

Interactive effects of charcoal and earthworm activity increase bioavailable phosphorus in sub-boreal forest soils

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Abstract The purpose of this study was to assess the effects of charcoal and earthworm presence in contrasting soil types of northern Japan using the biologically based phosphorus (BBP) extraction method, which employs a variety of plant P acquisition strategies. Using soils developed in serpentine and sedimentary parent materials, we tested the interactive effects of *Eisenia japonica* (Michaelson) earthworms and 500 kg ha⁻¹ of dwarf bamboo charcoal (*Sasa kurilensis* (Rupr.) Makino et Shibata) in a microcosm incubation that lasted four weeks. Soils were extracted in parallel after the incubation with the BBP method using 0.01 M CaCl₂ (soluble P), 0.01 M citric acid (chelate-extractable P), 0.02 phosphatase enzyme units ml⁻¹ (enzyme-extractable organic P), and 1.0 M HCl (mineral occluded P). Dwarf bamboo charcoal alone contained up to 444 mg total BBP kg⁻¹ prior to application to soil microcosms. Treatment effects in soil microcosms were highest in sedimentary soil types and where charcoal was combined with earthworms (15.97 mg P kg⁻¹ ± SE 1.23 total inorganic BBP). Recalcitrant inorganic P (HCl extracted) in combination treatments yielded the highest single inorganic BBP measure (12.41 mg kg⁻¹ ± SE 1.11). Our findings suggest that charcoal, as a legacy of wildfire, and native earthworm activity may help stimulate cycling of recalcitrant inorganic BBP pools.

Keywords Pyrogenic carbon · Black carbon · Biochar · Phosphate · Bioturbation

Abbreviations

P Phosphorus
BBP Biologically based phosphorus

Introduction

Phosphorus (P) limitations in forest soils are a major concern for co-limitation of nutrient availability and primary productivity, yet we lack a thorough understanding of P dynamics in context of common biotic and abiotic factors in forest ecosystems, such as soil fauna and fire disturbances. Forest soils of northern Japan are influenced by the high activity of native epigeic, geophagous earthworms and the presence of wildfire-deposited charcoal that, combined, provide an opportunity to measure the interactive effects of these two factors on nutrient availability. Soil P nutrient limitations are a particular concern in forests where andic properties further reduce the solubility of orthophosphate, the dominant form of P in the pedosphere (Shoji et al. 1994). However, P is rarely evaluated from the biological perspective of plant and microbial acquisition techniques, which may provide valuable insight into the shifts in P pools across complex environments (DeLuca et al. 2015a; Rowe et al. 2016). For example, changes in P pools over a 10-year period across a variety of ecosystem types in the UK showed a net decrease of plant-available inorganic P and net increase in organic, enzyme-extractable P as measured by the biologically based P (BBP) method (DeLuca et al. 2015a). An accurate assessment of P status and pools in complex environments, such as forest soils, incorporates a biologically based perspective in context of soil fauna and ecosystem disturbances.

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Soil P is a macronutrient that often exists in sparingly plant-available quantities. Apatite-type P minerals slowly undergo primary weathering to release small concentrations of the orthophosphate ion (H_2PO_4^- and HPO_4^{2-} , at $\text{pH} < 7.2$ and > 7.2 , respectively) (Walker and Syers 1976). Phosphorus solubility is limited in both alkaline and acidic soil conditions as well as in the presence of non-crystalline minerals associated with volcanic ash (Stevenson and Cole 1999). Organic P is generally cycled more rapidly than inorganic P as a result of biotic mineralization and immobilization of P in surface soil organic matter (Chapin et al. 2011; Stevenson and Cole 1999). As inorganic P is released into soil solution from organic matter, it is either immobilized into organic forms, strongly adsorbed by hydrous oxide clay surfaces, precipitated by aluminum (Al), iron (Fe), or manganese (Mn) in acidic soils, or precipitated as calcium (Ca) or magnesium (Mg) phosphates in alkaline soils (Stevenson and Cole 1999; Walker and Syers 1976). Total soil P concentrations were low in both serpentine and sedimentary derived soils of northern Japan, 0.09 and 0.08 g kg^{-1} , respectively (Makoto et al. 2016), and rapid uptake by plants and microbes is likely. Serpentine soils of northern Japan contain similar concentrations of Al and Fe as regional sedimentary soils, but contain 700% more Mn compared to sedimentary soils (Makoto et al. 2016), which may contribute to the exceptionally low P concentrations in serpentine soils. Contrasting soil types of northern Japan provide a pertinent comparison of P availability where previous research showed biological-context dependency of soil nitrogen (N) dynamics influenced by soil type (Makoto et al. 2016).

Anthropogenic and naturally ignited forest fires are common disturbances in Japanese sub-boreal forests (Takaoka and Sasa 1996), which may further exacerbate P solubility and uptake by combustion of forest floor organic matter, impacting soil micro- and macrofauna, and shifting vegetation dynamics (Certini 2014; Hart et al. 2005; Neary et al. 1999). While fire events can deplete soil P through combustion and erosion (Murphy et al. 2006), the deposition of charcoal during fire events is a legacy that continues to influence nutrient transformations months to years after a fire event (DeLuca and Aplet 2008; DeLuca et al. 2015b; Makoto et al. 2010, 2012b; Pluchon et al. 2014). Charcoal deposited by fires provides a modest source of available P for soil plant and microbial uptake and ash associated with fire tends to increase soil pH, which are all factors that may alleviate soil P limitations (Makoto et al. 2010; Pluchon et al. 2014). Charcoal deposited in soils after fire may contain a significant quantity of P that is subsequently released into solution and adsorbed by minerals or it may remain within the charcoal particle (DeLuca et al. 2015b). A small-scale prescribed burn in a dwarf bamboo (*Sasa kurilensis*)/Japanese white birch (*Betula platyphylla* var. *japonica*) forest stimulated an immediate increase in

plant-available P lasting no more than 1 month after the fire event, but one which was prolonged to four months in locales where charcoal remained on the forest floor (Makoto et al. 2012b). The amendment of soils with biochar, charcoal generated as an agricultural amendment, has been observed to increase citrate-extractable inorganic P by nearly 30% resulting in an increase in overall P retention in agriculture surface soils (DeLuca et al. 2015b; Gao et al. 2016).

