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Phosphorus characterization in wetland soils by solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy

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1 **Introduction**

2 The importance of phosphorus (P) availability in regulating productivity and diversity of
3 freshwater wetlands is well recognized (Newman and Robinson, 1999; Reddy et al., 1999), yet,
4 the forms and dynamics of P in such ecosystems remain poorly understood. This is in part due
5 to the difficulty of identifying and quantifying P compounds in wetland soils. With the
6 development of techniques such as ^{31}P nuclear magnetic resonance (NMR) spectroscopy,
7 researchers now have the tools to identify P compounds in the environment by their chemical
8 functionality (Figure 1). This has utility over methodological or operational classifications of soil
9 P since it provides information on biological sources (Bunemann et al., 2008; Koukol et al.,
10 2008; Makarov et al., 2005), chemical interactions with the abiotic environment (Heighton et al.,
11 2008), and susceptibility of compounds to enzymatic or abiotic hydrolysis (Cade-Menun,
12 2005a). The application of ^{31}P NMR spectroscopy allows researchers to define sources, identify
13 standing pools, and track transformations of biogenic P in the environment.

14 The theory behind NMR spectroscopy is covered in a number of comprehensive text
15 books (Berger et al., 1997; Canet, 1996; Claridge, 2009; Knicker and Nanny, 1997) and its
16 broad application to environmental samples reviewed in several comprehensive papers (Cade-
17 Menun, 2005a; Cade-Menun, 2005b; Condron et al., 1997). Here we summarize NMR
18 methodology and set out considerations needed for the application of ^{31}P NMR spectroscopy to
19 the study of wetland soils. Solid state (see below) ^{31}P NMR spectroscopy has been applied to
20 wetland soils (Delgado et al., 2000; Shand et al., 1999), but poor spectral resolution (Conte et
21 al., 2008; Dougherty et al., 2005) make its routine use on soils impractical and it is not
22 discussed further.

23 It should also be noted that, while not discussed here, ^{31}P NMR has been successfully
24 applied to other wetland ecosystem-components, including dissolved and particulate P forms
25 within the water column (Cade-Menun et al., 2006; Nanny and Minear, 1994b; Nanny and

Minear, 1997; Reitzel et al., 2009) and detrital plant material (Cheesman et al., 2010a; Pant and Reddy, 2001)

Although wetlands are commonly understood to represent a transitional ecotone between terrestrial and aquatic ecosystems, there is no single accepted definition for the term ‘wetland’. Federal institutions in the USA, at the recommendation of the Wetlands Subcommittee of the Federal Geographic Data Committee, use the Cowardin system (Cowardin et al., 1979) to define wetland and deepwater habitats (Federal Register 61, 29 July, 1996, 39465-39466). For the purposes of this chapter we consider wetlands as a transitional ecotone and draw on relevant literature and experience from both terrestrial and traditionally considered aquatic systems.

First applied to terrestrial systems over 30 years ago (Newman and Tate, 1980) the study of P dynamics in soils and sediments has been greatly enhanced by ^{31}P NMR spectroscopy. Studies in wetlands (Table 1) include lacustrine sediments in Europe (Ahlgren et al., 2005; Hupfer et al., 1995; Reitzel et al., 2007) and China (Bai et al., 2009; Liu et al., 2009; Zhang et al., 2009), as well as the highly organic palustrine systems of south Florida (Robinson et al., 1998; Turner and Newman, 2005; Turner et al., 2006b). Other wetlands studied to some degree have included ‘Carolina Bays’ (Sundareshwar et al., 2009), Australian ‘billabongs’ (Baldwin, 1996), New Zealand streams (McDowell, 2009), and Scottish blanket bogs (Bedrock et al., 1994). Despite this, it is clear that the use of ^{31}P NMR spectroscopy in wetlands has seen only limited application in comparison to terrestrial systems. As more researchers apply ^{31}P NMR to wetlands it is important that standardized procedures, based on an understanding of the issues, are used to provide both accurate and comparable results.

Brief Overview of the Principles of NMR Spectroscopy

NMR-sensitive nuclei

51 Certain nuclei (i.e. ^{1}H, ^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{19}F, ^{27}Al, ^{29}Si, ^{31}P etc.) exhibit the quantum
52 mechanical property of "spin". By analogy to classical electrodynamics their spin allows them to
53 be considered magnetic dipoles containing a magnetic moment, μ expressed as Equation 1
54 where $\hbar$ = [h (Planck's constant) / 2π], γ is the gyromagnetic ratio (a fundamental nuclear
55 constant) and I is the vector representation of the nuclear spin I.

56 $$\mu = \gamma \hbar I \qquad \text{(Equation-1)}$$

57 When nuclei with a nuclear spin (I) ≠ 0 are placed in a static magnetic field (B_0) the
58 magnetic moment (μ) aligns, and the nucleus precesses around the axis of the applied field with
59 a Lamor frequency (ω_0) as given by Equation 2.

60 $$\omega_0 = -\gamma B_0 \qquad \text{(Equation-2)}$$

61 Quantum mechanics states that an object with aforementioned spin has a discrete
62 number of spin states and energy levels described by the magnetic spin quantum number m_I. It
63 follows that 2I + 1 different spin energy levels are possible, each with an energy level as
64 described in Equation 3.

65 $$E = -\gamma \hbar m_I B_0 \qquad \text{(Equation-3)}$$

66 Therefore, nuclei with spin when placed in a magnetic field (B_0) have two distinct energy
67 states (Figure 2-A). During NMR spectroscopy this is achieved by placing the sample within a
68 powerful electromagnet. The distribution of nuclei between these two states is described by the
69 Boltzman distribution and is dictated by the ambient conditions of the sample and the strength of
70 the magnet. During an NMR experiment a radio frequency pulse (B_1), perpendicular to the B_0
71 field, is used to elevate nuclei to their higher energy, less stable state (Figure 2-B). The most
72 commonly used Fourier Transform (FT)-NMR spectrometer uses an intense B_1 pulse of fixed
73 frequency to excite all target (i.e., ^{31}P) nuclei within a sample. After the radio frequency pulse
74 ends the excited nuclei return to their equilibrium state emitting an oscillating current; this signal
75 is recorded as a free induction decay (FID). Although the NMR-sensitive isotope of P (^{31}P) is
76 100% abundant and has a relatively large magneticgyro ratio (γ), the low concentration of P

77 within most environmental samples requires that multiple scans (taking many hours) are
78 acquired and resulting FIDs summed before processing and interpretation. Basic processing of
79 the combined FID includes conditioning to optimize signal to noise ratio (S/N) and a Fourier
80 transformation (FT)- taking the time domain information (FID) to a frequency domain spectrum.
81 The resulting spectra are interpreted by comparison with known standards (see Data
82 Processing) allowing for the determination of indicative peak signals identified by their chemical
83 shift (Figure 1).

84 **Chemical shift**

85 Nuclei in a macroscopic sample, held within an applied field B_0 actually experience a
86 specific local magnetic field (B_{loc}) dependent upon a nucleus's interactions with its immediate
87 environment. These interactions alter a nuclei's perceived magnetic field and therefore modify
88 its Lamor frequency and transition energy. The change in the resultant frequency domain
89 spectrum can be interpreted to provide information on both the chemical bonding and
90 physicochemical nature found within the sample. Potential interactions include chemical shift,
91 spin-spin (scalar), dipole-dipole, and quadrupole interactions. The most readily interpreted
92 interaction, and most pertinent to ^{31}P NMR spectroscopy in wetland soils is that of chemical shift
93 interactions. Chemically nonequivalent nuclei experience different degrees of electron shielding
94 and an altered B_{loc}. The practical upshot of this is the ability to distinguish nuclei on the basis of
95 the atoms to which they are bound. The comparison of resulting spectra with standards allows
96 for the identification of both distinct functional groups (Figure 1) and specific P containing
97 compounds (Turner et al., 2003a). It should be noted that, given the impact of deprotonation on
98 chemical shift interactions, many peaks are pH-dependent (McDowell and Stewart, 2005a) and
99 comparison with spectral libraries should be carried out at a standard pH. In addition, given that
100 Lamor frequencies (ω_0) are dependent upon the size of the magnetic field being applied
101 (Equation 2) and magnet size is continuously evolving, resonance frequencies are routinely
102 reported in relation to a standard. In solution ^{31}P NMR the convention is to use 85% H_3PO_4 set

103 as zero, with chemical shifts (δ) reported as shift in frequency of the sample (v_s) from this

104 reference (v_{rf}) (Equation 4), normally in the units of parts per million (ppm) (Wilson, 1987).

105 $$\delta = [(v_s - v_{rf}) / v_{rf}] \times 10^6 \quad \text{(Equation-4)}$$

106 **Proton decoupling**

107 Spin-spin coupling is a phenomenon brought about by the interaction of the target nuclei,

108 in our case ^{31}P, with bonded nuclei that also have a half integer nuclear spin (e.g. the ubiquitous

109 ^{1}H). Although useful in garnering secondary and tertiary structural information in advanced

110 analytical chemistry (e.g., identifying structural stereoisomers of higher order inositol

111 polyphosphates; Murthy, 2007), the complex signal patterns that result from ^{31}P – ^{1}H coupling

112 are often hard to interpret within heterogeneous environmental samples. Therefore, broadband

113 heteronuclear (proton) decoupling is often applied. During decoupling, a secondary radio

114 frequency pulse is applied to saturate the ^{1}H nuclei. The rapid inter-conversion of ^{1}H spin states

115 results in a sample average, thereby negating the ^{1}H influence on the primary nuclei, ^{31}P (Figure

116 3). The decoupling pulse is often gated (i.e. is not held continuously on) to minimize both the

117 build-up of differential Nucear Overhauser Effect (NOEs) from ^{1}H to ^{31}P nuclei and heating of

118 the sample through ^{1}H irradiation.

119 **Ansiotropic electron distribution**

120 In any molecule, electron distribution is anisotropic. In solutions, rapid molecular

121 movement averages these distribution differences and the effective magnetic field experienced

122 by a nucleus is as discussed. In solids, or very viscous liquids, molecular movement is slowed,

123 leading to nuclei experiencing different localized fields and therefore a range of Lamor

124 frequencies. This leads to a broadening of the signal, and poor spectral resolution (Cade-

125 Menun, 2005a). As a result, most environmental studies extract P for identification by solution

126 ^{31}P NMR as opposed to applying NMR to the solid phase. For similar reasons, the concentration

127 of solutes/organics and viscosity of analyzed solutions may also impact spectral resolution; with

128 viscous solutions leading to broad signals and poor resolution (Cade-Menun, 2005a; Turner et
129 al., 2003b).

