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Influence of Triethyl Phosphate on Phosphatase Activity in Shooting Range Soil: Isolation of a Zinc-Resistant Bacterium with an Acid Phosphatase

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Abstract

Phosphatase-mediated hydrolysis of organic phosphate may be a viable means of stabilizing heavy metals via precipitation as a metal phosphate in bioremediation applications. We investigated the effect of triethyl phosphate (TEP) on soil microbial-phosphatase activity in a heavy-metal contaminated soil. Gaseous TEP has been used at subsurface sites for bioremediation of organic contaminants but not applied in heavy-metal contaminated areas. Little is known about how TEP affects microbial activity in soils and it is postulated that TEP can serve as a phosphate source in nutrient-poor groundwater and soil/sediments. Over a 3-week period, TEP amendment to microcosms containing heavy-metal contaminated soil resulted in increased activity of soil acid-phosphatase and repression of alkaline phosphatase, indicating a stimulatory effect on the microbial population. A soil-free enrichment of microorganisms adapted to heavy-metal and acidic conditions was derived from the TEP-amended soil microcosms using TEP as the sole phosphate source and the selected microbial consortium maintained a high acid-phosphatase activity with repression of alkaline phosphatase. Addition of 5 mM zinc to soil-free microcosms had little effect on acid phosphatase but inhibited alkaline phosphatase. One bacterial member from the consortium, identified as *Burkholderia cepacia sp.*, expressed an acid-phosphatase activity uninhibited by high concentrations of zinc and produced a soluble, indigo pigment under phosphate limitation. The pigment was produced in a phosphate-free medium and was not produced in the presence of TEP or phosphate ion, indicative of purple acid-phosphatase types that are pressed by bioavailable phosphate. These results demonstrate that TEP amendment was bioavailable

53 and increased overall phosphatase activity in both soil and soil-free microcosms
54 supporting the possibility of positive outcomes in bioremediation applications.
55

1. Introduction

A soil polluted with heavy metals alters the microbial community structure and biomass through the inhibition of microbial activity (Sas-Nowosielska et al., 2004; Kucharski et al., 2011, Kandeler et al., 1996; Utobo and Tewari, 2015). One of the common methods for remediation of metal-contaminated soils involve pump and treatment with chelating agents to enhance mobilization of heavy metals (Vulava and Seaman, 2000) and harnessing microbial-reductive processes to precipitate of heavy metals in anaerobic environments (Lovley and Coates, 1997). Drawbacks to these methods include the uncontrolled spread of metals/radionuclides and the alteration of soil chemistry involving removal of non-target elements, such as iron, needed for microbial metabolism.

Microorganisms serve as the foundation for nutrient cycling in any environment. Phosphorus is one of the key nutrients essential to all living things and though phosphorus is ubiquitously distributed, it is often biologically unavailable in an insoluble, inorganic form or organically bound, and must be released as phosphate ion via phosphatases. Phosphate comes from both natural and anthropogenic sources, and environmental phosphatases can play a major role in the phosphorus cycling and breakdown of synthetic organophosphates (Pansu & Gautheyrou, 2006). Microbial enzyme activity in soil can be used as one measure of soil quality (Amador et al., 1997; Kucharski et al., 2011; Utobo and Tewari, 2015). For example, soils exposed to tert-butylphenyl-diphenyl phosphate altered soil microbial phosphatase observed as an increase in monophosphoesterase activity (Heitkamp et al., 1986). However, laboratory

and field studies have demonstrated that phosphatases are inhibited by low levels of heavy metals such as zinc (Kucharski et al., 2011; Huang and Shindo, 2000). Where Kucharski et al. (2011) found that compared to alkaline soil, an acidic soil imposes greater metal toxicity to phosphatases than all other soil enzymes tested.

In light of microbial sensitivities to heavy metals, phosphate amendments have been shown to decrease heavy-metal or radionuclide bioavailability (Wilson et al., 2006; Knox et al., 2008). For example, phosphatase-mediated immobilization of uranium by microbial activity has been demonstrated in a *Citrobacter sp.* that utilized tributyl phosphate (TBP) as a sole phosphate source (Montgomery et al., 1995). In another study, lead provided as $Pb(NO_3)$ has been shown to be immobilized via phosphatase-mediated precipitation as $Pb(PO_4)$ on microbial surfaces (Levinson and Mahler, 1998). Recently, phosphatase-mediated bioprecipitation of lead by soil fungi can be applied for Pb recovery (Liang et al., 2016).

At many sites the subsurface saturated zones are nutrient-poor, depleted of phosphorus and contaminated with organics and heavy metals. Triethyl phosphate (TEP) has been applied through biosparging, serving both as a carbon and phosphorus source to stimulate endogenous microorganisms, with promising results to remediate organic-groundwater contaminants (Hazen et al., 1996). Although mildly toxic and a corrosive irritant, TEP is one of the safest phosphorus compounds known that can readily be gasified (Bogan et al., 2001). Biosparging employs forced air and gaseous distribution of TEP (carbon/nitrogen source), methane (carbon source) and nitrous oxide (nitrogen source) into the saturated subsurface zone as an effective means to stimulate anaerobic-driven bioremediation (Pfiffner et al., 1997). TEP enhanced the hydrocarbon degradation *in situ* and laboratory groundwater microcosms (Santo Domingo et al., 1997a & 1997b), yet the mechanism by which TEP stimulates microorganisms is unclear. Additionally,

