

Organic Acids Regulation of Chemical–Microbial Phosphorus Transformations in Soils

Daniel Menezes-Blackburn,^{*,†} Cecilia Paredes,[‡] Hao Zhang,^{*,†} Courtney D. Giles,[§] Tegan Darch,^{||} Marc Stutter,[§] Timothy S. George,[§] Charles Shand,[§] David Lumsdon,[§] Patricia Cooper,[§] Renate Wendler,[§] Lawrie Brown,[§] Martin Blackwell,[§] Catherine Wearing,[†] and Philip M. Haygarth[†]

[†]Lancaster University, Lancaster Environment Centre, Lancaster, LA1 4YQ, U.K.

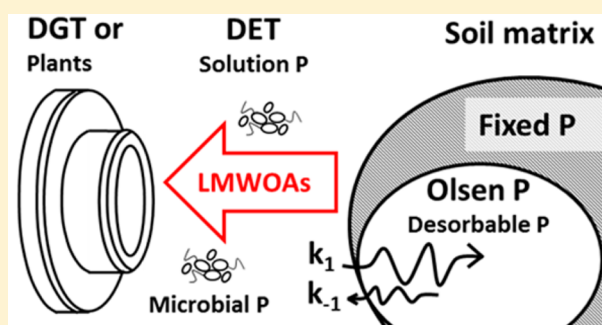
[‡]Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Temuco, Chile

[§]James Hutton Institute, The James Hutton Institute, Aberdeen, AB15 8QH and Dundee, DD2 5DA, Scotland, U.K.

^{||}Rothamsted Research, North Wyke, Okehampton, Devon, EX20 2SB, U.K.

Supporting Information

ABSTRACT: We have used an integrated approach to study the mobility of inorganic phosphorus (P) from soil solid phase as well as the microbial biomass P and respiration at increasing doses of citric and oxalic acid in two different soils with contrasting agronomic P status. Citric or oxalic acids significantly increased soil solution P concentrations for doses over 2 mmol kg⁻¹. However, low organic acid doses (<2 mmol kg⁻¹) were associated with a steep increase in microbial biomass P, which was not seen for higher doses. In both soils, treatment with the tribasic citric acid led to a greater increase in soil solution P than the dibasic oxalic acid, likely due to the rapid degrading of oxalic acids in soils. After equilibration of soils with citric or oxalic acids, the adsorbed-to-solution distribution coefficient (K_d) and desorption rate constants (k_{-1}) decreased whereas an increase in the response time of solution P equilibration (T_c) was observed. The extent of this effect was shown to be both soil and organic acid specific. Our results illustrate the critical thresholds of organic acid concentration necessary to mobilize sorbed and precipitated P, bringing new insight on how the exudation of organic acids regulate chemical–microbial soil phosphorus transformations.



INTRODUCTION

Modern agriculture is dependent on phosphorus (P) fertilizer applications to maintain crop productivity. As a major plant macronutrient, P is perhaps the most limited with respect to bioavailability due to its rapid precipitation and adsorption in soils.¹ Plants have evolved several mechanisms to increase P bioavailability in the rhizosphere.² The exudation of low-molecular-weight organic acids (LMWOA) is proposed as a key mechanism for increasing plant P uptake in soils.^{2–4} There is an increasing scientific interest in the selection and genetic engineering of plants that exudate LMWOAs into the rhizosphere (root–soil–interface) as a means to enhance P uptake and plant yields.^{5,6} However, some studies have shown difficulties in obtaining success in increasing plant P uptake with the expression of this trait in plants.⁷ Among the LMWOAs, the tribasic citric acid and the dibasic oxalic acid are reported to be among the most commonly produced root and microbial exudates affecting rhizosphere P availability.^{8,9} Citric and oxalic acids have been shown to induce higher P mobilization than other organic acids.^{10,11} The mechanism involved is generally assumed to be related to the ability of LMWOAs to complex metal cations and compete with P for adsorption sites on soil colloids, thereby releasing precipitated

and adsorbed P.^{3,12} Citric and oxalic acid may also destabilize soil organic matter and promote the cycling of organically bound soil P through the chelation of bridging cations.⁸ Citrate is reported to be most effective in promoting P release through the chelation of Al in acidic soils, whereas oxalate dominates in less acidic or calcareous soils.⁸ Though many studies have shown the effect of organic acids on P desorption in different soils, there remains a gap in knowledge concerning the critical threshold of organic acids concentrations necessary to produce a significant mobilization of P. Plant and microbial production of LMWOAs may contribute to the abiotic release of bioavailable P while simultaneously stimulating microbial growth and P sequestration into the microbial biomass, counteracting LMWOAs effect on plant P bioavailability. Additionally, little is known about how these LMWOAs affect the desorption kinetics of soil P once solid-to-solution phase equilibria are reached.

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Phosphorus supply from soil to plants is not just a matter of solution P concentration but also the capacity of the soil matrix to dynamically resupply the solution phase.¹³ Many previous experiments investigated the soil P mobilization by LMWOAs, nevertheless a considerable part of these studies are set up as incubation experiments using high doses of LMWOAs and soil suspensions at high liquid to solid ratios.¹⁴ These conditions are fundamentally dissimilar from those that exist in natural soils. In these conditions, equilibrium solution concentration may not necessarily represent plant availability since it does not account for the effect of LMWOAs in the kinetics of P resupply from the solid phase.¹³ The diffusive gradient in thin films (DGT) method represents a much better experimental setup that emulates the plant root depletion of soil solution P at field-like moisture content.¹⁵ Furthermore, the coupling of DGT with a dynamic numerical modeling (DIFS), mimicking exchange and diffusion processes in soils and uptake by DGT, provides a measurement of the dynamic resupply of P to soil solution adjacent to the DGT device in response to disturbances in the adsorbed-solution equilibrium.¹³

Our aim was to develop a holistic understanding of P dynamics in the rhizosphere, and the efficacy of plant traits such as LMWOA exudation for improving the mobilization of both organic and inorganic P in soils. The specific objectives of this study were (a) to determine the critical concentration of citric and oxalic acids sufficient to significantly increase P availability in soils with contrasting P status; (b) to evaluate the amount of inorganic and organic P that can be mobilized by increasing doses of citric or oxalic acid; (c) to evaluate the impact of citric acid on adsorbed-to-solution distribution coefficients, desorption rate constants and other parameters reflecting P resupply from soil solid phase; and (d) to evaluate to what extent the effect of LMWOAs on microbial biomass stimulation promotes the sequestration of released P, as well as the degradation of LMWOAs decreasing their net effect on P mobilization.

MATERIALS AND METHODS

Soil Samples and Characterization. Samples used in this study were topsoils (0–10 cm depth) from a site near the James Hutton Institute in Dundee (Tayport soil; 56°42'33.03"N –2°88'75.16"W) and from the Glensaugh Research Station in Laurencekirk, Aberdeenshire (Glensaugh soil, 56°53'42.29"N –2°32'00.42"W), both in Scotland, UK. The Glensaugh soil is a freely drained Podzol and the Tayport soil is a Cambisol (FAO 1994). These soils were chosen due to their contrasting agronomic P status, as measured by Olsen P and other soil P tests. The Tayport soil is an arable soil with a history of being cultivated with cereals (*H. vulgare*, *T. aestivum*) and annually fertilized with inorganic P and/or pig manure. The Glensaugh soil was collected from a natural pasture with low P status due to the lack of P fertilizer application. The initial chemical properties of the bulk soils (air-dried, <2 mm) are displayed in Table 1.