130 **Nuclear relaxation**

131 After the transitional energy pulse (B_1) is removed, and the spin system is relaxing to its
132 thermal equilibrium distribution, the dissipated energy is released through two processes spin-
133 spin relaxation and spin-lattice relaxation. Spin-spin is a randomized entropic process governed
134 by the spin-spin relaxation time constant T_2 and is important in determining the line width in an
135 NMR spectra, whereas during spin-lattice relaxation the energy is released to the surrounding
136 matrix, which is governed by the spin-lattice constant T_1. The process of relaxation is important
137 since the target nuclei must return to their ground state to avoid progressive saturation of the
138 sample, allowing for the collection of quantitative signals during repetitive acquisitions. Since the
139 relaxation of nuclei follows an exponential decay, a period of ~5 x T_1 is required for > 99% of
140 nuclei to return to an equilibrium state (Knicker and Nanny, 1997). The presence of natural
141 paramagnetic materials with unpaired electrons (i.e., iron and manganese), or the addition of
142 artificial transition metal complexes (e.g., ligand bound, or chloride salts, of gadolinium (III) in
143 aqueous solutions and chromium acetylacetonate in organic solvents; Claridge, 2009) provide
144 an efficient relaxation pathway. This greatly reduces T_1 constant and allowing for rapid pulses
145 (Cade-Menun et al., 2002; McDowell et al., 2006; Nanny and Minear, 1994a; Nanny and Minear,
146 1994b), although the presence of excessive quantities of “relaxation agents” can broaden
147 signals to an undesirable extent via the T_2 process. Conversely the analysis of soils with low
148 concentrations of paramagnetic ions (i.e., calcareous marshes or ombrotrophic bogs) may
149 require preliminary tests to confirm that experimental parameters allow for nuclei to return to
150 their ground state between repeated scans. Avoiding saturation of target nuclei can also be
151 achieved through the use of a reduced B_1 radio frequency pulse. If a calibrated 90° pulse is
152 applied, the nuclear magnetization vector is rotated from along the z axis of the magnet into the
153 XY plane (i.e. the tip angle = 90°) and the emitted signal is maximized. The use of a reduced

154 pulse length reduces the tip angle proportionally, while reducing the levels of energy returned on
155 a single iteration the nuclei achieve their ground state disproportionately faster. When
156 considering experiments of many thousands of combined scans this faster recovery time leads
157 to shorter total experiment times.

158 **Application of Solution ^{31}P NMR Spectroscopy to Wetland Soils**

159 **Sampling and pretreatment of soil**

160 As with all soil sampling, there is a need for clearly defined aims in studies applying ^{31}P
161 NMR spectroscopy. Considerations will include the depth of soil and increments to be sampled,
162 as well as distance to various abiotic or biotic influences (e.g. plant roots, hydrologic flow and
163 bioturbation). The time and costs associated with NMR spectroscopy (see below) often limit the
164 number of samples that can be practically analyzed, so sample amalgamation or analysis of
165 pooled extraction solutions is common. If this is carried out the sources of variance inherent to
166 specific sampling regimes and captured by sample amalgamation should be considered and
167 understood (Webster, 2007). In addition to the need for careful soil sampling (Kulmatiski and
168 Beard, 2004) and sample handling (Worsfold et al., 2005), the collection of wetland soils has a
169 number of distinct issues when considering potential sample alteration prior to analysis.
170 Pretreatment (lyophilization, air-drying or extraction of fresh samples) has been shown to have
171 significant, yet sample specific, effects on the P composition determined in wetland soils (Cade-
172 Menun, 2005a; Ding et al., 2010a; Turner et al., 2007). This may be associated with changes in
173 redox conditions, or P solubility during sample drying (Turner and Haygarth, 2001; Turner and
174 Haygarth, 2003). It is also possible that biotic changes in P forms (i.e., microbial senescence or
175 fungal sporulation) may occur during sample pretreatment. However, given the need for
176 consistency in sample pretreatment and practical constraints with rapid analysis of fresh
177 samples due to the inaccessibility of many wetland sites, we recommend the use of rapid air-
178 drying to stabilize samples. To this end samples should be spread out to dry in a thin layer
179 under conditions of elevated air flow, low humidity and warm temperatures no greater than

180 40°C. If extracting fresh samples it is important to account for water content of samples when
181 standardizing the final concentration of extraction solutions, since both the concentration of
182 extractant and solid:solution ratio influence the extraction efficiency and P composition
183 determined by NMR spectroscopy (Turner, 2008).

184 **Extraction of phosphorus from soil**

185 The extraction procedure used for ^{31}P NMR spectroscopy aims to maximize recovery of
186 P from soil while minimizing the alteration of forms present(Turner et al., 2005). Although some
187 studies apply compound-specific extractants (e.g., organic solvents to target phospholipids;
188 Bardygulanonn et al., 1995; Watts et al., 2002), most studies use a more general procedure to
189 examine all P compounds simultaneously (Cade-Menun, 2005a; Cade-Menun et al., 2002). The
190 application of ^{31}P NMR spectroscopy to sequential extractions may yield useful information on
191 the functional nature of P recovered by operational procedures (Baldwin, 1996; Condron et al.,
192 1985; Reitzel et al., 2006b; Robinson et al., 1998; Turner et al., 2006a), but can lead to
193 problems with low P concentrations in the extracts and the potential for stepwise modification of
194 P forms.

195 The most commonly used extraction procedure is a single step alkaline extraction. First
196 developed for organic P extraction from terrestrial soils (Bowman and Moir, 1993; Newman and
197 Tate, 1980), it is used to recover both organic P, and inorganic P associated with amorphous
198 metals (Cade-Menun and Preston, 1996; Turner et al., 2005). Initial extractions procedures such
199 as those of Bowman and Moir (1993) used alkaline (0.25 M NaOH plus 0.05 M
200 ethylenediaminetetraacetic acid [EDTA]) conditions at 85°C for 2 h. To reduce the risk of
201 hydrolysis at elevated temperature, however, most studies employ extractions of 4 or 16 h
202 under ambient lab temperatures (see Table 2). The alkaline degradation of some lipids and
203 RNA (Turner et al., 2003b) can also be minimized by using a lower concentration of NaOH,
204 although this can influence extraction of metals and subsequent spectral resolution (Turner,
205 2008). In the original procedure of Bowman and Moir (1993) a 1:50 solid to solution ratio was

used. Although 1:20 is now more commonly applied in mineral soils (Turner, 2008) care must be taken to ensure consistency. In fresh wetland soils high water content may preclude researchers applying a strict 1:20 ratio with potential implications upon P recovery (Turner, 2008). The use of metal chelators (i.e., EDTA, or Sephadex® and Chelex® resins) within the primary extraction solution have also been used to improve P recovery from metal-humic complexes and to reduce interference by paramagnetic species on spectral acquisition (Ahlgren et al., 2007; Cade-Menun, 2005a; Turner et al., 2005) (see below).

Treatment of paramagnetic species

To reduce the impact of sample associated paramagnetic species on spectral resolution (see Nuclear relaxation), researchers have applied both pre-extraction steps to soils, and post-extraction treatment to soil extracts (Table 2). These have included initial extracts with mineral acids (Sannigrahi and Ingall, 2005; Turner and Weckström, 2009) or metal chelators such as EDTA (Ahlgren et al., 2007; Hupfer et al., 1995; Khoshmanesh et al., 2002), and anaerobic extractions (Mahieu et al., 2000) or reducing agents such as dithionite (De Groot and Golterman, 1990), sometimes in concert with metal chelation (Carman et al., 2000; McDowell and Stewart, 2005b). Soil extracts have been treated with ion exchange media (Pant et al., 2002; Robinson et al., 1998), reducing agents (Ahlgren et al., 2005; Reitzel et al., 2007; Zhang et al., 2009) and organic precipitating agents (Ding et al., 2010b) to reduce the impact of both paramagnetic ions, and humic substances on spectral line broadening. It is worth noting that in wetland soils, where organic matter concentrations are typically high, the presence of high concentrations of dissolved organic compounds may reduce spectral resolution (see Anisotropic electron distribution).

Methods used to reduce the impact of paramagnetic ions on spectral resolution may have other implications for subsequent NMR spectroscopy. For example, EDTA used in the primary extraction step will retain any chelated paramagnetic species in solution, except at very high pH values where co-precipitation may occur (Turner, 2004). Although this reduces spin-

232 lattice relaxation times (T_1) and leads to more rapid nuclei relaxation, it may also lead to
233 undesired line broadening via spin-spin (T_2) interactions (McDowell et al., 2006; Riggle and von
234 Wandruszka, 2007). In contrast, the use of Chelex® resin can reduce line broadening by
235 removing paramagnetic species from the solution, but may necessitate an increased pulse
236 delay time (Cade-Menun and Preston, 1996; Cade-Menun et al., 2002). In addition, treatments
237 used to reduce the impact of paramagnetic ions may modify the P composition of the extract
238 (Ahlgren et al., 2007; Cade-Menun and Preston, 1996). Polyphosphates are stable under
239 alkaline extraction conditions, yet are catalytically hydrolyzed by the presence of divalent
240 cations (Harold, 1966). The routine use of sample pre-extraction to remove paramagnetic
241 species in the study of lake sediments (Ahlgren et al., 2006; Reitzel et al., 2007), as well as the
242 use of metal chelation in the primary extract (Hupfer et al., 1995), may preserve
243 polyphosphates, which would otherwise be lost from solution and the acquired spectrum.

244 **Preparation of extracts for NMR spectroscopy**

245 Although the observable nuclei ^{31}P is 100% abundant in nature, direct analysis at
246 environmental concentrations would require unfeasibly long run times. As a result, a process for
247 concentrating P is required. Rotary evaporation and lyophilization are most commonly applied to
248 wetland soils, although drying under a stream of N_2 has been used in organic terrestrial soil
249 extracts (Trasar-Cepeda et al., 1989). Given the known alkaline hydrolysis of some
250 phosphodiesters (Turner et al., 2003b), snap freezing (−80°C) to avoid prolonged alkaline
251 conditions during crystallization and lyophilization prior to re-suspension is suggested as the
252 best method to both concentrate and store samples prior to NMR spectroscopy (Cade-Menun,
253 2005a; Turner and Newman, 2005). Indeed tests a range of model compounds, (2-
254 aminoethylphosphonic acid, pyrophosphate, polyphosphate, D-glucose-6-phosphate, and
255 adenosine monophosphate) were found to be stable during lyophilization (using −20°C)
256 (Cheesman, 2010).

257 Typically, unless an internal capillary of a deuterated solvent is used, the resuspension
258 of lyophilized material must be into a liquid which contains at least a proportion of deuterium to
259 allow for NMR signal lock. Usually 10% D_2O, both 100% D_2O (Shafqat et al., 2009) and a
260 proportion of sodium deuteroxide (NaOD) (Sumann et al., 1998) have been used in studies of
261 terrestrial soils. In addition to a deuterated component, most standard techniques use either
262 deionized water or an alkaline solution (e.g. 1 M NaOH + 100 mM EDTA) to ensure a final pH
263 >13. If water is used to resuspend samples, care must be taken to note the final pH, since
264 inconsistencies in the ratio of 'organics' and salts in the lyophilized material from different
265 wetland soils may result in variation in the final pH, and alteration of chemical shifts, even in the
266 limited pH range of 10–13 (Crouse et al., 2000; McDowell and Stewart, 2005a). To ensure full
267 resuspension of lyophilized powder and the removal of particles that may otherwise disrupt
268 magnetic field homogeneity in the loaded NMR tube, solutions should be vortexed and either
269 centrifuged or filtered. The resulting solution is then loaded into a thin walled glass NMR tube
270 ready for NMR spectroscopy.