104 the use of TEP as an amendment to heavy-metal remediation has yet to be investigated.
105 TEP absorbs molecularly on both wet and dry iron-hydr(oxide) nanoparticles to the
106 longer aliphatic chain, stabilizing the loss of charge on the methoxy-CO bonds by charge
107 redistribution upon phosphoryl-O coordination to Fe surface atoms (Mäkie et al., 2013).
108 By absorption in soils and sediments, TEP is less likely to wash or leach out and thus
109 more likely to remain biologically available as a nutrient.
110 The spatial dependence of phosphatase activity in soils has important implications for
111 evaluating this enzyme activity (Amador et al., 1997). The application of TEP can
112 overcome these spatial heterogeneities, since it can be widely distributed in gas phase and
113 liquid phase and therefore stimulate microbial phosphatase in the area of interest. In this
114 way, remediation of heavy-metals/radionuclides via microbially-mediated release of
115 excess phosphate in subsurface soil could result in immobilization as metal phosphate.
116 We postulate that microorganisms adapt upon long-term exposure to heavy metals in an
117 acidic environment by upregulation or production of phosphatases in response to toxic
118 heavy-metal exposure and, given an adequate phosphate source in the form of TEP in a
119 nutrient-limited environment, the microbial population adapts to get the required
120 phosphorus nutrient and protection from heavy-metal exposure. Here will investigate the
121 selective impact of TEP on two major classes of microbial phosphatase and its effects on
122 the soil microbiota. We propose that specific classes of phosphatases can indirectly
123 relieve the toxic effects of heavy metals via chemical precipitation as metal phosphate
124 with acquisition of the essential phosphorus nutrient, increasing microbial activity.
125
126

2. Material and Methods

2.1 Soil Sampling

A soil sample consisting of approximately 10 kg was collected at the DoE's Savannah River Site, Aiken, SC, from its Small Arms Training Area (SATA) firing range, an area with high levels of heavy-metal contamination (Wilson et al., 2006). Surface soil was collected to a depth of 30 cm from the range center with a stainless-steel coring device and transported to the laboratory. Soils from the SATA area are generally considered to be within the Udorthents, Great Group (Rogers and Herren, 1990; USDA-NRCS, 2005). Soil samples were processed the same day as collection. One portion (100 gm) was assayed for acid and alkaline phosphatase; another was put on a tray to air-dry for elemental analysis. The air-dried soil was sieved through 4 mm mesh screening. Sieved soil was mixed and distributed into 100 mg aliquots for analysis of lead levels using multi-element X-Ray Fluorescence (XRF). The accuracy of the XRF analyses was verified by comparing results from selected samples using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Vulava and Seaman, 2000). Soil pH was averaged to a pH of 5.1, which was determined by a standard method previously described (Wilson et al., 2006).

2.2 Ion Chromatography

Phosphate concentrations were measured using a Dionex DX500 Ion Chromatograph (IC), operating at ambient temperatures and equipped with a conductivity detector and 250 mm Dionex IonPac AS14 Analytical Column (4 mm ID, 16 µm bead; Dionex Corp., Sunnyvale, CA). A 3.5 mM sodium carbonate/1 mM sodium bicarbonate buffer solution was used as the eluent (1.2 mL/min). Samples were taken from the supernatant of a solution consisting of: water, media or 5 gm of dry soil (dried at 121 °C for 24 h), and 5 mL of deionized water; vortexed for 1 min and then centrifuged for 5 min at 2,500 rpm. The ion detection limit for ions measured on this IC were 0.5 µg/L.

2.3 Soil Phosphomonoesterase Activities:

All phosphatase assays were measured in triplicate on both soil and soil free treatments. Based on the method of Eivazi and Tabatabai (1977), p-nitro phenyl phosphate was used to assay soil acid and alkaline phosphomonoesterase, bis-p-nitro diphenyl phosphate for phosphodiesterase, and tris-p-nitro triphenylphosphate for phosphotriesterase. A modified, universal buffer (TRIS-maleate) was used to maintain the pH for the assay of acid phosphatase (pH 6), alkaline phosphatase (pH 8), and phosphodiesterase and phosphotriesterase (pH 10). Absorbance units were converted to µmol p-nitrophenol (p-NP) produced per mL or gm soil per h. Saturating substrate at 3.0 mM was determined empirically. The assay concentration of p-NP released was calculated from a standard curve prepared with p-NP standards. Controls without substrate were extracted as described above, and values were subtracted as background. The apparent Michaelis-Menton enzyme constant (K_m) and maximum enzyme reaction

172 velocity (V_{max}) were calculated from the classical Lineweaver-Burk plot for all enzyme
173 assays.
174
175 *2.4 Microbial Growth Media*
176
177 Phosphate-free medium used in soil slurries and soil-free cultures was prepared in
178 g/L: TRIS base, 12.0; $(NH_4)_2SO_4$, 0.96; KCl, 0.62; $MgSO_4$, 0.063; $FeSO_4$. $7H_2O$. Culture
179 medium was sometimes amended with 1% glucose (w/v), 1-10% TEP (v/v), 10 mM
180 glycerol phosphate or 10 mM potassium phosphate. The pH of the medium was adjusted
181 to 6.0 or 8.0 with NaOH or HCl. Triplicate sets of soil slurries consisted of 100 mL
182 medium and 10 g soil, with a one-time amendment of 10% TEP and aerated on a shaking
183 incubator at 25 ^{o}C. Sampling for phosphatase assays was performed in triplicate. For
184 soil-free microcosms, 10 mL of soil-slurry supernatant was transferred to 40 mL, fresh
185 TRIS base medium (pH adjusted to 6 and 8), with or without 10% TEP and 1% glucose.
186 Zinc chloride was added (5 mM) to the soil-free consortium to observe the effect of metal
187 amendment on phosphatase activity. Tryptone-peptone broth (TSB) or Tryptic Soy Agar
188 (TSA) was purchased from Difco, Inc. (Detroit, MI) and prepared as per the
189 manufacturer. TBP was added at 10% final concentration. Dilutions of microcosm
190 solutions were made with 0.2 µm filter-sterilized, phosphate-buffered saline (PBS) and
191 plated onto 1% TSA medium to determine cultivable abundance and diversity. All
192 bacterial growth conditions were done in triplicate and phosphatase assays were
193 performed in triplicate as well.
194

2.5 Chemicals

TRIS and analytical controls (Pb, Cu, Fe, and Zn were purchased from Sigma (St. Louis, Mo.). TEP (99% pure) and TBP (98%) were purchased from Aldrich (Milwaukee, Wis.); N_2O (ultra-high purity) was from Matheson Gas Products (Joliet, Ill.). NH_4Cl and KH_2PO_4 were purchased from Mallinckrodt Chemicals (Paris, Ky.), and NaOH and HPLC-grade solvents purchased from Fisher Chemicals (Fairway, N.J.).