Preliminary Organic Acid Dose Survey and Their Effect on Equilibration Time. To determine the organic acid dose sufficient for mobilization of soil P, 1 g dry weight (DW) equivalent of each prewetted (50% of maximum water retention in the slurry - MR) soil was incubated with citric acid (Sigma-Aldrich, UK) doses ranging from 0 to 100 mmol kg⁻¹ soil at maximum slurry retention (MR; see DGT deployment section) for 24 h at room temperature (~20 °C). The slurry was then suspended in MQ water (18.2 MΩ cm) reaching a final 1:10 dry weight to liquid ratio, shaken at

Table 1. General Soil Properties and Phosphorus Indices

	Tayport	Glensaugh
Olsen P (mg kg ⁻¹)	77 (index 5)	6.7 (index 0)
modified Morgan P (mg kg ⁻¹)	21 (index high)	1.1 (index very low)
AER P (mg kg ⁻¹)	5.38	0.26
acetic acid P (mg kg ⁻¹)	207	2.5
calcium chloride P (mg kg ⁻¹)	2.2	0.08
water P (mg kg ⁻¹ ; 1:4 solid to liquid)	4.5	0.04
water P (mg kg ⁻¹ ; 1:10 solid to liquid)	9.7	0.19
water P (mg kg ⁻¹ ; 1:100 solid to liquid)	26.2	1.71
aqua regia P (mg kg ⁻¹)	1275	574
P saturation (oxalate)	49.5	10.5
microbial P (mg kg ⁻¹)	1.06	0.49
clay (%/w)	5.4	3.5
sand (%/w)	35.9	67.4
surface Area (m ² g ⁻¹)	0.77	0.46
C (%/w)	1.74	4.86
N (%/w)	0.16	0.43
C:N	11.12	11.37
C:P	13.61	84.72
N:P	1.22	7.45
pH (water)	6.2	5.1

30 rpm for 30 min in a rotatory shaker, decanted after 5 min settling and filtered through a Whatman 42 filter paper. The filtrates were analyzed for molybdate reactive phosphorus (deemed to be inorganic P (P_i)) as described by Murphy and Riley.¹⁶ The reaction volumes were proportionally reduced to a final 240 μL in order to be developed in 96 wells microplates and were read on a Multiskan spectrophotometer (Thermo Fisher Scientific Inc., UK). Maximum responses were obtained at 25 mmol kg⁻¹ citric acid (Figure S1). Based on this analysis, and considering that usual rhizosphere concentrations of LMWOAs are expected to be on the 0–1 mmol kg⁻¹ range, further studies were performed using doses from 0 to 10 mmol kg⁻¹ LMWOAs in order to simulate also the cases of high organic acids exuding plants (such as *Lupinus* spp.) and the possible cumulative effect of continuous LMWOA root exudation. The rhizosphere is a highly heterogeneous system due to chemicals, microbes and even enzyme activities. There are hotspots which may contain much higher concentration of organic acids compared to the bulk media.

The time needed for maximum P desorption after incubation with organic acids (equilibration time) was assayed by incubating 1g dry weight of each soil with the maximum citric or oxalic acid (ACROS Organics, UK) dose of 10 mmol kg⁻¹ at MR and at room temperature (~20 °C) for increasing times up to 4h. The slurry was then suspended in MQ water (18.2 MΩ cm) reaching a 1:10 dry weight to liquid ratio, shaken vigorously for 1 min and immediately filtered. The filtrates were assayed for P_i as described by Murphy and Riley.¹⁶ Equilibration of soil solution P appear to be reached at approximately 1 h (Figure S2) and the 24 h incubation used in the standard DGT deployment was considered sufficient time for the equilibration of soils with the LMWOAs.

Diffusive Gradient in Thin Films (DGT) Diffusive Equilibrium in Thin Films (DET) Analysis. Gel Preparation and Assembly of DGT and DET Devices. DGT cylindrical devices designed for soil deployment (DGT Research Ltd., Lancaster, UK) consisted of a binding and a diffusive gel layers

tightly packed into a plastic support comprised of a backing plate and a front plate with an exposure window ($A = 2.52 \text{ cm}^2$). Diffusive gels (0.78 mm) were placed on top of a ferrihydrite binding layer and a 0.13 mm thick poly(ether sulfone) filter (0.45 μm) was placed on top of the diffusive gel for physical protection. The filter layer has been shown to behave as an extension of the diffusive layer.¹⁷ The Diffusive Equilibrium in thin Films (DET) devices contained only the diffusive gel and the membrane filter tightly packed into a plastic support with similar dimensions to the ones used for the DGTs.

The diffusive gels containing acrylamide cross-linkers (DGT Research Ltd., Lancaster, UK) were prepared and cast according to published procedures.¹⁷ To prepare the ferrihydrite gel for the binding layer, diffusive gels (0.6 mm) were soaked for 2 h in a 0.1 mM FeCl_3 aqueous solution to allow uniform distribution of Fe inside and the surface of the gels.¹⁸ Each gel was then placed in a freshly prepared 0.05 M 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.7 buffer for 30 min to allow the ferrihydrite to precipitate. The gels were then washed 3 times (2 h intervals) with MQ water and stored at 4 °C in 0.01 M NaNO_3 solution.

DGT Deployment. Before the soil incubations with LMWOAs started, both soils were tested to determine the maximum water retention (MR) by adding MQ water and continuously mixing until MR was reached. A visual assessment of soil plasticity and the glistening of water on the soil surface was used to determine MR. Assuming that no air was trapped in the soil slurry, the final moisture concentration was used to calculate the particle concentration, porosity and tortuosity¹⁹ displayed in Table 2.

Table 2. Physical Properties from Dry Soils and Slurries Prepared for DGT Deployment^a

soil	MR g g^{-1}	P_c g cm^{-3}	ρ_s g cm^{-3}	ϕ	θ^2	D_s $10^{-6} \text{cm}^2 \text{s}^{-1}$
Glensnaugh	0.58	1.72	1.05	0.61	2.00	2.89
Tayport	0.30	3.33	1.47	0.44	2.62	2.2

^aMR, Maximum water retention; P_c , particle concentration, mass of particles in unit volume of soil solution; ρ_s , bulk density of the soil slurry; ρ_w , bulk density of the dry soil; ϕ , soil porosity; θ^2 , diffusive tortuosity according to Boudreau (1996).

For the incubation of soils with LMWOAs, samples (100 g DW) were adjusted to 50% of MR with MQ water 2 days before DGT and DET deployment. Twenty-4 h before deployment, the soil slurry was prepared by taking samples to 100% of MR with either citric or oxalic acid solutions, with six doses used in triplicate incubations: 0, 2, 4, 6, 8, and 10 mmol kg^{-1} LMWOAs. For each independent incubation, duplicates of DGT and DET devices were deployed after 24 h by gently pressing them against the soil slurry to ensure optimum surface contact. The air temperature was monitored every hour.

After 24 h of deployment the DGT and DET devices were removed and rinsed with MQ water to remove any adhering soil particles. The ferrihydrite gels from the DGTs and the diffusive gels from the DETs were retrieved and eluted with 2 mL of 0.25 M H_2SO_4 solution overnight before analysis. Three nondeployed DGT and DET “blanks” were prepared concurrently with each deployment and treated identically to the devices deployed on the soil samples. The concentration of the molybdate-reactive P in the DGT and DET eluents was

measured colorimetrically. Total P was measured by inductively coupled plasma mass spectrometry (XSERIES 2 ICP-MS, Thermo Fisher Scientific Inc., Hemel Hempstead, UK) and the molybdate-unreactive P in DGT and DET extracts was estimated as the difference between the total P and the molybdate-reactive P; this solution molybdate unreactive P is hereby termed organic P (P_o).

