Title: Microbially-mediated P fluxes in calcareous soils as a function of water-extractable phosphate

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Abstract

Soil phosphorus (P) tests are designed to indicate plant-available inorganic orthophosphate (P_i), but fail to account for P_i that may become available through organic phosphorus (P_o) mineralization. This P source may be especially important in soils with low concentrations of solution and labile P_i . We assessed gross P_o mineralization and immobilization using labeling with ^{33}P in four calcareous Alfisols with varying concentrations of Olsen soil test P that were collected from forage fields of dairy farms in Ontario, Canada. Rapid microbial ^{33}P uptake during incubation was found for the soils with the lowest available P_i as indicated by both Olsen soil test P and water-extractable P_i . The tracer incorporation into microbial P after 8 days ranged from 7 to 44% of applied ^{33}P and was negatively related to water-extractable P_i following a power-type relationship. As concentrations of microbial P were similar in all soils, this suggests faster turnover of P in the microbial biomass at water-extractable P_i below $0.1 \text{ mg P kg}^{-1} \text{ soil}$. Daily gross P_o mineralization rates ranged from 0.2 to $2.8 \text{ mg P kg}^{-1} \text{ soil d}^{-1}$ and contributed 7 to 56% of the isotopically-exchangeable P in 8 days. Based on these findings, microbial processes have the potential to make a significant contribution to forage P nutrition.

1.0 Introduction

A thorough understanding of processes that govern phosphorus (P) availability in soils is important as too little available orthophosphate (P_i) can limit crop yields, while excess P_i can result in off-site environmental degradation (Condon, 2004). P availability has generally been considered to be controlled by physical and chemical processes including sorption-desorption and precipitation-dissolution; however, biologically driven processes including organic P (P_o) mineralization and microbial P_i immobilization can also have a significant effect on P_i availability (Frossard et al., 2000; Bünemann, 2015). The ability of the microbial biomass to contribute to

plant available P depends on the quantity of the microbial P pool and its turnover time (time for a nutrient pool to completely renew itself) (Oberson and Joner, 2005).

Determination of the contribution of P_o mineralization to plant-available P would be useful, especially in systems with low concentrations of available P_i . For example, organic agricultural production systems often have low indices of plant-available P (Entz et al., 2001; Gosling and Shepherd, 2005; Oberson and Frossard, 2005; Knight et al., 2010). At the same time, the ability of commonly used soil tests to accurately predict plant-available P has been questioned because they do not account for biological contributions to plant-available P_i (Condrón, 2004; Fortune et al., 2005; Roberts et al., 2008; Nash et al., 2014). It can be assumed that relative to physico-chemical processes, P_o mineralization plays a greater role in contributing to plant-available P in systems that are low in solution P_i .

Organic P mineralization is difficult to measure due to the high reactivity of dissolved phosphate anions, which are often rapidly sorbed to soil surfaces following their release from organic matter (Frossard et al., 1996; López-Hernández et al., 1998). In the past, the measurement of total soil P_o over time was used to infer whether net mineralization occurred (Condrón et al., 2005), but extended time periods are needed for differences to become detectable. This method also does not allow for the detection of the simultaneous processes that are occurring (i.e. P_o may be mineralized and subsequently sorbed to soil constituents and/or immobilized again by soil microorganisms). Isotopic dilution techniques using either ^{32}P or ^{33}P have been developed to measure gross P_o mineralization under steady state conditions (López-Hernández et al., 1998, Oehl et al., 2001a). This is done by pairing a short-term (typically 80-100 min) Isotopic Exchange Kinetics (IEK) experiment with a longer term (several days or more) incubation experiment. The IEK experiment uses carrier-free radio-labeled P (e.g. $^{33}P_i$) and is based on the

assumption that unlabeled soil $^{31}\text{P}_i$ and labeled $^{33}\text{P}_i$ have the same fate in the system (Frossard et al., 2011). From the IEK experiment, a baseline is established which estimates the amount of isotopically exchangeable P (termed the *E*-value) due to physico-chemical processes. Using equations derived by Fardeau et al. (1985), *E*-values are extrapolated to calculate how much P_i would become available over a longer period of time. In a longer term incubation experiment, however, some of the P_i entering the soil solution results from biological mineralization processes which release unlabeled $^{31}\text{P}_i$ and thus lower the specific activity (SA), i.e. the ratio of $^{33}\text{P}_i$ to $^{31}\text{P}_i$ of the soil solution, providing a higher *E*-value than the one extrapolated from IEK data. Gross P_o mineralization is thus derived from the difference between measured and extrapolated *E*-values.

In addition to gross P_o mineralization, ^{33}P uptake by the microbial biomass can be measured using isotopic techniques (Oehl et al., 2001b). Assuming that soil solution P_i is the sole source of microbial P uptake, microbial P_i immobilization is calculated from the SA of the microbial biomass, the SA of the soil solution and the amount of microbial P (Bünemann et al., 2007). Net P_o mineralization can then be determined by subtracting microbial P_i immobilization from gross mineralization. Oehl et al. (2001b) found that P cycled faster through the microbial biomass in soils under biodynamic and organic management when compared to soil under conventional management. Achat et al. (2009a) and Bünemann et al. (2012) found rapid microbial P uptake under P-limited conditions, suggesting that microbial P uptake and subsequent turnover may occur more quickly in soils low in available P_i .

Bünemann et al. (2012) calculated gross P_o mineralization rates in grassland soils from a long term fertilization experiment in Switzerland and found that it was mainly governed by available P_i via a stimulation of microbial P_i immobilization at low P_i availability. This finding is probably

related to the fact that external P_i concentrations affect the expression of P uptake and transport genes in soil microorganisms (Saleh-Lakha et al., 2005). In a recent review, Bünemann (2015) outlined the need for P_o mineralization studies using high P-sorbing calcareous soils with low soil solution P_i concentrations. Here, we present microbially-mediated P transformations in calcareous Alfisols obtained from forage fields in Ontario, Canada, as assessed using isotopic techniques across a gradient of available P_i . Our main objective was to explore the effect of available P_i , as indicated by both Olsen soil test phosphorus (STP) (Olsen et al., 1954) and water-extractable P_i (P_w) concentrations on P_o mineralization and microbial P uptake.