271 **Nuclear magnetic resonance spectroscopy**

272 High field super-conducting magnets used for solution ^{31}P NMR are often referred to by
273 their field strength, B_0, referenced to the resonance of ^{1}H. Therefore, a 500-MHz spectrometer
274 has a 11.7-telsa magnet in whose field ^{1}H resonates at 500 MHz and ^{31}P at 202.47 MHz. Since
275 the energy difference between nuclei states in a B_0 field increases with an increasing field
276 (Figure 2 A), sensitivity will be improved when using a larger magnet. In addition, a larger
277 magnetic field will result in greater signal dispersion and potential spectra resolution.
278 Consideration should also be given to the bore diameter of magnets and probes available.
279 Since the signal to noise (S/N) ratio is proportional to the number of nuclei monitored, a probe
280 able to contain a larger NMR tube and, therefore, a greater volume of sample will provide a
281 better S/N ratio. If sample volume is not a constraint as in most soil studies the use of larger
282 diameter probes will result in faster acquisition times.

283 Although magnet and probe size should be considered when using ^{31}P NMR
284 spectroscopy, machine availability and access may dictate which equipment is used. Most large
285 academic institutions will have an NMR facility in Chemistry or Medical departments, while
286 institutions such as the US National High Magnetic Field Laboratory (www.magnet.fsu.edu), the
287 William R. Wiley Environmental Molecular Sciences Laboratory (www.emsl.pnl.gov/emslweb/),
288 and the National Magnetic Resonance Facility at Madison (www.nmrfam.wisc.edu) offer
289 facilities to external users.

290 **Probe conditions and experimental overview**

291 After the NMR tube has been loaded into the magnet, it must be allowed to equilibrate
292 with the probe temperature decided upon for the experiment. Temperature will impact a number
293 of aspects of solution ^{31}P NMR spectroscopy and often a compromise must be decided upon for
294 'standard conditions'. In addition to changes in chemical shift associated with temperature-
295 dependent conformational changes (Turner et al., 2003b) probe temperature is also likely to
296 impact T_1 constants (Ramarajan et al., 1981), spectral resolution (Crouse et al., 2000) and,
297 given its influence on the D_2O signal lock, may lead to significant changes in chemical shifts,
298 which must be accounted for when referencing spectra. After equilibration, the probe must be
299 locked and shimmed. This process uses the deuterated solvent as a frequency signal lock to
300 account for minor discrepancies in the B_0 field strength as well as homogenizing the field
301 experienced by the sample.

302 Subsequently, the experimental parameters can be selected based upon the chosen
303 spectral window and the nature of sample (i.e., P concentration and T_1-relaxation constant). The
304 majority of P compounds in the natural environment have a chemical shift in the region between
305 +25 and –25 ppm (Turner et al., 2003b), although certain phosphonates and exotic xenobitics
306 may fall outside of this region (Gurley and Ritchey, 1976). Therefore, for most wetland soil
307 studies a spectral window of 60 ppm centered on 0 ppm will be sufficient to capture all P
308 compounds of interest. Secondly, since the NMR signal is recorded digitally, the number of

309 data points to be captured must be decided, and adjusted to achieve good digitization of signal
310 peaks. These two parameters will then dictate the acquisition time used in the pulse program
311 (Figure 3). The pulse used to excite the ^{31}P nuclei is measured in μs at a given power level, but
312 is usually expressed as the angle to which the P nuclei are perturbed from the B_0 axis within a
313 given experiment. For solution ^{31}P NMR this tip angle usually ranges from 30 to 90° (see Nuclei
314 relaxation and Table 2). This parameter can be derived from optimization studies to determine
315 both 90° and 180° tip angles in standard P containing reference solutions, though since this
316 parameter is dependent on sample specific characteristics, it is best determined on a standard
317 soil extract which has been spiked with a concentrated P standard (e.g., orthophosphate). Once
318 the pulse width and acquisition time are set, a delay between sequential pulses should be
319 chosen dependent upon tip angle and rate of relaxation (T_1 constant) determined under specific
320 experimental conditions. The T_1 relaxation rate varies between P functional groups, but if
321 optimized for orthophosphate then delay times should also be sufficient for most other P nuclei
322 (McDowell et al., 2006). The number of scans required will be dependent upon the S/N ratio
323 required for good peak identification and quantification of the various P forms present, which is
324 itself dependent upon P concentration of the sample, and the availability of spectrometer time.

325 **Spectral processing and interpretation**

326 A number of programs can be used to interpret and analyze NMR data on personal
327 computers or data-stations. These include, but are not limited to, ACD/NMR Processor
328 (ACD/Labs), Mnova NMR (Mestrelab Research), Topspin (Bruker), VnmrJ (Agilent
329 Technologies), and wxNUTS (Acorn NMR). All such software allow the transformation of
330 combined FIDs to a frequency domain spectrum via a Fourier transformation, and then provide
331 tools allowing users to interpret the spectra by identifying peaks and quantifying their relative
332 proportions. As mentioned above, the assignment of spectra peaks requires a referencing of the
333 spectra against a known standard which by convention is 85% H_3PO_4 set as 0 ppm. This can be
334 achieved by running an ‘external standard’ within a coaxial insert (Figure 4) alongside and

335 separate from the sample solution, or by using ‘internal standards’ within the solution which
336 have themselves been related to H_3PO_4. The use of standardized experimental parameters and
337 internal standards such as methylenediphosphonic acid (MDP) allows clear and reproducible
338 spectral referencing (Bedrock et al., 1994; Cheesman et al., 2010b; Turner, 2008), although
339 care must be taken in choosing an internal standard. For example, MDP is not appropriate for
340 extracts that contain glyphosate or its degradation products due to potential peak overlap
341 (Castellino et al., 1989).

342 The quantification of solution ^{31}P NMR spectra relies upon the 100% natural abundance of
343 ^{31}P in the environment. Given full P relaxation during spectra acquisition (see above) the
344 integrated area under peaks can be related to total P in solution determined by parallel analysis
345 (e.g. inductively coupled-plasma optical-emission spectrometry (ICP–OES) or digestion and
346 molybdate colorimetry) or by comparison with the area of an internal standard (e.g., MDP)
347 spiked to the solution at a known concentration. The identification and quantification of specific
348 compounds, especially within the phosphomonoesters region of poorly resolved spectra, may
349 also be achieved by automated spectral deconvolution (Turner et al., 2003a). Authentic
350 compounds spiked into soil extracts provide additional confirmation on peak identity (Smernik
351 and Dougherty, 2007), although the use of a standard resuspension protocol and spectra
352 acquisition parameters allow confidence in peak assignments for certain P nuclei, such as *myo*-
353 inositol hexakisphosphate (Figure 5), when concentrations are high enough for peaks to be
354 resolved.

355 **Recommended Solution ^{31}P NMR Procedure**

356 We recommend the use of a single step alkaline extraction with an optional pre-
357 extraction dependent upon *a priori* knowledge of the sample and an understanding of how such
358 pre-extraction will impact P recovery and spectral composition. In organic or mineral dominated
359 soils containing high concentrations of paramagnetic species a pre-extraction with buffered
360 dithionite with or without EDTA (McDowell and Stewart, 2005b) may be appropriate, especially if

361 biologically derived polyphosphates are being studied (Ahlgren et al., 2007). In calcareous
362 mineral soils a pre-extraction with a dilute mineral acid may be applied to remove alkali-stable
363 (acid-soluble) inorganic P (Turner and Weckström, 2009).
364
365 **Chemicals:**
366 Sodium hydroxide (NaOH), FW 40.00.
367 Ethylenediaminetetraacetic acid (EDTA) disodium salt ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$), FW 372.24
368 Deuterium oxide (D_2O), FW 20.03
369 (Optional)
370 Methylenediphosphonic acid (MDP), $CH_2[P(O)(OH)_2]_2$, FW 176.00
371 Sodium bicarbonate ($NaHCO_3$), FW 84.01
372 Sodium dithionite ($Na_2S_2O_4$), FW 174.11
373 Hydrochloric acid (HCl), FW 36.46
374
375 **Reagents:**
376 • NaOH–EDTA extraction solution: Dissolve 10.00 g of NaOH and 18.61 g of EDTA in 1 L of
377 deionized water. The solution contains 0.25 M NaOH and 50 mM EDTA.
378 • NaOH–EDTA re-suspension solution: Dissolve 10.0 g of NaOH and 9.31 g of EDTA in 250
379 mL of deionized water. The solution contains 1 M NaOH and 100 mM EDTA

380 (Optional)
381 •MDP solution: Dissolve 19.52 mg of MDP in 100 mL deionized water to make a solution
382 containing 50 µg P mL^{-1}. *Note MDP is hydroscopic and often contains several moles of water*
383 *per mole of solid, which varies between batches and must be accounted for when preparing the*
384 *standard solution. The solution is stable for at least 6 months in the refrigerator.*
385 • Pre-extraction solutions

386 (i) 1 M HCl: Dilute 82.6 mL of conc. (12.1M) HCl to 1 L with deionized water,

387 (ii) 50 mM EDTA: Dissolve 18.61 g of EDTA in 1 L of deionized water.

388 (iii). Buffered dithionite: Dissolve 9.24 g $NaHCO_3$ and 19.15 g of $Na_2S_2O_4$ in 1 L of

389 deionized water. The solution contains 0.11 M $NaHCO_3$ and 0.11 M of $Na_2S_2O_4$.

390

391 **Extraction procedure:**

392 1. Weigh 5.00 ± 0.01 g of air-dried soil into a 250 mL centrifuge bottle.

393 Optional pre-extraction

394 (i) Add 100 mL of pre-extraction solution (1 M HCl, buffered dithionite, or EDTA),

395 cap and shake for 1 h at room temperature.

396 (ii) Centrifuge at approx 6,000 x *g* for 15 min and decant the supernatant

397 (iii) Retain an aliquot of the supernatant for determination of P concentration if

398 required.

399 2. Add 100 mL of NaOH–EDTA extraction solution in a 1:20 solid / solution ratio (see

400 Extraction of phosphorus from soil).

401 3. Cap the bottle and shake for 4 h (or 16 h overnight) at room temperature.

402 4. Centrifuge at approx. 6,000 x *g* for 15 min and decant the supernatant.