Calculation of P_{DGT} , P_E , K_d and R - R_{diff} . The concentration of solution P at the surface of the DGT device was calculated using eq 1,²⁰

$$P_{DGT} = \frac{M\Delta g}{(DA t)} \quad (1)$$

where M is the accumulated P mass in the DGT binding layer, A is the surface area of the DGT sampling window, t is the deployment time, Δg is the total thickness of the diffusive gel layer and the filter membrane, and D is the diffusion coefficient of P in the diffusive gel. The DGT is a passive sampler and, therefore, results from eq 1 reflect a time-averaged value of the soil solution immediately adjacent to the outer surface of the DGT device.

P_{DGT} was converted to an effective concentration using eq 2, to represent the P available from both the soil solution and the solid-phase labile pool.²¹

$$P_E = \frac{P_{DGT}}{R_{diff}} \quad (2)$$

R_{diff} is the hypothetical ratio of the P_{DGT} to the concentration in soil solution if no resupply from the solid phase occurred (only pore water P diffusion). R_{diff} was calculated using the DIFS dynamic numerical model of the DGT-soil system.²² Input parameters of particle concentration (P_c ; ratio between dry weight and soil solution), soil porosity (ϕ), and the diffusion coefficient of P in the soil (D_s) were calculated according to Harper et al. (2000).¹⁹ To simulate “diffusion only” conditions the system response time, T_c , was set to 1×10^{10} s and K_d was set as $1 \times 10^{-10} \text{ cm}^3 \text{ g}^{-1}$.¹³

The R ratio was calculated as the mean P concentration measured by DGT relative to the solution P concentration measured using DET (eq 3). Assuming that P_{Olsen} provides an estimate of the labile solid phase pool, the distribution coefficient between the solid and solution phases (K_d) was calculated as P_{Olsen}/P_{DGT} . The use of Olsen P as labile P concentration is still controversial and data resulting from these calculations must be taken with care.¹³ The value of T_c derived using DIFS corresponds to the time needed to bring the interfacial concentration of P, P_i , from 0 to 63% of its pseudo steady state value.¹⁹ Assuming that the desorption rate constants are much lower than the sorption rate constant ($k_1 \gg k_{-1}$), the kinetic constant of solid to solution phase resupply (k_{-1}) can be calculated as in eq 4.^{19,23} The desorption rate constant is independent of DGT deployment time.

$$R = \frac{P_{DGT}}{P_{DET}} \quad (3)$$

$$k_{-1} = \frac{1}{T_c(1 + K_d P_c)} \quad (4)$$

pH, P_{Olsen} , Water Extractable P. Before DGT and DET devices were deployed, the slurry was subsampled to quantify pH, P_{Olsen} , and water extractable P. To quantify soil pH, 10 g of slurry was taken to a final 1:4 soil to solution ratio using MQ

Table 3. pH and Phosphorus Concentrations in Water and Olsen Extracts, As Well As Determined by Diffusive Gradient in Thin Films (DGT) and Diffusive Equilibration in Thin Films (DET)

LMWOA (mmol kg ⁻¹)	pH	P _{water} (mg kg ⁻¹)		P _{Olsen}		P _{DET} (mg l ⁻¹)			P _{DGT} (μg l ⁻¹)		R
		P _i ^a	P _t	%Po ^b	(mg kg ⁻¹)	P _i	P _t	%Po	P _i	%Po ^c	
Glensaugh x citric acid											
0	5.24 ± 0.04	0.11 ± 0.02	0.17	38	9.52 ± 0.37	0.03 ± 0.00	0.14	76	6.38 ± 1.5	59	0.20
2	5.09 ± 0.03	0.10 ± 0.04	0.14	28	10.48 ± 0.30	0.04 ± 0.01	0.16	75	7.99 ± 1.6	56	0.20
4	4.94 ± 0.03	0.13 ± 0.02	0.17	25	10.37 ± 0.42	0.05 ± 0.00	0.17	73	6.84 ± 1.5	63	0.14
6	4.78 ± 0.01	0.13 ± 0.01	0.16	21	10.16 ± 0.42	0.07 ± 0.01	0.23	70	7.27 ± 2.2	62	0.10
8	4.67 ± 0.01	0.11 ± 0.04	0.15	23	9.61 ± 0.34	0.11 ± 0.00	0.46	76	7.80 ± 3.3	62	0.07
10	4.67 ± 0.01	0.11 ± 0.04	0.15	24	9.52 ± 0.39	0.13 ± 0.00	0.48	73	9.05 ± 3.0	58	0.07
Glensaugh x oxalic acid											
0	5.26 ± 0.06	0.11 ± 0.01	0.18	36	9.51 ± 1.78	0.03 ± 0.00	0.14	77	5.97 ± 1.7	59	0.19
2	5.19 ± 0.03	0.11 ± 0.01	0.20	42	9.19 ± 1.08	0.04 ± 0.00	0.20	80	6.08 ± 1.0	63	0.15
4	5.19 ± 0.02	0.12 ± 0.01	0.20	41	8.07 ± 0.98	0.08 ± 0.01	0.38	79	7.08 ± 1.9	61	0.09
6	5.14 ± 0.01	0.12 ± 0.01	0.22	46	9.09 ± 1.09	0.09 ± 0.00	0.39	76	7.60 ± 1.1	63	0.08
8	5.06 ± 0.01	0.12 ± 0.01	0.23	46	8.49 ± 1.01	0.11 ± 0.01	0.41	74	7.63 ± 2.2	64	0.07
10	5.02 ± 0.05	0.15 ± 0.01	0.27	47	8.10 ± 0.76	0.11 ± 0.01	0.51	78	7.87 ± 1.5	61	0.07
Tayport x citric acid											
0	6.43 ± 0.02	9.50 ± 0.46	13.70	31	79.66 ± 4.48	0.61 ± 0.10	2.76	78	141 ± 85.9	57	0.23
2	6.56 ± 0.02	10.70 ± 0.55	17.98	40	81.26 ± 5.00	0.76 ± 0.09	2.98	74	179 ± 22.4	40	0.24
4	6.64 ± 0.01	11.84 ± 1.21	20.35	42	85.99 ± 2.27	1.55 ± 0.23	3.44	55	194 ± 40.7	38	0.13
6	6.59 ± 0.06	14.55 ± 1.12	26.08	44	89.10 ± 3.18	2.66 ± 0.30	4.38	39	221 ± 36.4	31	0.08
8	6.16 ± 0.13	18.74 ± 4.37	24.84	25	95.11 ± 4.13	5.17 ± 0.34	6.28	18	260 ± 21.5	21	0.05
10	6.10 ± 0.20	19.10 ± 3.90	25.19	24	96.24 ± 3.25	5.41 ± 0.84	6.88	21	278 ± 54.1	21	0.05
Tayport x oxalic acid											
0	6.43 ± 0.02	9.50 ± 0.46	13.70	31	79.36 ± 1.13	0.67 ± 0.01	2.30	71	157 ± 2.0	61	0.24
2	6.56 ± 0.02	9.59 ± 0.51	14.08	32	79.04 ± 2.21	0.99 ± 0.03	2.60	62	202 ± 10.9	60	0.20
4	6.64 ± 0.01	9.96 ± 0.28	17.41	43	79.14 ± 1.41	1.14 ± 0.02	2.47	54	214 ± 16.5	58	0.19
6	6.59 ± 0.06	10.65 ± 0.58	17.75	40	81.98 ± 1.76	1.59 ± 0.11	2.44	35	218 ± 27.4	56	0.14
8	6.16 ± 0.13	11.41 ± 0.37	18.73	39	81.29 ± 2.85	1.73 ± 0.06	2.62	34	226 ± 17.7	52	0.13
10	6.10 ± 0.20	11.25 ± 1.75	20.54	45	83.07 ± 4.03	1.79 ± 0.09	2.50	28	229 ± 8.2	54	0.13

