

# Phosphorus Transformations from Reclaimed Wastewater to Irrigated Soil: A $^{31}\text{P}$ NMR Study

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Irrigation of soils with reclaimed wastewater (RW) is a common practice in arid regions, but may pose an environmental threat if labile phosphorus (P) forms accumulate at the soil surface. Soil P lability can be affected by P forms in the applied RW and by P composition and distribution in the soil. Solution  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy was employed to identify P forms in RW solutions, in whole soil extracts and in fractionated soil P pools in agricultural soils (maize crop, Acre, Israel) irrigated with the examined RW or with freshwater (FW) and a chemical fertilizer. The RW was rich with total P ( $\text{P}_T$ ) and molybdate-reactive P (MRP), consistent with high concentrations of MRP in the RW-irrigated soil. Identified compounds and compound classes in the RW and in the soils include orthophosphate, polyphosphate, orthophosphate monoesters, and orthophosphate diesters. However, there was a shift in P compound classes from the RW to the RW-irrigated soil; although the water sources were different, P forms in the soils of the different treatments were similar. The possible factors that might control this change are discussed, including biological and geochemical P recycling and crop inputs.

**Abbreviations:** DOC, dissolved organic C; EC, electrical conductivity; FW, freshwater; MRP, molybdate-reactive P; MUP, molybdate-unreactive P; NMR, nuclear magnetic resonance; Pi, inorganic P; Po, organic P;  $\text{P}_T$ , total P; RW, reclaimed wastewater; TOC, total organic C; WWTP, wastewater treatment plant.

Irrigation with RW is a common practice in arid and semiarid regions like Israel, simultaneously addressing FW shortage and crop requirements for nutrients. Reclaimed wastewater is also commonly diverted directly to streams, lakes, and seas, a practice with possible environmental implications. In Israel, irrigation with RW has resulted in increased soil bicarbonate P (Tarchitzky, 2007), a chemically labile, bioavailable form of P that is readily available to crops. However, long-term P application from RW or other organic wastes may cause a buildup of labile P at the soil surface (Hansen et al., 2004; Lehmann et al., 2005). This excess P is vulnerable to loss in runoff and erosion, which can contribute to water quality deterioration (e.g., Geohring et al., 2001; Elser and Bennett, 2011).

The concentration and composition of P in RW are key factors controlling the bioavailability and lability of the applied P, and may change with different reclamation processes in wastewater treatment plants (WWTP). Phosphorus compounds include organic P (Po) and inorganic P (Pi) forms, which differ in their bioavailability and sorption–fixation characteristics (McKercher and Anderson, 1989; Bar Yosef, 2003). This in turn affects the availability of P forms to soil flora and fauna (Celi and Barberis, 2005) and their susceptibility to leaching and loss.

Supplemental Material is available online.

Soil Sci. Soc. Am. J. 78:1884–1892

doi:10.2136/sssaj2014.01.0037

Received 27 Jan. 2014.

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Phosphorus in soil has an active biological cycle, which includes orthophosphate uptake and transformation to Po compounds or complex Pi forms, such as pyrophosphate. Degradation of organic compounds can be full or partial (Taranto et al., 2000) and can occur within cells or be extracellular (Oehl et al., 2004). These biogeochemical processes are impacted by soil physico-chemical conditions and management (e.g., Anderson, 1980), and the resulting changes in bioavailability can impact crop yield. On the other hand, crops will also contribute to the nature of P accumulation in the soil (Cade-Menun et al., 2010). Thus, knowing P composition and distribution in soil pools and in major sources of applied P (e.g., RW) may help to understand soil P dynamics and the factors that may lead to excess labile soil P.

Phosphorus composition has been characterized by using solution  $^{31}\text{P}$  NMR spectroscopy in a variety of environmental samples, including soils (intact samples, soil pools, and soil leachate) of various treatments (e.g., Rubaek and Sibbesen, 1993; Cade-Menun et al., 2002; Toor et al., 2003; Liu et al., 2013). Although application of organic wastes to soils has been examined in several studies (e.g., Guggenberger et al., 2000; Smith et al., 2006; Ajiboye et al., 2007), relatively little is known about the P composition of RW and of soils irrigated by RW, despite the prevalence of RW-irrigated soils and the potential threat of this practice to water bodies.

The objective of the overall project was to understand P dynamics in soils that are irrigated with RW. Components of the project were previously published in Zohar et al. (2010a, 2010b). The objective of this part of the study was to understand changes in specific P forms. To accomplish this, we used chemical analysis and solution  $^{31}\text{P}$  NMR spectroscopy to characterize P forms in RW that is used for irrigation, whole soils irrigated with RW, and fractionated P pools of RW-irrigated soil. The RW-irrigated soil was compared with a soil irrigated with FW and a chemical fertilizer. As far as we know, this is the first study to characterize P compounds in RW and RW-irrigated soils.

