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Environmental Impact

Among the materials to immobilize heavy metals, nano-hydroxyapatite (NHAp) was found to be effective in immobilizing heavy metals due to its moderate solubility and their high surface area and reactivity in soil. There are some new and significant results found in the manuscript. The results showed that NHAp could effectively reduce the CaCl2-extractable Pb, Cu, Cd, Zn and significantly reduce the metal content in ryegrass over time. Treatment with NHAp increased the *Stenotrophomonas sp.* and *Bacteroides* and enzyme activities including urease, phosphatase and dehydrogenase. The results from this study can be very useful for assess the role of NHAp on heavy metals remediation in soil.

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Nano-hydroxyapatite alleviates the detrimental effects of heavy metals on plant growth and soil microbes in e-waste-contaminated soil

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38 **Abstract**

39 The crude recycling activities of e-waste have led to the severe and complex
40 contamination of soil in e-waste workshop topsoil (0-10 cm) by heavy metals. After
41 nano-hydroxyapatite (NHAp) application in June 2013, plant and soil samples were
42 obtained in November 2013, December 2013, March 2014 and June 2014, respectively.
43 The results showed that NHAp effectively reduced the $CaCl_2$-extractable Pb, Cu, Cd, and
44 Zn in the topsoil, significantly reduced the metal content in ryegrass and also increased
45 the plant biomass compared with the control. Moreover, the concentrations of
46 $CaCl_2$-extractable metals in the soil decreased with the increasing NHAp. NHAp
47 application also increased the activities of soil urease, phosphatase and dehydrogenase.
48 Moreover, soil bacterial diversity and community structure were also altered after NHAp
49 application. Particularly, *Stenotrophomonas sp.* and *Bacteroides* percentages were
50 increased. Our work proves that NHAp application can alleviate the detrimental effects of
51 heavy metals on plants grown in e-waste-contaminated soil and soil enzyme activities, as
52 well as soil microbial diversity.

53 **Keywords**

54 e-waste, heavy metals, plant growth and biomass, soil microbial communities, enzyme
55 activity

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59 **1 Introduction**

60 The rapid development of electrical technology has markedly increased the production of

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61 electronic waste (e-waste). The majority of e-waste is exported to developing countries,
62 such as China, India, and Pakistan, for recycling and burning, where they are mostly
63 treated by land filling, cyanide leaching and open burning[1]. These crude recycling
64 activities have led to the severe and complex contamination of the soil by heavy metals
65 (Cd, Pb, Cu, and Hg)[2].Milojkovic & Litovski have reported that 70% of heavy metals
66 (including Hg and Cd) found in the soil are of electronic origin [3]. In an e-waste
67 recycling slum in Bangalore, India, the soil concentration of Cd, In, Sn, Sb, Hg, Pb and
68 Bi were up to39, 4.6, 957, 180, 49, 2850, and 2.7 mg.kg^{-1}respectively,which were approx.
69 100-fold higher than those at a nearby control site[4]. In 2005, Tang et al. have
70 investigated the soil heavy metal content in soil samples from farmlands nearest an
71 e-waste recycling area in Taizhou and found that the soil heavy metal contents exceeded
72 the standard levels by 100% for Cd, 87.5% for Cd, 37.5% for Hg, and 25% for Zn[5].
73 It is difficult and costly to remove heavy metals from soil and sediment[6]. As an
74 alternative, researchers have attempted to stabilize heavy metals in soil or sediment using
75 materials that make these contaminants less mobile and bioavailable, thereby reducing
76 the ecological risk of these metals. Among the materials used to immobilize heavy metals,
77 nano-hydroxyapatite (NHAp) is an efficient heavy metal-immobilizing agent because of
78 its high sorption capacity for heavy metal, low water solubility, high stability under
79 reducing and oxidizing conditions, availability and cost-effect[7]. Many studies have
80 confirmed the efficiency of NHAp in immobilizing Pb and Cd in the contaminated
81 sediment or soil [7, 8]. However, there is limited information on the effects of NHAp on
82 plant growth and soil microbes, especially the long-term effects of NHAp application.
83 This study was designed to investigate the long-term effects of NHAp on immobilizing

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84 heavy metals, plants growth and biomass, as well as on soil microbes in the e-waste
85 recycling area where the soil was contaminated by e-waste.

86 **2 Materials and methods**

87 **2.1 Measurement of soil properties**

88 The study area is located in north China (N 39°15′，E117°15′). Many simple household
89 e-waste recycling and burning workshops are distributed across farmlands and riversides
90 in this area, and most of them are currently operational. The bulk density, water content,
91 organic carbon content, cation exchange capacity (CEC) and pH of surface soil were
92 measured before the experiments were started. The bulk density was measured using an
93 soil density instrument (SDG200, TransTech, USA); pH was measured using a glass
94 electrode after the soil was suspended in H_2O (1:2.5 w/v). Water content was measured as
95 follows: 0.1 g of soil was collected and then dried in 105℃ for 6-8 h until a constant
96 weight was obtained, of the dried soil was used as the water content. Organic carbon
97 content was calculated by subtracting the inorganic carbon content from the total carbon
98 content, each being measured using a carbon measurement instrument. CEC was
99 measured as follows: First, 1.00 g of dried soil was weighed and then mixed in
100 EDTA-ammonium acetate solution repeatedly, followed by centrifugation at 3000g/min.
101 The precipitate was retained and was transferred into a 150 ml volumetric flash with
102 deionized water, with a final volume of 80-100ml. Then, 2 ml liquid paraffin and 1 g
103 MgO were added, followed by distilling using an azotometer. Finally, CEC was
104 calculated according to the equation: CEC (cmol/kg土)＝M×(V-V0)/soil sample weight.
105 The total concentrations of Cd, Cu, Pb, and Zn in soils were measured as follows: Briefly,
106 6 mL HNO_3 and 3 mL HF were added to each soil sample (1 g), and the mixture was