2.0 Materials and Methods

2.1 Soil sampling

Soils from four different forage fields from southwestern Ontario dairy farms were used in this experiment. The soil samples had been collected as part of a larger study (Schneider, 2014), which related Olsen STP and other soil properties to total forage yield and P uptake on paired organic and conventional dairy farm soils. The soils were all Grey-Brown Luvisols according to the Canadian System of Soil Classification (Hoffman and Richards, 1952; 1954; AAFC, 1998) and Alfisols according to the American soil taxonomy (Soil Survey Staff, 2014), and Luvisols according to the Food and Agriculture Organization World Reference Base (FAO, 2015).

In this study, soils from two organic (soils 1 and 2) and two conventional (soils 3 and 4) dairy farms were selected in order to provide a gradient of P_i availability. The soils (0-15 cm) were sampled from second or third year forage fields dominantly comprised of *Medicago sativa* and *Phleum pratense*. The forage fields ranged in size from 4-20 ha and were part of a regular crop rotation that also included annual crops. Additional information on farm management practices for each of the fields can be found in Supplementary material A. The

Olsen STP concentrations of soils used in this study ranged from 2.2 to 13.7 mg P kg⁻¹, total organic carbon ranged from 21 to 27 g kg⁻¹ and pH ranged between 7.2 and 7.5 (Table 1). After initial collection and sieving (2 mm), soils were frozen (-20°C) until needed for the incubation study. From an initial 12 soil samples collected separately per field (3 from each of 4 sub-plots), 4 composite samples were made representing each field at the sub-plot level.

2.2 Experimental Design

The experiment consisted of two parts: a short-term (100 min) isotopic exchange kinetics (IEK) experiment and a longer 7-d incubation experiment. The IEK experiment allowed the establishment of isotopic dilution over time as a result of physico-chemical processes. The longer incubation experiment with ³³P-labeled soil provided the measurement of isotopic dilution due to both physico-chemical and biological processes at two sampling times.

Before both experiments, the frozen soils were thawed at 4°C and then pre-incubated in the dark at 22°C for 28 d with gravimetric water content maintained at a level suitable for microbial activity (0.20-0.24 g H₂O g⁻¹ soil) (equivalent to ~65% field capacity, as estimated using the Soil Water Characteristics Hydraulic Properties Calculator version 6.02.74 [Saxton and Willey, 2006]). During this period, respiration was measured twice to confirm that the microbial activity was constant by the onset of the incubation experiment. This was necessary because basal gross organic P mineralization can only be determined under steady-state conditions (Oehl et al., 2001). Due to the time needed for the IEK experiments and the number of samples to process (n = 4 composite samples per field x 4 fields = 16) the incubation experiment was conducted in two sets. Each set contained half (eight) of the soils with two samples from each field.

2.3 Isotopic exchange kinetics (IEK) experiment

141 The IEK experiments were conducted in triplicate using unlabeled soil and were conducted in
 142 batches on day 5 and day 6 of the incubation experiment for each of the two sets of soils. The
 143 details of IEK methodology have been described elsewhere (Fardeau et al., 1985). Briefly, a
 144 soil:solution ratio of 1:10 was created by weighing moist soil equivalent to 10 g dry soil into 250
 145 mL centrifuge bottles (Nalgene) and adding nanopure water to bring the total volume (including
 146 soil water) to 99 mL. The soil solutions were brought to a steady state by shaking end-over-end
 147 for 16 h, after which the solutions were placed on magnetic stir plates and mixed for 10 min
 148 before the addition of 1 mL carrier-free (no ^{31}P) ^{33}P solution (Hartmann Analytics GmbH,
 149 Braunschweig, Germany) to start the experiment ($t = 0$). The target amount of initially added
 150 radioactivity (R) was 167 kBq (actual range was 164 -178 kBq). While maintaining constant
 151 stirring, ~ 5 mL of each solution was sampled at 1, 4, 10, 40, 60 and 80 minutes after the addition
 152 of the tracer and immediately syringe-filtered through a 0.2 μm cellulose acetate membrane. The
 153 concentration of P_i in the filtered solution (C_p) was determined colorimetrically at 610 nm using
 154 the malachite green method and a 1 cm cell path (Ohno and Zibilske, 1991). The radioactivity (r)
 155 in the filtrate was measured by liquid scintillation counting (2500TR, Packard Instrument
 156 Company Inc., Meriden, CT).
 157 From the IEK experiment, the following parameters were determined: 1) P_w concentration in the
 158 filtered soil extract (mg P kg soil^{-1}), where $P_w = 10 (\text{L kg}^{-1}) * C_p (\text{mg L}^{-1})$; 2) coefficients m and
 159 n , which are determined by nonlinear regression and account for the immediate and slow
 160 physico-chemical reactions, respectively, that describe the rate of decrease of radioactivity in soil
 161 solution with time (Fardeau et al., 1985; Achat et al., 2009b), and 3) the amount of isotopically
 162 exchangeable P during the first minute of the batch experiment (E_{Imin}) in mg P kg soil^{-1} ,
 163 determined as P_w / m .

Contrary to the common assumption that P dynamics during the short term batch experiment are controlled by physico-chemical processes only, Bünemann et al. (2012) found extremely rapid P uptake by the microbial biomass in a soil low in P_w , necessitating the use of $HgCl_2$, a known microbial inhibitor, during the IEK experiment. Therefore, using select samples of each of the four soils, a pre-test with $HgCl_2$ additions was made to determine whether potential rapid microbial P uptake had any significant effect on IEK parameters over the 80 min of the test (as described by Bünemann et al. 2012). There were no significant differences in water-extractable ^{31}P or ^{33}P due to $HgCl_2$ addition. Therefore, the use of a microbial inhibitor during the IEK was deemed unnecessary for these soils.