## MATERIALS AND METHODS

### Reclaimed Wastewater Sampling and Processing

Reclaimed wastewater used for irrigation in Acre Station, an experimental field site, was sampled annually for 3 yr (2006–2008). This RW was produced at the Shomrat WWTP, a secondary treatment plant. The RW was sampled before passing a field micron filter (~100-mm pore size) to represent the RW from the WWTP; samples collected after the field filter represented the actual RW applied. This has also allowed us to evaluate the impact of the field filter. All sampled RW solutions were kept cold until processing, which usually started the same day as sampling. To each 1 L of RW solution we added 1.68 g ethylenediaminetetraacetic acid disodium, dehydrate (EDTA) and 5 mL of 1 M  $\text{L}^{-1}$  NaOH to produce final concentrations of 4.5 mmol  $\text{L}^{-1}$  and 5 mmol  $\text{L}^{-1}$ , respectively. The RW solutions were shaken for 16 h, frozen, and lyophilized.

### Soil Sampling, Extraction, and Processing

Top soil (0–5 cm; dark, calcareous alluvial clay soil, Chromoxerert) was sampled in August 2006 at Acre Station from two plots receiving different treatments since 2002. The studied soils and their irrigation history are detailed in Zohar et al. (2010b). In brief, soils were collected from a maize plot irrigated with RW (40 kg P  $\text{ha}^{-1}$  each season) and from an adjacent plot, irrigated with FW and amended with fertilizers (10 kg P  $\text{ha}^{-1}$  in 2006). These are designated as RW soil and FW soil, respectively. From each plot, one hundred 20-g samples were collected and combined, air dried, crushed, and sieved (2-mm sieve). Soil subsamples from each plot were extracted by NaOH+EDTA: 10 g of soil was extracted with a 100-mL solution containing 0.25 mol  $\text{L}^{-1}$  NaOH and 0.05 mol  $\text{L}^{-1}$  EDTA by shaking for 16 h. Samples were centrifuged (15,000  $g \times 20$  min), frozen, and lyophilized (Cade-Menun et al., 2002). Soil subsamples were fractionated with a modified sequential procedure (Hedley et al., 1982), using water, bicarbonate, NaOH, and diluted and concentrated HCl solutions. More details are available in the Supplementary Material and Zohar et al. (2010b). After sequential extraction, the RW soil water extract and the RW- and FW-soil NaOH extracts were treated with 0.37 g of EDTA (to produce a final concentration of 1 mmol  $\text{L}^{-1}$  EDTA), shaken for 1 h, frozen, and lyophilized for  $^{31}\text{P}$  NMR analysis. The diluted HCl extracts were expected to include only orthophosphate (He et al., 2008) and thus were not analyzed by  $^{31}\text{P}$  NMR.

In 2003 through 2005, the two plots were also sampled (80 subsamples each year) as described above. A portion of each of those subsamples and of the 2006 subsamples before their combination into composite samples was extracted with 0.5 M  $\text{NaHCO}_3$  (“Olsen P”) in a 1:20 soil/solution ratio to measure plant-available P (Schoenau and O’Halloran, 1993).

### Chemical Analysis of Solutions

Concentrations of MRP in the RW and in the soil extracts were determined colorimetrically (Murphy and Riley, 1962), total P ( $\text{P}_T$ ) in RW was determined colorimetrically after acidic hydrolysis with sulfuric and nitric acids (Eaton and Franson, 2005), and  $\text{P}_T$  of soil extracts was determined colorimetrically after digestion with sulfuric acid and sodium persulfate (Tiessen and Moir, 1993). Total and dissolved organic C (TOC and DOC, respectively) were determined by the combustion–nondispersive infrared gas analysis method (Shimadzu Model 5000A). Additionally, solution pH (Thermo Orion, model 420) and electrical conductivity (EC) (Cyber scan 500) were measured in the RW solutions.

### $^{31}\text{P}$ Nuclear Magnetic Resonance Experiments Dissolution of Lyophilized Samples

Freeze-dried RW (1 g) and soil extracts (0.35 g) were dissolved in 3 mL  $\text{D}_2\text{O}$  and 1 mL  $\text{H}_2\text{O}$  (a minimum of 10%  $\text{D}_2\text{O}$ ). Samples were vortex mixed, shaken for 30 min, and left under frequent mixing for an additional 1.5 h. Before analysis, 0.4 mL of 10 M NaOH was added to the RW samples. Samples were centrifuged for 40 min at 6000  $g$  and filtered (0.45 mm). The

pH of prepared  $^{31}\text{P}$  NMR samples was confirmed to be  $>12.5$  (excluding the water extract) for optimal peak separation (McDowell and Stewart, 2005). The RW soil water extract was analyzed without pH adjustment to prevent degradation of alkaline-sensitive P compounds (Turner et al., 2003). The NMR experiments started within 3 h of sample preparation to minimize compound degradation.

### $^{31}\text{P}$ Nuclear Magnetic Resonance Spectroscopy

The  $^{31}\text{P}$  NMR experiments were conducted on a Bruker Avance 500 spectrometer, operating at 202.456 Hz, using a 5-mm probe. Other parameters: 12.1-ms pulse ( $90^\circ$ ); 0.806-s acquisition; dwell time 24.6 ms; 5.0-s delay time (e.g., Cade-Menun et al., 2002, 2006; McDowell et al., 2006); time domain 32,768 points; spectral width 20,325.203 Hz; proton decoupling;  $25^\circ\text{C}$ . The number of scans was 500 to 1600 for the RW solutions and 10,000 to 14,100 for the soil extracts. An external standard ( $85\% \text{H}_3\text{PO}_4$ ) was used for calibration.