Laboratory experiments show that P may be an important limiting nutrient in seedling growth that is facilitated by the presence of charcoal in soils (Makoto et al. 2010; Pluchon et al. 2014). Ectomycorrhizal fungi associated with coniferous trees have been found to directly influence the availability of P in forest soils. A study by Makoto et al. (2010) reported a significant increase in P concentrations in *Larix gmelinii* seedling needles when a buried layer of charcoal was added to potted plants (up to 2 g kg^{-1}) and with mycorrhizae infection (up to 1 g kg^{-1}), but no interactive effects, suggesting that sorption of P on charcoal surfaces may limit accessibility to mycorrhizae in the immediate zone surrounding charcoal particles or “charosphere” (Quilliam et al. 2013). Similarly, seedling growth of post-fire primary succession deciduous trees (*Populus tremula* and *B. pubescens*) was positively correlated to the PO_4^{3-} concentration of charcoal added to soil ($r = 0.686$ and 0.855, respectively) (Pluchon et al. 2014). Charcoal may function as a source of PO_4^{3-} in P-limited soils, which may be important for deciduous trees in acidic, P-poor soils of northern boreal forests.

The solubility and bioavailability of P can also be altered by activity of earthworms that are widely distributed across Japanese forests (Nakamura 1972). Earthworms re-inhabit post-fire forest soils, incorporate organic matter into mineral soils (bioturbation), create soil macropores and aggregation, and aid in the cycling and redistribution of nutrients (Edwards 2004). Clearly, earthworms may impact processes that have the capacity to directly or indirectly alter soil P availability. Soils of northern Hokkaido, Japan, contain approximately 47 earthworms per m^2 (Uchida et al. 2004) and represent the largest soil fauna biomass (Nakamura 1972). In a study of earthworm species and abundance in northern Japan, 50% of sampled individuals were identified as *Amyntas vittatus* ($3 \pm 11\%$), *Metaphire hilgendorfi* ($12 \pm 27\%$), and *Eisenia japonica* ($6 \pm 18\%$) (Uchida et al. 2004). Populations of earthworms are reduced or eliminated immediately after fire, but earthworms are known to re-inhabit these soils after a few months or years (Bhadoria et al. 2000). *Eisenia japonica* is a geophageous soil feeder but also an epigeic earthworm that resides in the surface soil, which renders it susceptible to wildfire soil heating that typically affects the top 5 cm of mineral soil (Ikeda et al. 2015; Ishizuka 2014; Monsanto and Agee 2008). Soil macrofauna-induced nutrient cycling asserts the importance of earthworms in plant-soil feedbacks and nutrient cycles, which is also highly dependent upon the

contrasting soil properties present in northern Japan (Kawakami and Makoto 2017; Makoto et al. 2014, 2016). The interactive effects of wildfire-deposited charcoal and re-inhabiting earthworms macrofauna activity can provide an ecological context for the cycling and availability of soil P in northern Japan that have yet to be adequately evaluated.

This research provides an innovative perspective of ecological context for P status in soils where interactive effects of charcoal presence and earthworm activity have the potential to alter P limitations, which are significant in many forest ecosystems (Herbert and Fownes 1995; Vitousek et al. 2010). Forest soils create a complex environment where mycorrhizal associations and plant adaptations to P-limited soils encourage the uptake of insoluble inorganic, organic, and soluble P pools via specialized mechanisms (Bielecki 1973; Dakora and Phillips 2002; Gahoonia et al. 1992; Hinsinger 2001; Pant and Warman 2000). We utilized a recently developed biologically based P (BBP) extraction approach that allowed us to quantify P pools solubilized by primary plant and microbial acquisition strategies and can be employed across complex landscapes (DeLuca et al. 2015a) (see Table 1 for details). We hypothesized that (1) soils developed on sedimentary parent material would yield higher overall concentrations of bioavailable P compared to soils developed on serpentine parent material due to the high Mn content in the latter; (2) the presence of earthworms would increase the bioavailability of organic P pools; (3) charcoal would increase the bioavailability of inorganic P pools; and (4) the interactive effects of charcoal presence and earthworm activity would result in the highest extractable P yields as measured by the BBP method.

Materials and methods

Soil sampling and earthworm collection took place during the last week of June 2016 at the Teshio Experimental Forest, Field Center for Northern Biosphere in northern Hokkaido, Japan, operated by Hokkaido University. Sampling locations were established on FAO classified Cambisols derived from sedimentary parent material and Lithosols formed from

serpentine parent material. Forest overstory was dominated by *Picea glehnii* and *Betula ermanii* at sites developed from serpentine parent material and *Picea jezoensis*, *Quercus crispula*, and *B. ermanii* at sites developed from sedimentary parent material. Forest understory at both sites was dominated by dwarf bamboo (*S. kurilensis*). At each site we collected bulk density samples ($n = 3$) and a > 1 kg composite sample of organic matter and surface mineral soil to approximately 20 cm in depth (O and A horizons). Soil samples were composited at each site to represent the variability of the sites. Three bulk density samples were collected at each field site at the same time of soil collection to a depth of 15 cm. Moisture content of bulk density and soil samples was determined by gravimetric dry weight basis after drying at 105 °C for 24 h (Gardner 1986).

Soils were homogenized, sieved to <5 mm, and kept at 14 °C in the dark for 1–3 days while earthworms were collected. In the field, epigeic and geophageous *E. japonica* earthworms were identified as mature by the presence of a clitellum, transported immediately to the lab, and allowed to acclimate to the respective soil type for 2 days (Blakemore 2012). This species is the most dominant species in northern Japan (Nakamura 1972) and provided a representative fauna effect due to habitation in both soils developed from serpentine and sedimentary parent materials (Makoto personal observation). Earthworms were washed and weighed before being added to microcosms, after the incubation period, and nine earthworms were weighed 24 h after separation from the microcosms without food to obtain depurative body weight. Charcoal was created using mature (at least 5 m tall) dwarf bamboo (*S. kurilensis*) feedstock prepared from a composite of 10 individual bamboo stalks. One-meter sections from each of the ten bamboo stalks were dried at 60 °C for 48 h, cut into 10 cm lengths, and combusted in a muffle furnace (Advantec, Tokyo, Japan) at 500 °C for 2 h under clean sand to limit oxygen flow.

In a fully factorial design, we combined two contrasting soil types (sedimentary and serpentine-derived soils, “Soil”), presence and absence of 500 kg ha⁻¹ charcoal (“Char”), the presence or absence of one earthworm (“EW”), and the

Table 1 Plant and microbial phosphorus (P) acquisition mechanisms emulated by the biologically based P method (DeLuca et al. 2015a). Each extraction method represents an acquisition mechanism that targets a specific pool and form of P in soil matrices