The temporal development of radioactive P remaining in a soil-solution system at steady state is described by Fardeau et al. (1985) as:

$$r_{(t)}/R = m * (t + (m)^{1/n})^{-n} + r_{inf}/R \quad (1)$$

where $r_{(t)}$ is the radioactivity remaining in soil solution of the initial amount added (R) after the time (t) of isotopic exchange (kBq) and r_{inf}/R is the maximum possible dilution of the isotope in solution at $t = \infty$ which is approximated by the ratio of P_w to total P_i (in our study calculated as total soil P minus total NaOH-EDTA extractable P_o). The parameters m and n are soil specific constants obtained from nonlinear regression as described above. The amount of isotopically exchanged P at a given time t , i.e. the E -value ($E_{(t)}$), is then calculated from $r_{(t)}/R$ as the inverse of the specific activity in solution:

$$E(t) \text{ (mg P kg}^{-1}\text{)} = P_w / (r_{(t)}/R) \quad (2)$$

under the assumption that the specific activity of isotopically exchanged P in solution is identical to the specific activity of isotopically exchanged P in the whole system.

2.4 Incubation experiment

In parallel with the short-term IEK experiment, a 7-d incubation experiment was conducted. A seven day incubation was selected as recommended by Oehl et al. (2001b), to limit effects of re-mineralization of ^{33}P -labeled microbial P. The soils were extracted at two sampling times and analysed for P_i in solution and in the microbial biomass. The ^{33}P in both pools was also determined to allow the calculation of gross P fluxes as well as to gain an indication of the rate of microbial P uptake and release. All analytical measurements were conducted in duplicate.

To label each sample, the equivalent of 190 g oven-dry soil was weighed into a bowl, five mL of a ^{33}P (as phosphoric acid) labeling solution was added, and the soil was mixed manually (with a spoon) for 5 min. Labeling resulted in a concentration of 2.49 (set 1) and 2.72 (set 2) kBq g^{-1} soil and increased the soil moisture content slightly to values ranging from 23 to 28% (~75% field capacity). Two portions equivalent to 10 g dry soil each were removed for the concurrent measurement of soil respiration in order to assess if respiration remained steady over the 7-d incubation. The remainder was weighed into plastic containers and incubated at 22°C . The moisture content was kept constant throughout the 7-d incubation.

The labeled soil was sampled (weighed and extracted) on day 0 (~7 h after labeling) and on day 7. Samples were analysed for ^{31}P and ^{33}P in P_w and in microbial P. All radioactivity measurements were corrected for decay to the time of soil labeling. The extraction period of 16 h was added to the sampling time point, since isotopic exchange continues during extraction (Bünemann et al., 2007). Therefore, all results from the incubation experiment are shown for 1.2 (day 1) and 8.1 d (day 8) after labeling.

In a pre-test, different methods were compared for measuring soil microbial P. The method of Morel et al. (1996) using a pulse of chloroform fumigation followed by water extraction was

compared with the method of Kuono et al. (1995) using liquid fumigation with hexanol and simultaneous extraction with anion exchange membranes, and with a modified version using liquid hexanol fumigation with water extraction. All methods yielded comparable estimates of fumigant labile P_i and the trends among samples were consistent (data not shown).

In this study, we chose to use the water extraction with hexanol fumigation because it allowed us to use the P_w concentrations determined for the non-fumigated samples (the control samples) as the P_w concentrations required to calculate E -values. For this method, analytical duplicates, each containing moist soil equivalent to 2 g dry soil and 20 mL of nanopure water were placed in 50 mL centrifuge tubes. Fumigated subsamples received an additional 1 mL of hexanol. Two non-fumigated, spiked samples were included: the first received 25 mg P kg⁻¹ (³¹P spike) and the second 25 mg P kg⁻¹ plus ~ 9.4 MBq ³³P kg⁻¹ soil (³³P spike), in order to calculate the recovery for both ³¹P and ³³P. Results of pre-tests showed the recovery to be linear, thus one P spike addition (for each ³¹P and ³³P) was determined to be adequate. Samples were shaken horizontally for 16 h, after which they were filtered through 0.2 µm cellulose acetate filters and solution P_i was measured as described earlier (Section 2.3). Hexanol-labile P (P_{hex}), a direct measure of microbial biomass P was calculated as:

$$^{31}P_{hex} \text{ (mg P kg}^{-1}\text{)} = (^{31}P_{fum} - ^{31}P_{nonfum}) / ^{31}P_{rec} \quad (3)$$

where $^{31}P_{fum}$ and $^{31}P_{nonfum}$ are the amounts of ³¹P extracted from fumigated and non-fumigated subsamples, respectively, and $^{31}P_{rec}$ is the fraction of the ³¹P spike that is recovered in the soil solution after shaking. No conversion factor was applied to account for incomplete extraction of soil microbial P, as K_p factors have been shown to be soil specific and can vary widely (Oberson and Jöner, 2005; Yevdokimov et al., 2016) and have not been determined for these soils and this method.

The radioactivity was measured in the filtrate of the non-fumigated and fumigated samples as described earlier. The release of ^{31}P from microbial cells as a consequence of fumigation affects the recovery of the label in the soil solution due to isotopic exchange reactions (McLaughlin et al., 1988). In order to determine the amount of ^{33}P in the microbial biomass, it is necessary to correct for the release of ^{31}P from the microbial P pool (which will release ^{33}P from the soil solid phase). This is simulated by the addition of a ^{31}P spike which has been shown to increase the recovery of the label (Oehl et al., 2001a). An equation was established for each soil sample using the non-fumigated subsamples (no P addition) and the subsamples receiving a 25 mg P kg^{-1} (^{31}P spike):

$$^{33}\text{P}_{\text{nonfum+P}} (\text{kBq g}^{-1}) = a (^{31}\text{P spike (mg P kg}^{-1})) + b \quad (4)$$

where $^{33}\text{P}_{\text{nonfum+P}}$ represents the ^{33}P (in kBq g^{-1}) extracted from non-fumigated subsamples receiving the ^{31}P spike and where a and b are the slope and intercept, respectively. The amount of P_{hex} (in mg P kg^{-1}) was then inserted as ^{31}P spike into equation (4) and the resulting value of extracted ^{33}P was used to calculate the incorporation of ^{33}P in P_{hex} ($^{33}\text{P}_{\text{hex}}$) as:

$$^{33}\text{P}_{\text{hex}} (\text{kBq g}^{-1}) = (^{33}\text{P}_{\text{fum}} - ^{33}\text{P}_{\text{nonfum+P}}) / ^{33}\text{P}_{\text{rec}} \quad (5)$$

where $^{33}\text{P}_{\text{fum}}$ is the ^{33}P extracted from hexanol-fumigated subsamples, and $^{33}\text{P}_{\text{rec}}$ the recovery of the ^{33}P spike described above.