### Data Analysis: Peak Assignment

Phosphorus compounds were identified by their chemical shifts after the orthophosphate peak in each spectrum was standardized to 6 ppm (Cade-Menun et al., 2010). Spectra were processed with 7-Hz line broadening for the overall spectrum and 2 Hz to preserve fine resolution in the orthophosphate monoester region, using NUTS software (2000 edition; Acorn NMR, Livermore, CA). Peak areas were calculated by integration and manual calculation. Peak assignments were based on previous reports (Cade-Menun, 2005; Doolette et al., 2009; Cade-Menun et al., 2010; Young et al., 2013). The water extract was analyzed at native pH and peak assignments in this sample were based on McDowell and Stewart (2005). The chemical shifts of some compounds (e.g., orthophosphate) are sensitive to pH changes, while other compounds are not as sensitive (McDowell and Stewart, 2005; Doolette et al., 2009). Spiking with model compounds was not included in the NMR analysis of any of these samples; however, spectra samples extracted with NaOH (to which EDTA was added after soil extraction) and NaOH+EDTA were compared with NaOH+EDTA extracts of similar soils spiked with various P compounds to confirm peak identification. A detailed description of peak assignments can be found in the Supplemental Material (Supplemental Table S1).

**Table 1. Phosphorus concentrations and related parameters in reclaimed water, collected at the outlet of a micromic field filter.**

	June 2006	July 2007	August 2007†	April 2008	Average ± standard deviation
$\text{P}_T$ , $\text{mg L}^{-1}$	13.1	17.7 (+1.8)‡	10.3 (+2.14)‡	16.4	$14.4 \pm 2.87$
Dissolved $\text{P}_T$ , $\text{mg L}^{-1}$	9.59	14.1		9.92	$11.2 \pm 2.03$
Particulate $\text{P}_T$ , $\text{mg L}^{-1}$ §	3.48	3.62		6.48	$4.53 \pm 1.38$
MRP, $\text{mg L}^{-1}$	3.02 (−1.74)‡	9.49 (+0.53)‡	7.93 (−0.25)‡	7.84	$7.07 \pm 2.43$
Total MUP, $\text{mg L}^{-1}$ ¶	10.1	8.18	2.39	8.56	$7.30 \pm 2.92$
MUP/ $\text{P}_T$	0.77	0.46	0.23	0.52	$0.50 \pm 0.22$
Dissolved MUP, $\text{mg L}^{-1}$ #	6.57	4.56		2.07	$4.40 \pm 1.84$
TOC, $\text{mg L}^{-1}$	45.70	121.8	25.1	60.42	$63.3 \pm 36.1$
DOC, $\text{mg L}^{-1}$		94.5	15.2	21.6	$43.8 \pm 36.0$
pH, no units	8.09	7.32		7.72	$7.71 \pm 0.31$
EC, $\text{dS cm}^{-1}$	1.32	2.55		1.93	$1.93 \pm 0.50$

† Sampling of reclaimed wastewater (RW) was at a different site in Acre experimental fields since irrigation in the maize plots finished.

‡ The difference from the value at the filter inlet, i.e., RW coming from Shomrat wastewater treatment plant (WWTP). MRP, molybdate-reactive P; MUP, molybdate-unreactive P; TOC, total organic C; DOC, dissolved organic C; EC, electrical conductivity.

§ The difference between total P ( $\text{P}_T$ ) and dissolved  $\text{P}_T$ .

¶ The difference between  $\text{P}_T$  and MRP.

# The difference between dissolved  $\text{P}_T$  and MRP.

## RESULTS

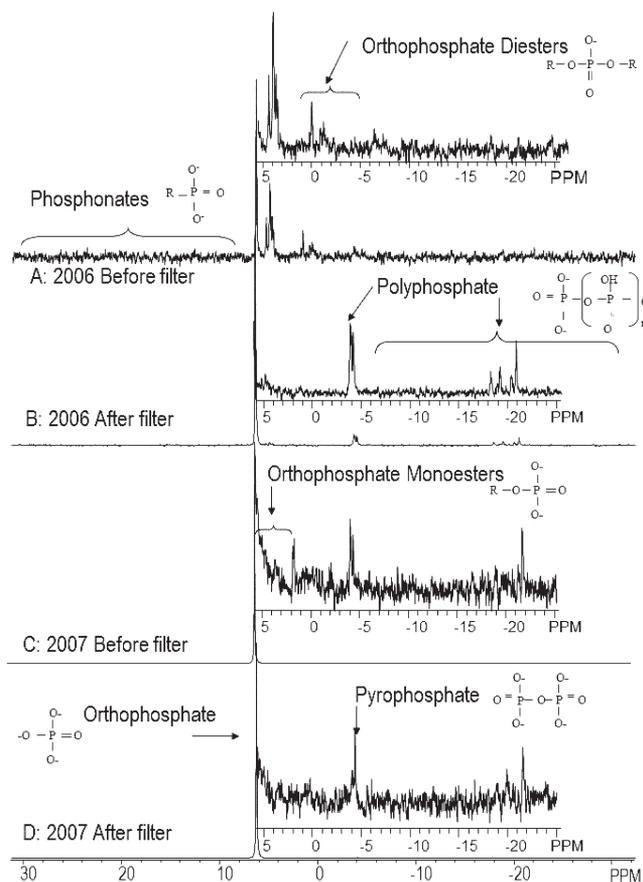
### Phosphorus in Acre Reclaimed Wastewater

The RW had high  $\text{P}_T$ , MRP and particulate P concentrations, and relatively high variability during the 2006 to 2008 sampling period ( $14.4 \pm 2.3$ ,  $7.1 \pm 2.4$ , and  $4.5 \pm 1.4 \text{ mg L}^{-1}$ , respectively, Table 1). Other parameters such as TOC, DOC, and EC also indicated the low quality of the RW, which was not improved by the micronic field filter (Table 1).