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107 subjected to microwave digestion (120°C for 3 min and 180°C for 15 min). Subsequently,
108 the acids were removed by using an acids-driving instrument (PH60-460, CIF, USA), and
109 the total heavy metal concentration was detected using ICP-MS (inductively coupled
110 plasma mass spectrometry).

111 **2.2NHAp application and sowing of ryegrass seeds**

112 Nano-hydroxyapatite (NHAp) (purity > 98%) was purchased from Nanjing Emperor
113 Nano Materials Co., Ltd (Nanjing, China).The average unit cell size of NHAp used in the
114 present study was 3 nm. Transmission electron
115 microscopy(TEM, Tecnai G2 20 S-TWIN, FEI, USA)revealed that the NHAp material
116 had a nano rod structure, with dimensions of 20 nm (i.d.) × 200 nm (length). The specific
117 surface area of NHAp was calculated as 130 $m^2 \cdot g$ according to its structural geometry.
118 A random block design was generated for three treatments with five replicates each. The
119 total field area was 560 m^2, including 9 plots and each plot had an area of 50 m^2(10
120 m×5m).NHAp was manually spread onto the topsoil in June 2013at $3 t \cdot ha^{-1}$ and
121 $5 t \cdot ha^{-1}$respectively; no NHAp was spread in the control. After spreading, the soil was
122 superficially tilled into interrows using a tiller at a 7-10 cm depth. The ryegrass seeds
123 were directly sown in soil at 1.5 $g \cdot m^{-2}$ in June 2013. Afterwards, the soil was never
124 plowed anymore.

125 **Measurement of plant biomass and heavy metal concentration in plants**

126 Ryegrass was harvested four times in November 2013, December 2013, March 2014, and
127 June 2014 respectively, and corresponding biomass was measured separately. Briefly, the
128 aboveground part of all ryegrass plants was cut directly in each plot and placed into paper
129 bags. Then, all the plants were washed first under tap water and then with deionized

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130 water, followed by drying in a 60□ oven for at least 48 h. The dried plants were then
131 finely ground and subjected to microwave digestion in nitric acid as described by
132 Mackieet al. (2015). Metal content in the plants was analyzed by in ductively coupled
133 plasma mass spectrometry (ICP-MS).

134 **Soil sampling and measurement of $CaCl_2$-extractable heavy metal**

135 Soil was sampled in the same day when plants were harvested. Briefly, 500 g of soil was
136 collected randomly from five locations in each plot at the depth of 10cm beneath, 100 g
137 each. Each sample was collected among ryegrasses not grown on the edge the plot to
138 reduce the edge effect. Then, the five samples were mixed to generate a mixed sample.
139 Among them,20 g was stored in a 4°C refrigerator for later soil enzyme activities
140 analyses; 10 g soil was stored in a -20°C freezer for subsequent microbial community
141 analyses. The rest soil samples treated with NHAp were sieved using a nylon mesh (2
142 mm in diameter) and homogenized, followed by air-drying and measurement of the
143 following parameters: pH, CEC and heavy metal content.

144 Heavy metal content was measured as previously described [9, 10]. Briefly, 2.5 g of each
145 soil sample was put in a polypropylene centrifugation tube containing 25 ml $CaCl_2$ (0.01
146 M) and shaken for 2 h at 20°C. Subsequently, the mixture was centrifuged at 3000 g for
147 15 min and the supernatant was retained. Finally, the concentration of each metal in the
148 supernatant was measured using Agilent 7500a ICP-MS instrument (Agilent, USA). The
149 detection limits for Cd, Zn, Pb and Cu were lower than 1.0 ng mL^{-1}.

150 **2.4 Measurement of soil enzyme activity**

151 Soil urease, alkaline phosphatase and dehydrogenase activities were determined as
152 described by Guan et al. (1986).

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For the assessment of soil urease activity, 5.0 g of soil was incubated with 1 mL toluene, 10 mL of 10% urea, and 20 mL citrate buffer (pH6.7) at 37°C for 24 h. Afterwards, 40 mL of deionized water, 4 mL of sodium phenolate and 3 mL of sodium hypochlorite were added. The blue products were measured using a spectrophotometer at λ= 578 nm within 1 h after a 30-min color reaction. Assays without soil and urea were examined as controls. The urease activity was expressed as milligrams NH_3-N generated from 1 g of soil at 37°C per 24 h.