403 5. Retain an aliquot of the supernatant for determination of total P by persulfate digestion

404 or ICP–OES after suitable dilution (e.g., 1:20 – 1:100) *(Caution: highly organic wetland soil*

405 *extracts may clog tubing or nebulizer during ICP analysis without proper dilution or prior*

406 *digestion.)*

407 Optional internal standard

408 (i) To a 20 mL aliquot of the remaining supernatant add 1 mL of the MDP solution.

409 6. Freeze the sample at –30°C or less and lyophilize (freeze-dry). Homogenize the

410 lyophilized powder by gently crushing and mixing.

411

412 **NMR spectroscopy:**

413 1. Transfer approximately 300 mg of lyophilized material to a 15 mL centrifuge tube. Re-
414 dissolve in 2.7 mL of NaOH–EDTA re-suspension solution and 0.3 mL D_2O. *(Note: Scale*
415 *proportionally for use with smaller NMR tube volumes).*

416 2. Cap and vortex for at least 1 min. Filter using 0.45 µm GF-B syringe filter prewashed
417 with NaOH–EDTA re-suspension solution.

418 3. Transfer to a 5 or 10-mm NMR tube and analyze by solution ^{31}P NMR spectroscopy.
419 Approximate machine parameters for wetland soil extracts are: broad-band decoupling
420 gated on during acquisition and off during inter-pulse delay (e.g. Brucker nomenclature –
421 zgig, or Agilent/Varian – dm = 'nny'), a 30° pulse, 2.0 s delay, 0.8 s acquisition time and
422 25°C probe temperature. These general parameters can be further optimized for the specific
423 probe and soils being used, particularly in the adjustment of pulse delay times (McDowell et
424 al., 2006). The number of scans required to obtain a well-resolved spectrum will vary
425 depending on the P concentration in the sample, but between 5,000 and 30,000 scans are
426 typical (i.e., up to 24 h).

427 4. Determine chemical shifts of signals in parts per million (ppm) relative to an external
428 standard of 85% H_3PO_4 ($\delta = 0.0$). Assign signals to individual P compounds or functional
429 groups (Turner et al., 2003b). Determine signal area by integration or spectral
430 deconvolution, and calculate P concentrations based on either total P determined in the
431 extract or the MDP internal standard.

432 Table 1. Studies employing ^{31}P nuclear magnetic resonance spectroscopy in wetland soils and sediments

Reference	Focus	Wetland type[†]	Location
Bedrock et al., 1994	Forms of P in blanket peat under different management and vegetation	Palustrine	Scotland
Hupfer et al., 1995	Detection of polyphosphate as a transient sink in benthic sediment	Lacustrine	Switzerland
Baldwin, 1996	NMR analysis coupled to modified SEDEX (Ruttenberg, 1992) sequential extraction scheme	Lacustrine	Australia
Robinson et al., 1998	NMR analysis coupled to pre-extraction of labile P in organic soils	Palustrine	FL, USA
Shand et al., 1999	Potential for use of solid state ^{31}P NMR in peats	Palustrine	Scotland
Carman et al., 2000	Oxic/anoxic conditions and presence of various cations suggested as source of variability in P composition	Lacustrine, Marine	Sweden
Delgado et al., 2000	Solid state analysis of calcareous marsh soils	Palustrine	Spain
Mahieu et al., 2000	P composition in soils under intensive lowland rice cropping	Palustirne	Philippines
Sundareshwar et al., 2001	Pyrophosphate accumulation associated with anthropogenic impact of coastal systems	Estuarine	SC, USA
Pant et al., 2002	P composition within surface sediments of a submerged aquatic vegetation (SAV) treatment wetland	Palustrine	FL, USA
Turner et al., 2003c	P composition in upland peats	Palustrine	England
Hupfer et al., 2004	Origin and diagenesis of polyphosphates in lakes with various trophic states	Lacustrine	Europe
Ahlgren et al., 2005	Attenuation of P forms with depth in sediments; half-life times estimated for pyrophosphate and organic P forms	Lacustrine	Sweden
McDowell and Stewart, 2005b	Use of a Ca-EDTA-dithionite pre-extraction step to reduce line broadening in samples with high paramagnetic ion concentrations	Riverine	New Zealand
Turner and Newman, 2005	Importance of phosphodiesters in subtropical wetlands	Palustrine	FL, USA
Ahlgren et al., 2006	Analysis of three oligotrophic lakes showing high variability in the presence of polyphosphate	Lacustrine	Sweden
Reitzel et al., 2006a	Changes in P groups with time in sediment and with addition of Al as a lake management strategy	Lacustrine	Denmark
Reitzel et al., 2006b	P composition within lake sediments from arange of trophic states	Lacustrine	Denmark
Turner, 2006	Analysis of soils under rice cultivation, including flood irrigation	Palustrine	Madagascar
Turner et al., 2006a	Orthophosphate association with organic molecules preventing detection by standard molybdate colorimetric methods	Palustrine	FL, USA
Turner et al., 2006b	Biogenic P forms within treatment wetlands dominated by phosphodiesters	Palustrine	FL, USA
Ahlgren et al., 2007	Comparison of NaOH and NaOH + EDTA extraction using bicarbonate buffered dithionite or EDTA as a pre-extraction step	Lacustrine	Sweden
Reitzel et al., 2007	Sources and degradation of polyphosphates and organic P in sediments	Lacustrine	Sweden

Turner et al., 2007	Comparison of sample handling procedures prior to extraction and identification of P forms	Palustrine	FL, USA
El-Rifai et al., 2008	Parallel analysis with mass spectroscopy	Palustrine	FL, USA
Bai et al., 2009	Presence of organic P in a eutrophic lake	Lacustrine	China
Liu et al., 2009	Dominance of orthophosphate and phophomonoesters in heavily eutrophic lake systems	Lacustrine	China
McDowell, 2009	Changes in stream sediment P forms as a result of surrounding land use change	Riverine	New Zeland
Simon et al., 2009	Changes in P forms associated with *Aphanizomenon flos-aquae* bloom	Lacustrine	OR,USA
Sundareshwar et al., 2009	Diversity of P forms used as a measure of ecosystem function	Palustrine	NC & SD USA
Turner and Weckström, 2009	Use of phytate in brackish sediments as a paleo-indicator	Lacustrine	Denmark
Zhang et al., 2009	Surface sediments from 7 shallow lakes of various trophic status	Lacustrine	China
Cheesman et al., 2010a	Phosphorus forms in surface soils across a nutrient gradient	Palustrine	FL, USA
Cheesman et al., 2010b	Phosphorus forms across the upland-wetland transition	Palustrine	FL,USA
Ding et al., 2010a	Consideration of EDTA pretreatement	Lacustrine	China

433 † = Dominant system type as designated by Cowardin et al. (1979)

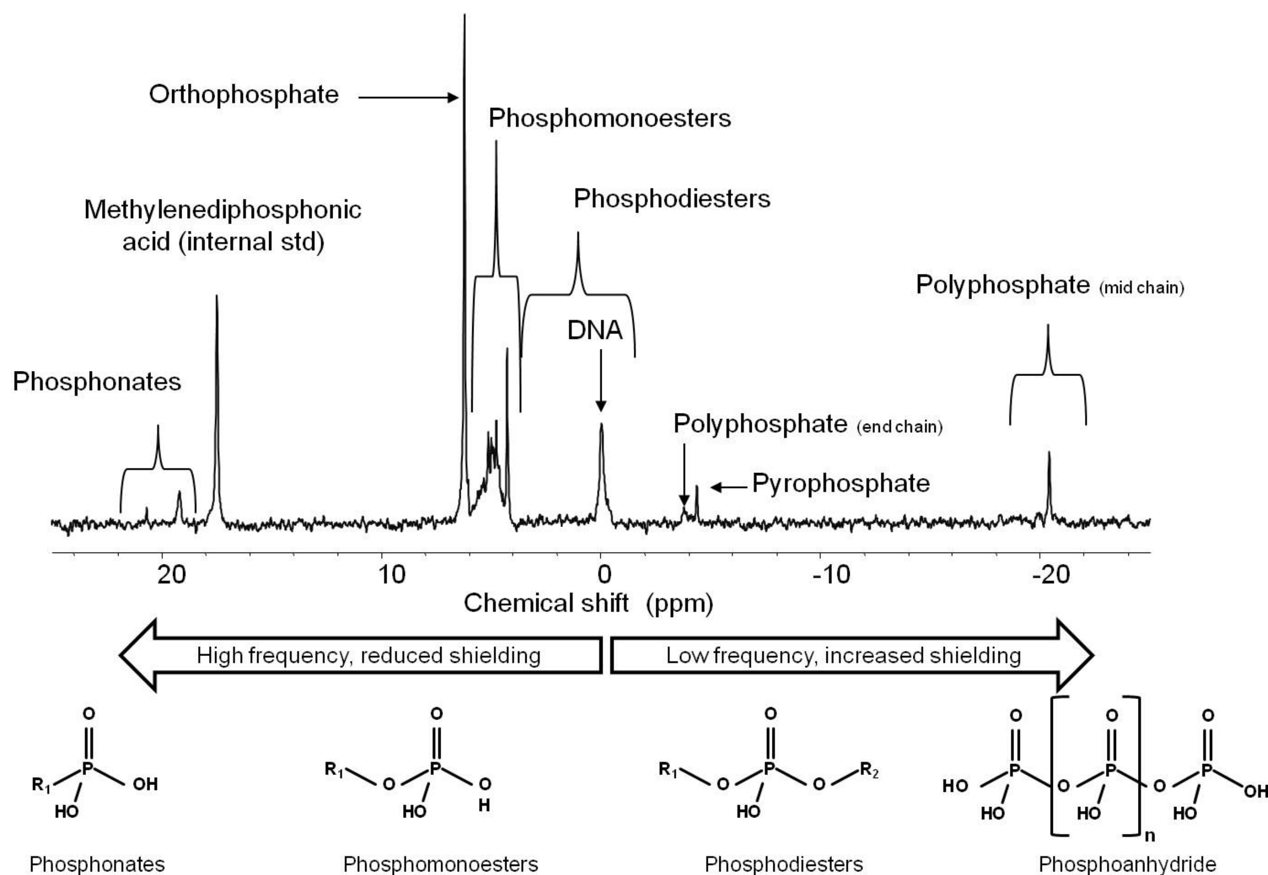
434 Table 2. Methodological details of studies employing ^{31}P nuclear magnetic resonance spectroscopy in wetland soils