The majority of P in the  $^{31}\text{P}$  NMR spectra of the RW samples was found as orthophosphate (Fig. 1, Tables 2 and 3); the exception was the sample collected before field filtration in 2006. For that sample, a number of P forms were detected: orthophosphate monoesters such as mononucleotides and  $\alpha$ - and  $\beta$ -glycerophosphates; orthophosphate diesters, pyrophosphate and polyphosphate (Table 2). Organic P forms predominated in this sample (Table 3). Many of the orthophosphate monoesters likely result from the degradation of orthophosphate diesters (see the Supplementary Material for more details). After correcting for this degradation, orthophosphate diesters were the predominant compound category in the samples collected before filtration in 2006. The sample collected after filtration in 2006 contained fewer organic P compounds, but had the highest polyphosphate percentage of the RW samples (Tables 2 and 3). The P in RW samples collected before and after filtration in 2007 was predominantly inorganic P, with only traces of organic P forms of any kind (Fig. 1, Tables 2 and 3).

### Reclaimed Wastewater-Irrigated and Freshwater-Irrigated Soils

The predominant P form determined by  $^{31}\text{P}$  NMR in all the extracts of the two soils was orthophosphate (Fig. 2), with a slightly higher percentage in the RW-irrigated soil (Tables 2 and 3). However, the percentages of orthophosphate and thus



**Fig. 1.**  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectra of reclaimed wastewater (RW), sampled before and after a field micronic filter in 2006 and 2007. All spectra were processed with 7-Hz line broadening, including the expanded insets. Full spectra are scaled to the height of the orthophosphate peaks; insets are scaled to the height of the pyrophosphate peaks.

total Pi were higher in the spectra for bulk NaOH–EDTA extracts than in the NaOH sequential extracts. In contrast to the RW samples, the alkaline soil extracts contained a wide range of organic P forms, but a lower percentage of inorganic P (Tables

**Table 2.** Percentages of phosphorus species in reclaimed water and alkaline soil extracts, determined by solution  $^{31}\text{P}$  NMR spectroscopy.†

	Orth	Pyro	Poly	Phon	myo-IHP	$\alpha$ glyc	$\beta$ glyc	Nucl	Pchol	scyllo-IHP	Mono1	Mono2	Mono3	OthDi	DNA
Reclaimed water															
Before, 2006‡	31.8	1.5	3.1	0.8	nd§	5.0	7.4	12.4	nd	nd	1.2	12.9	0.6	23.3	nd
After, 2006‡	71.9	3.0	17.7	0.7	nd	nd	nd	1.6	nd	nd	0.6	3.9	0.6	nd	nd
Before, 2007	94.3	0.3	1.9	0.7	nd	nd	nd	nd	nd	nd	0.7	1.4	nd	0.7	nd
After, 2007	94.2	0.5	1.7	0.7	nd	nd	nd	nd	nd	nd	0.7	1.4	nd	0.8	nd
Soil															
RW, N/E¶	81.6	0.2	1.8	1.7	4.1	0.7	1.3	1.3	0.7	0.7	0.7	2.7	1.3	0.8	0.7
FW, N/E	78.4	0.3	1.0	0.7	4.1	0.7	1.3	3.4	0.7	1.3	1.3	2.0	1.3	2.5	1.0
RW, NaOH	78.7	0.3	0.7	0.7	nd	nd	nd	6.1	nd	nd	1.6	9.4	0.8	1.4	0.3
FW, NaOH	74.2	0.3	0.8	0.7	nd	nd	nd	6.2	nd	nd	1.4	11.7	1.4	2.7	0.6

† Orth, orthophosphate; Pyro, pyrophosphate; Poly, polyphosphate; Phon, phosphonate; myo-IHP, myo-inositol hexakisphosphate (phytate);

$\alpha$ glyc,  $\alpha$ -glycerophosphate;  $\beta$ glyc,  $\beta$ -glycerophosphate; Nucl, various mononucleotides; Pchol, Choline phosphate; scyllo-IHP, scyllo-inositol hexakisphosphate; Mono1, 2, and 3, various monoesters excluding specifically identified peaks; OthDi, unspecified diesters. See text for more details.

‡ Reclaimed water collected before or after field filter. Reclaimed water before filter represents the WWTP product; the RW after filter are the actual applied RW.

§ nd, not detected.

¶ RW, irrigated with reclaimed wastewater; FW, irrigated with freshwater; N/E, extracted with NaOH–EDTA; NaOH, the NaOH step of the Hedley fractionation. See text for more details.