Alkaline phosphatase activity was assayed as follows: 5.0 g of soil was incubated with1 mL of toluene and 20 mL of 0.5% disodium phenylphosphate in acetate buffer (pH6.7) at 37°C for 24 h. The phenol produced was extracted and oxidized using 0.5 mL potassium hexacyanoferrate in alkaline buffer. The products were determined using 0.5 mL of4-aminoantipyrine through colorimetry at λ= 510 nm. An assay without soil was examined as a control. The phosphatase activity was expressed as milligrams of hydrolyzedphenol generated from 1 g soil at 37°C per 24 h.

Dehydrogenase activity was determined after incubating 5.0 g of soil with 5 mL of 2,3,5-triphenyltetrazolium chloride (TTC) solution at 30°C for 6 h in the dark. Following incubation, the soil was extracted with 40 mL of methyl alcohol for 1 h to produce tetrazole red formazan (TRF). The filtrate was colorimetrically determined at λ= 485 nm. The dehydrogenase activity was expressed as microliters of hydriongenerated from 5 g of soil at 30°C per 6 h.

2.5 PCR-DGGE analysis of soil bacteria community

After incubation for 360 days, the total microbial DNA was extracted from 0.5g of soil sample by the bead beating method following the manufacturer's instructions using

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176 FastDNA SPIN Kit for Soil(Bio101 Inc., USA).The purified DNA extract was
177 fluorometrically quantified using Quantity One 4.0.1,(Fluor-S MultiImager, Bio-Rad,
178 USA). A fluorescent DNA quantification kit (Bio-Rad, CA, USA) was used to generate
179 the standard curve. The quantified DNA extracts were stored at -20°Cfor subsequent
180 analysis.

181 The V3 region of 16S rDNA was amplified by PCR on Hybrid PCR Express thermal
182 cycler (Bio-Rad, USA) using the primers 357F-GC clamp
183 (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAG
184 GCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'). PCR was performed in a
185 50-Ml volume containing 10-15 ng of DNA template, 25 pM of each primer, 2.5 mM
186 deoxynucleotide triphosphates (dNTPs, Promega, USA), PCR buffer (Applied
187 Biosystems, USA), 0.1 mM $MgCl_2$ solution (Sigma) and 1 U of Taq polymerase (Applied
188 Biosystems, USA). The negative controls contained no template DNA. Parameters for
189 amplification were initial denaturation at 94°C for 3 min, followed by 34 cycles of
190 denaturation at 94°C for 50 s, annealing at 56°C for 1 min and DNA extension at 72°C
191 for 30 s, with a final extension step at 72°C for 10 min. The amplified DNA was verified
192 on 1% agarose gels stained with SYBR™ Green I (Sigma, USA) and visualized using a
193 Fluor-S MultiImager (Bio-Rad, USA).

194 DGGE (denaturing gradient gel electrophoresis) was performed using a Dcode™
195 Universal Detection System instrument following the manufacturer's instructions
196 (Bio-Rad, USA). Briefly, a polyacrylamide gel (8% acrylamide/bisacrylamide (37.5:1) in
197 1X TAE buffer (pH 8.0) was prepared with a denaturing gradient of 35~60% (100%
198 denaturant contained 7 M urea and 40% (v/v) formamide). Thirty microliters of each

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199 PCR product was mixed with loading dye (0.08% bromophenol blue (w/v), 0.08% xylene
200 cyanol (w/v) and 30% glycerol (v/v)), loaded onto the gels and electrophoresed in 1X
201 TAE buffer at 60°C for 5 h at a constant voltage of 160 V (DcodeTM Universal
202 Detection System, Bio-Rad, USA). After electrophoresis, the gels were stained with
203 SYBR™ Green I (Sigma, USA) for 30 min and photographed under UV light using a
204 Fluor-S MultiImager (Bio-Rad, USA).
205 Cluster analysis of the 9 samples based on all DGGE fingerprints was performed using
206 the SAS program (SAS Institute, Cary, NC).The Shannon diversity index of each
207 replicate was calculated following the equation, $H' = \sum_{i=1}^{S} p_i \ln p_i$, where S is richness or
208 the total number of band, pi is the proportion of total intensity accounted for by the ith
209 band, and ln is the natural logarithm. The mean value in the three replicates was used as
210 final Shannon diversity index.
211 **2.6Identification of featured bands by sequencing**
212 After DGGE, the bands that varied notably between NHAp-treated soil samples and the
213 control soil samples were excised from the gel. DNA from each band was extracted using
214 the FastDNA SPIN Kit (Bio101 Inc., USA). Then, the extracted DNA was re-amplified
215 with the primer set without a GC clamp. The qualified PCR products were sent to the
216 Beijing Huada Gene Company (Beijing, China) for sequencing. The sequences were
217 aligned using the software MAGE4.0 (Tokyo, Japan).
218 **2.7 Quantitative real-time PCR assays**
219 Quantitative real-time PCR (qPCR) was performed on an iCycler IQ(BioRad, Hercules,
220 CA) using the SYBR Green Jump Start™ Taq Ready Mix™ (Sigma, Milan, Italy)
221 following the manufacturer's instructions. Amplification of 16S rRNA genes was