Reference	Extraction method‡		Treatment of Paramagnetic Species§		Concentration¶	Acquisition Pulse	
			Pre-extraction	Post-extraction		Pulse width	Delay (s)
Bedrock et al., 1994	0.5 M NaOH	5min, 16 h	na	na	Rotary evap.	90°	0.2
Hupfer et al., 1995	0.2 M NaOH + 67 mM EDTA	2 h	67 mM EDTA	na	Rotary evap	-	10
Baldwin, 1996	SEDEX sequential (Ruttenberg, 1992)	-	na	na	na	-	-
Robinson et al., 1998	0.25 M NaOH + 50 mM EDTA	2 h 85°C	1M KCl/ $NaHCO_3$	Chelex X-100 Column	Lypholization	-	1.5
Carman et al., 2000	0.5 M NaOH	24 h	CDB $MgCl_2$	na	Rotary evap.	90°	1
Mahieu et al., 2000	0.25 M NaOH	20 h	na	HCl/ HF Dialysis	Lyophilization	30°	<2
Sundareshwar et al., 2001	0.5 M NaOH + 100 mM EDTA	16 h	na	na	na	45 °	2.1
Pant et al., 2002	0.4 M NaOH	4 h	na	G-25 Sephadex	Rotary evap.	90°	5
Turner et al., 2003c	0.25 M NaOH + 50 mM EDTA	16 h	na	na	Lypholization	30°	1
Hupfer et al., 2004	0.2 M NaOH + 67 mM EDTA	16 h	67 mM EDTA	na	Rotary evap.	-	2
Ahlgren et al., 2005	0.1 M NaOH	16 h	na	dithionite	Rotary evap.	72°	0.2
McDowell and Stewart, 2005b	0.25 M NaOH + 50 mM EDTA	16 h	Ca-EDTA-dithionite	na	Lypholization	45°	5
Turner and Newman, 2005	0.25M NaOH + 50 mM EDTA	4 h	na	na	Lypholization	45°	1
Ahlgren et al., 2006	0.125 M NaOH + 25 mM EDTA	16 h	na	dithionite	Rotary evap.	63°	1.2
Reitzel et al., 2006a	0.125 M NaOH + 25 mM EDTA	16 h	na	dithionite	Rotary evap.	63°	1.2
Reitzel et al., 2006b	Sequential (Psenner and Pucsko, 1988)	-	na	na	Rotary evap.	63°	1.2
Turner, 2006	0.25 M NaOH + 50mM EDTA	16 h	na	na	Lypholization	45°	2
Turner et al., 2006a	0.5 M NaOH	16 h	0.5 M $NaHCO_3$ 1 M HCl	na	Lypholization	45°	2
Turner et al., 2006b	0.25M NaOH + 50mM EDTA	4 h	na	na	Lypholization	45°	1
Ahlgren et al., 2007	0.1 M NaOH, 0.125 M NaOH + 0.25 M EDTA	16 h	dithionite or EDTA	na	Rotary evap	63°	1.2
Reitzel et al., 2007	0.1 M NaOH and Sequential	-	dithionite	dithionite	Rotary evap	63°	1.2

	(Psenner and Pucsko, 1988)						
Turner et al., 2007	0.25 M NaOH + 50mM EDTA	16 h	na	na	Lypholization	45°	2
El-Rifai et al., 2008	0.25 M NaOH + 50mM EDTA	16 h	HF	na	Lyphilization	45°	1.5
Bai et al., 2009	0.1 M NaOH	16 h	EDTA−dithonite	na	Rotary evap	90°	2
Liu et al., 2009	0.25 M NaOH + 50mM EDTA	16 h	na	na	Lypholization	45°	2
McDowell, 2009	0.25 M NaOH + 50 mM EDTA	16 h	na	na	Lyophilization	45°	4
Simon et al., 2009	0.25 M NaOH + 50 mM EDTA	16 h	CDB $MgCl_2$ 1M HCl	na	Lyophilization	30°	1
Sundareshwar et al., 2009	0.25M NaOH + 0.1M EDTA	-	na	na	Lypholization	45°	2.1
Turner and Weckström, 2009	0.25 M NaOH + 50mM EDTA	16 h	1 M HCl	na	Lypholization	45°	2
Zhang et al., 2009	0.25 M NaOH	16 h	na	BD	Lypholization	90°	4
Cheesman et al., 2010a	0.25 M NaOH + 50 mM EDTA	4 h	na	na	Lyophilization	30°	2
Cheesman et al., 2010b	0.25 M NaOH + 50 mM EDTA	4 h	na	na	Lyophilization	30°	2
Ding et al., 2010a	0.25 M NaOH + 50 mM EDTA	16 h	EDTA	na	Rotary evap	90°	2

435 ‡ extraction method; single step extraction or steps used within ^{31}P NMR studies
436 § Treatment of paramagnetic species; method applied to minimize effect of paramagnetic species, pre or post extraction; (EDTA)=
437 Ethylenediaminetetraacetic acid, (CDB)= Citrate+ Dithonite+ Bicarbonate, (BD)= Bicarbonate + Dithonite, (HF) = hydrofluoric acid
438 ¶ Concentration; method used to concentrate sample prior to ^{31}P NMR analysis
439 Acquisition parameters used in ^{31}P NMR analysis
440 na = not applicable
441 - = not reported

442 Figure 1. Example solution ^{31}P nuclear magnetic resonance spectrum showing commonly identified peaks. Phosphorus nuclei are
443 separated due to differences in shielding from the applied magnetic field. The sample is a surface soil from a Carolina Bay, SC
444 extracted using 0.25 M NaOH + 50 mM EDTA, with pre-concnetration by lypholization and resuspension in 1 M NaOH + 100 mM
445 EDTA and 10% D_2O. Spectra were acquired using a Bruker Avance 500 Console with a Magnex 11.75 T/54 mm magnet using a 10
446 mm BBO probe at a stabilized 25° C with a calibrated (~30°) pulse length, a zgig pulse program, and a 2 s pulse delay.



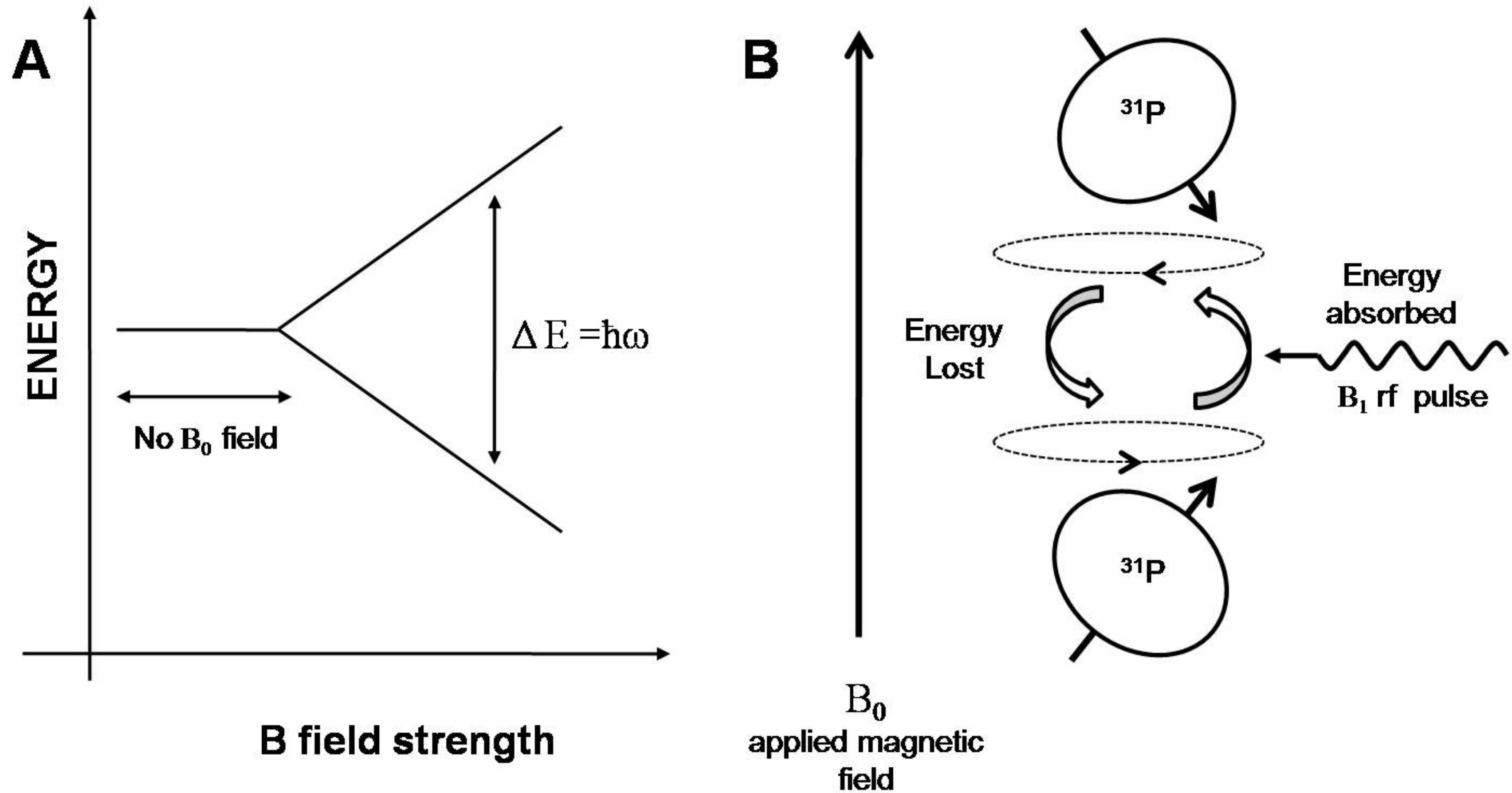
447

448 Figure 2 - Response of phosphorus nuclei (I = ½ ; γ = 10.829 x 10^7 rad/T/sec) to an applied magnetic field. A) Zeeman splitting of a

449 m_I = ½ system, B) graphical representation of precessional orbit of ^{31}P nuclear magnetic dipole around the applied magnetic field

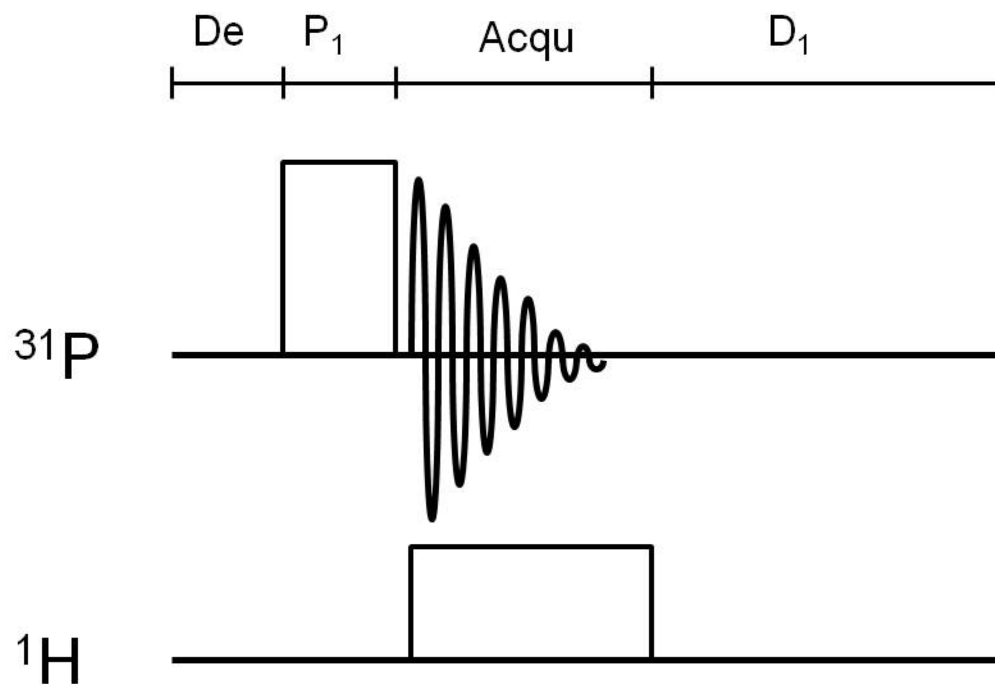
450 with transition due to applied B_1 radio frequency (rf) pulse (Adapted from (Cade-Menun, 2005a; Knicker and Nanny, 1997).