2 and 3). In general, peaks in the spectra for the NaOH–EDTA extracts were better resolved, allowing the identification of more peaks, including myo-inositol hexakisphosphate (myo-IHP) and its stereoisomer scyllo-inositol hexakisphosphate (scyllo-IHP) (Supplemental Fig. S1). It is possible that  $\alpha$ - and  $\beta$ -glycerophosphate, myo-IHP, scyllo-IHP, and choline phosphate are also present in the NaOH extracts. However, we were not confident in our peak identifications for those samples, and thus included peak areas in those regions of the NaOH spectra with the Mono2 category. The nucleotides, which we were more confident about identifying, were higher in the NaOH fraction than the bulk soil, which also had a higher percentage of P in the Mono2 category. This resulted in a higher proportion of P as orthophosphate diesters in the NaOH fractions relative to the bulk soil extracts after correction for degradation (Table 3). In general, more orthophosphate degradation peaks were present in the FW-irrigated samples than in those receiving RW, indicating a lower percentage of orthophosphate diesters in the RW-irrigated soils relative to the FW soils.

The RW soil water extract was analyzed at native pH. Orthophosphate resonated at 1.53 ppm, and two more peaks appeared at  $-0.02$  and  $-6.94$  ppm (Supplemental Fig. S2). We can only speculate about peak identifications for spectra of these samples due to limited information on P speciation under these conditions. Based on McDowell and Stewart (2005), the 1.53-ppm peak was assigned to orthophosphate, the  $-0.02$ -ppm peak was assigned to the phospholipid  $\alpha$ -phosphatidyl choline and the  $-6.94$ -ppm peak was assigned to pyrophosphate. Orthophosphate was the predominant P form in this sample.

As demonstrated in Zohar et al. (2010b), the RW soil had significantly higher MRP and  $P_T$  concentrations in the five tested fractions compared with the FW soil (e.g.,  $P_T$  in the water extracts of the two soils was  $44.2 \pm 4.5$  and  $14.9 \pm 4.2$  mg kg $^{-1}$  soil, respectively, Supplemental Table S2). Molybdate-unreactive P (MUP) was lower than the MRP in all fractions, in both soils. The MUP was in similar levels in the two soils. These results are

**Table 3. Percentages of phosphorus compound classes in reclaimed water and alkaline soil extracts, determined by solution  $^{31}\text{P}$  NMR spectroscopy.†**

	Pi	Po	Pi/Po	Mono	Di	M/D	Deg	cMono	CDi	cM/cD
Reclaimed water										
Before, 2006‡	36.4	63.6	0.6	39.5	23.3	1.7	31.3	8.3	54.6	0.2
After, 2006	92.6	7.6	12.5	6.7	nd§	nd	3.6	3.2	1.4	0.9
Before, 2007	96.5	3.5	27.6	2.1	0.7	3.0	0.7	1.4	1.4	1.0
After, 2007	96.4	3.6	26.8	2.1	0.8	2.6	0.7	1.4	1.5	0.9
Soil										
RW, N/E	83.6	16.4	5.1	13.5	1.5	9.0	4.7	8.9	6.2	1.4
FW, N/E	79.7	20.3	3.9	16.1	3.5	4.6	6.4	9.7	9.9	1.0
RW, NaOH¶	79.7	20.3	3.9	17.9	1.7	10.5	10.8	7.1	12.5	0.6
FW, NaOH	75.3	24.7	3.0	20.7	3.3	6.3	12.1	8.7	15.4	0.6

† Pi, total inorganic P; Po, total organic P; Mono, total orthophosphate monoesters, uncorrected for diester degradation peaks; Di, total orthophosphate diesters, uncorrected for diester degradation peaks; M/D, ratio of monoesters to diesters, uncorrected for degradation peaks; Deg, degradation peaks (diesters converted to monoesters:  $\alpha$ -glyc,  $\beta$ -glyc, Nucl, half of monoester 2); cMono, corrected orthophosphate monoesters, excluding diester degradation peaks; CDi, corrected orthophosphate diesters, including diester degradation peaks; cM/cD, ratio of monoesters to diesters, corrected for degradation peaks. See text for more details.

‡ Reclaimed water collected before or after field filter. Reclaimed water before filter represent the WWTP product; the RW after filter are the actual applied RW.

§ nd, not detected.

¶ RW, irrigated with reclaimed wastewater; FW, irrigated with freshwater; N/E, extracted with NaOH-EDTA; NaOH, the NaOH step of the Hedley fractionation. See text for more details.