^aP_i values in water extract were slightly below the detection limit to the Glensaugh soil and therefore are assumed as inaccurate. ^bPo was calculated as the difference of P_t and P_i, and corresponds to the molybdate unreactive P, here assumed to be mostly organic P. ^cMolybdate unreactive P mass at the ferrihydrite binding layer eluate from the DGT device. Citric and oxalic acid doses of ≤1 mg kg⁻¹ did not show any effect on the tested parameters and were omitted.

water, shaken vigorously for 5 min and settled for 2 h before pH was assayed in the supernatant. Water extractable P was determined by shaking the slurry suspended in MQ water (1:10 w/v) for 30 min. After filtration using Whatman 42 filter paper the total and inorganic P in the extract was quantified by molybdenum blue colorimetry. Additionally, the inorganic P concentration was also assayed on sodium hydrogen carbonate extracts (P_{Olsen}) according to Sim (2000).²⁴

Microbial Biomass P and Microbial Respiration.

Microbial P was measured by a procedure from McLaughlin and Alston (1986) modified by Stutter et al. (2015).^{25,26} Quadruplicates of soil slurry as prepared for DGT measurements (2 g DW equivalent) were extracted for 16 h in 20 mL of MQ with anion exchange resin (AER) strips either with or without addition of 0.8 mL hexanol. Hexanol was the biocide agent responsible for the release of intracellular P into soil solution. The phosphate collected in resin strips was eluted with 0.1 M HCl and the concentrations of molybdate reactive P determined colorimetrically. Microbial P was estimated as the difference between samples extracted with and without hexanol. A correction factor to account for sorption of P to soil solid phase during extraction was determined from soil samples spiked with 20 mg P g⁻¹ immediately before adding the resin strips.

Microbial respiration was measured using the MicroResp system as described in detail by Campbell et al. (2003).²⁷ Each well of the deep-well microplate (96 wells, 1.2 mL per well) was filled with 0.41 ± 0.02 or 0.58 ± 0.03 g of the Glensaugh or Tayport dry soil soils, respectively. The moisture content was chosen to match the maximum water retention for the slurry for the Glensaugh (0.58 g/g) and Tayport (0.30 g/g) soils (Table 2). Soils were moistened with half of the total volume of liquid to be added using sterile deionized water 24 h prior to the addition of the remaining volume, which contained increasing concentration of LMWOAs, equivalent to the conditions used during the DGT and DET assays. A second microplate holding a CO₂ detection gel (12.5 mg kg⁻¹ cresol red, 150 mM KCl, and 2.5 mM NaHCO₃ in 1% purified agar) was assembled on top to the soil microplate using an airtight sealing system. The system was incubated in the dark at room temperature and detection plates were replaced every 6 h. The absorbance of the indicator plates was measured at 570 nm using an Emax microplate reader (Molecular Devices,) before and after incubation with soils. The CO₂ released from soils (% CO₂) was converted to respiration rate (μg CO₂-C g⁻¹ dry soil h⁻¹) as described by Campbell et al. (2003).²⁷

The degradation of LMWOA was estimated using the cumulative measured microbial respiration response at the beginning and the end of DGT deployment (24 and 48 h

respectively). The priming effect was not directly measured, and for a matter of calculations, a 10% of the increase in microbial respiration due to LMWOA addition was assumed as coming from pre-existing soil organic carbon based on existing literature.²⁸ The estimated degradation of LMWOAs was then deducted from the total added dose to represent the concentration of LMWOA remaining in soil, and will be further referred to as the effective LMWOA dose.

Statistical Analyses. The data were subjected to full factorial analysis of variance analysis using IBM SPSS Statistics for Windows 22.0 software. For every data set the standard deviation ($n = 3$) was calculated and used as data dispersion indicators in tables and figures.

RESULTS

pH and Phosphorus Concentrations in Soil Extracts.

Both citric and oxalic acid resulted in the acidification of soils, but the extent of this effect was both soil and LMWOA type specific. The 0.5 and 0.05 M stock solutions of LMWOAs were respectively at pHs 2.12 and 2.43 for the citric acid, and 1.5 and 1.98 for the oxalic acid. The pH of Glensaugh soil was 5.1 initially, after incubation as the slurry prepared for DGT this pH raised to 5.2 in absence of LMWOAs, when the slurry contained 10 mM of either citric acid or oxalic acid the soil was acidified to 4.7 and 5.0 respectively (Table 3). In the Tayport soil, the slurry preparation raised the pH from 6.2 to 6.4, and the presence of 10 mM kg⁻¹ LMWOA resulted in a pH decrease to 6.1 and 5.6 for citric acid and oxalic acid, respectively. Acidification increased with increasing doses of LMWOAs, with the exception of the Tayport soil incubated with citric acid, in which pH increased at doses up to 4 mM kg⁻¹ and decreased up to 10 mM kg⁻¹ (Table 3).

Inorganic P in water extracts of the Glensaugh soil was below the detection limit. For the purposes of calculating the P_o concentrations in extracts, P_i was assumed to be equivalent to the detection limit of the colorimetric assay (0.13 mg kg⁻¹). In the Glensaugh soil, citric acid did not affect P_{water} and P_{olsen} while oxalic acid caused a 30% increase in P_{water} and a 15% depletion in P_{olsen} at 10 mM kg⁻¹ dose (Table 3). In the Tayport soil both LMWOAs induced a significant increase in P_{water} and P_{olsen} . The effective concentration (P_E) was 29% and 24% greater in the Glensaugh soil incubated with 10 mg kg⁻¹ citric and oxalic acid, respectively, compared to the 0 dose (Figure 1A and B). In contrast, P_E in the Tayport soil increased by 49% and 31% when incubated with 10 mg kg⁻¹ citric and oxalic acid, respectively. The proportion of P_E resupplied from the soil solid phase ($P_E - P_{DET}$) decreased continuously with increasing LMWOA doses, with the exception of the Tayport soil incubated with oxalic acid, where this increase tended to a plateau from 2 to 10 mM kg⁻¹. Citric acid caused a greater increase in P_E than the oxalic acid at similar doses.