Extract solution and molarity	Mechanism	Pool of P	Form of P
0.01 M calcium chloride (CaCl ₂)	Root hairs and arbuscular mycorrhizas remove P directly from solution	Labile P easily accessible to plants	Soluble inorganic
0.01 M citric acid	Adsorbed P to clay or as compounds of Ca, Fe, and Al released with organic acids	Labile inorganic, actively cycled P	Soluble to moderately insoluble inorganic
Phosphatase and phytase enzymes 0.02 enzyme units ml ⁻¹	Enzyme hydrolysis of organic P compounds	Labile organic, actively cycled P	Soluble and insoluble organic
1.0 M HCl	Solubilized by proton excretion in roots and microbes	Recalcitrant P occluded by apatite-type minerals	Soluble to insoluble inorganic

combination of charcoal with earthworms (“Char + EW”) with five replicates per treatment to total 40 microcosms. For each earthworm treatment (“EW” and “Char + EW”), one extra replicate was added in case of earthworm mortality, which was assessed weekly with a visual check. The homogenized, field-moist soil was added to 350 cm³ microcosms at the equivalent bulk density of field samples (1.18 g cm⁻³ in sedimentary and 1.50 g cm⁻³ in serpentine). The dwarf bamboo feedstock was crushed and sieved to range 2–1.2 mm, dried at 60 °C for 48 h, then applied at a rate of 500 kg ha⁻¹ or 0.2% (w/w) to soils. The charcoal application rate represented a lower content for boreal and Japanese forest soils (Bélanger and Pinno 2008; Zackrisson et al. 1996). Bamboo charcoal pH was alkaline at pH 9.60 (SE 0.19). Pre-incubation soil and charcoal characteristics are provided in Table 2.

After thorough mixing, all treatments were added to microcosm containers. Earthworms were placed on the surface of mixed soil in microcosm containers. The microcosms were kept in the dark at 15 °C for four weeks under monitoring to ensure that earthworms were alive. Approximately 2 mL of deionized water was added to microcosms on the second week of the incubation to adjust the microcosm weight to the original field-moist weight. During the incubation period of four weeks, three earthworms perished (Serp Char + EW, Serp EW, and Sed Char + EW), however, we substituted the extra replicated earthworm microcosm for each treatment in microcosms with earthworm mortality to maintain a balanced experimental design ($n = 5$). The pH of pre- and post-incubation soils as well as charcoal were determined by combining roughly 1:1 ratio of distilled water to soil (or ground and sieved charcoal) to create a saturated slurry (Thomas 1996). We allowed the slurry to equilibrate for 30 min before reading the pH on a benchtop meter (LAQUAtwin, Horiba, Japan).

Pre- and post-incubation soils were homogenized and extracted with the four BBP solutions that act as a proxy for plant and microbial acquisition strategies, which access P across varied conditions of P limitation (Table 1). Approximately 2.00 g of soil were added to 40 ml of 0.01 M CaCl₂ (to determine soluble and weakly sorbed inorganic P), 0.01 M citrate (to determine active inorganic P weakly bound to clay), 0.02 phosphatase enzyme unit ml⁻¹ in acetic acid (to determine organic P readily mobilized by phosphatase enzymes), and 1.0 M HCl (to determine soluble inorganic P in adsorbed

to minerals or precipitates) (DeLuca et al. 2015a). Soils and extraction solutions were added to 50 mL polyurethane centrifuge tubes oriented horizontally and shaken on a rotating table for 3 h at 200 rpm. Samples were filtered by gravity through Whatman #42 filter paper. Filtrate samples were stored at -10 °C for 14 days, then analyzed on a spectrophotometer at 630 nm (U-1500 Hitachi, Tokyo, Japan) for BBP with the malachite green method in order to avoid organic acid contamination (Ohno and Zibilske 1991). Charcoal samples were extracted separately before the incubation by combining 0.5 g charcoal with 10 mL of the BBP solutions and following the same method as detailed above. Process blanks were extracted and analyzed alongside soil and charcoal sample extractions. Blank concentrations were subtracted from final sample P concentrations for all samples.

Three replicate bulk density soil samples were subdivided and used for elemental C and N, particle size, and organic matter analyses. Total P of charcoal samples was determined by ashing ~5 g at 400 °C for 5 h and digesting with concentrated HNO₃ and H₂O₂ (Bakirdere and Yaman 2008) and analyzed on an ICP-OES (Thermo Scientific 6300, Waltham, MA, USA). Charcoal and soil samples were ground to a fine powder and analyzed in triplicate for total C and N by dry combustion (PerkinElmer 2400 CHN Analyzer, Waltham, MA, USA). Soil organic matter was measured by loss-on-ignition at 400 °C (Nelson and Sommers 1996). After pre-treatment to remove organic matter and iron oxides, soil particle size was determined by the hydrometer method with an electric mixer (Gee and Or 2002).

Statistical analysis

Soil characteristics (bulk density and pH) were tested with a separate one-way analysis of variance (ANOVA) to compare pre-treatment soil differences, which met the assumptions of normal distributions and homoscedasticity. Gravimetric water content and soil pH were tested in separate two-way ANOVAs against soil type and incubation time to determine the effect of these factors, which also met the assumptions of normal distributions and homoscedasticity. Earthworm body weight changes over the four-week incubation period met the assumptions of normality and homoscedasticity and were tested in a

Table 2 Chemical and physical characteristics of soils and charcoal utilized in the four-week incubation experiment. Average values are reported with standard error in parentheses ($n = 3$). Total phosphorus (P) of soils were originally reported in Makoto et al. (2016) ($n = 5$)

Material	Total P (g kg ⁻¹)	% C	% N	% OMatter	% Sand	% Silt	% Clay
Sedimentary	0.09 (0.01)	7.33 (2.02)	0.47 (0.13)	15.43 (4.39)	48 (14)	31 (19)	21 (6)
Serpentine	0.08 (0.01)	3.62 (0.97)	0.21 (0.06)	6.67 (0.8)	31 (9)	56 (13)	12 (4)
Charcoal	0.59 (0.02)	69.90 (0.42)	0.33 (0.02)				

Table 3 Physical and chemical characteristics of soils utilized in the four-week incubation experiment. Average values are reported with standard error in parentheses (pre-incubation soils $n = 3$, post-incubation soils $n = 5$). Results from separate one-way ANOVAs to compare pre- and post-incubation soil (“Soil”) characteristics are provided for pH and gravimetric moisture content as grouped by soil type (p value < 0.05)

Incubation Time	Material	Treatment	pH	Gravimetric Moisture Content
Pre	Sedimentary	Soil	4.87 (0.18) _a	0.45 _{ab} (0.01)
	Serpentine		6.05 (0.04) _c	0.39 _b (0.00)
Post	Sedimentary	Soil	4.42 (0.13) _b	0.47 _a (0.01)
	Serpentine		6.67 (0.04) _c	0.42 _{ab} (0.01)
	Sedimentary	Charcoal	4.29 (0.07)	0.49 (0.01)
	Serpentine		5.93 (0.04)	0.41 (0.00)
	Sedimentary	Earthworm	4.43 (0.11)	0.47 (0.01)
	Serpentine		6.38 (0.15)	0.40 (0.02)
	Sedimentary	Charcoal + Earthworm	4.37 (0.03)	0.49 (0.00)
	Serpentine		6.46 (0.19)	0.41 (0.01)

two-way ANOVA against soil type crossed with treatment. The enzyme-extractable soil samples were not included in the statistical analyses due to non-detectable concentrations; therefore, we use inorganic BBP to express the sum of all inorganic P pools from extraction with CaCl_2 , citrate, and HCl.