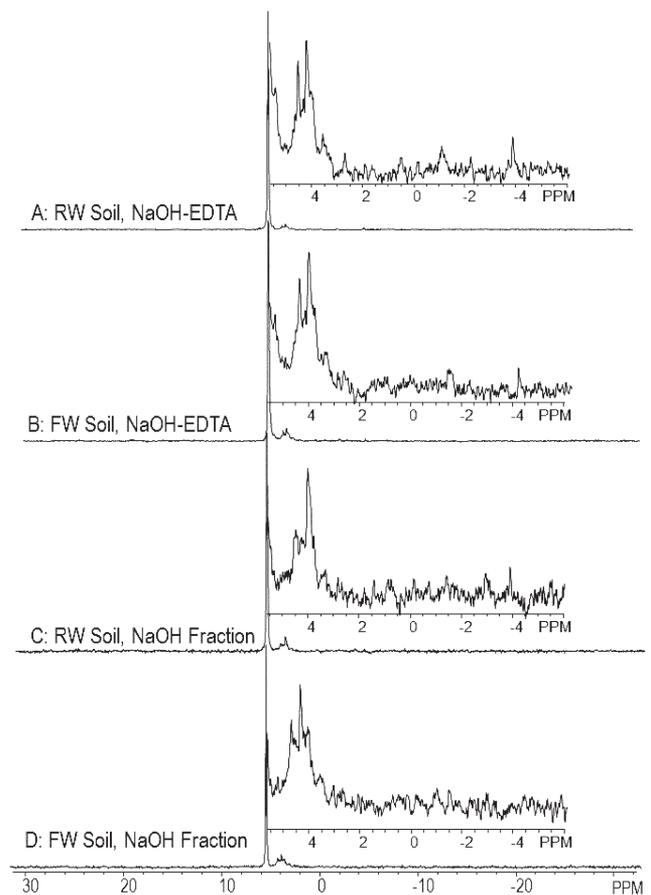
consistent with the determination of Olsen P in these soils (Fig. 3). For all years, Olsen P concentrations were higher in RW than FW soils, and for all samples but the 2003 FW soil, Olsen P exceeded the recommended Olsen P value of 10–15 mg P kg<sup>-1</sup> soil for optimal production of irrigated maize (Leikam, 2008).

## DISCUSSION

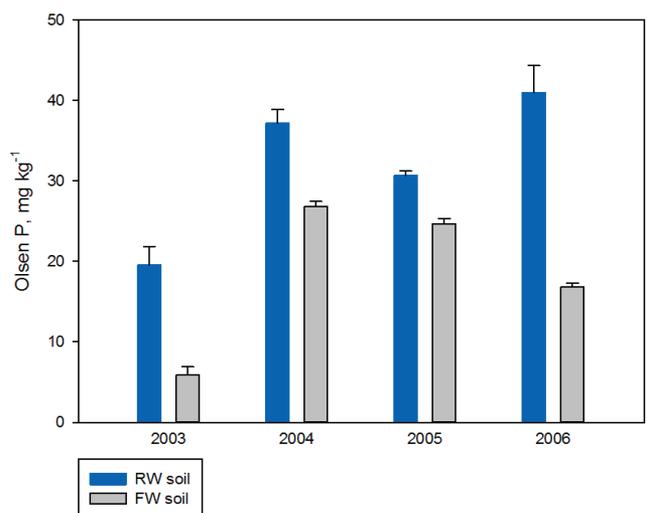
### Phosphorus in Reclaimed Wastewater

In this study, RW solutions were processed as whole samples such that the alkaline extraction released the P adsorbed on particles, as well as the P already in solution. Other  $^{31}\text{P}$  NMR studies of aqueous solutions separated the dissolved and particulate P (Cade-Menun et al., 2006), examined only the dissolved fraction (Fuentes et al., 2012), or extracted the P only after lyophilizing the entire aqueous sample (Toor et al., 2003). In the case of particulate P-rich RW used for soils irrigation, both the dissolved and the particulate P fractions are applied simultaneously and are likely involved in P reactions in the soil. As such, we chose to analyze the RW sample as a whole, rather than separating the dissolved and particulate fractions. We also chose to extract the entire sample directly, rather than freeze-drying it before extraction, to eliminate the number of steps in the procedure, potentially minimizing degradation of sensitive P forms. However, further testing is warranted to determine if degradation is indeed minimized.

The RW samples before the field filter represent the product of the Shomrat WWTP, delivered directly to Acre experimental station in the irrigation season. There was considerable annual variation in the RW, with higher percentages of polyphosphates



**Fig. 2.  $^{31}\text{P}$  NMR spectra of alkaline soil extracts. All spectra are plotted with 7-Hz line broadening, including the expanded insets. Full spectra are scaled to the height of the orthophosphate peaks; insets are scaled to the height of the tallest peak in the orthophosphate monoester region. RW, irrigated with RW; FW, irrigated with freshwater; NaOH-EDTA, bulk soil extracted with NaOH-EDTA; NaOH, the NaOH step of the Hedley fractionation, with EDTA added after soil extraction and before freeze-drying. The pH of all spectra was adjusted to >12 before  $^{31}\text{P}$  NMR analysis.**



**Fig. 3. Bicarbonate extracted P (Olsen P) in the RW and FW soils from 2003 to 2006. Values are means ( $n = 80$  for 2003–2005 and  $n = 100$  for 2006); error bars represent standard error.**

and total Po in the 2006 samples than the 2007 samples, even after filtration (Tables 2 and 3). This and other parameters (Table 1) emphasize the variability of the reclamation process in Shomrat WWTP. The 2006 RW sample was compared with wastewater samples from the reclamation process, sampled in January 2006 (Supplemental Fig. S3). In the wastewater samples, polyphosphate was an important constituent, especially in the second stage of treatment (the clarification pond), increasing its fraction 10 fold compared with the first stage. The presence of polyphosphates and their role in the cycling of organic compounds in wastewater has been described in several studies (e.g., Fuhs and Chen, 1975; Hill et al., 1989; Pereira et al., 1996; Hupfer et al., 2007). Organic P forms, including orthophosphate monoesters and diesters, are also present in both the wastewater samples.