451



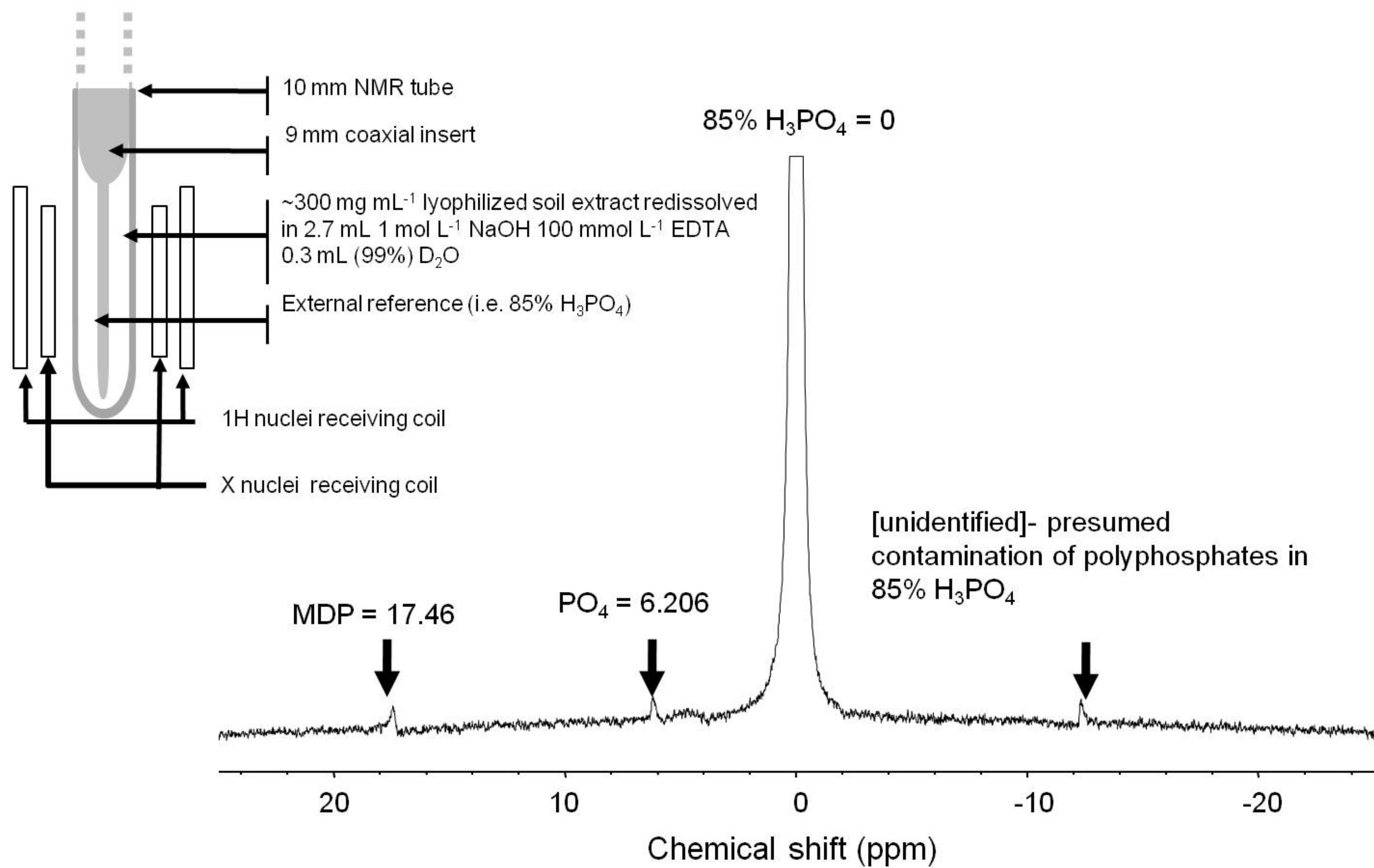
452

453 Figure 3. Pulse program for solution ^{31}P NMR experiment with heteronuclear proton decoupling. Pulse program represents
454 zero-gated inverse-gated (zgig) profile for two channels (^{31}P, ^{1}H) (Berger and Siegmar, 2004). For the purposes of illustration Bruker
455 nomenclature is used, De = prescan delay, P_1 = excitation pulse, Acqu = signal acquisition, D_1 = pulse delay.
456



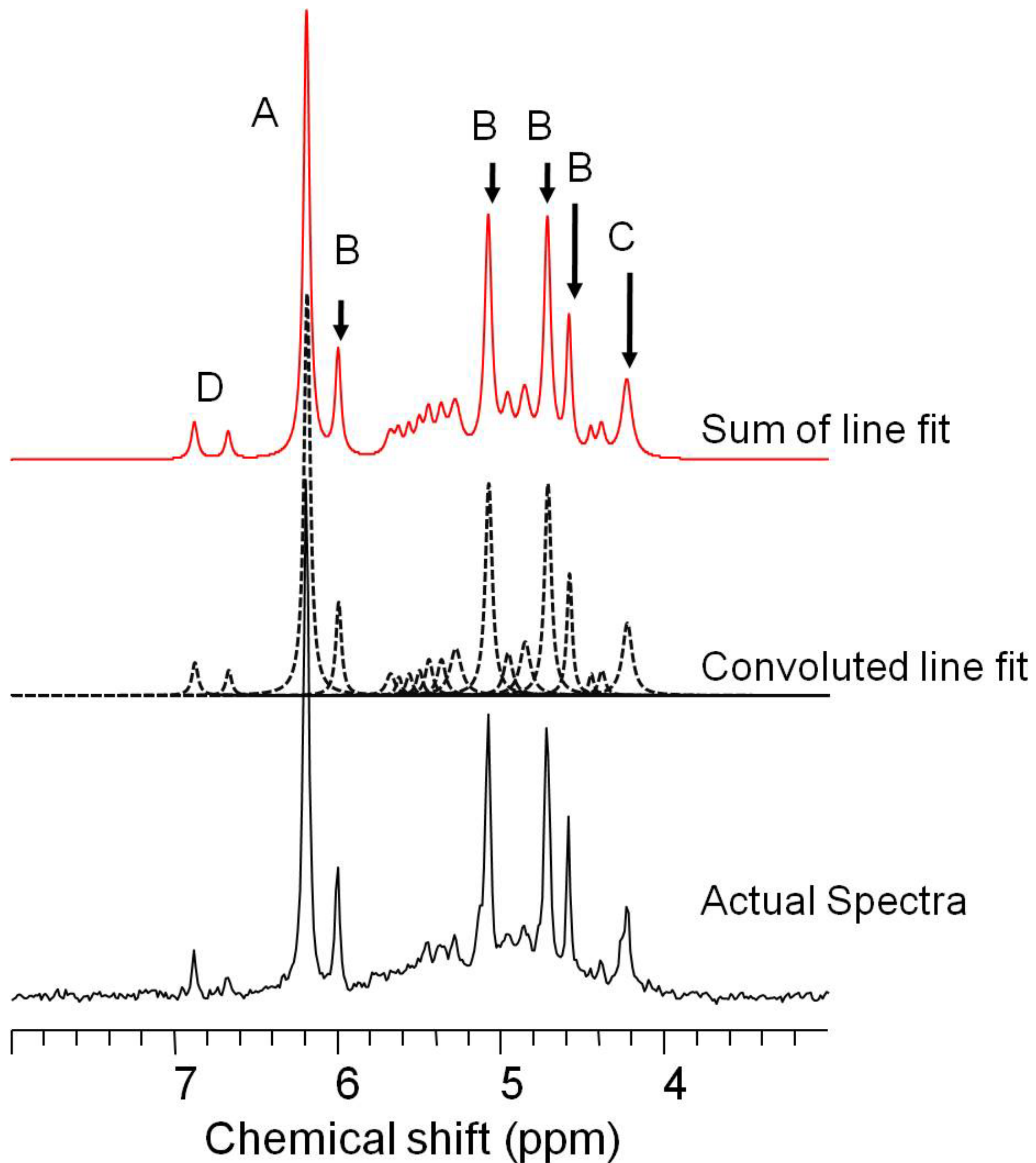
457

458 Figure 4. Referencing of solution ^{31}P NMR comparing externally held H_3PO_4 and internal standard, methylenediphosphonic
459 acid (MDP).



460

461 Figure 5. Solution ^{31}P spectral deconvolution to identify selected P peaks within
462 phosphomonoester region. Sample is a surfacre soil from a Carolina Bay wetland plotted using
463 2 Hz line broadening, with automatic peak picking and deconvolution algorithm applied. A =
464 orthophosphate B = *myo* - inositol hexakisphosphate, C = *scyllo* - inosito hexakisphosphate, D =
465 unidentified phosphomonoesters



466

References

Ahlgren J., K. Reitzel, R. Danielsson, A. Gogoll, and E. Rydin. 2006. Biogenic phosphorus in oligotrophic mountain lake sediments: Differences in composition measured with NMR spectroscopy. Water Res. 40:3705-3712.

Ahlgren J., L. Tranvik, A. Gogoll, M. Waldeback, K. Markides, and E. Rydin. 2005. Sediment depth attenuation of biogenic phosphorus compounds measured by ^{31}P NMR. Environ. Sci. Technol. 39:867-872.

Ahlgren J., H. De Brabandere, K. Reitzel, E. Rydin, A. Gogoll, and M. Waldeback. 2007. Sediment phosphorus extractants for phosphorus-31 nuclear magnetic resonance analysis: A quantitative evaluation. J. Environ. Qual. 36:892-898.

Bai X.L., S.M. Ding, C.X. Fan, T. Liu, D. Shi, and L. Zhang. 2009. Organic phosphorus species in surface sediments of a large, shallow, eutrophic lake, Lake Taihu, China. Environ. Pollut. 157:2507-2513.