Inorganic BBP pools did not meet the assumptions of normality; therefore, we used a multivariate permutational analysis of variance (PERMANOVA) with the *adonis* function in the vegan package to explore the effects of incubation time and soil type (Oksanen et al. 2013). Data were first relativized by maxima and a distance matrix was built with Euclidean distances for the PERMANOVA test, which permuted freely within and between observations and repeated 9999 times (Anderson 2001). A Kruskal-Wallis rank-sum test was employed to test the effect of incubation time on soil without treatments.

The post-incubation BBP pools of CaCl_2 and citrate were log transformed and tested against soil type and treatment with separate one-way ANOVAs due to the interaction between the

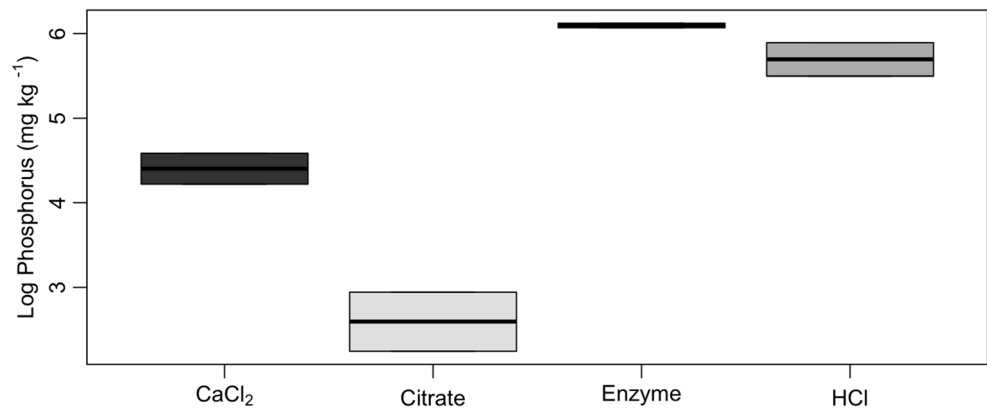
two factors. Post-incubation P as measured by HCl-extractions were also log transformed, but were compared to soil type and treatment with separate tests for equal means in a one-way design and subsequent two-sided pairwise *t* tests with a Bonferroni adjustment to relax the assumption of homoscedasticity, which was not met with a log transformation (Reimann et al. 2008). Inorganic BBP pools showed significant interactions between soil type and treatment, which suggests that inorganic BBP is affected by the combination of these factors. Therefore, treatment effects were evaluated within each soil type and differences of soil inorganic BBP were tested within treatment groups using a Tukey’s honest significant differences (HSD) after an ANOVA of each log transformed inorganic BBP pool.

Data were also analyzed using a multivariate distance matrix in separate PERMANOVA tests for soil type and treatments with free permutations within the data groups due to the fully factorial design of the experiment (Oksanen et al. 2013). Data were standardized by the maxima and distance matrices

Table 4 Inorganic biologically based phosphorus (BBP) extraction results and total inorganic BBP reported in mg phosphorus (P) kg dry soil^{-1} for pre-incubation soils and after the four-week incubation experiment for each treatment. Average values are reported with standard error in parentheses ($n = 5$)

Incubation Time	Soil Type	Treatment	CaCl_2	Citrate	HCl	Total BBP
Pre	Sedimentary	Soil	0.08 (0.01)	1.73 (0.27)	9.14 (0.73)	10.54 (0.91)
	Serpentine		0.09 (0.01)	0.27 (0.02)	1.41 (0.16)	1.48 (0.16)
Post	Sedimentary	Soil	0.09 (0.01)	1.67 (0.14)	2.18 (0.10)	3.47 (0.19)
	Serpentine		0.10 (0.01)	0.32 (0.03)	1.98 (0.03)	1.99 (0.03)
	Sedimentary	Charcoal	0.13 (0.02)	2.88 (0.24)	10.01 (0.44)	12.55 (0.37)
	Serpentine		0.11 (0.00)	0.34 (0.03)	3.40 (0.38)	3.42 (0.40)
	Sedimentary	Earthworm	0.11 (0.01)	3.70 (0.97)	2.94 (0.77)	6.28 (1.09)
	Serpentine		0.10 (0.01)	0.78 (0.24)	3.77 (0.57)	4.29 (0.82)
	Sedimentary	Charcoal + Earthworm	0.16 (0.02)	3.89 (0.25)	12.41 (1.11)	15.97 (1.23)
	Serpentine		0.08 (0.01)	0.62 (0.11)	4.93 (0.27)	5.27 (0.20)

Fig. 1 Biologically based phosphorus (BBP) ($\log \text{mg kg}^{-1}$) as measured by extractions with CaCl_2 , citrate, enzyme, and HCl solutions of dwarf bamboo (*Sasa kurilensis*) charcoal combusted at 500°C for 2 h ($n = 2$)



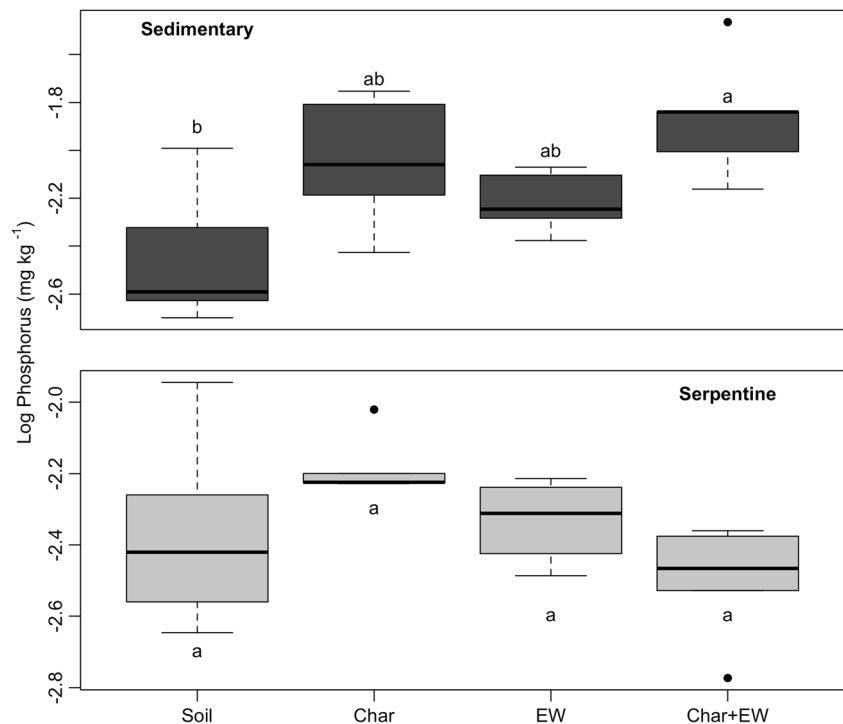
were created using Euclidean distances in order to compare the different scales across inorganic BBP pools with the *decostand* function in the vegan package (Anderson 2001; Oksanen et al. 2013). All analyses were conducted in the R statistical environment (R Core Team 2016).