For the incubation experiment, E -values were calculated as the inverse of the specific activities measured in the water extract. To calculate cumulative gross P_o mineralization, E -values were extrapolated from the short-term batch experiment using equations (1) and (2) for each sampling time in the incubation experiment and subtracted from the respective measured E -values. As these soils are calcareous, it is possible that some of this difference could be a result of microbial solubilization of recalcitrant calcium phosphate minerals (Arcand and Schneider, 2005). While it

cannot be ruled out completely, we are assuming this contribution to be low to negligible, and that the difference between measured and extrapolated E -values is solely a result of gross P_o mineralization.

The quantity of P in a soil pool that has been derived from a labeled P source can be calculated from the specific activities of the pool and the source, and the amount of P in the pool (Fardeau et al., 1996). Assuming that P_w is the sole source of microbial P uptake, microbial P_i immobilization at each sampling point was calculated according to Bünemann et al. (2007) as:

$$P_i \text{ immobilization (mg P kg}^{-1}\text{)} = (SAP_{\text{hex}}/SAP_w) * (P_{\text{hex}}) \quad (6)$$

where SAP_{hex} (kBq μg^{-1} P) is the specific activity of hexanol-labile (microbial) P, SAP_w (kBq μg^{-1} P) is the specific activity in P_w , and P_{hex} is the amount of hexanol-labile (microbial) P (in mg P kg^{-1}). Net P_o mineralization was then derived as gross P_o mineralization minus P_i immobilization.

During the incubation experiment respiration was determined by trapping CO_2 released from 10 g of soil in 0.2 M NaOH (Alef, 1995) and precipitating it as SrCO_3 , followed by back titration of the unreacted NaOH with 0.2 M HCl, using phenolphthalein as an indicator solution. In all cases, the CO_2 was collected over a period of 7 days.

2.5 Statistics

Statistical analyses were performed using the statistical software program SAS ver. 9.2 for Windows (SAS Institute, Cary, NC, USA). In this study, available P_i in the soils was indicated by both Olsen STP and P_w , which were positively correlated for these soils ($r = 0.96$, $p < 0.0001$). Regression statistical methods were used to evaluate relationships between P flux parameters and P_w concentrations. Relationships were also analyzed for Olsen STP, but the data are not shown since results were similar to those obtained with P_w . After visual inspection of scatterplots, the SAS procedures Proc Glm or Proc Nonlin were used for linear and nonlinear

regression, respectively (Bowley, 2008). Using the SAS Proc Univariate procedure (Fernandez 1992; Bowley2008), homogeneity of error (residuals) was tested for each model. The residuals were also tested for normality as determined by the Shapiro–Wilk statistic. In the case of non-linear regression analysis, pseudo R^2 values were calculated according to the method of Efron (1978). Analysis of variance (ANOVA) was used to determine significant differences among the data using the Proc GlmSAS procedure (Bowley, 2008). A multiple mean comparison test was performed using Tukey’s HSD (honest significance difference) test. A type I error rate of $p = 0.05$ was used for all statistical evaluations.

3.0 Results

3.1 Isotopic exchange kinetics batch experiment

Water-extractable P_i (P_w) concentrations ranged from a mean of 0.03 (soil 1) to 0.24 (soil 4) mg P kg^{-1} , and $E_{1\text{min}}$ ranged from 0.40 (soil 1) to 1.62 (soil 4) mg P kg^{-1} (Table 2). The P_w and $E_{1\text{min}}$ concentrations were significantly correlated with previously measured Olsen STP concentrations ($r = 0.96$ and $r = 0.93$, respectively). The parameter m ranged from 0.09 (soil 1) to 0.14 (soil 4), while calculated n values ranged from 0.32 (soil 4) to 0.38 (soil 1) (Table 2). The full list of IEK parameters from individual soil samples can be found in Supplementary Material B.

3.2 Incubation experiment

Soil respiration during the last week of the pre-incubation was similar to that during the week of the incubation experiment (data not shown), indicating that the respiration rate had stabilized during pre-incubation and was not increased again by soil mixing during labeling. Mean CO_2 release rates for the four soils were not significantly different from each other, with mean respiration rates during the incubation experiment ranging from 13.1 to 17.1 $\text{mg C kg}^{-1} \text{ soil d}^{-1}$ (Table 2). The P_w and P_{hex} concentrations (Supplementary Material C) showed minimal variation

from the first sampling point (1 day after soil labeling) to the end of the incubation experiment (8 days after soil labeling), again supporting that the system was at a steady-state and in the absence of flush effects.

3.2.1 Amount of ^{33}P in different P pools

The proportion of the added ^{33}P that was detected in soil solution one day after soil labeling ranged from 0.4 to 1.7% (Figure 1). After eight days, this proportion had decreased slightly to 0.2 to 1.3%. At both sampling times, the recovery of ^{33}P in P_w was positively related to P_w concentrations ($p < 0.001$ for both day 1 and day 8). In the P_{hex} pool, recovery of the initially added radioisotope one day after soil labeling ranged from 1.4 to 32.4 % and increased to a range of 6.9 to 43.7 % after eight days. Soil 1, with the lowest P_w concentrations, had the highest recovery of ^{33}P in the P_{hex} pool (Figure 1). One day after soil labeling, this was the only soil with notably high (>20 %) amounts of the label present in the P_{hex} pool. Eight days after soil labeling, the negative relationship between the proportion of ^{33}P in the P_{hex} pool and the P_w concentrations was significant ($p < 0.0001$ [based on the log-log relationship where ^{33}P in $P_{\text{hex}} = -3.45(P_w) + 1.59$, $R^2 = 0.82$]), and 11 of 16 samples had $>20\%$ of the label in the P_{hex} pool (Figure 1). The SA of the P_{hex} pool was notably higher in three samples from soil 1 that had very low solution P_i concentrations (Figure 2, Table 3). After eight days, the SA of the P_{hex} pool had increased across the soils (Table 3) and the power-type relationship between the SA of the P_{hex} pool and P_w was significant with a rapid increase below P_w concentrations of $\sim 0.1 \text{ mg kg}^{-1}$ (Figure 2). Finally, the ratio of the SAP_{hex} to the SAP_w at day 8 was significantly greater for soil 1 than for the other three soils (Table 3).