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222 performed the universal primers 341F and 534R [27], respectively. Amplification was
223 performed in a 25 mL total volume containing 12.5 mL of 2X SYBR Green Jump-Start
224 Taq mix, 2.5 mL of each primer (0.05 and 0.9 mM for the primers 341F and 534R
225 respectively), and 7.5 mL of template DNA. To avoid PCR amplification problems due to
226 the presence of inhibitors, the environmental DNA samples were diluted 10 to 100
227 times.The amplification cycle included an initial denaturation step at 95°C (5 min),
228 followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and
229 elongation at 72°C for 30 s, with a final extension step at 72°Cfor 7 min. At the end of
230 the qPCR, the melting curve analysis was conducted, measuring the SYBR Green I signal
231 intensities for a 0.5°C temperature increment every 10 s from 50°C to 95°C. The target
232 gene abundance in the microcosms was investigated, and the results were expressed as
233 changes (fold) with respect to the relative zero-time point, according to the expression:
234 $\text{Fold} = 2^{(Ct_x)-(Ct_0)}$
235 where Ct_0 and Ct_x are the threshold cycles for the zero and successive time-points,
236 respectively. The threshold cycle (Ct) is the cycle number at which the fluorescence
237 generated within are action crosses the threshold. The specificity of the qPCR assays was
238 confirmed based on the occurrence of both single melting peaks and the unique bands of
239 expected sizes on agarose gels.

240 **2.6 Statistical analysis**

241 All data was expressed as Mean± SD. Differences between different treatments or groups
242 were statistically calculated using ANOVA and Tukey's *t*-testusing SPSS 11.5 (SPSS for
243 Windows, Version 11.5, USA). $P<0.05$ was considered to indicate a significant
244 difference. Pearson correlation analyses between heavy metal concentrations and

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245 enzyme activities were also performed using SPSS 11.5.

246 **3 Results**

247 **Soil properties prior to the experiments**

248 The bulk density, water content, organic carbon content, cation exchange capacity (CEC)
249 and pH of the soil before any treatment were 1.03 $g \cdot cm^3$, 47.3%, 2.54%, 17.3 $cmol \cdot kg^{-1}$
250 and 5.16, respectively. The soil contained 2.65 $g \cdot kg^{-1}$ total N and 0.47 $g \cdot kg^{-1}$ total P. The
251 total concentrations of Cd, Cu, Pb, and Zn in the soil were 3.76, 472.7, 2016.3 and 3076.5
252 $mg \cdot kg^{-1}$, respectively.

253 **Effect of NHAp on $CaCl_2$-extractablemetal concentration in the soil**

254 Before the experiments started, pH was 5.16±0.1, 5.77±0.1 and 6.96±0.2 in the control
255 soil, in the 3 $t \cdot ha^{-1}$ NHAp-treated soil and 5 $t \cdot ha^{-1}$ NHAp-treated soil respectively (Table
256 1). Generally, the pH in each soil was increasing, and that of the soil treated with
257 NHAp was larger than that of the control soil at each time point,suggesting that after
258 NHAp application, the soil pH was affected and the metal immobilization in the soil was
259 enhanced.

260 With reference to the thresholds in table 2, the concentration of each heavy metal before
261 NHAp treatment exceeded the values of Grade II soil quality standards of the State
262 Environmental Protection Administration (SEPA) of China.

263 And there was no significant difference in the concentration of each metal among
264 different groups before NHAp treatment (Fig.1). The concentration of each metal
265 decreased markedly over time in the soil treated with 3 $t \cdot ha^{-1}$ or 5 $t \cdot ha^{-1}$ NHAp, each
266 being significant lower as compared to that in the control soil in June 2014.

267 **Effect of NHAp on plant growth and metal accumulation in ryegrass**

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268 Symptoms of toxicity were observed in the control ryegrass, such as filemotnecrotic spots
269 on the young leaves; by contrast, no symptoms of toxicity were visually observed in the
270 ryegrass grown in the soil treated with NHAp. Meanwhile, the biomass of the control
271 ryegrass harvested at each time point was lower than that of ryegrass grown in soil
272 treated with NHAp, although without significant difference (Table 1).
273 Overall, ryegrass grown in the control soil had lower metal contents as compared to those
274 grown in the NHAp-treated soil, with significant difference in Cu, Pb and Zn content at
275 each time point (Table 1).
276
277 **Effects of NHAp on soil enzyme activity**
278 Changes in soil dehydrogenase, urease, and acid phosphatase after the addition of NHAp
279 were determined in the present study (Table 3).
280 Either dehydrogenase, urease or phosphatase activity in the soil to be treated by 5 $t \cdot ha^{-1}$
281 was the highest prior to NHAp treatment. However, the enzyme activity in the control
282 soil was lower than its initial level, indicating a detrimental effect of heavy metals on soil
283 enzyme activities, whereas the enzyme activity in the soil treated by either NHAp was
284 higher than their initial level, indicating a beneficial role of NHAp treatment. Thus, the
285 dehydrogenase activity in soil treated by $3t \cdot ha^{-1}$ NHAp, urease activity in the soil treated
286 with 5 $t \cdot ha^{-1}$ NHAp, and phosphatase activity in the soil treated with either 3 or 5 $t \cdot ha^{-1}$
287 NHAp was significantly higher than their counterpart in the control soil in June 2014.
288 Pearson correlation analyses revealed a significant positive correlation between
289 dehydrogenase and phosphatase (R^2 =0.758), indicating a similar sensitivity of the two
290 enzymes to heavy metal contamination in the study area. It was also found that there was

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291 a significant negative correlation between urease activity with either soil Cu ($R^2 = -0.897$)
292 or Cd ($R^2 = -0.911$) content (Table 4).