Results

Prior to the incubation experiment, serpentine soils had significantly higher bulk density compared to sedimentary soils at 1.50 (SE 0.07) and 1.18 (SE 0.08), respectively ($p < 0.05$), and no significant difference in gravimetric water content (w/w) (Table 3). Serpentine soils had a significantly higher pH compared to sedimentary soils ($p < 0.001$) prior to the

incubation (Table 3). Acidity of the sedimentary soils increased (pH decreased) significantly ($p < 0.05$) over the incubation period with no change being observed in the serpentine soils (Table 3). Extraction of sedimentary soils with HCl at the end of the incubation period resulted in significantly lower P (mg kg^{-1}) than the pre-incubation samples, while serpentine soils yielded significantly higher HCl-extractable P (mg kg^{-1}) as compared to pre-incubation samples (Table 4). A multivariate, non-parametric PERMANOVA of all inorganic BBP extractions (CaCl_2 , citrate, and HCl) resulted in significant correlations with soil type ($p < 0.001$), incubation time ($p < 0.001$), and the interaction term ($p < 0.001$), which was due to the trend in HCl-extractable P. When the HCl extracts were excluded from the PERMANOVA, only soil type was significantly correlated to inorganic BBP pools ($p < 0.001$).

Fig. 2 Inorganic biologically based phosphorus (BBP) (mg kg^{-1}) as measured by 0.01 M CaCl_2 extraction from contrasting soils derived on sedimentary (dark gray, top) and serpentine (light gray, bottom) parent materials after a four-week incubation. This extraction solution represents that which plant root hairs and arbuscular mycorrhizae can directly remove in the soluble inorganic, labile P form from soil solution. Soils were treated with no amendments (Soil), 500 kg ha^{-1} dwarf bamboo (*Sasa kurilensis*) charcoal (2–1.2 mm) combusted at 500°C for 2 h (Char), one *Eisenia japonica* earthworm (EW), and a combination of the charcoal and earthworm treatments (Char + EW). Different letters indicate significant differences with an ANOVA and Tukey's HSD post hoc comparisons ($n = 5$, $p < 0.05$)



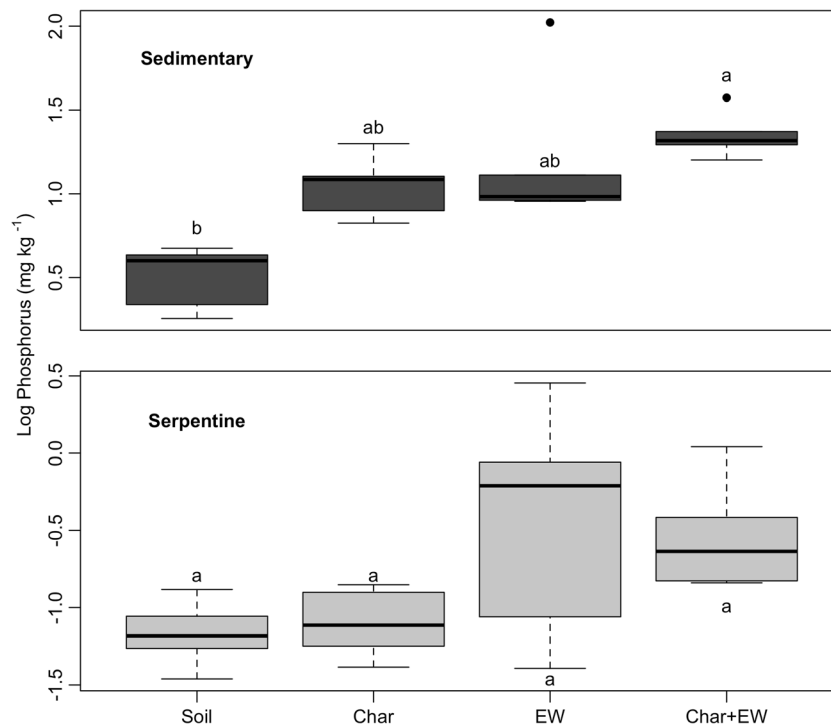


Fig. 3 Inorganic biologically based phosphorus (BBP) (mg kg^{-1}) as measured by 0.01 M citric acid extraction from contrasting soils derived on sedimentary (dark gray, top) and serpentine (light gray, bottom) parent materials after a four-week incubation. This extraction solution represents the release of organic acids by plant roots and microorganisms to solubilize labile inorganic, actively cycled P adsorbed onto clay surfaces or as

compounds with Ca, Fe, Al. Soils were treated with no amendments (Soil), 500 kg ha^{-1} dwarf bamboo (*Sasa kurilensis*) charcoal (2–1.2 mm) combusted at 500 °C for 2 h (Char), one *Eisenia japonica* earthworm (EW), and a combination of the charcoal and earthworm treatments (Char + EW). Different letters indicate significant differences with an ANOVA and Tukey's HSD post hoc comparisons ($n = 5$, $p < 0.05$)

All earthworms weighed an average of 1.86 (SE 0.1) g at the beginning of the incubation experiment. After the four-week incubation period, body weight of the earthworms changed by +0.06 (SE 0.07) g in sedimentary EW treatment, +0.07 (SE 0.06) g in sedimentary Char + EW treatment, +0.15 (0.06) g in serpentine EW treatment, and −0.04 (0.16) g in serpentine Char + EW treatment. Also after the incubation period, a subset of nine earthworms weighed after 24 h without food averaged 1.91 (SE 0.12) g and the change in body weight after the incubation period did not differ significantly between soil type or treatment ($p > 0.05$).

When all BBP extractions (CaCl_2 , citrate, HCl, enzyme) were performed on the dwarf bamboo charcoal, higher total BBP concentrations were recorded in the charcoal compared to that in soil (Soil) or soil treatments (Char, EW, Char + EW). Dwarf bamboo charcoal extracted with CaCl_2 solution released an average of 83 mg P kg^{-1} charcoal, 14 mg P kg^{-1} charcoal with citrate solution, 444 mg P kg^{-1} charcoal with enzyme solution, and 303 mg P kg^{-1} charcoal with the HCl solution to average a total BBP of 211 mg P kg^{-1} charcoal (Fig. 1).