Baldwin D.S. 1996. The phosphorus composition of a diverse series of Australian sediments. Hydrobiologia 335:63-73.

Bardygulanonn L.G., J.L. Kaster, and T. Glonek. 1995. Phospholipid profiling of sediments using ^{31}P nuclear-magnetic- resonance. Lipids 30:1047-1051.

Bedrock C.N., M.V. Cheshire, J.A. Chudek, B.A. Goodman, and C.A. Shand. 1994. Use of ^{31}P NMR to study the forms of phosphorus in peat soils. Sci. Total Environ. 152:1-8.

Berger S., and S. Siegmar. 2004. 200 and More NMR experiments: a practical course. John Wiley & Sons, Hoboken, NJ.

Berger S., S. Braun, and H.O. Kalinowski. 1997. NMR Spectroscopy of the non metallic elements. John Wiley & Sons, Hoboken, NJ.

Bowman R.A., and J.O. Moir. 1993. Basic EDTA as an extractant for soil organic phosphorus. Soil Sci. Soc. Am. J. 57:1516-1518.

Bunemann E.K., R.J. Smernik, P. Marschner, and A.M. McNeill. 2008. Microbial synthesis of organic and condensed forms of phosphorus in acid and calcareous soils. Soil Biol. Biochem. 40:932-946.

Cade-Menun B.J. 2005a. Using phosphorus-31 nuclear magnetic resonance spectroscopy to characterize organic phosphorus in environmental samples, in: B. L. Turner, et al. (Eds.), Organic phosphorus in the environment, CABI Publishing, Wallingford UK. pp. 21-44.

Cade-Menun B.J. 2005b. Characterizing phosphorus in environmental and agricultural samples by ^{31}P nuclear magnetic resonance spectroscopy. Talanta 66:359-371.

Cade-Menun B.J., and C.M. Preston. 1996. A comparison of soil extraction procedures for ^{31}P NMR spectroscopy. Soil Sci. 161:770-785.

Cade-Menun B.J., J.A. Navaratnam, and M.R. Walbridge. 2006. Characterizing dissolved and particulate phosphorus in water with ^{31}P nuclear magnetic resonance spectroscopy. Environ. Sci. Technol. 40:7874-7880.

Cade-Menun B.J., C.W. Liu, R. Nunlist, and J.G. McColl. 2002. Soil and litter phosphorus 31 nuclear magnetic resonance spectroscopy: Extractants, metals, and phosphorus relaxation times. J. Environ. Qual. 31:457-465.

Canet D. 1996. Nuclear Magnetic Resonance: Concepts and Methods. John Wiley & Sons, Hoboken, NJ.

Carman R., G. Edlund, and C. Damberg. 2000. Distribution of organic and inorganic phosphorus compounds in marine and lacustrine sediments: a ^{31}P NMR study. Chem. Geol. 163:101-114.

Castellino S., G.C. Leo, R.D. Sammons, and J.A. Sikorski. 1989. ^{31}P, ^{15}N, and ^{13}C NMR of glyphosphate - comparison of pH titrations to the herbicidal dead-end complex with 5-enolpyruvoylshikimate - 3-phosphate synthase Biochemistry 28:3856-3868.

513 Cheesman A.W. 2010. Biogenic phosphorus in palustrine wetlands:sources and stabilization. PhD Thesis,
514 Soil and Water Science Department, University of Florida, Florida, Gainesville. pp. 319.
515 Cheesman A.W., B.L. Turner, P.W. Inglett, and K.R. Reddy. 2010a. Phosphorus transfomrations during
516 decomposition of wetland macrophytes. Environ. Sci. Technol. 44:9265-9271.
517 Cheesman A.W., E.J. Dunne, B.L. Turner, and K.R. Reddy. 2010b. Soil Phosphorus Forms in Hydrologically
518 Isolated Wetlands and Surrounding Pasture Uplands. J. Environ. Qual. 39:1517-1525.
519 Claridge T.D.W. 2009. High-resolution NMR techniques in organiic chemistry. Elsevier Science, Oxford.
520 Condron L.M., K.M. Goh, and R.H. Newman. 1985. Nature and distribution of soil-phosphorrus as
521 revelaled by a sequential extraction method followed by ^{31}P nuclear magnetiic-resonance
522 analysis. J. Soil Sci. 36:199-207.
523 Condron L.M., E. Frossard, R.H. Newman, P. Tekely, and J.L. Morel. 1997. Use of ^{31}P NMR in the study of
524 soils and the environment, in: M. A. Nanny, et al. (Eds.), Nuclear Magnetic Resonance
525 Spectroscopy in Environmental Chemistry, Oxford University Press, Oxford. pp. 247-271.
526 Conte P., D. Smejkalova, A. Piccolo, and R. Spaccini. 2008. Evaluation of the factors affecting direct
527 polarization solid state ^{31}P NMR spectroscopy of bulk soils. Eur. J. Soil Sci. 59:584-591.
528 Cowardin L.M., V. Carter, F.C. Golet, and E.T. LaRoe. 1979. Classification of wetlands and deepwater
529 habitats of the United States. U.S. Fish and Wildlife Service, Washington.
530 Crouse D.A., H. Sierzputowska-Gracz, and R.L. Mikkelsen. 2000. Optimization of sample pH and
531 temperature for phosphorus-31 nuclear magnetic resonance spectroscopy of poultry manure
532 extracts. Commun. Soil Sci. Plant Anal. 31:229-240.
533 De Groot C.J., and H.L. Golterman. 1990. Sequential fractionation of sediment phosphate. Hydrobiologia
534 192:143-148.
535 Delgado A., J.R. Ruiz, M.D. del Campillo, S. Kassem, and L. Andreu. 2000. Calcium- and iron-related
536 phosphorus in calcareous and calcareous marsh soils: Sequential chemical fractionation and ^{31}P
537 nuclear magnetic resonance study. Commun. Soil Sci. Plant Anal. 31:2483-2499.
538 Ding S.M., X.L. Bai, C.X. Fan, and L. Zhang. 2010a. Caution Needed in Pretreatment of Sediments for
539 Refining Phosphorus-31 Nuclear Magnetic Resonance Analysis: Results from a Comprehensive
540 Assessment of Pretreatment with Ethylenediaminetetraacetic Acid. J. Environ. Qual. 39:1668-
541 1678.
542 Ding S.M., D. Xu, B. Li, C.X. Fan, and C.S. Zhang. 2010b. Improvement of ^{31}P NMR spectral resolution by
543 8-hydroxyquinoline precipitation of paramagnetic Fe and Mn in environmental samples.
544 Environ. Sci. Technol. 44:2555-2561.
545 Dougherty W.J., R.J. Smernik, and D.J. Chittleborough. 2005. Application of spin counting to the solid-
546 state ^{31}P NMR analysis of pasture soils with varying phosphorus content. Soil Sci. Soc. Am. J.
547 69:2058-2070.
548 El-Rifai H., M. Heerboth, T.E. Gedris, S. Newman, W. Orem, and W.T. Cooper. 2008. NMR and mass
549 spectrometry of phosphorus in wetlands. Eur. J. Soil Sci. 59:517-525.
550 Gurley T.W., and W.M. Ritchey. 1976. Analysis of organophosphorus compounds at parts-per-million
551 level by phosphorus-31 Fourier transform nuclear magnetic-resonance spectroscopy. Anal.
552 Chem. 48:1137-1140.
553 Harold F.M. 1966. Inorganic polyphophates in biology- structure metabolism and function. Bacteriol.
554 Rev. 30:772-794.
555 Heighton L., W.F. Schmidt, and R.L. Siefert. 2008. Kinetic and equilibrium constants of phytic acid and
556 ferric and ferrous phytate derived from nuclear magnetic resonance spectroscopy. J. Agric. Food
557 Chem. 56:9543-9547.
558 Hupfer M., R. Gachter, and H. Rüegger. 1995. Polyphosphate in lake-sediments ^{31}P NMR spectroscopy as
559 a tool for its identification. Limnol. Oceanogr. 40:610-617.

560 Hupfer M., B. Rube, and P. Schmieder. 2004. Origin and diagenesis of polyphosphate in lake sediments:
561 A ^{31}P-NMR study. Limnol. Oceanogr. 49:1-10.
562 Khoshmanesh A., B.T. Hart, A. Duncan, and R. Beckett. 2002. Luxury uptake of phosphorus by sediment
563 bacteria. Water Res. 36:774-778.
564 Knicker H., and M.A. Nanny. 1997. Nuclear magnetic resonance spectroscopy, in: M. A. Nanny, et al.
565 (Eds.), Nuclear Magnetic Resonance Spectroscopy in Environmental Chemistry, Oxford
566 University Press, Oxford. pp. 3-15.
567 Koukol O., F. Novak, and R. Hrabal. 2008. Composition of the organic phosphorus fraction in
568 basidiocarps of saprotrophic and mycorrhizal fungi. Soil Biol. Biochem. 40:2464-2467.
569 Kulmatiski A., and K.H. Beard. 2004. Reducing sampler error in soil research. Soil Biol. Biochem. 36:383-
570 385.
571 Liu J.Y., H. Wang, H.J. Yang, Y.J. Ma, and O.C. Cai. 2009. Detection of phosphorus species in sediments of
572 artificial landscape lakes in China by fractionation and phosphorus-31 nuclear magnetic
573 resonance spectroscopy. Environ. Pollut. 157:49-56.
574 Mahieu N., D.C. Olk, and E.W. Randall. 2000. Analysis of phosphorus in two humic acid fractions of
575 intensively cropped lowland rice soils by ^{31}P-NMR. Eur. J. Soil Sci. 51:391-402.
576 Makarov M.I., L. Haumaier, W. Zech, O.E. Marfenina, and L.V. Lysak. 2005. Can ^{31}P NMR spectroscopy be
577 used to indicate the origins of soil organic phosphates? Soil Biol. Biochem. 37:15-25.
578 McDowell R.W. 2009. Effect of land use and moisture on phosphorus forms in upland stream beds in
579 South Otago, New Zealand. Mar. Freshw. Res. 60:619-625.
580 McDowell R.W., and I. Stewart. 2005a. Peak assignments for phosphorus-31 nuclear magnetic resonance
581 spectroscopy in pH range 5-13 and their application in environmental samples. Chem. Ecol.
582 21:211-226.
583 McDowell R.W., and I. Stewart. 2005b. An improved technique for the determination of organic
584 phosphorus in sediments and soils by ^{31}P nuclear magnetic resonance spectroscopy. Chem. Ecol.
585 21:11-22.
586 McDowell R.W., I. Stewart, and B.J. Cade-Menun. 2006. An examination of spin-lattice relaxation times
587 for analysis of soil and manure extracts by liquid state phosphorus-31 nuclear magnetic
588 resonance spectroscopy. J. Environ. Qual. 35:293-302.
589 Murthy P.P.N. 2007. Identification of inositol phosphates by nuclear magnetic resonance spectroscopy:
590 unravelling structural diversity, Inositol phosphates: linking agriculture and the environment,
591 CABI, Wallingford UK. pp. 7-22.
592 Nanny M.A., and R.A. Minear. 1994a. Use of lanthanide shift-reagents with ^{31}P FT-NMR spectroscopy to
593 analyze concentrated lake-water samples. Environ. Sci. Technol. 28:1521-1527.
594 Nanny M.A., and R.A. Minear. 1994b. Organic phosphorus in the hydrosphere - Characterization via ^{31}P
595 fourier transform nuclear magnetic resonance spectroscopy in: L. A. Baker (Ed.), Environmental
596 Chemistry of Lakes and Reservoirs, Amer Chemical Soc, Washington. pp. 161-191.
597 Nanny M.A., and R.A. Minear. 1997. 31P FT-NMR of concentrated Lake Water Samples, in: M. A. Nanny,
598 et al. (Eds.), Nuclear Magnetic Resonance Spectroscopy in Environmental Chemistry, Oxford
599 University Press, Oxford. pp. 221-246.
600 Newman R.H., and K.R. Tate. 1980. Soil-phosphorus characterization by ^{31}P nuclear magnetic-resonance.
601 Commun. Soil Sci. Plant Anal. 11:835-842.
602 Newman S., and J.S. Robinson. 1999. Forms of organic phosphorus in water, soils, and sediments, in: K.
603 R. Reddy, et al. (Eds.), Phosphorus biogeochemistry of subtropical ecosystems, CRC Press LLC,
604 Boca Raton, Florida. pp. 207-223.
605 Pant H.K., and K.R. Reddy. 2001. Hydrologic influence on stability of organic phosphorus in wetland
606 detritus. J. Environ. Qual. 30:668-674.