Both soil solution concentration (P_{DET}) and the time-averaged solution concentration at the surface of the DGT device (P_{DGT}) continuously increased in response to increasing LMWOA doses (Table 3). Nevertheless, the slope of this increase was much more pronounced for P_{DET} values in comparison to P_{DGT} . The proportional P_{DET} response (as % of 0 mM kg⁻¹) to LMWOAs was more pronounced in the Tayport soil than in the Glensaugh soil. The latter showed a similar response of P_{DET} (also P_{DGT} and P_E) to citric and oxalic acids, whereas the Tayport soil had a greater response to the citric acid treatment for these parameters. In general, the proportion of P_o in the Glensaugh soil extracts was not altered

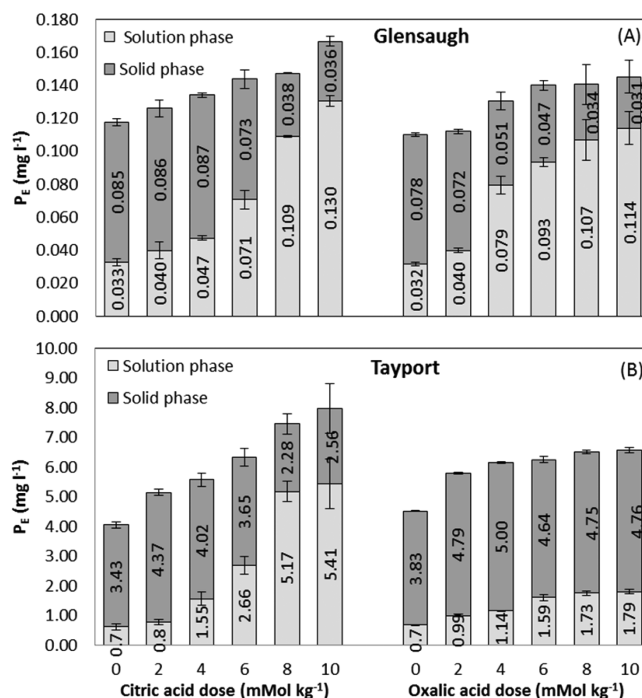


Figure 1. Effective phosphorus concentration (P_E) in soils as affected by increasing citric and oxalic acid doses to Glensaugh (A) and Tayport (B) soils. The soil solution phase (P_{DET}) and solid phase ($P_E - P_{DET}$) contributions are detailed in light and dark gray, respectively. Error bars represent \pm one standard deviation.

by addition of LMWOAs, indicating a similar solubilization of inorganic and organic P. In contrast, the proportion of P_o measured by DGT and DET in the Tayport soil tended to decrease in response to increasing LMWOA doses, indicating a significant solubilization of inorganic P in this soil. No P mobilization was detected at doses under 1 mmol kg⁻¹ in water extract, DGT, and DET measurements, for both soils and organic acids.

Phosphorus Desorption and Resupply from Solid Phase. The ratio of P_{DGT} to P_{DET} (R) corresponds to the contribution of both P_i diffusion through soil pores and the replenishment of pore water P_i due to its desorption from solid phase (Table 1). To isolate the contribution from solid phase, the relative contribution of diffusion (R_{diff}) was estimated using the DIFS model by artificially setting diffusion only conditions. R_{diff} was equivalent to 0.035 in the Tayport soil and 0.054 in the Glensaugh soil. The difference between R and R_{diff} represents a quantitative measure of soil P_i resupply from the solid phase, relative to the initial solution concentration. Since R_{diff} is constant for each soil, the $R - R_{diff}$ values rapidly decreased in response to the decreasing R values, which were associated with increasing LMWOAs doses (Figure 2A). The $R - R_{diff}$ values tended to stabilize to a minimum of ~ 0.015 at LMWOA doses over 8 mmol kg⁻¹ with the exception of Tayport soils incubated with oxalic acid, which showed stable $R - R_{diff}$ values of ~ 0.1 across similar doses.

The K_d is the ratio between adsorbed-to-solution concentrations and, since solution concentration increased in response to LMWOAs, K_d values decreased continuously with increasing LMWOA doses (Figure 2B). This pattern reflects a displacement of the equilibrium toward the solution phase, due to the P desorption induced by the LMWOAs. Due to the DIFS model assumptions, T_c responds to variations in R and K_d . For

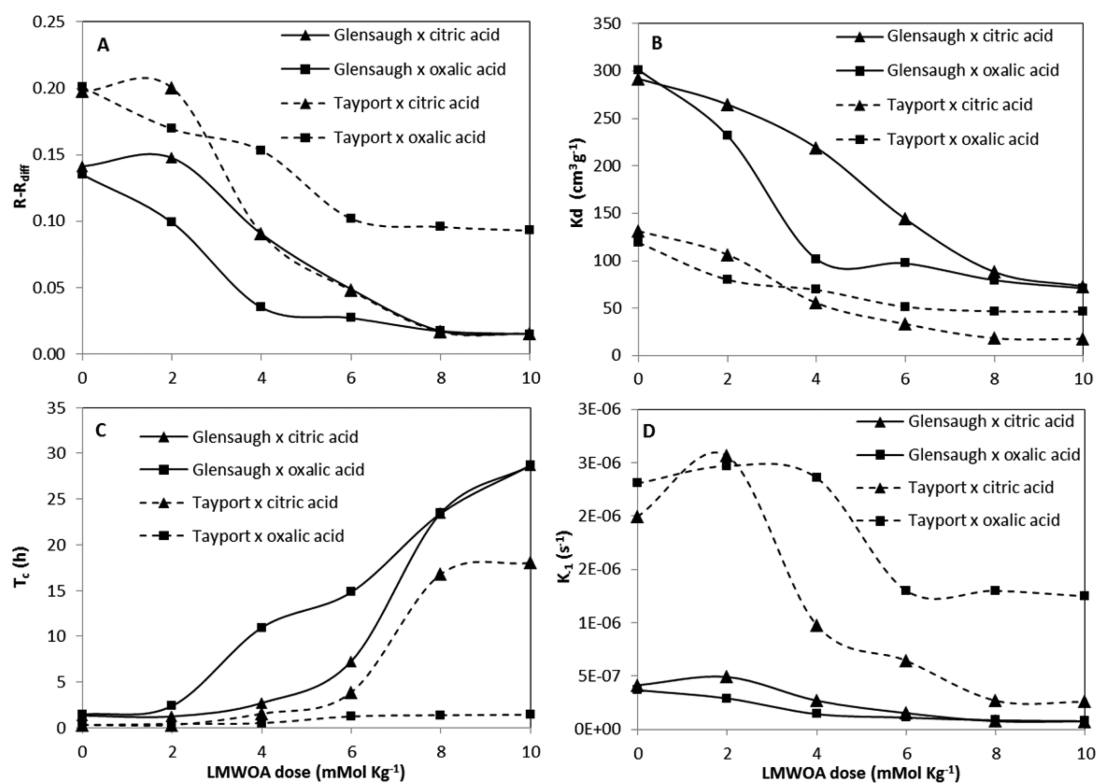


Figure 2. Effect of citric and oxalic acids on soil relative P resupply from solid phase ($R-R_{diff}$), resupply potential (K_d), Response time of the system (T_c) and desorption rate constant (K_{-1}). Model calculations were performed with average ($n = 3$) values for input variables.

example, T_c values are expected to exponentially increase with decreasing R values (Table 1). A sharp increase in T_c values at increasing LMWOA doses (Figure 2C) was observed, most likely in response to the sharp decrease in both K_d and R values for the same doses. In the Tayport soil, citric acid caused a more significant increase in T_c than oxalic acid. Little increase was observed for oxalate acid.

Desorption rate constants (k_{-1}) decreased in response to increasing LMWOA doses, mirroring the pattern observed in $R-R_{diff}$ values, with a lower decrease observed in the Tayport soil incubated with oxalic acid in comparison to the other combinations of soils and LMWOAs (Figure 2D).