2.6. *Biolog GN identification*

Two days before the inoculation of Biolog GN or GP plates (Biolog, Inc.), depending on the Gram stain, microbial isolates from the shooting range enrichments were streaked on R2A agar plates. The wells of the Biolog GN plates were inoculated with 150 μL of the bacterial suspension, adjusted to the appropriate density, and incubated at 30 °C for 24 and 48 h. The corresponding growth and associated color development indicating redox activity was automatically recorded using a 96-well microplate reader with a 590 nm wavelength filter. Identification (Biolog Microlog 3.70 database) and ASCII-file output of test results applying the automatic-threshold option were performed using Biolog software.

***2.7** Statistical analysis*

All differences in independent treatment response between TEP amended and controls were analyzed with JMP Pro Version 11.2.1. Significant differences between

219 treatment groups and the controls were determined using one-way analysis of variance
220 (ANOVA) supported by a Tukey-Kramer HSD evaluation for situations involving these
221 treatments. Treatment means were considered different when the differences were
222 statistically different at a 5% level.
223

224 **3. Results**

225

226 *3.1 Soil Characterization*

227 The shooting range soil has been in use for over 20 years and was acidic (pH 5.1),
228 with high levels of lead (200-600 mg/kg) and other heavy metals (Wilde et al., 2005). It
229 was expected that acid phosphomonoesterase would predominate over alkaline
230 phosphatase due to the soil acidity selecting for microorganisms with upregulated acid-
231 phosphatase (Sharma et al., 2013). The soil was collected from an area lacking in surface
232 vegetation to a depth of 30 cm in a hot, dry climate during early spring and appeared as a
233 weathered, sandy soil. The lead (Pb), copper (Cu), zinc (Zn), and iron (Fe)
234 concentrations in the soil sample used in this study (means mg/kg ± 1 SD) were 233± 51
235 14±43, 3 ± 11, and 10,565± 1960 (n=6).
236 Soil analysis found the soil as nutrient-poor, with mg/kg of: NH_4^+ 1.6, Na^+ 1.4, K^+ 2.7,
237 Ca^{2+} 7.4, Cl^- 1.2, NO_3^- 3.7, SO_4^{2-}14.8, PO_4^{2-} 1.5. Soil pH was 5.7.
238

239 Table 1 summarizes assay data for two classes of phosphatases in acidic (pH 6) or
240 alkaline (pH 8) -adjusted soil conditions. The soil pH was adjusted to pH 6 by the
241 addition of 0.5 mol HCl or NaOH. In alkaline-adjusted soil, the specific activity of
242 alkaline phosphatase of control soil was significantly higher than acid phosphatase. In

243 acidic soil, the specific activity of acid phosphatase was significantly higher than alkaline
244 phosphatase, as previously described (Pansu & Gautheyrou, 2006). When phosphate ion
245 (10 mM) was added to soil slurries, it was observed that alkaline-phosphatase activity
246 decreased by 77% in the alkaline soil, as compared to a 40% decrease in the acidic soil.
247 This indicates the soil was phosphate limited, and the addition of phosphate ion was
248 sufficient to relieve phosphate stress and repress alkaline phosphatase (Turner and
249 Haygarth, 2005).
250 Applying a 15 mM p-NPP to the microcosms ensured a saturating substrate (*e.g.*, to
251 overcome sorption due to clay fraction). This was verified with control soil amended
252 with product (p-NP). In acidic soil, addition of phosphate ion resulted in a 51% increase
253 in acid phosphatase, and a small but significant decrease (18%) in alkaline soil. Addition
254 of phosphate ion can inhibit soil phosphatase via the repression of regulatory phosphatase
255 genes (Nannipieri, et al., 2011). The inverse relationship between alkaline-phosphatase
256 activity and acid-phosphatase activity is consistent with that reported, since the soil
257 source/environment selects for expression of either alkaline or acid phosphatase (Pansu &
258 Gautheyrou, 2006). Addition of TEP to alkaline soil resulted in no significant difference
259 in either acid or alkaline phosphatase activity, indicating that TEP neither inhibited nor
260 stimulated phosphatase.
261 In contrast, TEP additions to acidic soil decreased alkaline phosphatase (~38% decrease),
262 with less effect on acid phosphatase (~16% decrease). The fact that TEP addition
263 lowered alkaline-phosphatase activity to a value equivalent to that observed with
264 phosphate-ion addition is an indication that TEP may be serving as a phosphate source
265 sufficient to repress alkaline phosphatase. Phosphodiesterase and phosphotriesterase

activity was consistently low in both acidic and alkaline soil conditions (data not shown) consistent with previous reports (Pansu & Gautheyrou, 2006). However, there was no significant difference between treatments, suggesting TEP may not be a suitable substrate for this class of enzymes.

Table 1. Microbial phosphatase activity[a] (umol p-NPP g^{-1} h^{-1}) in freshly-sampled shooting range soil.

alkaline soil pH 8		
	Alkaline Phosphatase[a]	**Acid Phosphatase**[a]
Control[b]	**4.31 (+/- 0.57)**[e]	**1.20 (+/- 0.14)**
K_2PO_4[c]	**0.97 (+/- 0.26) ***	**2.45 (+/ - 0.27)***
TEP[d]	**4.07 (+/- 0.32)**	**1.64 (+/- 0.32)**
acidic soil pH 6		
	Alkaline phosphatase[a]	**Acid Phosphatase**[a]
Control	**2.84 (+/- 0.10)**	**6.61 (+/- 0.49)**
K_2PO_4	**1.69 (+/- 0.42)***	**5.36 (+/- 0.47)**
TEP	**1.74 (+/- 0.31) ***	**5.54 (+/- 0.64)**

One gram of soil was incubated for one hour at 37^{o}C with substrate and buffer to maintain pH for the phosphatase class of interest (Eivazi, & Tabatabai 1977).