293 **Effect of NHAponsoil microbial diversity**

294 DGGE revealed that the DGGE fingerprints were similar in the three replicates in the soil
295 subject to the same treatment, indicating a relatively higher reproducibility(Fig. 2).
296 Cluster analysis showed that the bacterial communities treated with NHAp (3t.ha^{-1} and 5
297 t.ha^{-1}) were separated from the control group (Fig. 3), indicating similar microbial
298 composition in the soil treated with NHAp.

299 The Shannon diversity index of microbial commnunities in the control soil was 3.41,
300 while that in the soil treated with 3 t·ha^{-1} and 5t·ha^{-1} NHAp was 3.69 and 3.88,
301 respectively, indicating that soil microbial diversity is increasing with NHAp
302 concentration.

303 **Identification of featured bands in the NHAp-treated soil microbial samples**

304 Sequence analysis showed that most clones in the soil treated by NHAp belonged to
305 *Stenotrophomonas sp* and *Bacteroides*accounting for 40% and 28% respectively (Fig. 3);
306 and the rest bands mostly belonged to *Enterobacter sp.* and *Acidobacteria*.

307 **4 Discussion**

308 **Effects of NHAp on $CaCl_2$-extractablemetal concentration in the soil and metal**
309 **concentration in plants**

310 pH affects the chemical forms of the metals in the soil [11]. CEC is a commonly used
311 indicator of soil fertility, nutrient retention capacity[12]. In the present study, it was found
312 that NHAp didn't alter the change in pH over time, while higher concentration NHAp
313 altered the change in CEC over time. *in situ* immobilization of heavy metals using NHAp
314 is a cost-effective and environmentally sustainable remediation approach by reducing

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315 their mobility and availability. Here, we examined the effect of NHAp on immobilizing
316 heavy metals by using $CaCl_2$ to extract exchangeable metal heavy metals that were not
317 immobilized in the soil. For each$CaCl_2$-extractable metal, its concentration in the soil was
318 significantly lower than that in the NHAp-treated soil, thus it seemed that NHAp
319 especially 5 $t{\cdot}ha^{-1}$ NHAp can significantly decreased the content of exchangeable heavy
320 metals, which were available by plants. This was consistent with the finding that lower
321 heavy metal content was detected in plants grown in the NHAp-treated soil, as well as
322 better performance of ryegrass in growth and biomass in the soil treated by NHAp.
323 Previously, Boisson et al. have also reported that hydroxyapatite decreased the
324 concentrations of `toxic' metals in the leaves of the test plants; however, they also found
325 that too higher hydroxyapatite application rate reduced the uptake of some essential trace
326 elements, thus leading to deficiency problems[13].

327 **Effects of NHAp on soil enzyme activity**

328 Dehydrogenase is an intercellular enzyme in the soil, which catalyzes the removal of
329 hydrogen atom from different metabolites[14], urease hydrolyzes urea intracellularly,
330 leading to a shift in soil pH Phosphatase activity. Alkaline phosphatase is involved in soil
331 phosphorus metabolism[15]. Numerous studies have confirmed that the activities of soil
332 enzymes were decreased with the increasing heavy metal pollution
333 [16-18].Dehydrogenase activity was particularly sensitive to heavy metals [19].Kandeler
334 et al. have reported that phosphatase activities were dramatically decreased by heavy
335 metal pollutants [17].Tyler has declared that urease and acid phosphatase activity was
336 closely negatively correlated with log Cu+Zn concentration[20]. Decrease in the
337 activities of three soil enzymes was also observed in the present study. Heavy metals in

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338 the soils react with active the protein groups of enzymes, such as sulfhydryl groups, to
339 form metal-sulfide equivalents, or sequester the enzyme binding sites through the
340 formation of complexes with the substrates, thereby inactivating or inhibiting enzyme
341 activity[21]. By contrast, the beneficial role of NHAp was also observed here. Previously,
342 Bert et al. (2012) have reported that addition of hydroxyapatite can reduce sediment
343 ecotoxicity and improved the growth of the total bacterial population.
344

345 **Effect of NHAp on soil microbial diversity**

346 Here, comparison of Shannon diversity indices between the soil subject to different
347 treatments indicates that microbial diversity was decreased in the soil contaminated by
348 heavy metals, while NHAp has a beneficial effect on soil microbial diversity. Previously,
349 Oliveira et al. have also reported that quantitative analysis of soil microbial populations
350 shows a marked decrease in total culturable numbers of the different microbial groups of
351 the soil samples contaminated by Hg and As[19].Du et al. further reported that the
352 microbial diversity index of microbial community in the treatments amended with NHAp
353 was significantly higher than that of control[22]. Thus, it can be concluded that NHAp
354 can improve the microbial diversity in the metal-contaminated soil. Furthermore,
355 sequencing discovered that microbes belonging to *Stenotrophomonas sp* and *Bacteroides*
356 showed an elevated abundance in the NHAp-treated soil as compared to the control soil.
357 Previously, Pages et al. have presented that another *Stenotrophomonas* species *S.*
358 *maltophilia* can develop tolerance to overcome metal toxicity in the presence of heavy
359 metals[23]. Thus, it is presumably that NHAp may increase the microbial populations
360 that are tolerant to metal toxicity.