Across all treatments, sedimentary soils yielded higher inorganic BBP for each of the extraction methods and, as expected, the highest values were extracted by HCl solution

(Table 4). The final results from this study were evaluated statistically for each combination of extraction method and soil type (e.g., $P \sim \text{treatment}$; Figs. 2, 3, 4, and 5) and for each combination of treatment and soil type (e.g., $P \sim \text{soil type} * \text{extraction method}$; Fig. 5). This statistical method allows evaluation of treatment effects separately from soil types as previous research has shown that nutrient availability and soil macrofauna activity is highly dependent on contrasting soil types (Makoto et al. 2014, 2016).

Phosphorus solubilized by the CaCl_2 solutions, which represents labile inorganic P easily accessible to plant roots and mycorrhizae, showed no differences within serpentine soils and averaged 0.10 (SE 0.01) mg kg^{-1} (Table 4 and Fig. 2) across all treatments. Sedimentary soils yielded higher values of extractable P (0.12 SE 0.01 mg kg^{-1}) compared to serpentine soils across all treatments. Inorganic BBP was most affected by treatments that incorporated charcoal and earthworms into the microcosm experiment, with the highest values shown in the Char + EW > Soil treatment ($p > 0.05$) (Table 4 and Fig. 2). Although the effects of Char and EW treatments were not significantly different, the combination of the two amendments (Char + EW) yielded the highest values for soluble P in CaCl_2 -extracted soils ($p > 0.05$) (Table 4 and Fig. 2).

Citrate-extracted P is a proxy for the solubilized inorganic, actively cycled P after secretion of organic acids by plant roots and microbes. In this study, sedimentary soil types released higher concentrations of citrate BBP than serpentine soil types, 3.04 (SE 0.01) mg P kg⁻¹ and 0.52 (SE 0.08) mg P kg⁻¹ across all treatments, respectively (Table 4). We observed no differences in treatment effects within the serpentine soils, but citrate-extractable BBP in the amended sedimentary soils were influenced by treatments, notably the combination treatment Char + EW as compared to Soil microcosms (Fig. 3).

As noted above, the HCl extraction yielded the highest P concentration of the three inorganic BBP methods tested here (Fig. 4). The HCl extraction imitates proton excretion by plant roots and microbes solubilizing recalcitrant, inorganic P in soils. Sedimentary soils released higher P concentrations compared to serpentine soil types (6.88 SE 1.06 mg kg⁻¹ and 3.52 SE 0.30 mg kg⁻¹) across all treatments (Table 4 and Fig. 4). Serpentine soils and sedimentary soils had similar treatment effects in Char and the Char + EW treatments resulting in significantly higher P released compared to the Soil and EW treatments (Fig. 4). In both soil types, the combination treatment Char + EW had the greatest effect on P concentrations.

Total inorganic BBP, measured as the sum of CaCl₂, citrate, and HCl-extractable P, showed significant treatment effects in sedimentary soils with the highest average values measured by the Char and Char + EW treatments, 12.55 mg P kg⁻¹ and 15.97 mg P kg⁻¹, respectively (Table 4 and Fig. 5). In serpentine soils, the combination Char + EW treatment resulted in significantly higher total inorganic BBP relative to Soil (5.27 mg P kg⁻¹), but non-significant differences with EW (4.29 mg P kg⁻¹), and Char treatments (3.42 mg P kg⁻¹) (Table 4 and Fig. 5). Mean total inorganic BBP values were highest in the Char + EW treatments relative to Soil only microcosms in both soil types (Fig. 5).

Charcoal and earthworm treatment effects (Char, EW, Char + EW) were consistently higher in sedimentary soil types compared to serpentine soils (Fig. 6). Soils with no amendments (Soil) yielded high citrate and HCl-extractable P concentrations with significant differences between soil type in the citrate-extracted P (Fig. 6). Incubation of soils with charcoal (Char) resulted in significant differences in citrate and HCl-extracted P between the two soil types (Fig. 6). The presence of earthworms (EW) in the microcosm experiments only influenced citrate-extractable P (Fig. 6). And the combination treatment (Char + EW) had the greatest effect on all inorganic BBP-extracted P between soil types (Fig. 6).

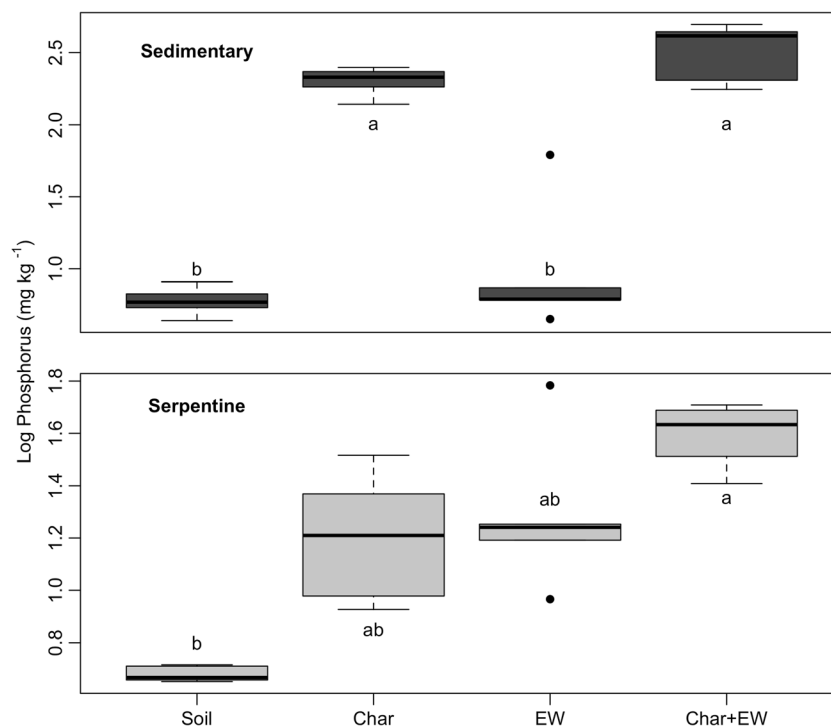


Fig. 4 Inorganic biologically based phosphorus (BBP) (mg kg⁻¹) as measured by 1.0 M HCl acid extraction from contrasting soils derived on sedimentary (dark gray, top) and serpentine (light gray, bottom) parent materials after a four-week incubation. This extraction solution is a proxy for proton excretion by plant roots and microorganisms that solubilize recalcitrant inorganic P occluded by apatite-type minerals. Soils were treated with no amendments (Soil), 500 kg ha⁻¹ dwarf bamboo (*Sasa*

kurilensis) charcoal (2–1.2 mm) combusted at 500 °C for 2 h (Char), one *Eisenia japonica* earthworm (EW), and a combination of the charcoal and earthworm treatments (Char + EW). Different letters indicate significant differences from separate tests for equal means in a one-way design and subsequent two-sided pairwise *t* tests with a Bonferroni adjustment ($n = 5$, $p < 0.05$)