3.2.2 P fluxes

For all 16 samples, measured E -values were greater than extrapolated E -values at both 1 and 8 d after labeling (Supplementary Material D; Table 4). Across all soil samples, gross P_o mineralization accounted for 4.2 to 60.9 % and 7.0 to 56.2 % of the total isotopically exchangeable P (E_{meas}) at 1 and 8 d after soil labeling, respectively. Daily gross P_o mineralization and P_i immobilization rates ranged from 0.2 to 2.8 and 0.5 to 1.6 mg P kg⁻¹ d⁻¹ across all soil samples, while daily net P_o mineralization rates were calculated to range from -0.8 to 2.4 mg P kg⁻¹ d⁻¹ (Supplemental Material, Table D1).

There were no significant differences found among soils for cumulative or daily gross P_o mineralization, P_i immobilization or net P_o mineralization (Table 4). However, when all 16 samples were included, regression analysis revealed a significant positive linear relationship between gross P_o mineralization and P_w concentrations for both day 1 ($p = 0.006$), and day 8 ($p = 0.0024$) (Figure 3). Gross P_i immobilization was not significantly related to P_w concentrations for either sampling day ($p = 0.779$ for day 1, and 0.057 for day 8) (Figure 3), while there was a significant positive linear relationship between net P_o mineralization and P_w concentrations for both day 1 and day 8 ($p = 0.0003$ and 0.0009, respectively) (Figure 3). Because the daily fluxes were calculated from the cumulative rates after 8 days, the relationships with P_w concentrations were similar to those at the 8 day sampling time and are therefore not shown.

4.0 Discussion

4.1 Isotopic Exchange Kinetics Parameters

Overall, the soils in this study were found to have relatively low P_w and E_{lmin} concentrations. Morel et al. (1992) observed that wheat yield was responding to P fertilizer in soils presenting E_{lmin} values less than 5 mg P kg⁻¹ soil. This observation was confirmed for other

crops by Galletet al. (2003). The E_{Imin} concentrations in this study ranged from 0.3 to 2.2 mg P kg⁻¹ soil and are similar to the range from an 18-year grassland study where 0.8 mg P kg⁻¹ was the E_{Imin} for a field which was not P-fertilized and 1.6 mg P kg⁻¹ was the E_{Imin} derived from a P-fertilized soil (Bünemann et al., 2012); however, the E_{Imin} concentrations found here are low compared to other studies of P_o mineralization using field crop soils, even in the absence of mineral P fertilization (e.g. Oehl et al., 2001b). Most of the soils in this study are categorized as low (<10 mg P kg⁻¹) in Olsen STP for the growing of perennial forages (OMAFRA, 2009); however, the fields they were collected from were providing satisfactory yields to the farmers that were above conventional county averages (Schneider, 2014).

The relationships between IEK parameters and soil P_w concentrations were in agreement with the literature. For example, the finding of a positive correlation of $m (r_{Imin}/R)$ with P_w is consistent with the results of other authors (e.g. Achat et al., 2009b). Accordingly, m was lowest in soil 1 and greatest in soil 4, while n was greatest in soil 4 and lowest in soil 1, which indicates the greatest fast and slow sorption in soil 1 and the least in soil 4 (Sen Tran et al., 1998).

4.2 Incubation experiment

4.2.1 ³³P recovery in the hexanol-labile (microbial) P pool

The percentage of the added ³³P remaining in soil solution was very low (range was 0.4 to 1.7%) for all soils after just 1 day of soil labeling, decreasing further by 8 days. These results support that the soils in this study probably have a strong P-sorption capacity, especially those with low solution Pi concentrations (Figure 1).

Soil 1 with the lowest P_w concentrations had the highest recovery of ³³P in the P_{hex} pool (Figure 1), with notably high (>20%) amounts of the label present in the P_{hex} pool already after one day.

370 After eight days, the negative relationship between ^{33}P recovery in the P_{hex} pool and
 371 P_w concentrations was significant and 11 of 16 subsamples had >20% of the label in the P_{hex} pool.
 372 The recovery of ^{33}P in the P_{hex} pool is in the upper end of recovery percentages reported by
 373 similar studies, where recovery of ^{33}P in the P_{hex} or microbial P pool in soils labeled with carrier-
 374 free $^{33}\text{P}_i$ have ranged from ~2 to 46% at comparable sampling times (7-8 d) (Achat et al., 2009a;
 375 Bünemann et al., 2004, 2012; Oehl et al., 2001b, 2004; Oberson et al., 2001). This potentially
 376 indicates a rapid and extensive uptake of solution P_i by the microbial biomass in these soils
 377 under steady state conditions.
 378 The proportion of label recovered in the P_{hex} pool may depend on the size of the microbial P
 379 pool. Some studies have found greater recoveries of ^{33}P in treatments that had the highest
 380 microbial biomass (e.g. Bünemann et al., 2004), but others have not (Bünemann et al., 2012). In
 381 this study, greater P uptake was not significantly related to the size of the microbial P pool ($p =$
 382 0.10 for linear regression) (Figure E1, in Supplementary Material E). In fact, some of the soils
 383 with the lowest microbial biomass had the greatest recoveries of ^{33}P in P_{hex} , indicating an
 384 increased rate of P uptake by the soil microbial biomass.
 385 The soils in the present study were collected from alfalfa-grass forage fields and some of the
 386 legume-derived organic matter may have helped to support rapid microbial P uptake. For
 387 example, Oberson et al. (2001) found more rapid uptake of added ^{33}P by the P_{chl} pool in soil from
 388 a grass-legume pasture, when compared to soil from native savannah and continuous rice
 389 systems. Oberson et al. (2011) stated that even in low available P soils, microbes are limited by
 390 C and N rather than P and that legume residues, by supplying N-rich organic matter, may help
 391 increase microbial P cycling. While this may help to explain some of the overall high levels of

microbial P uptake, it does not explain differences in ^{33}P uptake by P_{hex} pools across the soils, which appear to be inversely dependent on P_w concentrations.