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361 **Conclusion**

362 In the present study, NHAp significantly decreased the exchangeable heavy metals
363 contents in the e-waste-contaminated soil, also reduced the metal concentration in plants
364 and increased plant biomass, suggesting NHAp has a good performance on immobilizing
365 the heavy metals in produced by e-waste, accordingly alleviating the detrimental effects
366 of heavy metals on plant growth. NHAp has also alleviated the detrimental effects of
367 heavy metals on soil enzyme activities. In addition, NHAp application also has a positive
368 role on soil microbial diversity and microbial composition possibly via increasing the
369 percentage of metal- tolerant populations, such as *Stenotrophomonas sp* and *Bacteroides*.

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Table legends

Table 1. Plant biomass, pH, CEC, and plant metal content between soil with and without NHAp treatment

Table 2.Thresholds for Grade II soil quality standards of the State Environmental Protection Administration (SEPA) of China

Table 3.Enzyme activities in soil with and without NHAp treatment at different time points

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Table 4.Pearson correlation analyses between heavy metal concentrations and enzyme activities

Figure captions

Figure 1.Effect of NHAp on the $CaCl_2$-extractable heavy metal concentration in the soil over time.

Figure 2. (A) DGGE profiles of bacterial 16S rRNA genes in biochar-amended soil after NHAp treatment. (B) Similarity relationships among different DGGE results.

Figure 3. Cluster analysis of bacterial 16S rRNA gene DGGE profiles after NHAp treatment.

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501 Table 1

variable	Treatment	June 2013 (no plants)	November 2013	December 2013	March 2014	June 2014
Plant biomass (kg DW ha^{-1})	Control	0	2066±103	996±87	1524±112	1429±340
	NHAp(3t)	0	2657±69	1012±33	1877±96	2019±210
	NHAp(5t)	0	3398±159	1147±93	2739±76	3570±270
pH	Control	5.16±0.1a	5.66±0.1a	5.79±0.1a	6.03±0.2b	6.11±0.1b
	NHAp((3t)	5.77±0.1a	6.12±0.1b	6.33±0.1b	6.65±0.1b	6.91±0.1b
	NHAp(5t)	6.96±0.2*a	7.14±0.1a	7.36±0.1*a	7.75±0.2b	7.69±0.1b
CEC (cmol kg^{-1} soil)	Control	17.3±0.3a	19.8±0.1a	20.9±0.2a	21.2±0.4a	22.1±0.3a
	NHAp(3t)	18.11±0.2a	19.9±0.1a	21.3±0.2b	21.9±0.2b	22.7±0.2b
	NHAp(5t)	24.5±0.2*a	20.3±0.2b	23.5±0.4*a	22.5±0.3a	23.8±0.4a
Plant Cu ($mg.kg^{-1}$)	Control	0	16.4±0.2*a	15.3±0.1*a	16.9±0.3*a	17.2±0.2*a
	NHAp(3t)	0	13.2±0.1a	13.1±0.1a	11.3±0.2a	12.3±0.2a
	NHAp(5t)	0	12.1±0.1a	10.7±0.2a	8.7±0.2b	8.1±0.2b
Plant Pb ($mg.kg^{-1}$)	Control	0	37.2±0.6*a	36.1±0.3*a	34.9±0.5*a	34.7±0.3*a
	NHAp(3t)	0	33.4±0.3a	32.1±0.2a	31.7±0.2a	27.9±0.3b
	NHAp(5t)	0	29.3±0.3a	27.6±0.2a	26.8±0.3b	23.5±0.2b
Plant Zn ($mg.kg^{-1}$)	Control	0	43.7±0.6*a	40.9±0.5*b	42.5±0.3*a	39.7±0.4*b
	NHAp(3t)	0	37.8±0.3a	32.1±0.3b	30.5±0.2b	29.6±0.3b
	NHAp(5t)	0	33.2±0.3a	27.6±0.4b	23.7±0.6b	19.6±0.3c
Plant Cd ($mg.kg^{-1}$)	Control	0	0.98±0.01*a	1.03±0.01*a	0.87±0.04*a	1.11±0.02*a
	NHAp(3t)	0	0.79±0.01a	0.63±0.01a	0.64±0.02a	0.77±0.03a
	NHAp(5t)	0	0.66±0.01a	0.52±0.02a	0.39±0.02b	0.43±0.09b

502 Note: Different letters represent significant difference between different sampling time points
503 according to the results of ANOVA and Tukey test (P<0.05);* indicates significant differences
504 between different treatments at the same sampling time point according to the results of ANOVA and
505 Tukey test(P<0.05).