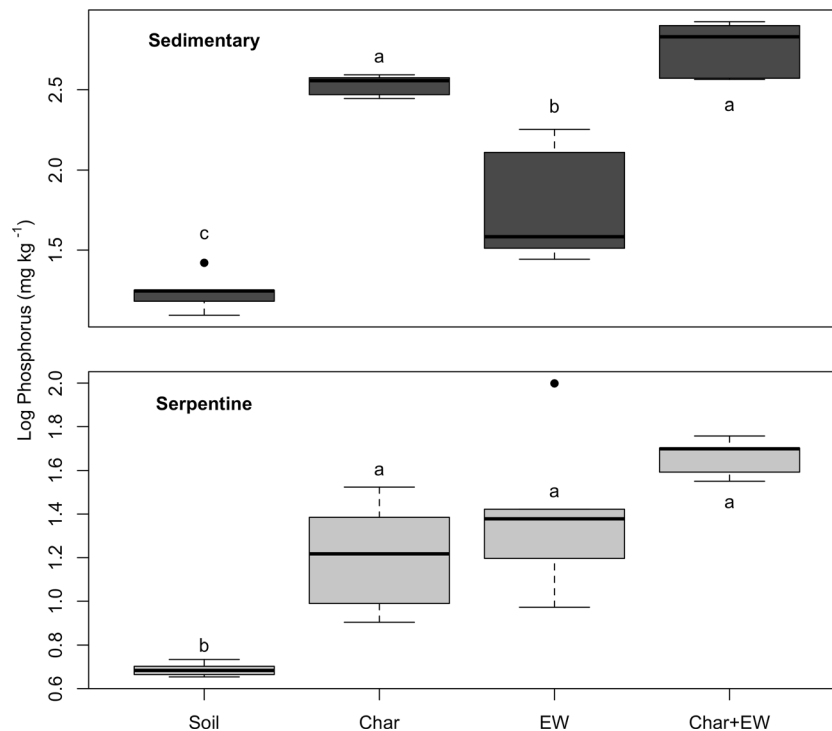


Fig. 5 Total inorganic biologically based phosphorus (BBP) (mg kg^{-1}) as measured by the sum of all inorganic BBP extraction methods in contrasting soils derived on sedimentary (dark gray, top) and serpentine (light gray, bottom) parent materials after a four-week incubation. This sum represents the total biologically available P acquired by plant roots and microorganisms. Soils were treated with no amendments (Soil),

500 kg ha^{-1} dwarf bamboo (*Sasa kurilensis*) charcoal (2–1.2 mm) combusted at 500 °C for 2 h (Char), one *Eisenia japonica* earthworm (EW), and a combination of the charcoal and earthworm treatments (Char + EW). Different letters indicate significant differences from separate tests for equal means in a one-way design and subsequent two-sided pairwise *t* tests with a Bonferroni adjustment ($n = 5$, $p < 0.05$)

Discussion

The combination of earthworm activity and charcoal incorporated into soils has the potential to increase and diversify biologically available inorganic P in northern sub-boreal forests of Japan (Table 4, Figs. 5 and 6). In this region, volcanic ash soils (Andisols) are prevalent and the weathering products of ash, non-crystalline aluminosilicates, strongly adsorb phosphate in soils (Shoji et al. 1994). Despite the lower, less favorable pH for P availability, sedimentary soils consistently yielded significantly higher inorganic BBP concentrations compared to serpentine soils (Table 4, Fig. 6), most notably when amended with the combination Char + EW treatment. Previous studies in these soils reported high Mn concentrations in serpentine soils (546 mg kg^{-1}) compared to sedimentary soils (71 mg kg^{-1}) (Makoto et al. 2016), which would likely account for low P availability as phosphate can be precipitated as mineral Mn-P (Lindsay 1979). In serpentine soil microcosms, the significant treatment differences were found in soils extracted with 1.0 M HCl (Fig. 4). Although serpentine soils contain lower concentrations of inorganic BBP, the presence of charcoal and earthworm activities have the potential to release recalcitrant P adsorbed onto clay and provide available P to plants and microbes in a highly limited soil

environment (Figs. 4 and 5). These results provide support for the notion that biologically available P indices are more representative of the potential for biological acquisition of inorganic BBP in soils of northern Japan compared to assays for total P (DeLuca et al. 2015a).

Due to non-detectable phosphatase enzyme P concentrations, we were not able to measure biologically available organic P by enzymatic hydrolysis and could not test the second hypothesis relating to the treatment effects on organic P. It is possible that phosphatase inhibitors were present in soils or soil extracts, but this interaction was not addressed in this study (Juma and Tabatabai 1977). We found levels of enzyme-extracted soil P to be below the minimum detection in all soil samples, yet enzyme-extracted charcoal samples contained very high concentrations of available P (444 mg kg^{-1}) approximately 2–900 times higher than as released by extracted soils (Table 4, Fig. 1).

Total BBP reported in this study for dwarf bamboo charcoal agree with total charcoal-extractable P as previously reported (DeLuca et al. 2015b; Gundale and DeLuca 2006; Pluchon et al. 2014). Soluble phosphate concentrations extracted from Douglas-fir (*Pseudotsuga menziesii*) wood charred at 350°C yielded up to 50 mg P kg^{-1} (Gundale and DeLuca 2006) and total P after digestion of charcoal prepared

at 450°C from various boreal plant species (conifer, deciduous, and shrub) ranged from 1100 to 3900 mg P kg⁻¹ (Pluchon et al. 2014). Charcoal P concentrations have been shown to be related to plant growth in deciduous primary succession seedlings and suggest that charcoal P may be an important source of P in the post-fire soil environment (Pluchon et al. 2014). The addition of biochar as an agriculture amendment has also been shown to increase P uptake, mycorrhizal colonization, and microbial P biomass (Madiba et al. 2016; Zhai et al. 2015). Our current study shows that dwarf bamboo charcoal can be a significant source of bioavailable P derived from inorganic P in soil solution and recalcitrant inorganic P in acidic, low P soils.

The presence of charcoal in this microcosm experiment had a significant effect on total inorganic BBP concentrations in both soil types relative to Soil only microcosms ($p < 0.05$) (Fig. 5), supporting the third hypothesis that the presence of charcoal would have an effect on the bioavailability of inorganic P pools. The presence of charcoal was significantly effective at increasing extractable inorganic BBP concentrations when combined with earthworms (Char + EW), especially for labile, inorganic P pools (Figs. 4 and 5). Total extractable inorganic BBP concentrations were highest in sedimentary and serpentine soil types amended with the combination of charcoal and earthworms (Char + EW) (Fig. 6). These findings support our last hypothesis, that the interactive effects of charcoal presence and earthworm activity would yield the highest P concentrations. A recent study of *E. japonica* suggests that the activity of this geophageous species may increase net nitrification rates due to cast production (Kawakami and Makoto 2017), which may indirectly influence P solubility (Vitousek et al. 2010).