[a]µmol p-nitrophenol produced per gram dry weight of soil per hour.

[b]no amendment

[c]phosphate ion was provided as 10 mM KPO_4.

[d]triethyl phosphate (TEP) 10% v/v

[e]standard deviation of the mean

* Significantly different from the control ($p \leq 0.05$).

Longer-term exposure of TEP on soil microbial acid and alkaline monophosphoesterase activity was assayed in microcosms over a three-week period (Fig.1, a & b). Control microcosms had a decrease in acid phosphatase (Fig. 1a) and an increase in alkaline phosphatase (Fig. 1b), indicative of a microbial population under phosphate stress. In contrast, TEP appeared to relieve phosphate limitation, as demonstrated by an increase in acid phosphatase (Fig. 1a) and a corresponding decrease in alkaline-phosphatase activity (Fig. 1b). This data can support a previous finding that environmental exposure of synthetic sources of organophosphates lead to altered phosphatase activity found as a positive correlation between phosphomonoesterase activity and the rate of tert-butylphenyl-diphenyl phosphate biodegradation in sediments (Heitkamp et al., 1986). We speculate that the microbial population may be using different strategies for utilizing TEP in the phosphate-depleted soil. Variability in phosphatase activity could be explained by shifts in soil microbial population, differential expression of phosphatase genes, and influence of inorganic/organic phosphate from soil. Prolonged exposure of soil to TEP resulted in a comparable pattern of acid-phosphatase and alkaline-phosphatase activity (Fig. 1, a & b), which indicates TEP stimulated the microbial population. The acid phosphatase in the TEP soil microcosms was not significantly higher than the control soils until 96 hours (Figure 1a). The TEP microcosm alkaline phosphatase activity demonstrated a significant increase over control soils from 48 hours to one week and then the control soils were higher weeks two and three (Figure 1b).

3.2 Soil-Free Enrichment

316

317 The supernatant from soil slurries that was exposed to TEP for three weeks was
318 transferred into three separate flasks amended with additional TEP, in order to maintain
319 the more stable consortium of the TEP-amended medium without the influence of the soil
320 matrix. When TEP was provided as a sole carbon source, the activity of acid phosphatase
321 and alkaline phosphatase declined rapidly and bacterial numbers declined (not shown).
322 This pattern reflects carbon starvation, indicating TEP did not provide suitable carbon for
323 growth. The slow decrease in growth as determined by optical density, as well as a slow
324 decline in cultivable abundance, demonstrated that the soil matrix may have had a
325 positive influence on the microbial population by microbial-cell soil surface interactions
326 via the contribution of residual carbon and phosphate sources.
327 When TEP was provided as a sole phosphate source with 1% glucose as a carbon
328 supplement, a significant increase in phosphatase activity was observed by 1 week (Fig.
329 2, a & b). The TEP treatment phosphatase concentration was significantly elevated as
330 compared to the control soil and the Zn-TEP treatment for weeks 1-3 (Figure 2a). For
331 weeks four and five the TEP and TEP-Zn treatments were comparable (Figure 2b).
332 Over time, microbial abundance and diversity stabilized as the same colony morphotypes
333 and proportions were obtained as observed with the soil microcosms. Additionally, there
334 was a consistency in BIOLOG patterns (Garland and Mills, 1991) that indicated a stable
335 microbial population (not shown). Alkaline-phosphatase activity was low as compared to
336 acid phosphatase, indicating this microbial consortium was active using TEP as a sole
337 phosphate source, and this was a pattern consistent with the soil microcosms (Figs. 1 &
338 2). That acid phosphatase remained high over a five-week period indicates a robust,

active microbial population was maintained with TEP as a sole phosphate source. The acid phosphatase produced by the 10% TEP soil treatments was not significantly different from the soil controls until 96 hrs after which there was an increase. The alkaline phosphatase produced by the 10% TEP soil microcosms was not significantly different from the soil controls until 48 hrs. Then after the second week the 10% TEP soil microcosm demonstrated a significant decrease in alkaline phosphatase at week three. When a high level of zinc (5 mM) was added to the soil-free microcosms, acid-phosphatase activity remained significantly high as compared to alkaline phosphatase (Fig. 2, a & b). The small but significant decrease in acid phosphatase may be due to elimination of populations of zinc-sensitive bacteria and, consequently, the elimination of these population groups' contribution to acid phosphatase.

Another possibility is that the precipitation of zinc as zinc phosphate via phosphatase hydrolysis of TEP and release of phosphate ion may have removed a significant amount of Zn from the solution, reducing the toxic effect on microorganisms which could also lower acidic-phosphatase level. Though enzyme-kinetic studies were not evaluated, Martinez et al. (2007) used pure cultures of bacteria isolated from metal/radionuclide-contaminated soil to assay to directly the phosphatase-mediated removal of soluble uranium (U-IV), where 73-95% of the U-IV was precipitated as $Ca(UO_2)_2(PO_4)_2$. Bioremediation of uranium and other radionuclides in groundwater through bioreduction and phosphate biomineralization through long-term precipitation for groundwater remediation has been reviewed by Newsome et al. (2014), thus our observations may be due to microbial precipitation of zinc.