The relationship between the amount of ^{33}P found in P_{hex} and P_w concentration follows a power type relationship and it appears that rather than a gradual development, there is a threshold P_w concentration (somewhere $< 0.1 \text{ mg P kg}^{-1}$) where a dramatic increase in the amount of label found in P_{hex} occurs (Figure 1). These results indicate that when soil solution P_i concentrations are very low, a rapid uptake of P into the microbial biomass occurs. Bünemann et al. (2012) also reported rapid P uptake by the microbial biomass for a P-limited soil (fertilized only with N and K). In their study, a microbial inhibitor was used during the 100 min IEK batch experiment to ensure that the P_i removal effects were solely from physico-chemical processes. In this study, the necessity of using a microbial P inhibitor was investigated during an IEK pre-test on composite soil samples and was determined to be unnecessary. However, the rapid tracer uptake observed by three soil samples (1a, 1b and 1c) in the incubation experiment after 1 day of labeling suggests that the use of a microbial inhibitor would have been desirable for these individual soil samples. Without the use of a microbial inhibitor, rapid ^{33}P uptake by the microbial P pool during the IEK experiment likely would have resulted in an overestimation of the extrapolated E -values, which would in turn result in an underestimation of gross P_o mineralization for these three samples. The possible effects of this on our results is discussed in the P fluxes section, however, it is evident that further research on the necessity and the practical use of microbial inhibitors (many of which are highly toxic) is needed.

The observed dramatic increase in microbial P uptake could be explained by a biological response of soil microbes to low P_w concentrations. It has been established that soil microorganisms have developed systems to assist them in P uptake, including the induction of

phosphate-specific transport systems, production of phosphatase enzymes, and promotion of the dissolution of insoluble P minerals (Saleh-Lakha et al., 2005). In this study, it is plausible that high-affinity phosphate transport systems were activated by low available P_i conditions, accelerating the uptake of P_i from solution and hence the uptake of the ^{33}P label. It would be informative for future studies to combine studies measuring gross P_o mineralization with molecular biology approaches to quantify gene expression and proteins related to microbial P uptake.

As discussed earlier, the relationship observed between ^{33}P incorporation into P_{hex} and P_w concentrations (Figure 1) supports that rapid P uptake is promoted under low available P_i conditions. If the microbial P (P_{hex}) pool remains constant (as was the case in this experiment), then re-mineralization of P_{hex} should be equal to the immobilization of P_i by the microbial biomass (Achat et al., 2009a). Thus, our results indicate that faster microbial P turnover occurs under conditions of low soil solution P_i concentrations. This is further supported by SAP_{hex} being negatively and linearly related to P_w concentrations ($R^2=0.42$, $p = 0.0068$; Figure 2) at the 8 day sampling time. Further, Oehl et al. (2001a) discusses how the convergence of the SAP_{mic} (represented here by SAP_{hex}) and SAP_w corresponds with the turnover time of microbial P. Here we found that the ratio of the SAP_{hex} : SAP_w for soil 1 was 0.77 after 8 days of incubation which was significantly greater than for the other three soils (Table 3).

However, it is interesting that despite the indication of an increased rate of microbial P turnover in soil 1 with the lowest P_w concentrations, we do not see differences in respiration rates among the soils. Thus, it is possible that high-affinity phosphate transport systems were upregulated (Saleh-Lakha et al., 2005), but did not require extra carbon costs. Further, P mineralization may not be as closely linked to C mineralization as N mineralization as a result of P being

mineralized by both biological and biochemical (enzyme driven) mineralization (McGill and Cole, 1981; Oehl et al., 2001a). In support of this concept, Fraser et al. (2015) have recently shown that alkaline phosphatase activity and *phoD* gene abundance are negatively related to soil available inorganic P concentrations.

The significance of a rapid uptake of P by the microbial biomass under low available P_i concentrations is not fully clear. Oehl et al. (2001b) suggest that a rapid P uptake could be an early sign of P deficiency for soil microbes. Faster microbial P turnover may also help to keep P in a more available form for plants, especially in high P-fixing soils (Oberson et al., 2011). Further research is needed to explore the significance of microbial P turnover rates in relation to plant-available P_i .

4.3 P fluxes

Gross P_o mineralization rates were found to range from 0.2 to 2.8 mg P kg⁻¹ d⁻¹, which is within the range of rates (0.1 to 12.6 mg P kg⁻¹ d⁻¹) determined in other studies using similar isotopic dilution techniques (Bünemann, 2015). P_i immobilization rates and net P_o mineralization rates were also close to the range of rates previously reported (Bünemann, 2015). Six out of the sixteen soil samples were calculated to have slightly negative net P_o mineralization rates, indicating net immobilization (Supplemental Material, Table D1). We acknowledge that calculated P_i immobilization and net P_o mineralization rates may be less accurate than gross P_o mineralization rates due to the fact that we did not apply a K_p factor to convert P_{hex} into microbial biomass P (Brookes et al., 1982). This could have resulted in the underestimation of microbial P_i immobilization and relatedly an overestimation of net P_o mineralization, making immobilization even more important (Bünemann et al., 2012).

Gross P_o mineralization was found to account for an average of 39.9% and 32.4% of the total isotopically exchangeable P (E_{meas}), which was on average 19.2 and 30.2 mg P kg⁻¹ soil after 1 and 8 days of soil labeling, respectively. Bünemann (2015) compared and summarized the absolute and relative contributions of physico-chemical and biological processes to total isotopically exchangeable P determined after 5-10 days of incubation from 6 different isotopic dilution studies. The proportions of isotopically exchangeable P_i derived from gross P_o mineralization in our study, are at the upper end of values reported for arable land but at the low end of those reported for permanent grassland sites (Bünemann et al., 2015). In the sole permanent grassland study, after 8 days of incubation, Bünemann et al. (2012) found gross P_o mineralization to account for 68% (65 mg kg⁻¹) and 51% (25 mg kg⁻¹) in non-P-fertilized and P-fertilized soils, respectively. Based on the high contributions of P_o mineralization to available P_i , the authors emphasized the importance of microbial processes in P dynamics in grassland soils. The soils in the present study were collected from second and third year perennial forage fields, and the significant biological contributions to total isotopically exchangeable P indicate that soil microbial P cycling is important in such forage systems. Our results also indicate that biological contributions accounted for a greater proportion of total isotopically exchangeable P when P_w concentrations were the lowest (soil 1). Soil 1 averaged an E_{meas} of 23.7 mg kg⁻¹ after eight days with gross P_o mineralization accounting for 10.2 mg kg⁻¹ (43%), which was a greater proportion than in the three other soils (range was 34-35%). More studies are needed to better delineate the relative contributions of gross P_o mineralization under different cropping systems and different soil test P concentrations.