Microbial Biomass P and Microbial Respiration Response to Organic Acid Addition. Microbial biomass P was investigated in order to evaluate if P mobilized by LMWOAs was simultaneously being fixed by soil microbes before the DGTs could accumulate them. At low doses of LMWOAs (0.5 to 1 mmol kg⁻¹), there was a steep increase in microbial biomass P in the Tayport soil (Figure 3A), but this effect was less pronounced in Glensaugh soil (Figure 3B). Curiously, this increase in microbial biomass P was not seen at doses higher than 4 mmol kg⁻¹ of citric acid, whereas doses of oxalic acid greater than 1 mmol kg⁻¹ led to a gradual decrease in microbial biomass P in both soils (Figure 3B).

Microbial respiration increased in response to LMWOA addition, and this increase was more pronounced with increasing incubation time (Figure 3C–F). The maximum respiration response for the Tayport soil incubated with citric acid was reached at doses of 4 mmol kg⁻¹ (Figure 3C), while for the Glensaugh soil the respiration rates seemed to continuously increase with increasing citric acid doses until 8 mmol kg⁻¹ (Figure 3D). When oxalic acid was added, maximum respiration was reached at doses of 2 mmol kg⁻¹

for both soils at all times (Figure 3E and F). Analysis of variation indicated that the microbial respiration significantly increased ($p < 0.05$) in response increasing LMWOA doses in both soils, nevertheless, a significant ($p < 0.01$) effect of the soil type on microbial respiration was only seen after 6 h of incubation (Table S1).

When a fresh labile carbon source (such as our used LMWOAs) is added to soils, the increase in microbial respiration cannot be entirely attributed to the added C, but a fraction of this comes from the pre-existing soil carbon. This phenomenon is commonly known as a priming effect. In order to estimate the degradation of the LMWOAs in soils, 90% the cumulative respiration rates were assumed to be coming from the freshly added organic acids and 10% from the priming of pre-existing labile soil carbon based on existing literature.²⁴ The effective LMWOA dose was calculated as the difference between the added dose minus the estimated LMWOA loss by respiration, for the both soils incubated with both the organic acids at increasing doses (Figure 4).

DISCUSSION

The pH changes in soils are expected to be affected not just by the low pHs of the added LMWOAs and their reaction in soils, but also to some extent due to the effect of the microbial community stimulated by both moisture and LMWOAs. Citric and oxalic acids are considered to be easily available carbon sources, acting as a substrate for microorganisms in soil.³ When reactive metals such as Fe and Al are present in the soil, the expected complexation reaction will release protons from the hydrated organic molecule, decreasing the pH. This pH decrease was observed as a general case, except at low citric acid doses in the Tayport soil, where the pH increased (Table 3). We speculate that this pH increase may have been due to a

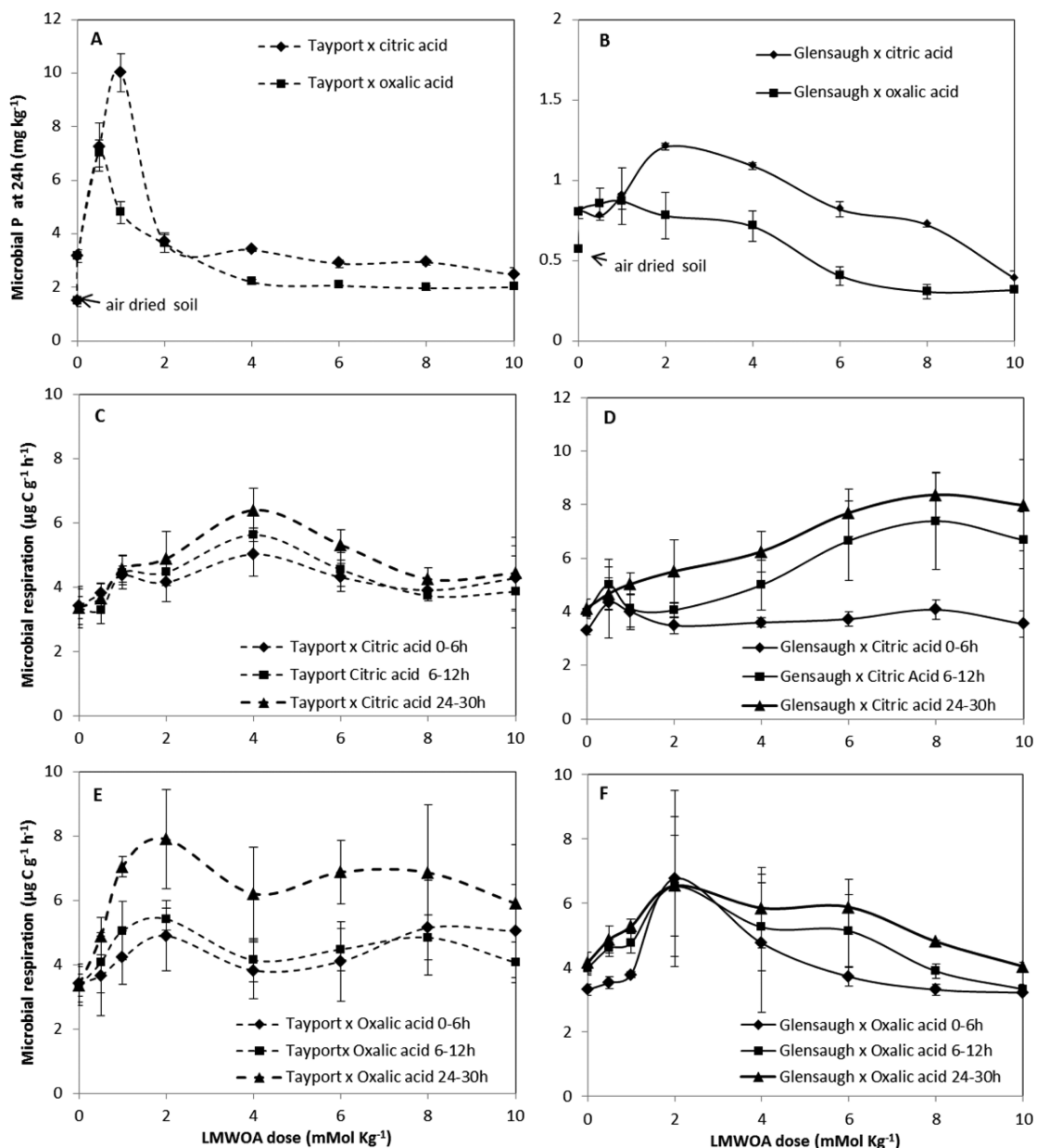


Figure 3. Microbial Biomass phosphorus (A and B) and microbial respiration (C, D, E, and F) response to increasing doses of low molecular weight organic acids (LMWOAs): citric and oxalic acid. Error bars represent \pm one standard deviation.

biotic effect as it was associated with a strong increase in the microbial biomass P and microbial respiration. The link between organic acid addition and pH increase, has been previously attributed to the microbial metabolism of organic acids into carbon dioxide.²⁹ The phenomenon does not occur at high citric acid concentrations most likely due to short-term (days) changes in microbial biomass and community function, which could also explain why this effect was not observed in the Glensough soil. We did not perform additional tests under sterile conditions or further characterization of additional microbial parameters to settle this subject.

Little is known about the exudation processes of LMWOAs from the roots of different plant species, regarding if these are exuded as acids or their salts. This is a fundamentally important issue when it comes to soil pH changes and their possible associated effects on P mobility. In our experiments the acid form of LMWOAs was used causing a pH decrease as expected.

Nevertheless, in a complementary experiment using the Tayport soil incubated with sodium citrate, soil pH showed substantial increases from 6.2 to over 8 after 3 days. This behavior may be explained by the citrate metabolism by soil microbes leaving the sodium hydroxide in solution.