362 The amount of zinc used in our studies was within the critical concentration-range
363 identified in previous field studies to be toxic to microbial enzymes such as phosphatases
364 (Kucharski et al., 2011). Doelman and Haanstra (1989) demonstrated a 30-60%
365 inhibition of soil phosphatase at a zinc concentration of 500 mg 1 kg^{-1} Zn. Additionally,
366 it has been observed that soil acidification increases the negative effects of zinc on soil
367 phosphatase (Kucharski et al., 2011), where a significant negative correlation exists
368 between zinc levels (70-10,000 mg 1 kg^{-1}) and soil acid-phosphatase, which had the
369 longest recovery time to normal enzyme status as compared to other enzyme indicators of
370 soil quality. In our study, the shooting range soil was highly contaminated with heavy
371 metals and was acidic (pH 5.1). It is suggested that the microbial population enriched in
372 our microcosm studies that expressed a constitutive production of soil acid-phosphatase
373 resistant to zinc is a reflection of environmental-selection pressure under long-term
374 exposure to heavy metals at low pH.
375
376 *3.3 Isolation of TEP-Utilizing Bacteria and Identification of a Zinc-Resistant Bacterium*
377
378 Bacteria were isolated from the TEP-exposed soil enrichments on minimal agar
379 medium, using TEP as the sole phosphate source supplemented with 1% glucose. A total
380 of 14 bacterial-colony types were observed. One of the isolates produced a blue pigment
381 in the agar medium with TEP as a sole phosphate source (Fig. 3, a & b). This bacterium
382 was identified as *Burkholderia cepacia*. The pigment it produced was soluble in water,
383 and appeared indigo in color with a metallic sheen when growing on agar with low levels
384 of phosphate. The pigment was not produced in the presence of inorganic phosphate or

TEP in solution (Fig. 3c). The *Burkholderia cepacia* sp. isolate was tested for growth on various media, including Tryptic Soy Broth (TSB), potassium phosphate (KPO_4), TEP, and tributylphosphate (TBP). Pigment production was qualitatively correlated to phosphatase activity of cultures exposed to various phosphate sources (Fig. 3), where we observed a significant repression of alkaline phosphatase but not acid phosphatase (Fig. 4). This pattern of pigment production in response to phosphate limitation is consistent with purple acid-phosphatases (PAPs) found in many plants, eukaryotic cells and some prokaryotes (Shenk et al., 2000; Olczak et al., 2003). PAPs are part of the superfamily of metallophophoesterases (Matange et al., 2015) that share structural similarity and functional versatility (i.e., bone re-absorption in animals, microbial virulence factors, plant-organophosphate degradation, cell signaling, and utilization of a wide range of substrates). Though the role of PAPs in environmental microorganisms is not fully understood, several prokaryotic genera have been identified which contain genes for these types of phosphatases.

4. Discussion

Microorganisms that have been identified that produce PAPs include several species of *Burkholderia*, *Mycobacterium*, and *Frankia* along with several genera of fungi and cyanobacteria (Yeung et al., 2009). Further investigation is needed to determine if the pigment produced by the bacterial isolate identified in our study is a PAP-type phosphatase, and whether it is directly related to phosphate starvation and zinc tolerance.

Zinc (5 mM) had a small effect on acid-phosphatase activity of the *Burkholderia cepacia* strain, while alkaline phosphatase was significantly inhibited by Zn (Table 2). The acid phosphatase of this bacterium may be a non-specific acid phosphatase, since both TEP and TBP were utilized; we cannot distinguish whether the direct mechanism by which the acid phosphatase was uninhibited by Zn, as it may be removed from solution as $ZnPO_4$. We did not determine the contribution of the PAP-like enzyme, as several classes of phosphatases may be represented (Rossolini et al., 1998) and thus the assay reflects the weighted average of soil phosphatase. Recently, a *Burkholderia gladioli* strain has been identified with a zinc-resistant acid phosphatase that was also uninhibited by tartrate (positive-identification assay for PAPs) (Rombola et al., 2011) and is among one of the first studies demonstrating regulation of acid phosphatase by phosphorus in a *Burkholderia spp.*

Many species of microorganisms that can degrade different types of phosphate compounds, including those that metabolize organophosphates, have been characterized (Abe et al., 2011). Phosphate-solubilizing microorganisms can help metabolize available organic phosphate that stimulates overall microbial activity (Sharma et al., 2013). Specific microbial activities and selected enzymes associated with biodegradation have biotechnological potential for use in industrial, bioremediation, waste-treatment and agriculture applications (Rao et al., 2010). In our study, TEP stimulated a microbial population in an acidic, heavy-metal contaminated soil, with TEP being metabolized as a sole phosphate source. These phosphatases from microbial activity may serve as bioindicators of the soil ecosystem status (Utobo and Tewari, 2015). Expression of soil acid-phosphatase uninhibited by zinc may indicate an alternate path of reducing zinc

toxicity via precipitation as $ZnPO_4$. A previous study has demonstrated that aerobic heterotrophic bacteria within a uranium-contaminated environment can hydrolyze organophosphate at low pH conditions, and this can support development of a bioremediation application of uranium via a specific biomineralization process (Beazley et al., 2007).

A *Burkholderia cepacia* strain was purified that grew with TEP as a sole phosphate source and expressed an acid phosphatase uninhibited by zinc. This bacterium appears to have a unique response to phosphate starvation by producing an indigo pigment characteristic of purple acid-phosphatase. An interesting association is that this metabolic activity is characteristic of the oxidation of the hydrocarbon indole to indigo by naphthalene dioxygenase in an efficient reaction that does not generate waste products (Kauppi et al., 1998). The microcosms amended with 10 mM phosphate tested here did not repress acid-phosphatase fraction at low pH, but inorganic phosphate (Pi) did inhibit alkaline phosphatase. The application of acid-phosphatase-constitutive activity in the indigenous soil microbial community can be used in mineral recovery as well as bioremediation as an immobilization technology (Liang, 2016).