4.3.1 Relationships of P fluxes with available P_i

Overall, regression analysis revealed that gross P_o mineralization increased with P_w concentrations (Figure 3). This was unexpected, since the literature suggests that gross P_o mineralization may be inversely related to P_w concentrations because enzyme activity and/or microbial P cycling may increase as available P_i decreases (Quiquampoix and Mousain, 2005; Oehl et al., 2001b). Reasons for the increasing trend of gross P_o mineralization with P_w concentrations are not fully clear; and we did not find a positive relationship between P_w and P_{hex} or P_w and P_{org} , which might support greater P_o mineralization at higher P_w concentrations. Although regression analysis found a positive relationship, as seen in Table 4, there were no significant differences in gross P_o mineralization across the four soils after 8 days of incubation. A larger sample size comprising a greater range of P_w concentrations might help to further understand the observed relationship. As discussed above (section 4.2.1), it is possible that gross P_o mineralization was underestimated in three of the soils with the lowest available P_i , which would weaken the positive relationship between gross P_o mineralization and P_w . Further, it appears that there could be a change-point at a P_w concentration $\sim 0.10 \text{ mg kg}^{-1}$ where below this concentration, gross P_o mineralization could be inversely related to P_w concentrations (Figure 3). However, more data points would be required to substantiate this trend.

Although not significant, a weak negative relationship between P_i immobilization and P_w was observed. This is in agreement with the rapid uptake of the label at low P_w concentrations (Figure 1). Bünemann et al. (2012) found gross P fluxes in grassland soils to be dominated by microbial P_i immobilization, especially in the absence of P fertilization.

One day after soil labeling, net P_o mineralization was shown to increase with P_w concentrations. This was expected as gross P_o mineralization increased and P_i immobilization decreased slightly with increasing P_w concentrations. If gross P_o mineralization was underestimated for samples in

the low end of the range of available P_i concentrations, then net P_o mineralization (gross P_o mineralization – P_i immobilization) would also be underestimated, potentially removing the positive relationship between net P_o mineralization and P_w concentrations.

4.5 The use of the isotopic dilution method in calcareous soils with low water-extractable P_i concentrations

In a recent review, Bünemann et al. (2015) discuss how very low P_i availability can impede the determination of IEK parameters needed to establish the baseline for isotopic dilution due to physico-chemical processes over time, and comment that more isotopic dilution studies using high-P sorbing acid or calcareous soils are needed. Low P_w concentrations run the risk of introducing more error as a small change in concentrations can lead to greater variation of extrapolated E -values (Bünemann et al., 2007). In this study, some soil P_w concentrations were very low, with about half of the samples being below the detection limit of 0.01 mg P L⁻¹ or 0.1 mg P kg⁻¹ for the malachite green method as determined recently (Randriamanantsoa et al., 2013). However, because many measurements were done per sample and at different points of the IEK and incubation experiment (for example see supplemental material Tables B1 and C1), we are convinced that our concentrations were consistently and reliably measured even at these low concentrations. Future isotopic dilution studies with low soil P_w concentrations could consider using a hexanol concentration method for determining phosphate in solution which has been recently described by Randriamanantsoa et al. (2013) and applied in an isotopic dilution study (Randriamanantsoa et al., 2015).

4.6 Conclusions

In this study, for the first time soil microbial P fluxes were explored across a gradient of water-extractable P_i concentrations in calcareous soils. The rapid incorporation of ³³P into the microbial

P pool (P_{hex}) indicates that important P uptake occurs under steady-state conditions and that this uptake is accelerated under low available P_i conditions. A very rapid uptake of the radioisotope, without changing microbial P concentrations, suggests an accelerated turnover of the microbial P pool when available soil P_i is low. Rapid microbial P turnover may help to provide P to plants under low P_i conditions, by promoting a rapid uptake and also release of P from the microbial P pools. Gross P_o mineralization was shown to release significant amounts of P, accounting for on average 32% of total isotopically exchangeable P at the end of the 8 d incubation experiment. By better understanding P_o contributions to plant-available P, more accurate soil P fertility recommendations can be made, thus promoting overall greater P-use efficiencies in agriculture.

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Table 1: Select soil properties and productivity indicators for the four field soils used in the study. Mean (standard deviation) of 4 subplots per field.

Soil no.	Soil Texture ^a	Total Organic C ^b (g C kg ⁻¹)	Total Inorganic C ^c (g C kg ⁻¹)	Total N ^d (g N kg ⁻¹)	pH ^e	Olsen STP ^f (mg P kg ⁻¹)	Total P ^g (mg P kg ⁻¹)	Organic P ^h (mg P kg ⁻¹)	Microbial C ⁱ (mg C kg ⁻¹)
1	silt loam/loam	20.7 (1.1)	4.1 (0.4)	2.2 (0.1)	7.4 (0.05)	2.8 (0.6)	684 (61)	216(32)	539 (30)
2	loam	27.3 (3.5)	4.5 (1.5)	2.6 (0.3)	7.2 (0.33)	5.5 (1.6)	781 (109)	222 (51)	744 (60)
3	silt loam	24.3 (1.2)	6.0 (1.0)	2.5 (0.1)	7.3 (0.17)	6.8 (3.3)	809 (81)	273 (23)	813 (137)
4	silt loam	23.8 (3.0)	6.9 (2.9)	2.1 (0.2)	7.5 (0.52)	11.2 (1.8)	729 (135)	145 (45)	522 (54)

^aParticle size distribution was measured by the pipette method (Sheldrick and Wang, 1993)

^bTotal organic C was measured using a Costech ECS4010 elemental analyzer (Costech Analytical, Valencia California) at the University of Saskatchewan Stable Isotope Facilities after the removal of inorganic carbonates by HCl fumigation for 48 hrs (Ramnarine et al., 2011)

^cTotal inorganic C was calculated as the difference between total C measured on bulk soil using a Costech ECS4010 elemental analyzer at the University of Saskatchewan Stable Isotope Facilities and total organic C measured as described above.