The field filter appears to have altered the P composition in the 2006 applied RW (Fig. 1), decreasing the proportion of Po from the filter inlet (63.3%) to its outlet (7.6%) and subsequently increasing the fraction of Pi (Table 3). Possible explanations for these differences include the following: preferable sorption of Po onto the filter cake due to the higher adsorption capability of some Po forms (e.g., Leytem et al., 2002); the presence of different microorganism groups, which may produce different P compounds (Bünemann et al., 2008) in the RW and in the filter; or the filter-retention of particles, to which Po and microbes are preferably attached (Banfield et al., 1999; Mills, 2003).

Very few speciation data for RW exist, because most  $^{31}\text{P}$  NMR studies of WWTP samples have focused on the solid product of WWTP, namely the sludge. For example, Escudey et al. (2004) claimed that in anaerobically digested sewage sludge, inorganic orthophosphate is the main signal seen, but when the sewage sludge is aerobically digested, the main signal corresponds to orthophosphate diester. Smith et al. (2006) identified more P forms (orthophosphate, orthophosphate monoesters [e.g., myo-IHP], phospholipids, and DNA) during the sludge stabilization process and tracked the change in their proportions, and Bartoszek et al. (2008) followed changes in P forms during the humification of the sludge. More study is required to elucidate the connection between P forms in RW and in the respective sludges that are produced by different reclamation processes.

### Acre Soils: Phosphorus Distribution and Speciation

The concentration of Olsen P in the RW soil was higher than in the FW soil throughout the tested years (Fig. 3), indicating higher P availability (Schoenau and O'Halloran, 1993) in this soil. This is in accordance with the results from the sequential extraction (Zohar et al., 2010b), which demonstrated that the 2006 RW soil contained a higher concentration of labile P than the FW soil: total P in the two most labile soil fractions (i.e., the water and the bicarbonate sequential extracts) sums to 114 and 50 mg kg<sup>-1</sup> soil, consisting mostly of MRP (Supplemental Table S2). This is consistent with other studies that found higher P lability in organically amended soils compared with unamended soils (e.g., Sui et al., 1999). Although MUP constituted 77% of the P<sub>T</sub> in the applied RW in 2006 and an average of approxi-

mately 50% from 2006 to 2008 (Table 1) and is assumed to be 0% in the FW irrigation water, the concentration of MUP in the labile fractions was lower in the RW soil sampled in 2006, after 3 mo of RW irrigation (including the last irrigation event a few days before soil sampling) than in the FW soil (Supplemental Table S2). The proportion of MUP to P<sub>T</sub> in the two labile fractions of the FW soil, calculated based on Supplemental Table S2, was higher than in the RW soil labile fractions (FW soil, 11.2%; RW soil, 1.9%). The concentration of MUP in the RW soil in the NaOH fraction (considered to be the strongly adsorbed P pool) was higher in the RW than FW soils (Supplemental Table S2). Labile MUP compounds in the RW soil were probably very bioavailable and more susceptible to rapid recycling than in the FW soil (see discussion below).

The  $^{31}\text{P}$  NMR data of the soil extracts may elucidate the possible MRP and MUP compounds attributed to the soil fractions. In the RW soil water extract, analyzed at native pH, the largest peak (1.53 ppm, Supplemental Fig. S2) was assigned to orthophosphate based on the high concentration of MRP in this extract (Supplemental Table S2). This indicates that the pH of the concentrated sample was ~6.2 (according to McDowell and Stewart [2005]), which was moderate enough to minimize the degradation of sensitive labile Po compounds. We speculate that the sharp peak at -0.02 ppm is the phospholipid  $\alpha$ -phosphatidyl choline, although resonance of protonated orthophosphate at this pH is also an option. The smaller peak at -6.94 ppm may be pyrophosphate. The compound  $\alpha$ -phosphatidyl choline is common in soils in which plants are growing (Kowalenko and Mc Kercher, 1971), but is easily degraded to glycerophosphates in alkaline conditions (Turner et al., 2003).

The spectra from the sequential soil NaOH extracts of the RW and FW soils were very similar to one another, as were the spectra from the NaOH+EDTA bulk soils extracts (Fig. 2). In the spectra of the NaOH sequential extracts, orthophosphate and orthophosphate monoesters were the major species. Degradation products (nucleotides and glycerophosphates) may originate from living cells extracted in this step (lysed under the experimental conditions) or directly from the soil constituents, after their formation during degradation of orthophosphate diesters in situ (Taranto et al., 2000); these would be included in the Mono2 category. NaOH+EDTA solution will extract labile and stable soil P pools (Turner et al., 2005); myo-IHP was not confidently identified in the NaOH extracts, thus its appearance in the NaOH-EDTA extracts (Table 2) may be attributed to its removal in the fractions extracted before NaOH in the sequential fractionation (i.e., the bicarbonate extract) or to the residual Po pool in the soil. The last sequential soil extraction step (the hot and concentrated HCl) addresses this pool, and indeed a substantial concentration of MUP was found in this extract, in both soils (Supplemental Table S2).