Bacillus and *Rahnella spp*. isolated from subsurface acidic soils contaminated with heavy-metals and radionuclides have been found that can immobilize uranium (IV) via phosphatase activity (Martinez et al., 2007). Differentiating the specific class of phosphatase involved in heavy-metal precipitation was not a priority in this work, but more recently there has been a renewed interest in functionally-diverse, non-specific acid phosphohydrolases (Gandi and Chandra, 2012; Rossolini et al., 1998) and other phosphatases grouped as metallophosphoesterases (MPEs) (Schenk et al., 2013; Matange

et al., 2015), where MPEs have been found to be useful for the remediation of heavy-metals, radionuclides, and anthropogenic sources of organophosphates.

5. Conclusion

TEP applications can have a significant impact in remediation of heavy-metal contaminated environments via the stimulation of growth of microorganisms by providing a key nutrient, Phosphate that is often limiting and indirectly resulting in the stabilization of heavy metals as an insoluble metal-phosphate. The selective stimulation of microbial activity can influence metal behavior, including precipitation, biomineralization and sorption across a wide array of ecosystems. These results are relevant to the bioremediation of heavy metal contaminated soils over potentially large areas. Our research indicates that greater emphasis should be placed on understanding the role of phosphomonoesterase activity and—more specifically—acid phosphatases in the cycling of organic phosphates, including synthetic forms and their role in metal chemistry in the environment. Selective biostimulation of soil microbial activity with TEP application could be used for bioremediation.

Table 2. Kinetic values[+*] of acid and alkaline phosphatase for *Burkholderia cepacia** isolated from a TEP-enriched consortia**.**

Enzyme	**Control**		**5 mM zinc**	
	K_m	**V_{max}**	**K_m**	**V_{max}**
Acid phosphatase	0.87 (+/- 0.081)	6.9 (+/- 1.530)	0.81 (+/- 0.135)	5.02 (+/- 1.322)
Alkaline phosphatase	0.44 (+/- 0.017)	4.33 (+/- 1.634)	0.05 (+/- 0.078)[**]	1.77 (+/- 0.435)

[+]K_m and V_{max} values were determined from Lineweaver-Burk plots. K_m (mM), V_{max} (µmol p-NP . mL^{-1} . h^{-1})

[*]standard deviation of the mean

[**]Significantly different from the control ($p \leq 0.05$).

A better understanding of biogeochemical processes is essential for contaminated-site remedial activities as well as the subsurface, where ongoing efforts should be evaluated case-by-case when amendments such as TEP are applied. Microbial metabolic processes, including phosphatases, have a significant impact on contaminant mobility in diverse environments and will be important in managing contaminated land sites as well as in subsurface biogeochemical remedial processes (Liang et al., 2016). More study is needed to determine if metal-resistant acid phosphatases are common, specific activity can be sustained, and whether they enhance microbial survival and metabolic activities in metal-contaminated soils, groundwater, and sediments.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Figure 1. Acid (**a**) and alkaline (**b**) phosphatase activity in soil microcosms over a three-week period in response to TEP amendment. Means with the same letter are not significantly different from the control ($p \leq 0.05$).

Figure 2. Acid (a) and alkaline (b) phosphatase activity, with and without Zn & glucose, in soil-free microcosms over a three-week period in response to TEP amendment. Means with the same letter are not significantly different from the control ($p \leq 0.05$).

Figure 3a. Growth of a Burkholderia cepacia strain isolated from the soil-free TEP enrichment with 10% (v/v) TEP in 1% glucose minimal medium with phosphate limitation.

Figure 3b. Growth with 10 mM glycerol phosphate in 1% glucose minimal medium.

Figure 3c. Exposure of a Burkholderia cepacia strain to different phosphate sources in glucose minimal medium containing 1% glucose. Left to right: phosphate-free medium (control), 10% TEP, 10% TBP, 10 mM glycerol phosphate.

Figure 4. Effect of different phosphate sources and on the specific activity of

652 acid and alkaline phosphatase of a Burkholderia cepacia strain isolated from a
653 TEP-enriched consortia. Means with the same letter are not significantly different
654 from the control ($p \leq 0.05$).
655

Figures

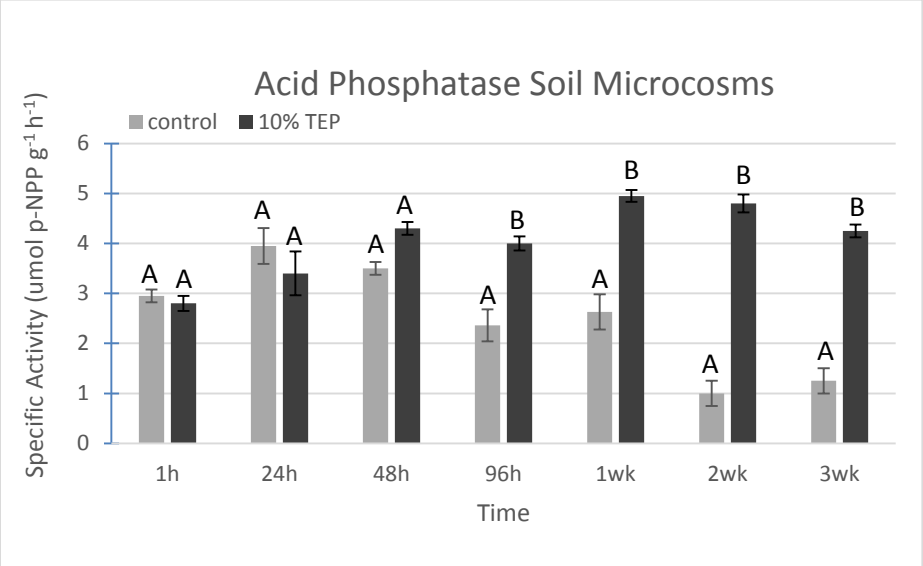

Figure 1a.