607 Pant H.K., K.R. Reddy, and F.E. Dierberg. 2002. Bioavailability of organic phosphorus in a submerged
608 aquatic vegetation-dominated treatment wetland. J. Environ. Qual. 31:1748-1756.
609 Psenner R., and R. Pucsko. 1988. Phosphorus fractionation advantages and limits of the method for the
610 study of sediment P origins and interactions. Ergebnisse der Limnologie 30:43-60.
611 Ramarajan K.R., M.D. Herd, and K.D. Berlin. 1981. Spin-lattice relaxation phoenomena (T1 values) of the
612 ^{31}P nucleus in certain classes of organo-phosphorus comounds. Phosphorus Sulfur Silicon Relat.
613 Elem. 11:199-209.
614 Reddy K.R., R.H. Kadlec, E. Flaig, and P.M. Gale. 1999. Phosphorus retention in streams and wetlands: A
615 review. Crit. Rev. Environ. Sci. Technol. 29:83.
616 Reitzel K., J. Ahlgren, A. Gogoll, and E. Rydin. 2006a. Effects of aluminum treatment on phosphorus,
617 carbon, and nitrogen distribution in lake sediment: A ^{31}P NMR study. Water Res. 40:647-654.
618 Reitzel K., H.S. Jensen, M. Flindt, and F.O. Andersen. 2009. Identification of dissolved nonreactive
619 phosphorus in freshwater by precipitation with aluminum and subsequent ^{31}P NMR Analysis.
620 Environ. Sci. Technol. 43:5391-5397.
621 Reitzel K., J. Ahlgren, A. Gogoll, H.S. Jensen, and E. Rydin. 2006b. Characterization of phosphorus in
622 sequential extracts from lake sediments using ^{31}P nuclear magnetic resonance spectroscopy.
623 Can. J. Fish. Aquat. Sci. 63:1686-1699.
624 Reitzel K., J. Ahlgren, H. DeBrabandere, M. Waldeback, A. Gogoll, L. Tranvik, and E. Rydin. 2007.
625 Degradation rates of organic phosphorus in lake sediment. Biogeochemistry 82:15-28.
626 Riggle J., and R. von Wandruszka. 2007. ^{31}P NMR peak width in humate-phosphate complexes. Talanta
627 73:953-958.
628 Robinson J.S., C.T. Johnston, and K.R. Reddy. 1998. Combined chemical and ^{31}P-NMR spectroscopic
629 analysis of phosphorus in wetland organic soils. Soil Sci. 163:705-713.
630 Ruttenberg K.C. 1992. Development of a sequential extraction method for different forms of phosphorus
631 in marine-sediments. Limnol. Oceanogr. 37:1460-1482.
632 Sannigrahi P., and E. Ingall. 2005. Polyphosphates as a source of enhanced P fluxes in marine sediments
633 overlain by anoxic waters: evidence from ^{31}P NMR. Geochem. Trans. 6:52-59.
634 Shafqat M.N., G.M. Pierzynski, and K. Xia. 2009. Phosphorus source effects on soil organic phosphorus: a
635 ^{31}P NMR study. Commun. Soil Sci. Plant Anal. 40:1722-1746.
636 Shand C.A., M.V. Cheshire, C.N. Bedrock, P.J. Chapman, A.R. Fraser, and J.A. Chudek. 1999. Solid-phase
637 ^{31}P NMR spectra of peat and mineral soils, humic acids and soil solution components: influence
638 of iron and manganese. Plant Soil 214:153-163.
639 Simon N.S., D. Lynch, and T.N. Gallaher. 2009. Phosphorus Fractionation in Sediment Cores Collected In
640 2005 Before and After Onset of an Aphanizomenon flos-aquae Bloom in Upper Klamath Lake,
641 OR, USA. Water Air Soil Pollut. 204:139-153.
642 Smernik R.J., and W.J. Dougherty. 2007. Identification of phytate in phosphorus-31 nuclear magnetic
643 resonance spectra: the need for spiking. Soil Sci Soc Am J 71:1045-1050.
644 Sumann M., W. Amelung, L. Haumaier, and W. Zech. 1998. Climatic effects on soil organic phosphorus in
645 the North American Great Plains identified by phosphorus-31 nuclear magnetic resonance. Soil
646 Sci. Soc. Am. J. 62:1580-1586.
647 Sundareshwar P.V., C.J. Richardson, R.A. Gleason, P.J. Pellechia, and S. Honomichl. 2009. Nature versus
648 nurture: functional assessment of restoration effects on wetland services using nuclear
649 magnetic resonance spectroscopy. Geophys. Res. Lett. 36:L03402.
650 Sundareshwar P.V., J.T. Morris, P.J. Pellechia, H.J. Cohen, D.E. Porter, and B.C. Jones. 2001. Occurrence
651 and ecological implications of pyrophosphate in estuaries. Limnol. Oceanogr. 46:1570-1577.
652 Trasar-Cepeda M.C., F. Gil-Sotres, W. Zech, and H.G. Alt. 1989. Chemical and spectral-analysis of organic
653 P forms in acid high organic matter soils in Galacia (NW Spain) Sci. Total Environ. 81-2:429-436.

654 Turner B.L. 2004. Optimizing phosphorus characterization in animal manures by solution phosphorus-31
655 nuclear magnetic resonance spectroscopy. J. Environ. Qual. 33:757-766.
656 Turner B.L. 2006. Organic phosphorus in Madagascan rice soils. Geoderma 136:279-288.
657 Turner B.L. 2008. Soil organic phosphorus in tropical forests: an assessment of the NaOH-EDTA
658 extraction procedure for quantitative analysis by solution ^{31}P NMR spectroscopy. Eur. J. Soil Sci.
659 59:453-466.
660 Turner B.L., and P.M. Haygarth. 2001. Biogeochemistry - Phosphorus solubilization in rewetted soils.
661 Nature 411:258-258.
662 Turner B.L., and P.M. Haygarth. 2003. Changes in bicarbonate-extractable inorganic and organic
663 phosphorus by drying pasture soils. Soil Sci. Soc. Am. J. 67:344-350.
664 Turner B.L., and S. Newman. 2005. Phosphorus cycling in wetland soils: the importance of phosphate
665 diesters. J. Environ. Qual. 34:1921-1929.
666 Turner B.L., and K. Weckström. 2009. Phytate as a novel phosphorus-specific paleo-indicator in aquatic
667 sediments. J. Paleolimnol. 42:391-400.
668 Turner B.L., N. Mahieu, and L.M. Condron. 2003a. Quantification of *myo*-inositol hexakisphosphate in
669 alkaline soil extracts by solution ^{31}P NMR spectroscopy and spectral deconvolution. Soil Sci.
670 168:469-478.
671 Turner B.L., N. Mahieu, and L.M. Condron. 2003b. Phosphorus-31 nuclear magnetic resonance spectral
672 assignments of phosphorus compounds in soil NaOH-EDTA extracts. Soil Sci. Soc. Am. J. 67:497-
673 510.
674 Turner B.L., S. Newman, and K.R. Reddy. 2006a. Overestimation of organic phosphorus in wetland soils
675 by alkaline extraction and molybdate colorimetry. Environ. Sci. Technol. 40:3349-3354.
676 Turner B.L., S. Newman, and J.M. Newman. 2006b. Organic phosphorus sequestration in subtropical
677 treatment wetlands. Environ. Sci. Technol. 40:727-733.
678 Turner B.L., J.A. Chudek, B.A. Whitton, and R. Baxter. 2003c. Phosphorus composition of upland soils
679 polluted by long-term atmospheric nitrogen deposition. Biogeochemistry 65:259-274.
680 Turner B.L., B.J. Cade-Menun, L.M. Condron, and S. Newman. 2005. Extraction of soil organic
681 phosphorus. Talanta 66:294-306.
682 Turner B.L., S. Newman, A.W. Cheesman, and K.R. Reddy. 2007. Sample pretreatment and phosphorus
683 speciation in wetland soils. Soil Sci. Soc. Am. J. 71:1538-1546.
684 Watts E.E., P.A.W. Dean, and R.R. Martin. 2002. P-31 nuclear magnetic resonance study of sediment
685 microbial phospholipids. Can. J. Anal. Sci. Spectrosc. 47:127-133.
686 Webster R. 2007. Analysis of variance, inference, multiple comparisons and sampling effects in soil
687 research. Eur. J. Soil Sci. 58:74-82.
688 Wilson M.A. 1987. NMR techniques and applications in geochemistry and soil chemistry. Pergamon
689 Press, Oxford UK.
690 Worsfold P.J., L.J. Gimbert, U. Mankasingh, O.N. Omaka, G. Hanrahan, P.C.F.C. Gardolinski, P.M.
691 Haygarth, B.L. Turner, M.J. Keith-Roach, and I.D. McKelvie. 2005. Sampling, sample treatment
692 and quality assurance issues for the determination of phosphorus species in natural waters and
693 soils. Talanta 66:273-293.
694 Zhang R.Y., F.C. Wu, Z.Q. He, J.A. Zheng, B.A. Song, and L.H. Jin. 2009. Phosphorus composition in
695 sediments from seven different trophic lakes, China: a phosphorus-31 NMR study. J. Environ.
696 Qual. 38:353-359.

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