low-molecular-weight organic acids into rhizosphere soil, which promoted the microbial activity and recovered the soil biochemical prosperties (43). After bioremediation by *C. maxima*, a significant increase of invertase activity was detected in each incubated contaminated soil, increments were however found to be minor in non-incubated treatments. It was proved directly the bioremediation potential of C. maxima for co-contaminated soil, and the incubation of *C. maxima* effectively

alleviated the toxicity of contaminants in soil.

Soil enzyme played an essential role in the overall bioremediation process of 2,4,5-TCP decompositions in soil. And it was regarded as potential indicators of the adverse effects of various contaminants on soil quality. Soil remediation cannot be simply evaluated by the removal efficiency of pollutants, and complete detoxific effectiveness of contaminated soil should be evaluated based on environmental risk. Therefore, soil enzyme activity was a valid indicator for assessments of soil toxicity after bioremediation. (11, 22, 23, 43, 45)

5 Conclusions

The interaction of copper and 2,4,5-TCP on their fate and soil biochemical properties in co-contaminated soil were investigated in present study. Co-contamination level had no significant effect on biomass of fruiting body but on the fruiting time of *C. maxima* due to an interaction of copper and 2,4,5-TCP on mushroom growth. Copper was accumulated in the fruiting body of *C. maxima* and a significant positive correlation was found between copper spiked in soil and copper bioaccumulation. Dissipation of 2,4,5-TCP enhanced distinctly when incubated with *C. maxima*, and removal ratios varied with the level of co-contaminations. Invertase, urease and dehydrogenase activities can be valid biochemical indicators to reflect the remediation effect of co-contaminated soil during the process. The study first reported the interaction of metal and organic pollutants on mycoremediation effects in co-contaminated soil by macro fungi. *C. maxima* has been proved to possess the bioremediation potential of metal bioaccumulation and organic biodegradation, the latter of which is however scarce for plants and phytoremediation technology. The results are of importance for a better understanding of bioremediation on co-contamination.

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Figure captions:

Fig. 1. Copper bioaccumulation and BCF in fruiting body of *C. maxima* under various levels of pollutants. All values are mean of three replicate ± S.D. Different letters on the top of the bar indicate different significance at $P<0.05$ according to one-way ANOVA.

Fig. 2. (A) 2,4,5-TCP removal ratio and residues in soils among 2,4,5-TCP-contaminated soil samples and (B) comparison of 2,4,5-TCP removal percentages among non-incubated or sterilized treatments to incubated and non-sterilized treatment (CK). All values are mean of three replicate ± S.D. Different letters on the top of the bar indicate different significance at $P<0.05$ according to one-way ANOVA.

Fig. 3. Laccase and MnP activity in rhizosphere soil at fifteenth day during the bioremediation of various concentrations of pollutants among all the treatments. All values are mean of three replicate ± S.D. Different letters on the top of the bar of the same enzyme indicate different significance at $P<0.05$ according to one-way ANOVA test.

Fig. 4. Changes of soil activities in rhizosphere sediments before and after bioremediation. (A) Dehydrogenase activity. (B) Urease activity. (C) Invertase activity. All values are mean of three replicate ± S.D. Different letters on the top of the bar of the same soil enzyme indicate different significance at $P<0.05$ according to one-way ANOVA test.

496

497 **Table 1**

498 The design and contaminant contents of all treatments

Treatment		Concentration		Treatment		Concentration		Treatment		Concentration	
		Cu	TCP			Cu	TCP			Cu	TCP
T1	CK	0	0	T5	Cu_{100}	100	0	T11	Cu_{300}	300	0
T2	TCP_{40}	0	40	T6	$Cu_{100}TCP_{40}$	100	40	T12	$Cu_{300}TCP_{40}$	300	40
T3	TCP_{80}	0	80	T7	$Cu_{100}TCP_{80}$	100	80	T13	$Cu_{300}TCP_{80}$	300	80
T4	TCP_{120}	0	120	T8	$Cu_{100}TCP_{120}$	100	120	T14	$Cu_{300}TCP_{120}$	300	120
				T9	$Cu_{100}TCP_{80}$+Un	100	80	T15	$Cu_{300}TCP_{40}$+Un	300	40
				T10	$Cu_{100}TCP_{80}$+ St	100	80	T16	$Cu_{300}TCP_{40}$+St	300	40

499 There are three replicates for each treatment.

Table 2

Fruiting time (growing days till fruiting body harvested), biomass (g dry weight pot^{-1}, mean ± SD, n=3) of *C.maxima* harvested in soil and percentage of control (%) for each treatment.