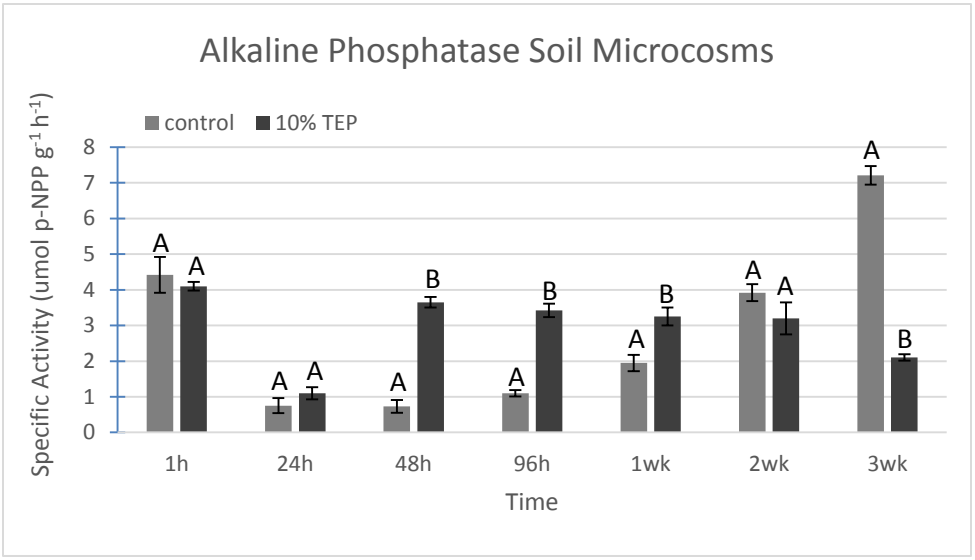

Figure 1b.

Figure 1. Acid (**a**) and alkaline (**b**) phosphatase activity in soil microcosms over a three-week period in response to TEP amendment. Means with the same letter are not significantly different from the control ($p \leq 0.05$).

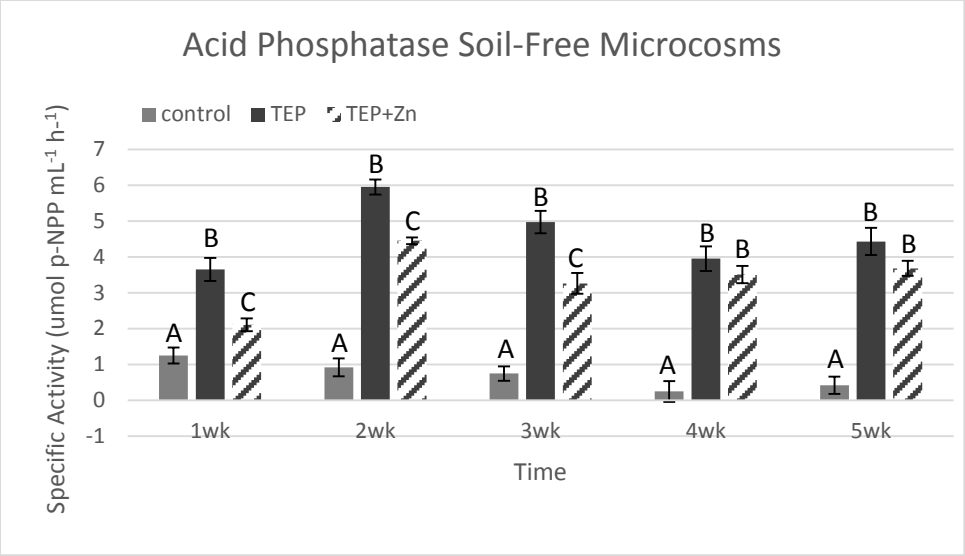

Figure 2a.

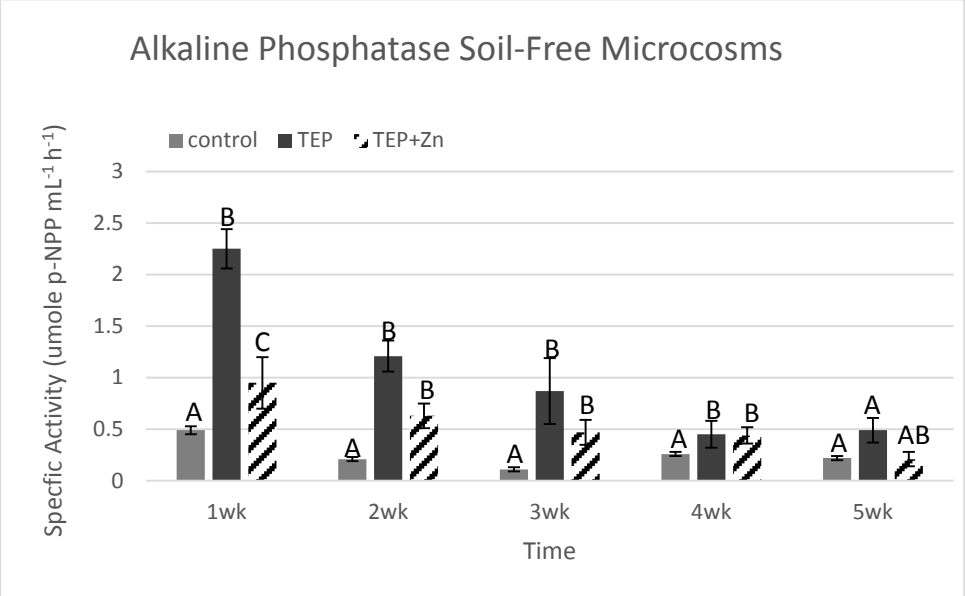

Figure 2b.

Figure 2. Acid (**a**) and alkaline (**b**) phosphatase activity, with and without Zn & glucose, in soil-free microcosms over a three-week period in response to TEP amendment. Means with the same letter are not significantly different from the control ($p \leq 0.05$).