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Table 2

	Heavy metal threshold			
pH	Cd(≤)	Cu(≤)	Pb(≤)	Zn(≤)
<6.5	0.30	50	250	200
6.5-7.5	0.30	100	300	250
>7.5	0.60	100	350	300

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Sampling time	Dehydrogenase (μl $5g^{-1}$ soil 6 h^{-1}, dw)			Urease ($mg.kg^{-1}$soil $24h^{-1}$, dw)			Phosphatase activity ($mg.kg^{-1}$.soil 24 h^{-1}, dw)		
	NHAp(5t)	NHAp(3t)	Ck	NHAp(5t)	NHAp(3t)	Ck	NHAp(5t)	NHAp(3t)	Ck
0	4.88±0.45*a	4.01±0.02a	3.66±0.17a	485.02±2.73a	369.77±0.69a	321.7±2.73a	205.36±5.92*a	165.79±1.77a	130.2±3.19a
Nov 2013	6.02±0.09*b	4.69±0.11*a	3.71±0.11a	489.21±3.47*a	375.36±1.38a	305.7±1.49a	244.41±3.59*b	177.46±1.49b	123.7±1.76a
Dec 2013	6.11±0.25*b	5.21±0.04*b	3.43±0.05a	495.22±7.98*a	378.69±1.47a	291.4±1.38a	263.40±9.00*b	185.63±1.66*b	120.9±1.84a
Mar 2014	6.32±0.34*c	5.56±0.03*c	3.37±0.01a	484.26±7.81*a	377.44±2.11a	301.5±0.88a	254.43±5.66*c	199.48±1.09c	121.3±2.11a
June 2014	5.09±0.09a	5.41±0.03*b	3.29±0.09a	485.61±6.22*a	379.81±3.17a	289.6±1.66a	241.02±7.39*b	211.42±2.04*c	124.4±2.66a

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31 581 Note: Mean values followed by the same letter are not significantly different between different sampling time in the same treatment plot according to
32
33 582 ANOVA and multiple comparisons with Tukey test ($p \geq 0.05$). * means the significantly different between different treatment at the same sampling time
34
35 583 according to ANOVA and multiple comparisons with Tukey test ($p \geq 0.05$).
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Table 4

	Cu	Pb	Zn	Cd	Urease	Phosphatase	Dehydrogenase
Cu	1	-0.271	0.309	0.446	−0.897	-0.469	-0.557
Pb		1	0.266	-0.254	-0.527	-0.556	-0.364
Zn			1	0.284	-0.338	-0.396	-0.285
Cd				1	−0.911	-0.639	-0.439
Urease					1		0.223
Phosphatase						1	0.758
Dehydrogenase							1

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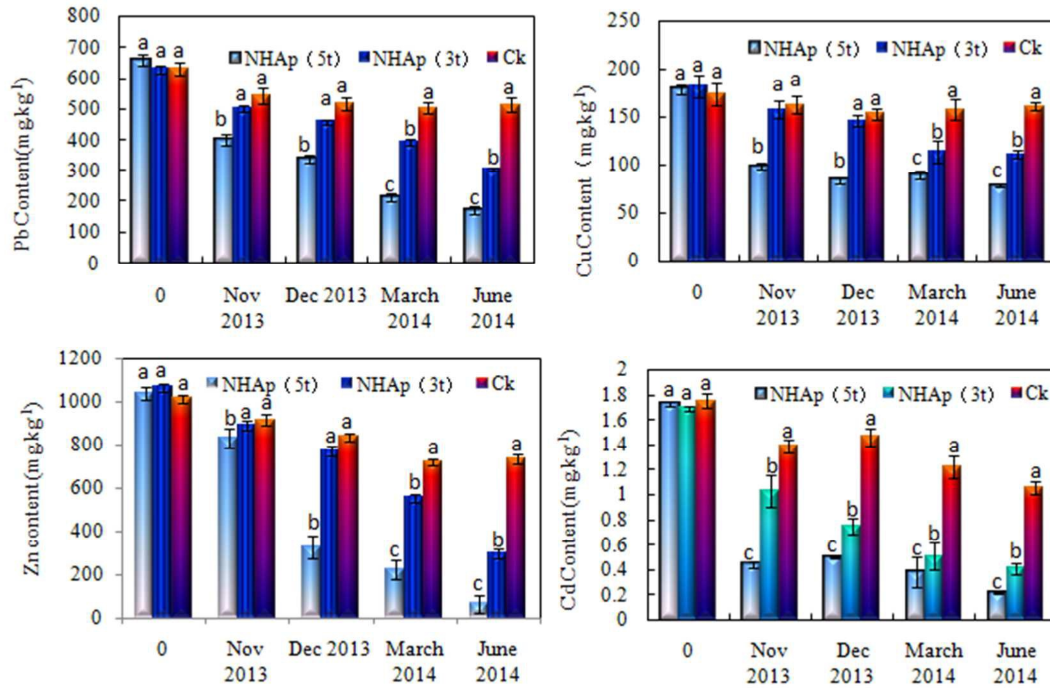