^dTotal N was measured by dry combustion using an automated Dumas method according to LECO FP428 protocol (LECO Corporation; Rutherford et al. 2007)

^epH was measured as a soil-water saturated paste (Miller et al. 1997)

^fSoil test phosphorus as indicated by NaHCO₃ extractable P (Olsen STP)

^gTotal P was determined according to the wet digestion method of Parkinson and Allen (1975) using H₂SO₄ and H₂O₂

^hNaOH-EDTA extractable organic P (Cade-Menun and Preston, 1996)

ⁱMicrobial biomass C was determined according to the chloroform fumigation method using a k_C factor of 0.35 (Voroney et al., 2008)

Table 2: Parameters determined from the isotopic exchange kinetics experiment, along with soil respiration rates measured during the 7-day incubation experiment for each of the four soils. P_w = water-extractable P_i (mg kg^{-1}), where $P_w = 10 (\text{L kg}^{-1}) * C_p (\text{mg L}^{-1})$; m and n are coefficients accounting for the immediate and slow physico-chemical reactions, respectively that describe the rate of decrease of radioactivity in the soil solution with time; E_{1min} = amount of isotopically exchangeable P during the first minute of the experiment. Mean (standard error) of $n=4$ subsamples. Within columns, means followed by the same letter are not significantly different ($p < 0.05$).

Soil no.	P_w (mg P kg^{-1})	n	m	E_{1min} (mg P kg^{-1})	CO_2 release rate (mg C kg^{-1} soil d^{-1})
1	0.03a (0.03)	0.38a (0.01)	0.09a(0.01)	0.40a (0.15)	15.3a (2.0)
2	0.07a (0.03)	0.37a (0.01)	0.13b (0.01)	0.55a (0.15)	13.1a (2.0)
3	0.10a (0.03)	0.36a (0.01)	0.12ab (0.01)	0.78a (0.15)	15.9a (2.0)
4	0.24b (0.03)	0.32b (0.01)	0.14b (0.01)	1.62b (0.15)	17.1a (2.0)

Table 3: Specific activity (SA), which is the ratio of $^{33}\text{P}_i$: $^{31}\text{P}_i$ of the water-extractable P_i pool (P_w) and the microbial P pool (indicated by hexanol-labile P) (P_{hex}) and the ratio of SAP_{hex} to SAP_w for the four field soils at 1 and 8 days after soil labeling. Mean and standard deviation (n=4); within columns, means followed by the same letter are not significantly different (p <0.05).

Soil no.	SAP_w		SAP_{hex}		$\text{SAP}_{\text{hex}} : \text{SAP}_w$	
	d1 ^a	d8	d1 ^a	d8	d1 ^a	d8 ^a
1	2.3b (1.3)	0.9a (0.2)	0.4b(0.2)	0.6b (0.1)	0.2b (0.1)	0.8b (0.3)
2	1.2ab (0.3)	0.8a (0.2)	0.03a (0.03)	0.2a (0.1)	0.03a (0.02)	0.3a (0.1)
3	1.3ab (1.0)	0.7a (0.3)	0.1a (0.03)	0.3a (0.2)	0.1ab (.03)	0.4a(0.1)
4	0.7a (0.2)	0.5a (0.1)	0.1a (0.04)	0.2a (0.1)	0.1ab (.03)	0.3a (0.1)

^astatistical analysis was performed using log-transformed data.

Table 4: Extrapolated and measured E-values, gross P_o mineralization (P min), P_i immobilization (P imm) and net P_o mineralization determined for four field soils at the end of an 8-day incubation experiment. Daily P fluxes are also shown. Mean and standard deviation (n=4); within columns, means followed by the same letter are not significantly different ($p < 0.05$).

Soil no.	Extrapolated E-value	Measured E-value	Gross P min - mg P kg ⁻¹ soil	Gross P imm -	Net P min	Daily gross P min - mg P kg ⁻¹ d ⁻¹	Daily gross P imm -	Daily net P min
1	13.8a (4.5)	23.7a (4.6)	9.9a (2.1)	8.6a (3.1)	1.3a (3.6)	1.20a (0.26)	1.10a (0.39)	0.16a (0.44)
2	17.8a (5.5)	24.7a (7.3)	6.9a (5.4)	6.3a (1.7)	0.6a (6.2)	0.86a (0.68)	0.78a (0.21)	0.08a (0.77)
3	20.5ab (5.3)	30.0a (8.8)	9.5a (4.2)	6.6a (1.3)	2.9a (5.0)	1.17a (0.52)	0.81a (0.16)	0.36a (0.61)
4	29.8b (5.7)	42.6b (9.2)	12.8a (7.3)	5.7a (2.1)	7.0a (9.2)	1.58a (0.90)	0.71a (0.26)	0.87a (1.13)

^aValues for all 16 soil samples, including those after 1 day of incubation can be found in supplemental information (Table D1).

Figure Captions

Figure 1: Recovery of ^{33}P in the water-extractable P_i pool (P_w) (top) and the hexanol-labile P pool (P_{hex}) (bottom) versus water-extractable P_i concentrations (mg kg^{-1}) at 1d (left) and 8 d (right) after soil labeling. If the relationship between the variables was found to be significant, the linear regression equation is shown. The shapes indicate the different soils in the study: soil 1 (■), soil 2 (▲), soil 3 (○), soil 4 (+).

Figure 2: Specific Activity (ratio of $^{33}\text{P}_i:^{33}\text{P}_i$) of the hexanol-labile P pool (SA P_{hex}) versus water-extractable P_i concentrations (mg kg^{-1}) at 1d (top) and 8 d (bottom) after soil labeling. The relationship between the variables was found to be significant for day 8 and the regression equation is shown. The shapes indicate the different soils in the study: soil 1 (■), soil 2 (▲), soil 3 (○), soil 4 (+).

Figure 3: Gross P_o mineralization (top), P_i immobilization (middle), and net P_o mineralization (bottom) in relation to water-extractable P_i after 1(left) and 8 (right) days of soil labeling with ^{33}P . If the relationship between the variables was found to be significant, the linear regression equation is shown. The shapes indicate the different soils in the study: soil 1 (■), soil 2 (▲), soil 3 (○), soil 4 (+).

Figure 1

Figure 2

Figure 3