Although we cannot directly extrapolate these findings to a natural post-fire soil environment, where organic matter in organic and surface soil horizons are likely to be partially or completely volatilized and soil microbial activity may be limited, these results do provide some insight to the capacity for charcoal and earthworms to influence concentrations of BBP in soils. The incubation experiment also induced soil mixing and altered soil chemical characteristics that are not equivalent to field conditions. Concentrations of HCl-extractable P significantly decreased during the incubation in sedimentary soils and increased in serpentine soils, possibly due to Mn precipitation of P (Lindsay 1979). Alternatively, short-term changes in soil chemical and physical properties due to charcoal or biochar amendments have been documented in agricultural soils and may help explain pH and extractable inorganic BBP changes in microcosms after the incubation period (Barrow 2012; Zhai et al. 2015). Post-incubation P concentrations should not be extrapolated to soil BBP values in situ. Future studies are necessary to assess field-based biologically available P and relate these values to specific species known to utilize the P acquisition strategies.

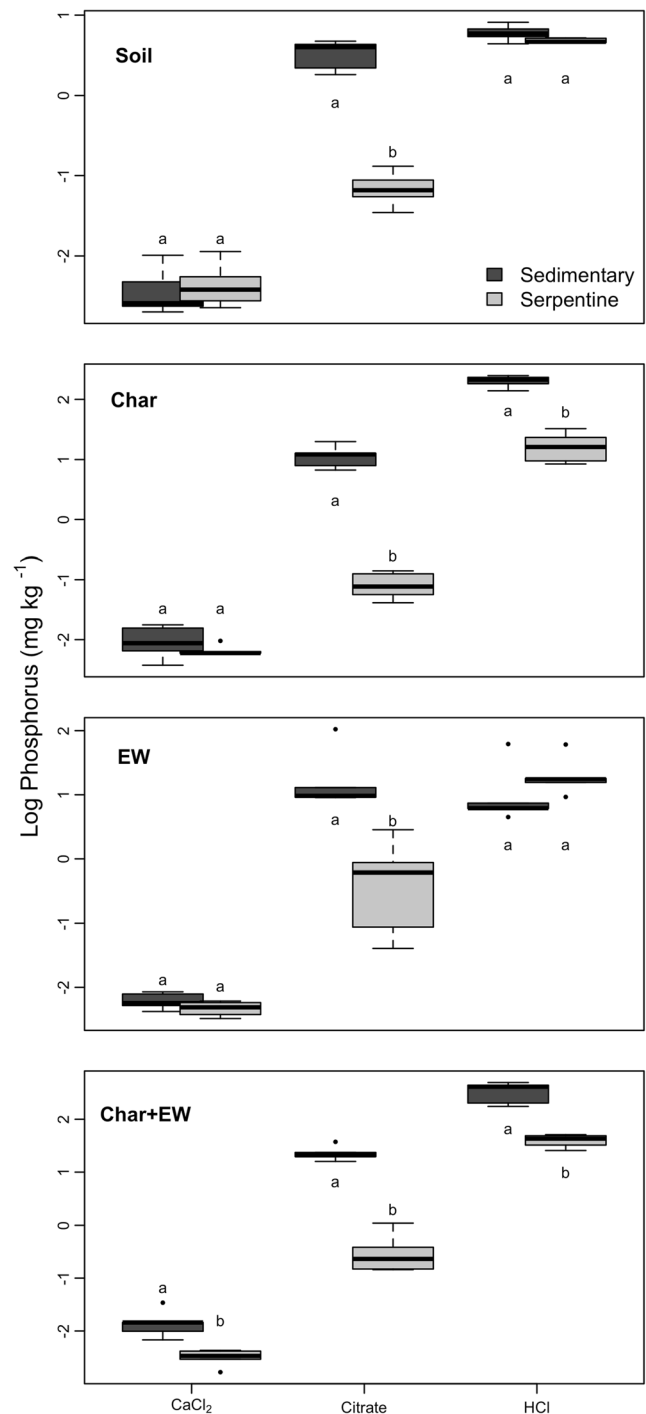


Fig. 6 Inorganic biologically based phosphorus (BBP) (mg kg⁻¹) in a side-by-side comparison of sedimentary (dark gray) and serpentine (light gray) parent materials for each treatment after a four-week incubation. Soils were treated with no amendments (Soil), 500 kg ha⁻¹ dwarf bamboo (*Sasa kurilensis*) charcoal (2–1.2 mm) combusted at 500 °C for 2 h (Char), one *Eisenia japonica* earthworm (EW), and a combination of the charcoal and earthworm treatments (Char + EW). Different letters indicate significant differences with an ANOVA and Tukey's HSD *post hoc* comparisons for each treatment ($n = 5$, $p < 0.05$)

Wildfires may influence soil P cycling via charcoal deposition into the post-fire soil environment. Our study suggests

that plants and microbes that release protons into the rhizosphere have the greatest potential to solubilize P in serpentine soils, whereas sedimentary soils may favor a higher diversity of species or individual species with a greater variety of P acquisition techniques. Interestingly, these results also suggest that recalcitrant, slowly cycled inorganic P may be a substantial source of bioavailable P in soils of northern Japan. Root proton excretion to reduce P limitations is most effective in mildly acidic soils and is likely to affect serpentine-derived soils evaluated in this study, which measured an average pH of 6.4 (Table 2) (Hinsinger 2001; Neumann and Römheld 1999). However, HCl-extractable P has also been associated with the dominant form of inorganic N in soil (Gahoonia et al. 1992), which may further alter in situ extrapolation of our findings.

The relationship between post-fire forest regeneration and P bioavailability may be especially important in soils developed on serpentine parent material despite the less pronounced effects of microcosm treatments in this study. These forests are dominated by slow-growing *P. glehnii* and are often completely replaced by dwarf bamboo after wildfires, which are extensive due to the gentle topography created by the serpentine formation (Takaoka and Sasa 1996). Our findings support previous research that charcoal may serve as a legacy in post-fire soils by providing a source of inorganic P, which may ultimately stimulate seedling germination and growth (Makoto et al. 2010; Pluchon et al. 2014). Soils developed on serpentine parent material have been shown to harbor a more active earthworm population, possibly a result of compensation feeding in the low-nutrient soil or higher nutrient efficiency, both mechanisms would be likely be further stimulated in a post-fire soil environment where nutrients are lost by partial or complete combustion of nutrients and nutrient-bound organic matter (Makoto et al. 2016). Our results from this laboratory-based experiment suggest P bioavailability can be driven by the interactive effects of earthworm activity and charcoal presence in forest soils, which is dependent on soil type.

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