Figure 1

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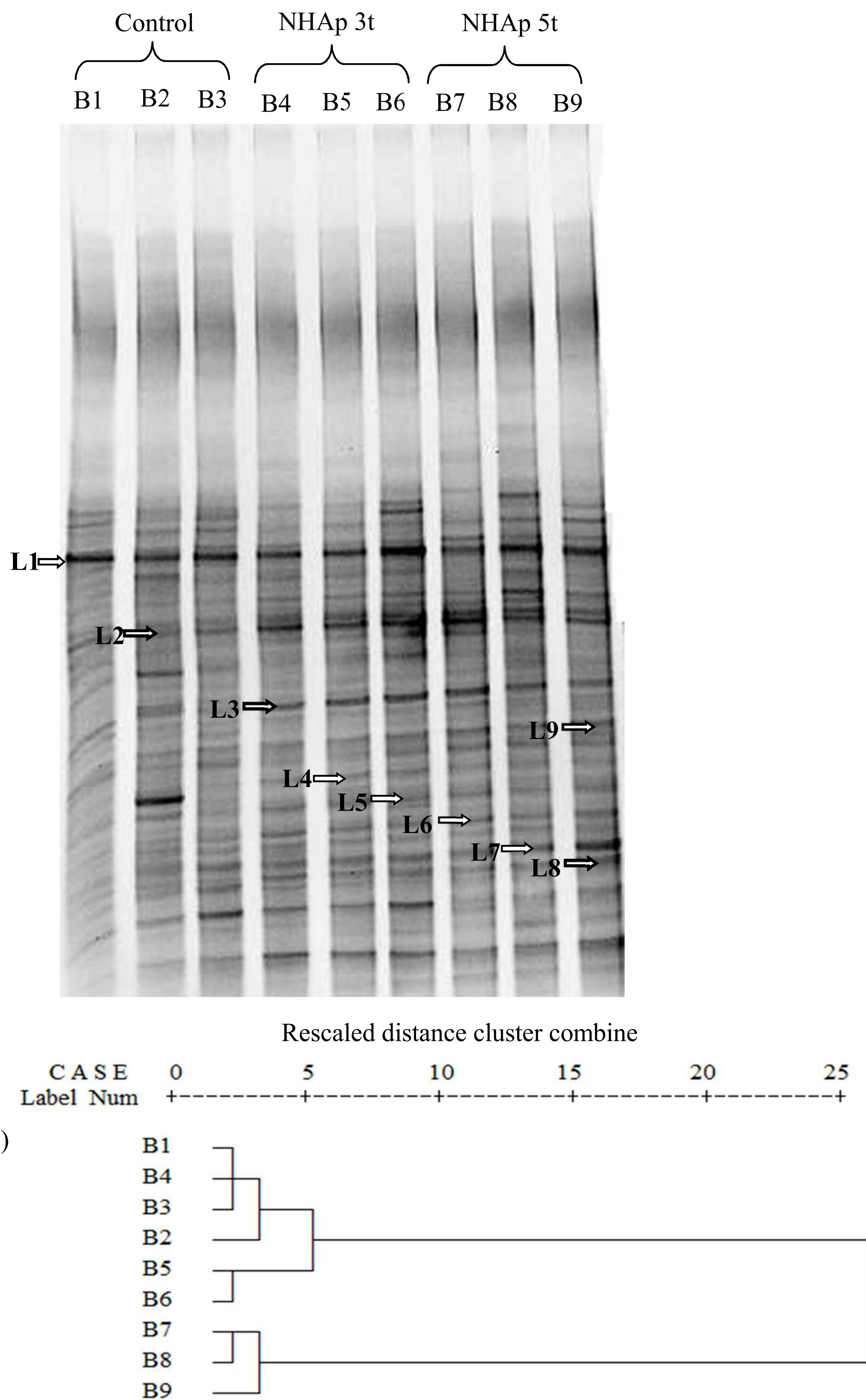

690 Figure 2

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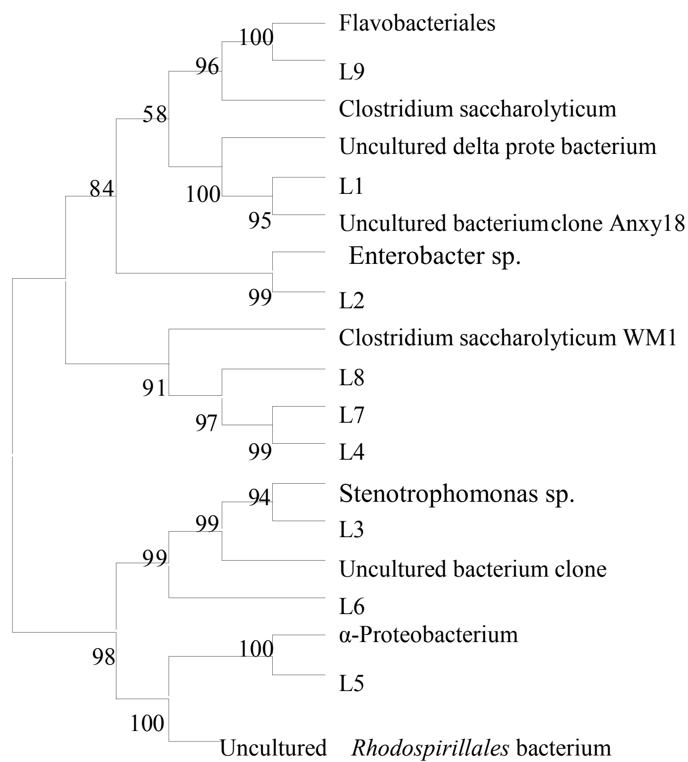

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