Group	Contaminant spiking		Nonsterilized or sterilized soil	Incubated or non-incubated	Fruiting time	% of Control	Biomass
	Cu	2,4,5-TCP					
T1	0	0	Nonsterilized	Incubated	25.5±0.71	100	29.53±5.61
T2		40	Nonsterilized	Incubated	27.33±2.52	107.19	24.268±1.62
T3		80	Nonsterilized	Incubated	33±3.46	129.41	28.0866±2.44
T4		120	Nonsterilized	Incubated	41±5.29	160.78	23.22±0.23
T5	100	0	Nonsterilized	Incubated	35±4.00	137.25	22.902±1.19
T6		40	Nonsterilized	Incubated	34.67±3.79	135.95	22.025±3.42
T7		80	Nonsterilized	Incubated	33.67±6.11	132.03	26.443±1.06
T8		120	Nonsterilized	Incubated	38±8.19	149.02	25.151±2.60
T11	300	0	Nonsterilized	Incubated	36.33±2.08	142.48	22.875±1.70
T12		40	Nonsterilized	Incubated	35.67±2.52	139.87	30.212±2.97
T13		80	Nonsterilized	Incubated	39±1.73	152.94	24.905±2.92
T14		120	Nonsterilized	Incubated	32.33±2.52	126.80	30.382±2.88
T10	100	80	Sterilized	Incubated	nd	0	nd
T16	300	40	Sterilized	Incubated	nd	0	nd
T9	100	80	Nonsterilized	Non-incubated	nd	0	nd
T15	300	40	Nonsterilized	Non-incubated	nd	0	nd
Significance of:							
Copper					F=3.211 P=0.059		F=1.088 P=0.353
2,4,5-TCP					F=2.620 P=0.075		F= 0.135 P=0.938
Cu × 2,4,5-TCP					F=3.543 P=0.012*		F=1.413 P=0.252

Percentage of the control (%) = Growing days of fruiting body in treatment/Growing days in control × 100%. Two-way ANOVA was used to compare the interaction of copper and 2,4,5-TCP. nd: not determined as mushroom was not cultivated in several treatments or none of fruiting body was harvested.

507 **Table 3**

508 Linear regressions between 2,4,5-TCP removal and three related enzyme activities in rhizosphere

509 soil.

Enzyme Name	Linear equation	R^2
Manganese peroxidase	Y = 0.01X + 0.944	0.891**
Dehydrogenase	Y=0.41X + 0.785	0.617*
Laccase	Y= 0.02X + 0.574	0.329

510 Y denotes 2,4,5-TCP removal ratio, and X denotes enzyme activities.

511 *Correlation is significant at the 0.05 level,

512 **Correlation is significant at the 0.01 level.

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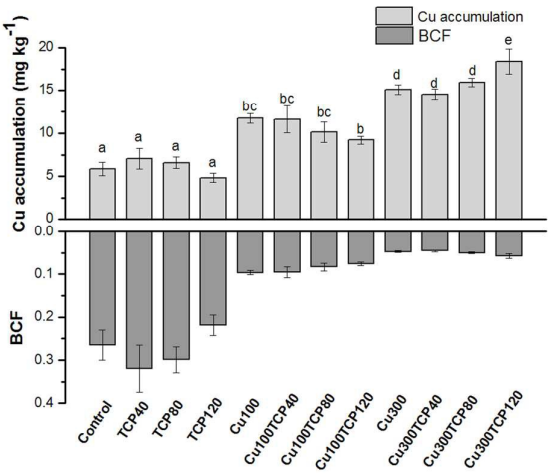
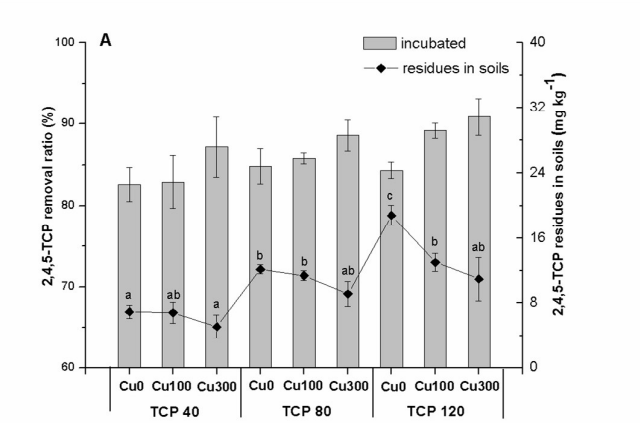
514