The distribution of P forms among the soil pools, based on sequential fractionation, is relatively similar to other studies of soil P distribution in organic waste-treated soils (He et al., 2008) and is in agreement with what is expected based on the sorption

capabilities of various P forms (e.g., Rubaek and Sibbesen, 1993; Cade-Menun and Preston, 1996; Cade-Menun et al., 2002). For example, although there is variation among specific P forms within the broad compound classes of orthophosphate monoesters and diesters, many orthophosphate monoesters (e.g., myo-IHP) tend to sorb strongly on soil minerals (McKercher and Anderson, 1989; Giles et al., 2011, and references therein), while adsorption of orthophosphate diesters in neutral to alkaline soils is considered relatively weak (Taranto et al., 2000, and references therein). The extracted nucleotides, DNA, and phospholipids and their breakdown products may represent living cells (Cade-Menun et al., 2010); thus, their presence is expected in the more labile fractions. Consistent with the results of this study, Liu et al. (2013) reported that orthophosphate diesters were significantly correlated with the concentration of MUP in the soil labile fraction (NaHCO<sub>3</sub> extract) in relatively acidic Ultisols. Weakly sorbed compounds are more susceptible to leaching and this may have environmental implications, especially for orthophosphate diesters that are thought to be more bioavailable than orthophosphate monoesters (Taranto et al., 2000).

### Phosphorus Transformations from Applied Reclaimed Wastewater to Soil Phosphorus

Orthophosphate was the major P compound in the applied RW and in all the soil fractions. We expect that the excessive application of orthophosphate in the RW compared with the chemical fertilizer application in the FW soil has contributed to the higher content of P in the RW soil (Supplemental Table S2). Orthophosphate is the preferred compound for uptake by microorganisms and plants. The shift in values for the ratio of Pi/Po from that in RW to that in the soil extracts (Table 3) suggests that the applied orthophosphate in the RW may be removed from soil by plant uptake, or may be transformed to other P forms. Moreover, the Po nature in the applied RW changed in the soil, as the ratios of orthophosphate monoesters to diesters were found higher in the soil than in the RW, even when considering the correction for degradation (Table 3). The soils treatments and P content are different and yet P composition in the RW soil is very similar to the FW soil. These results are consistent with those of Soinnie et al. (2011), who also obtained very similar NMR spectra for soils of organic and conventional treatments. This suggests that the RW and FW soils are affected by similar processes, which overshadow the applied P forms. The RW signature was probably erased by enhanced P recycling in the RW soil, which is expected to be very nutrient-rich and bioreactive after 3 mo of RW irrigation and plant growth, as was found for other organically amended soils (e.g., Crouse et al., 2002; Oehl et al., 2004). In the companion paper to this study (Zohar et al., 2010b), the isotopic signature of oxygen in phosphate (d<sup>18</sup>O<sub>p</sub>) of the RW was erased very rapidly in the soil solution by what was identified as enzymatic processes; trends in the d<sup>18</sup>O<sub>p</sub> of soil pools were very similar in the two soils, treated with RW or with a chemical fertilizer. The results of the isotopic and the <sup>31</sup>P NMR studies complement each other and both suggest that P recycling is very fast in the RW soil, leading to overshadowing

of the applied P forms and to the final P status being governed by similar processes as in the FW soil. A lower proportion of orthophosphate diesters in the soil extracts of the RW soil compared with the FW soil (Table 3) may be the result of intense P turnover and utilization of less stable P species, preventing them from accumulating (Guggenberger et al., 2000; Taranto et al., 2000; Akhtar et al., 2002; Crouse et al., 2002; Oehl et al., 2004; He et al., 2008). Polyphosphate was a dominant P compound in the irrigating RW (17.7% in the applied RW in 2006), but its presence in the soil is relatively low (1.8% in the NaOH-EDTA extract of the RW soil at the same year). The limited presence of polyphosphate and pyrophosphate in the RW soil probably reflects their relative geochemical instability, leading to their dissociation into orthophosphate (Lindsay et al., 1989). Another important factor in Acre agricultural soils is the maize growth, which has probably contributed to the accumulation of myo-IHP and other P compounds in the soils (Cade-Menun et al., 2010; Noack et al., 2012).

We conclude that P transformations in the soil, post application of RW, appear to include biological and geochemical cycling together with crop inputs. Prior research shows that in some soils, external inputs govern soil P, rather than internal microbial cycling (Cade-Menun et al., 2010), while in other cases, P transformations in receiving soils and sediments, post application, predominate (e.g., McDowell and Stewart, 2005; McDowell et al., 2006; He et al., 2008; Reitzel et al., 2009). Nevertheless, soil type, treatment, and ambient conditions affect P speciation and distribution and thus more research is required. In particular, more research is required for RW-irrigated soils in context of different conditions, to assess the potential environmental threat in this practice.

### SUPPLEMENTAL MATERIAL

Additional information on P concentrations in the soil extracts (Supplemental Table S2), the chemical shifts of peaks detected in <sup>31</sup>P NMR spectra (Supplemental Table S1), details of peak identification in the orthophosphate monoester region (Supplemental Fig. S1), and <sup>31</sup>P NMR spectra of the water soil extracts (Supplemental Fig. S2) and the WWTP RW (Supplemental Fig. S3) are available in the Supplemental Materials.

### ACKNOWLEDGMENTS

This research was supported by The United States-Israel Binational Agricultural Research and Development Fund (BARD fund no. IS 3963-07). We thank Yael Balazs and Shifi Kababya at the Magnetic Resonance Laboratory at The Technion, and Ronit Faran and Malik Cochava at the Soil Chemistry Laboratory at the Technion. Finally, we thank the anonymous reviewers of this paper for their helpful comments.

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