The resulting pH change from LMWOA addition does not explain P mobilization as measured by the concentration of P in the soil extracts. The influx of P into the DGT device is a holistic representation of multiple interactions of the LMWOAs in soils including (a) the resulting soil solution equilibrium P concentration; (b) the kinetics of P desorption after soil solution is depleted; and (c) the response of soil microbes to both P and LMWOA concentrations (Figure 5). The greater accumulation of microbial P in Tayport soil is probably related to its higher concentration of available P_i in comparison to the Glensough soil. The nonlinear response of microbial biomass P and respiration to increasing LMWOA dose (mismatching the

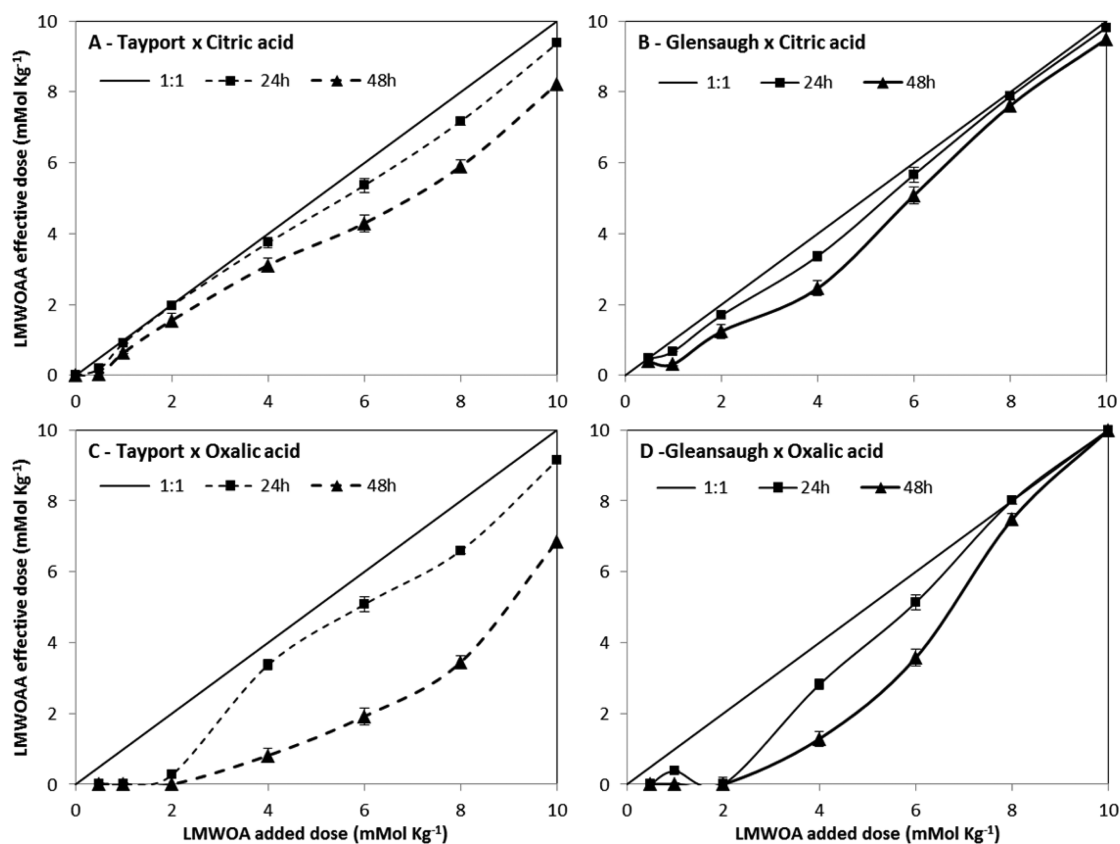


Figure 4. Estimation of effective low molecular weight organic acid (LMWOA) dose (added minus estimated degradation) based on cumulative measured microbial respiration response at beginning and end of DGT deployment (24 h and 48 h, respectively) for the Tayport and Glensnaugh soils incubated with increasing doses of citric and oxalic acids.

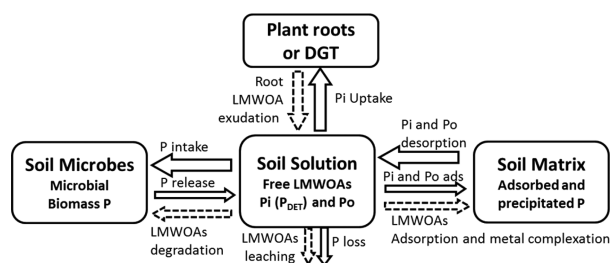


Figure 5. Conceptual model of the effect low molecular weight organic acids (LMWOAs) on the desorption/solubilization of soil P, microbial biomass P and plant P uptake. Dashed arrows represent the movement of LMWOAs and solid arrow represent movement and redistribution of soil phosphorus.

linear P_{DGT} and P_{DET} increase) suggests that the microbial responses are nonstoichiometric, in other words, independent of the C:P ratios. The amount of carbon added could only cause marginal increases in the C:P or C:N ratios, nevertheless these are considered carbon sources readily available to the soil microbes when compared to the existing soil organic carbon. In this study it is hard to quantify the extent to which the P mobilizing effect is chemical (metal chelation and competition for P adsorption sites) or indirectly biological (changes caused by the stimulated microbial community). Nevertheless, our data suggest that at low LMWOA doses the microbial component is dominant while at higher doses the chemical component is prevailing. No P losses are expected in our incubations; nevertheless, they may occur in natural systems in response to a higher solution P concentration.

It is known that soil microbes play a major role in redistributing and changing soil P fractions.³⁰ The microbial biomass P substantially increased as a result of low LMWOA doses in the Tayport soil, but not in the Glensnaugh soil. Since this P mobilization was only seen on the microbial biomass and not on DGT and DET extracts, it is fair to speculate that some of the mobilized P by the lower doses of LMWOAs may have been sequestered by soil microbes, and remained relatively unavailable to plants. Curiously, the effect of LMWOAs on boosting microbial biomass was not observed at higher doses. Additionally at lower doses nearly all of LMWOAs may have been degraded by soil microbes at 24h incubation (Figure 4), nullifying any chemical effects on P desorption and solubilization. The addition of phytochemicals, either from plants or as synthetic exudate mixtures, has been shown to affect the composition and functions of microbiota in soils.^{31–33} In addition to the potential changes in microbial community function due to the addition of citric and oxalic acids, other conceivable causes for the unexpected decrease in microbial biomass P and respiration with high LMWOA doses may be the possible release of adsorbed and precipitated compounds toxic to microbes, or the sequestration of micronutrients through LMWOA complexation reactions in soil. Further studies are needed to understand the underlying causes of this behavior.