515

516 **Fig 1**

517



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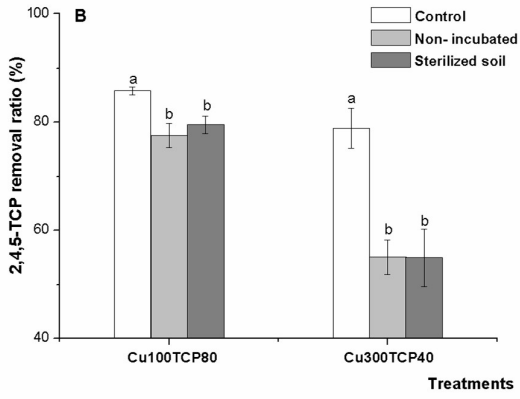

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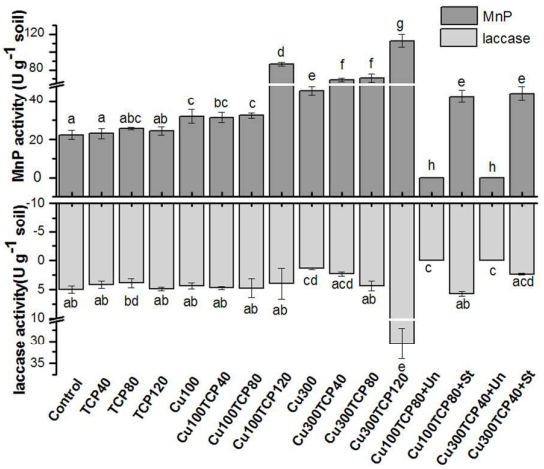
520 **Fig 2**



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522 **Fig 3**

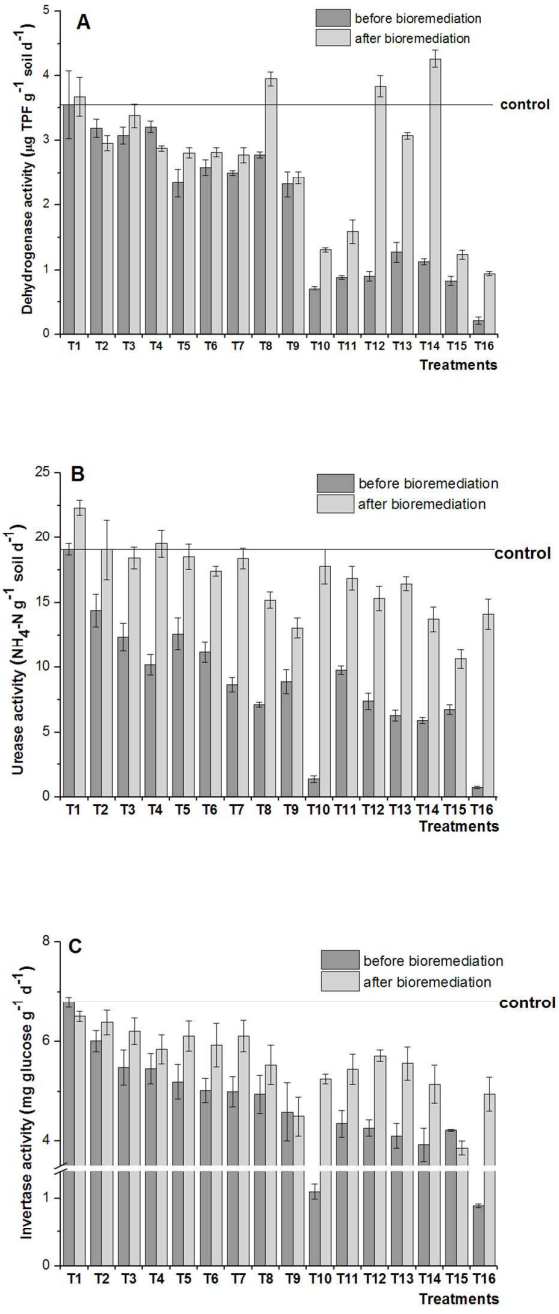

Fig. 4