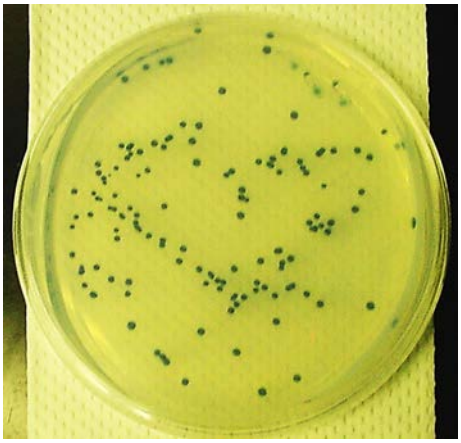

Figure 3a. Growth of a *Burkholderia cepacia* strain isolated from the soil-free TEP enrichment with 10% (v/v) TEP in 1% glucose minimal medium with phosphate limitation.

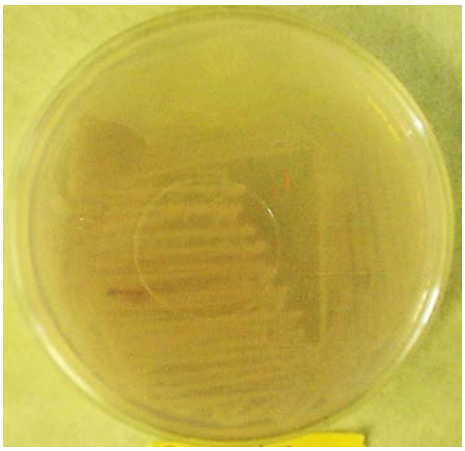

Figure 3b. Growth with 10 mM glycerol phosphate in 1% glucose minimal medium.

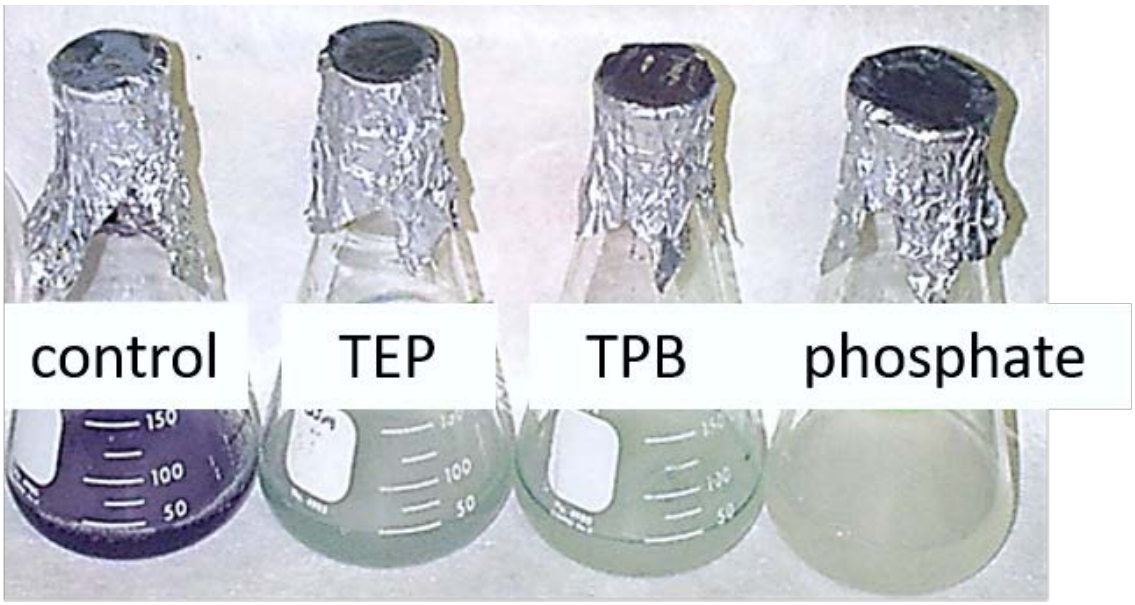

Figure 3c. Exposure of a *Burkholderia cepacia* strain to different phosphate sources in glucose minimal medium containing 1% glucose. Left to right: phosphate-free medium (control), 10% TEP, 10% TBP, 10 mM glycerol phosphate.

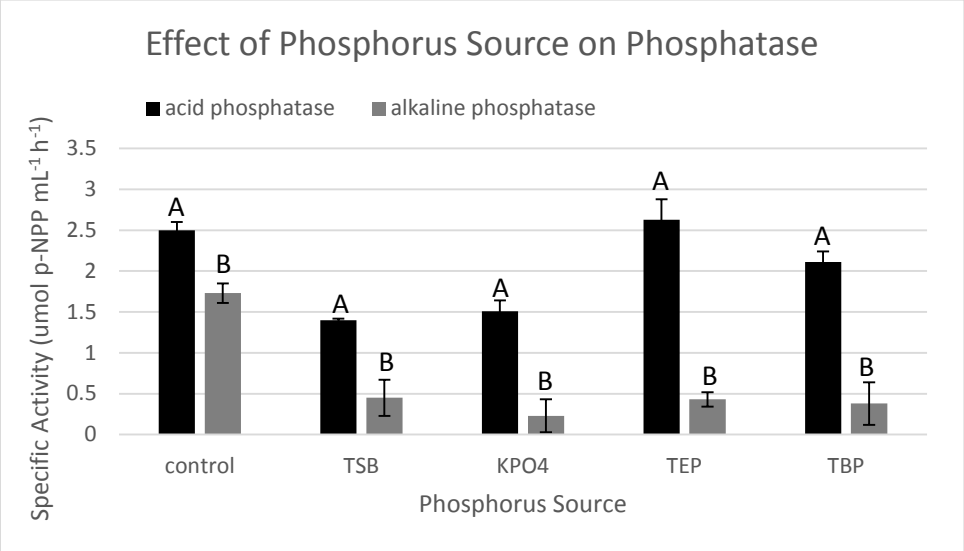

Figure 4. Effect of different phosphate sources and on the specific activity of acid and alkaline phosphatase of a *Burkholderia cepacia* strain isolated from a TEP-enriched consortia. Means with the same letter are not significantly different from the control ($p \leq 0.05$).