The effective concentration of P (P_E ; calculated from the P_{DGT}) is a much smaller pool than the P_{water} and P_{Olsen} , and it is thought to be a better representation of plant P bioavailability than other indicators such as the P_{Olsen} .^{13,15} This concentration is equivalent to the sum of solution P_i concentration and the desorbable/solubilizable P determined through the deployment

of the DGT device and its disturbance of equilibrium between solid and solution P concentrations. Citric acid caused a greater increase in P_E , P_{DGT} , and P_{DET} than oxalic acid at the same doses. This effect was expected based on previous comparisons of tricarboxylate and dicarboxylate compounds in soils³⁴ and synthetic minerals.¹¹ On the other hand, this response is not universally seen across different soils due to the differential effect of organic acids on P mobilization in soils with contrasting pH and abundance of metal-P precipitates.^{8,35} Citric acid is a better chelator of Fe and Al while oxalic acid is a better chelator of Ca.⁸ In a similar case study to our Glensaugh podzolic soil, citrate addition led to a cumulative desorption of P, which was equivalent to a 20 times increase in the rate of P desorption and was closely related to the release of Fe+Al.³⁶

Considering the pool partition hypothesis illustrated in Zheng and Zhang (2012),³⁷ our data showed that in the Tayport soil, the LMWOAs not only induced the replenishment of the soil solution concentrations but led to an increase in the lability of fixed P as reflected by the increase in P_{Olsen} concentrations.³⁷ In the case of the Glensaugh soil, the incubation with LMWOAs causes a depletion of the labile P pool in the soil, reflected by the unchanged or reduced P_{Olsen} . Even in cases where the P_{Olsen} is increased, this response was not as steep as the increase in the solution concentration, and in all cases, the distribution coefficient K_d decreases with increasing LMWOA doses. Similarly, P_{DET} increase was much greater than P_{DGT} increases, and R values decreased with increasing LMWOA doses. This behavior is opposite to the expected pattern of interdependency between P_{DET} and P_{DGT} when no organic added LMWOAs are added, where R ratios increase at greater P_{DGT} values.¹³ Since R_{diff} is constant for each soil, a sharp decrease in P resupply from solid phase ($R-R_{diff}$) at increasing LMWOA doses was also observed, reflecting the imbalance in the adsorbed-to-solution ratio. The decrease in K_d and $R-R_{diff}$ does not indicate a decrease in P bioavailability with increasing LMWOA doses, but only that the increase in bioavailable P (P_{DGT} and P_E) is not as steep as the increase in solution P concentration (P_{DET}) due to the reduction of the P desorption.

The doses at which the LMWOAs had an effect on soil P desorption were at least 4 fold greater than what we previously considered as a high rhizosphere LMWOA concentration. Our expectations of what would be high values of LMWOA rhizosphere concentrations ($\sim 0.5 \text{ mmol kg}^{-1}$) were calculated based on maximum in vitro root exudation³⁸ and in situ organic acid measurements.^{39,40} Rhizosphere concentrations of LMWOAs are a product of the spatial distribution of rates of root exudation and their fate thereafter: adsorption/desorption, diffusion and ultimately their degradation by the microbial community and extracellular enzymes (Figure 5). The presence of root exudation hotspots in the rhizosphere is well-known, and in these hotspots, the concentration of organic acids would be much higher than in the bulk media. Therefore, rhizosphere LMWOAs concentrations at high exudation hotspots on the order of 2–10 mmol kg^{-1} , however unlikely, are still within reason.

The increase in respiration rates measured after addition of LMWOAs was sufficient to cause a significant effective dose reduction at 24 and 48h. Oxalic acid was completely degraded at doses up to 2 mmol kg^{-1} in both soils, and further doses were significantly reduced by microbial respiration. Citric acid degradation was much lower than oxalic acid, since it has 3 fold more carbon atoms for the same molar base dose. Lower

overall degradation in addition to a greater binding capacity of citrate with soil metals could explain the overall larger effect of citric acid in mobilizing soil P in comparison with oxalic acid. Further studies are needed to clarify what LMWOA concentrations are likely to occur in the rhizosphere of different plant species in order to accurately frame the results of this and other similar studies. Importantly, this study identifies the critical thresholds of organic acid concentration necessary to mobilize sorbed and precipitated P and control the partitioning of P between biotic (microbe, plant) compartments in soil.

It is commonly hypothesized that the direct exudation of organic acids into soil (from root and soil microorganisms) is related to a nutrient mobilization mechanism, which primarily affects poorly soluble P in complex with metals, minerals and organic matter.^{8,14} Our study supports the hypothesis that low rates of exudation of citric and oxalic acid are not related to a P mobilization mechanism, but the stimulation of microbial activity in the rhizosphere,² which may have many indirect implications on nutrient uptake by plants (positive or negative). Considering our case studies, only conditions of high organic acid exudation (e.g., proteoid roots of lupins) would be able to overcome the degradation of LMWOAs observed at low LMWOA doses and directly contribute to the mobilization of plant-available P from the soil solid phase.⁴¹

In a nutshell, the simultaneous study of the influence of increasing organic acids doses on P desorption kinetics and the partitioning of P between microbes and plant compartments in soil allowed a holistic understanding of the complex interactions and multiple regulations involved in rhizosphere P dynamics. Citric and oxalic acids, common root exudates, induced a strong mobilization of soil P, but only at doses considered to be much greater than those found in most rhizospheres. After soil equilibration with these organic acids, a strong reduction in the adsorbed-to-solution distribution coefficient (K_d) and desorption rate constants (k_{-1}) was observed whereas the response time of solution P equilibration (T_c) increased. Our study suggests that low LMWOAs concentrations in soils ($< 2 \text{ mg kg}^{-1}$ soil) which have a limited direct effect on plant available P concentrations in soils, may be quickly degraded through the activity of soil microbes and lead to the accumulation of microbial biomass P. Therefore, a critical concentration threshold may exist for the ability of LMWOAs to mobilize soil P for plant use, which will depend on an effective dose (LMWOA concentration after correcting for microbial degradation) as well as the capacity of soils to resupply P from the solid phase. In practice, this means that low rates of LMWOA exudation are not enough to cause significant alteration of plant available P in soils, indicating a resistance of the soil system to disturbances caused by citric and oxalic acids. So, in order for the plants to increase their P uptake from soil recalcitrant P sources, the roots must exudate enough organic acids to raise the LMWOA concentration above this critical threshold. This represents new insight on the organic acid regulation of the chemical–microbial phosphorus transformations in soils.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03017.

Citric acid dose survey using water extractable P as response variable, Kinetics of P desorption for citric and oxalic acids using water extractable P as response variable, Analysis of variance for soil type, low molecular weight organic acids type and dose(PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: d.blackburn@lancaster.ac.uk.

*E-mail: h.zhang@lancaster.ac.uk.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS AND SYMBOLS

P_i	Inorganic phosphorus
P_o	Organic phosphorus measured as the difference between total and inorganic P
DGT	Diffusive gradients in thin films using a ferrihydrite containing gel as a P binding layer
DET	Diffusive equilibration in thin films (same DGT setup without the binding layer)
DIFS	'DGT Induced Fluxes in Soils and sediments' model
P_{DET} (mg l ⁻¹)	Soil solution (dissolved) P concentration determined using DET
P_{DGT} (mg l ⁻¹)	DGT measured time average soil solution P concentration at the surface of DGT device
P_E	Effective P concentration – DGT estimated soil solution P + labile P concentration from the solid phase
P_{Olsen} (mg kg ⁻¹)	Phosphorus concentration (solid phase) measured using NaHCO ₃ extraction
D_0 (cm ² s ⁻¹)	Diffusion coefficient in diffusive layer of DGT device
D_s (cm ² s ⁻¹)	Diffusion coefficient in soil
k_{-1} (s ⁻¹)	Desorption rate constant
k_1 (s ⁻¹)	Sorption rate constant
K_d (cm ³ g ⁻¹)	Equilibrium distribution coefficient between solid phase and soil solution
R	Ratio of P_{DGT} to P_{DET}
R_{diff}	Ratio of P_{DGT} to P_E in the case where there is no P resupply from the solid phase, estimated using DIFS for diffusion only case
T_c (s)	Response time of (de)sorption process

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